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Cloning and Expression of *Acinetobacter calcoaceticus* Catechol 1,2-Dioxygenase Structural Gene *catA* in *Escherichia coli*

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Catechol 1,2-dioxygenase (EC 1.13.1.1), the product of the *catA* gene, catalyzes the first step in catechol utilization via the β-ketoadipate pathway. Enzymes mediating subsequent steps in the pathway are encoded by the *catBCDE* genes which are carried on a 5-kilobase-pair (kbp) *Eco*RI restriction fragment isolated from *Acinetobacter calcoaceticus*. This DNA was used as a probe to identify *Escherichia coli* colonies carrying recombinant pUC19 plasmids with overlapping sequences. Repetition of the procedure yielded an *A. calcoaceticus* 6.7-kbp *Eco*RI restriction fragment which contained the *catA* gene and bordered the original 5-kbp *Eco*RI restriction fragment. When the *catA*-containing fragment was placed under the control of the *lac* promoter on pUC19 and induced with isopropylthiogalactopyranoside, catechol dioxygenase was formed in *E. coli* at twice the level found in fully induced cultures of *A. calcoaceticus*. *A. calcoaceticus* strains with mutations in the *catA* gene were transformed to wild type by DNA from lysates of *E. coli* strains carrying the *catA* gene on recombinant plasmids. Thus, *A. calcoaceticus* strains with a mutated gene can be used in a transformation assay to identify *E. coli* clones in which at least part of the wild-type gene is present but not necessarily expressed.

Catechol 1,2-dioxygenase plays a central role in pathways for catabolism of many aromatic growth substrates. The compounds are converted to catechol which is cleaved by the dioxygenase to form *cis,cis*-muconate and further metabolized to tricarboxylic acid cycle intermediates via the β-ketoadipate pathway (Fig. 1) (25). The dioxygenase has been purified from diverse microbial sources and invariably contains trivalent nonheme iron (4, 7, 11, 16–20). Different subunit sizes and oligomeric structures have been reported for the protein; these properties apparently depend on the biological source of the enzyme. Catechol 1,2-dioxygenase from *Acinetobacter calcoaceticus* is a dimer formed by association of identical subunits and has a molecular weight of 81,000 (20).

The structural gene for the enzyme, *catA*, is expressed in response to muconate. Expression of *catB*, the structural gene for the enzyme that acts upon muconate, is also induced by this metabolite. A number of physiological observations of *A. calcoaceticus* (previously designated *Moraxella calcoaceticus* [1]) indicate that, although they share a common regulatory metabolite, *catA* and *catB* are transcribed independently (3). In general, *catA* appears to be relatively sensitive to catabolite repression. Transductional analysis of *Pseudomonas aeruginosa* showed that the two structural genes were linked on the chromosome and clustered with independently regulated genes coding for enzymes having related metabolic functions (21). Similar supraoperonic clustering of genes for aromatic dissimilation was observed in *Pseudomonas putida* (15).

The possibility of a similar arrangement in *A. calcoaceticus* was raised by the observation of deletions extending through both the *catA* and *catB* genes in a number of spontaneous mutants unable to oxidize benzoate (M. E. Rae, unpublished observations). Isolation of a 5.0-kilobase-pair (kbp) *Eco*RI fragment containing the *catBCDE* genes from *A. calcoaceticus* (23) allowed us to test this possibility by isolating neighboring DNA and examining it for the presence of the *catA* gene. As we report here, this effort was successful. Furthermore, we were able to use a simple transformation procedure to test for the presence of the cloned *catA* gene even in the absence of its expression in *Escherichia coli*. This technique may prove useful in the identification of *E. coli* colonies carrying genes from *A. calcoaceticus*.

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MATERIALS AND METHODS

Bacterial strains and plasmids. *A. calcoaceticus* strains were derived from strain BD413 (9, 10). ADP134 is a spontaneous mutant of wild-type strain ADP1 selected on agar containing 10 mM succinate and 5 mM *p*-fluorobenzoate at 37°C (R. Lerud, unpublished data). ADP139 was also selected for resistance to *p*-fluorobenzoate toxicity (M. E. Rae, unpublished data). Both ADP134 and ADP139 carry mutations in the *catA* structural gene. *E. coli* JM101 has the following genotype: *supE thi* Δ(*lac-proAB*) F′ *traD36 proAB lacI*^{q}ZΔM15 (14). The cloning vector used in this study was pUC19 (Ap *lacp/o*) (27). Plasmid pAN4, which carries the *catBCDE* genes (23), has been renamed pPAN4.

Media and growth conditions. Cultures of *A. calcoaceticus* and *E. coli* were grown as described previously (23). Succinate (10 mM) or benzoate (5 mM) was provided as the carbon source. Supplements were added as needed at the following final concentrations: ampicillin, 50 μg/ml; 5-bromo-4-chloro-3-indolyl-β-D-galactoside 0.006%; isopropyl-β-D-thiogalactopyranoside (IPTG), 1 mM.

Isotopes, enzymes, and chemicals. *cis,cis*-Muconate was a gift from the Celanese Research Co. 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 supplier.

DNA purifications. Chromosomal DNA was purified from a 50 ml pelleted culture suspended in 10 ml of 50 mM Tris hydrochloride–20 mM EDTA (pH 8.0) by the method of Berns and Thomas (2). Plasmid DNA was purified by the method of Holmes and Quigley (8) and by the method of Schleif and Wensink (22).

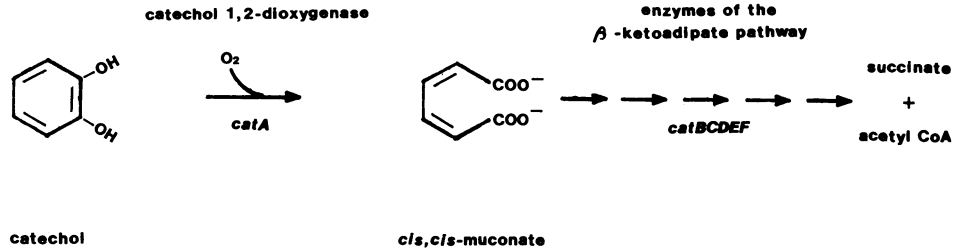

FIG. 1. Reaction catalyzed by catechol 1,2-dioxygenase. CoA, Coenzyme A.

DNA size fractionation. Wild-type chromosomal DNA (50 μg) was completely digested with a restriction endonuclease and electrophoresed on a 0.7% SeaPlaque low-melting-temperature agarose (FMC Corp., Marine Colloids Div., Rockland, Maine) horizontal gel. Gel slices were taken containing DNA of the approximate size desired, and DNA was extracted from the gel slices (13).

Subcloning and restriction mapping. Subcloning and restriction mapping were accomplished by established techniques (13). Particular DNA fragments were recovered from low-melting-temperature agarose and were ligated to linearized pUC19. *E. coli* was transformed by the method of Hanahan (6). Two plasmids, pIB1341 and pIB3 (see Fig. 3), are deletion derivatives of pIB1362.

***Acinetobacter* transformation assay.** *A. calcoaceticus* was transformed by the method of Juni (9) with several modifications. Donor DNA was prepared from a 5-ml overnight liquid culture. After being harvested, cells were suspended in 0.5 ml of sterile prewarmed lysis buffer (0.05% sodium dodecyl sulfate [SDS] in 0.15 M NaCl–0.015 M citrate, trisodium salt) and were incubated in a 65°C water bath for 1 to 3 h. Donor DNA isolated in other ways could also be used to transform the recipient *Acinetobacter* strain.

The recipient *Acinetobacter* mutant strain was grown overnight in 5 ml of succinate medium. In the morning, 0.01 ml of sterile 1 M succinate was added, and the cells were incubated at 37°C for 30 min at which point they were ready for transformation. The recipient (0.1 ml) was plated on a selective medium plate on which it could not grow. Samples of donor DNA (25 μl) were spotted directly onto the recipient plate. After incubation at 37°C, colonies appeared in the spots where the *Acinetobacter* mutant had been transformed to wild type (see Fig. 5). The donor DNA was diluted in 1× SSC buffer (0.15 M NaCl and 0.015 M citrate, trisodium salt), and when different dilutions of the same DNA were used, the number of transformants was proportional to the amount of DNA added. As controls, the recipient was plated on a selective medium plate to which no DNA was added. The donor DNA and dilution buffer were checked for sterility by incubating samples on a rich medium (L agar) plate.

Southern blots and colony hybridizations. Southern hybridizations were done as described previously (23). The hybridization and wash conditions used for Southern blots were also used for colony hybridizations (13).

Measurement of catechol dioxygenase enzyme activity. Cultures for enzyme assays were prepared as described previously (23). Cells were sonicated by four 20-s bursts with a Braun-Sonic 2000 apparatus. Enzyme activity was measured spectrophotometrically by monitoring the increase in A_{260} (7). Protein concentrations were determined by the method of Lowry et al. (12) with bovine serum albumin as the standard.

A qualitative pH indicator assay was used to follow acid production colorimetrically. A stock solution of 10 mM catechol, 0.004% phenol red, and 1 mM EDTA was adjusted with ammonium hydroxide to form a deep red color (pH 6 to 7). Several drops of toluene were added to 1 ml of the stock solution. A loopful of cell paste from the surface of a suitable growth medium was assayed by suspension in the substrate-indicator dye mix. The presence of catechol dioxygenase resulted in a color change from deep red to yellow in several minutes at room temperature.

High-performance liquid chromotography. Catechol dioxygenase was partially purified by the following method. Frozen cells (about 2.5 g) were suspended 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 × *g* for 15 min. The supernatant fraction was then centrifuged at 100,000 × *g* for 30 min in an ultracentrifuge. The supernatant fraction was filtered through a 0.22-μm pore-size Millex GV (low binding) unit, and the filtrate was termed crude extract.

The crude extract was separated on a Toya Soda TSK DEAE-5PW anion-exchange column (7.5 mm by 7.5 cm). The column was equilibrated with ethylenediamine buffer, and 1 ml of the crude extract (20 to 25 mg/ml) was injected. Elution (1 ml/min) was carried out with a 0 to 0.25 M NaCl gradient in 90 min. The eluant was monitored by UV detection at 280 nm, and 1-ml fractions were collected. Fractions were assayed for enzyme activity and were checked by SDS-polyacrylamide gel electrophoresis (26). Samples were run on a 12% total, 2.7% cross-linked, 0.1% SDS denaturing polyacrylamide gel.

RESULTS

Cloning an *Xba* I fragment overlapping the *catBCDE* genes. Southern blot analysis of *Xba*I-cut chromosomal *A. calcoaceticus* DNA probed with various radioactive segments of the *catBCDE* genes showed that a chromosomal *Xba*I fragment spanned most of the 5.0-kbp *catBCDE Eco*RI fragment and extended a little beyond. An appropriate size fraction of *Xba*I-cut *A. calcoaceticus* chromosomal DNA was ligated to pUC19, which had been linearized and dephosphorylated, and was used to transform JM101. One of 100 screened recombinant plasmid-containing clones demonstrated colony hybridized with a probe from the *catBCDE* gene region (Fig. 2). The plasmid from the hybridizing clone, having a 6.3-kbp *A. calcoaceticus* insert in pUC19, was designated pIB1. One of the ends of this cloned insert, however, could not be regenerated as an *Xba*I end. Further characterization by Southern blot and restriction analyses revealed no other irregularities.

Characterization of pIB1. Comparative restriction analyses revealed that the pIB1 insert has 4.8 kbp with the same restriction pattern as the pPAN4 insert. In addition, pIB1 has 1.5 kbp of newly cloned DNA. Southern blot analysis of

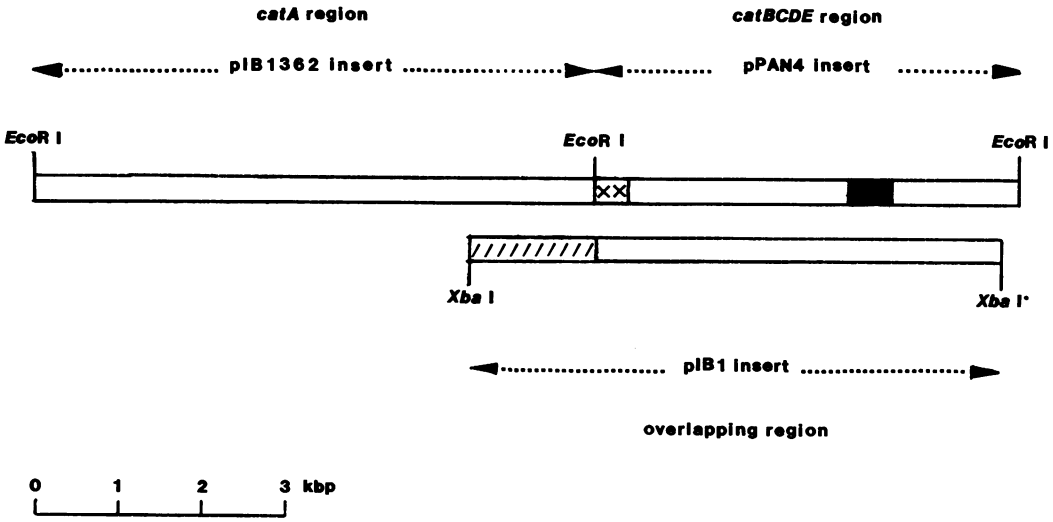

FIG. 2. Relative chromosomal positions of cloned fragments from plasmids pIB1362, pPAN4, and pIB1. Inserts ligated to pUC19 form the corresponding plasmids. DNA probes used for hybridizations are represented by black, slashed, and crossed areas. ■, Used to isolate pIB1. ▨, Used to isolate pIB1362; and ⊠, used to probe genomic digests. The site designated *Xba*I* could not be regenerated.

*Eco*RI-cut chromosomal DNA with a probe from pIB1 showed that the pIB1 insert overlaps two chromosomal fragments which have approximately 5 and 7 kbp of DNA.

Cloning the *Eco*RI fragment adjacent to the 5-kbp *catBCDE* *Eco*RI fragment. A purified 6.6- to 9.4-kbp size fraction of *Eco*RI-cut wild-type *A. calcoaceticus* DNA was ligated to *Eco*RI-cut dephosphorylated pUC19. After transformation of JM101, 220 colonies having recombinant plasmids were screened by colony hybridization with a probe from the pIB1 insert. Restriction analysis of plasmids from the four hybridizing colonies showed that each had the same 6.7-kbp fragment inserted in pUC19 and also showed that plasmids with both of the possible orientations of the insert relative to the *lac* promoter of pUC19 had been isolated. The plasmid

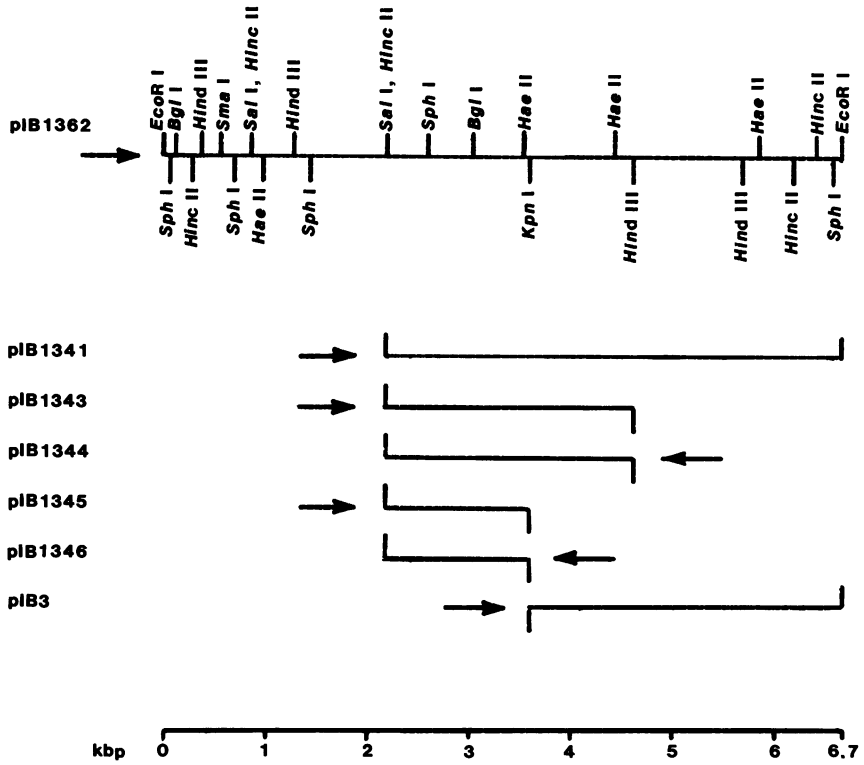

FIG. 3. Restriction map of the cloned *A. calcoaceticus* DNA segment containing *catA*. Horizontal lines represent DNA fragments which form the plasmids named at left when ligated to pUC19. Arrows indicate the direction of transcription starting from the *lac* promotor of pUC19. pIB1345 is the smallest plasmid from which *catA* is expressed in *E. coli*.

TABLE 1. Expression of *catA* gene in *E. coli*

Strain	Expression of *catA* gene[a] in:	
	L broth-grown cells	L broth + IPTG-grown cells
JM101(pUC19)	<0.001	<0.001
JM101(pIB1361)	0.002	0.003
JM101(pIB1362)	0.004	0.02
JM101(pIB1341)	0.16	0.8
JM101(pIB1343)	0.48	2.0
JM101(pIB1344)	0.003	<0.001
JM101(pIB1345)	0.05	0.11
JM101(pIB1346)	0.003	<0.001

[a] Expressed as the ratio of the observed specific activity of catechol dioxygenase to the specific activity of the enzyme found in fully induced (benzoate grown) cultures of *A. calcoaceticus* (0.75 μmol/min per mg of protein).

having an insert transcribed from left to right (Fig. 3). when expressed from the *lac* promoter of pUC19 was designated pIB1362. The plasmid having an insert of opposite orientation was designated pIB1361. When the 6.7-kbp cloned fragment was used to probe *Eco*RI-cut chromosomal DNA from a mutant strain deleted for the *catA* gene, no strongly hybridizing bands were detected.

Southern blot analyses are consistent with the 6.7-kbp cloned fragment being the chromosomal fragment adjacent to the *catBCDE* region. Cloned fragments from the end regions of the pPAN4 and pIB1 inserts (the crossed and slashed areas in Fig. 2) were used to probe ADP1 chromosomal DNA digested with various restriction endonucleases including *Hin*cII, *Hin*dIII, *Sal*I, *Sma*I, and *Sph*I. When either probe was used, the sizes of hybridizing chromosomal fragments were the same as those predicted by the pPAN4 and pIB1362 restriction maps for chromosomal fragments overlapping the *catBCDE* region.

Expression and subcloning of *catA* gene in *E. coli*. Cultures of JM101(pIB1361) and JM101(pIB1362) were grown in the presence or absence of IPTG and were checked for catechol dioxygenase by the pH indicator assay. Neither strain expressed the enzyme at detectable levels when grown in the absence of IPTG; the inducer elicited expression of detectable levels of the oxygenase in JM101(pIB1362) but not in JM101(pIB1361). Spectrophotometric assays confirmed that *catA* is expressed at low levels from the *lac* promoter in pIB1362 and that expression of the enzyme is enhanced by the addition of IPTG to the growth medium (Table 1). Levels of catechol dioxygenase in JM101(pIB1361), barely detectable in the spectrophotometric assay, were 10-fold lower than those found in induced cultures of JM101(pIB1362). Muconate, the inducer of *catA* in *A. calcoaceticus*, and adipate, an inducer analog of muconate, did not elicit synthesis of the enzyme in the recombinant *E. coli* strains.

Subclones derived from pIB1361 and pIB1362 were isolated to locate the *catA* gene on the 6.7-kbp fragment (Fig. 3). The highest specific activity of catechol dioxygenase in *E. coli* crude extracts was found in JM101(pIB1343) whose plasmid contains a 2.5-kbp *Sal*I-*Hin*dIII *A. calcoaceticus* insert (Table 1). The smaller 1.6-kbp *Sal*I-*Kpn*I insert of pIB1345 was expressed at a lower level in *E. coli*. However, when partially purified catechol dioxygenases from *A. calcoaceticus*, JM101(pIB1345), and JM101(pIB1343) were compared by SDS-polyacrylamide gel electrophoresis, there were no detectable size differences in the proteins (Fig. 4).

Use of transformation to detect DNA carrying the *catA* gene. Mutant *A. calcoaceticus* ADP134 and ADP139 do not grow at the expense of benzoate because they carry mutations in the *catA* structural gene. DNA carrying the wild-type *catA* gene from the parental *A. calcoaceticus* strain transforms the mutant cells, and the transformation takes place readily on solidified medium containing benzoate as a growth substrate (Fig. 5). Thus, the mutant cells can be used to detect the presence of the functional allele of the wild-type *catA* gene in crude lysates of recombinant *E. coli* strains. The spot transformation assay revealed DNA from the *catA* gene in lysates of JM101(pIB1361), JM101(pIB1362), JM101 (pIB1341), JM101(pIB1343), JM101(pIB1344), JM101 (pIB1345), and JM101(pIB1346). Lysates of JM101(pIB3) and JM101(pPAN4) did not confer the ability to grow with benzoate upon the *catA* mutant strains.

Limits of spot transformation assay. Crude lysates made from cultures of mixed colonies, in which the appropriate plasmid was present in 1% of the *E. coli* cells, could transform *A. calcoaceticus catA* mutants to wild type. The mutants also could be transformed by appropriate DNA isolated in any of the following ways and spotted directly on the recipient strain: plasmid DNA purified by cesium chloride-ethidium bromide density gradient centrifugation, plasmid DNA isolated by the rapid boiling method, and wild-type DNA size fractionated and purified from low-melting-temperature agarose. When purified recombinant plasmid DNA was used as a donor, transformation frequencies of between 10^8 and 10^9 transformants per μg of DNA were observed in the presence of 10^9 recipient cells. The major source of variability is likely to be in the competence of the recipient strain.

In accord with the observations of Singer et al. (24), no evidence of an *A. calcoaceticus* restriction system was observed when the transformation frequency with donor DNA purified from *E. coli* was compared with that with DNA purified from *A. calcoaceticus*. Transformation frequencies (per microgram of DNA) were lower by 1,000-fold for *Eco*RI-cut ADP1 chromosomal donor DNA than for *Eco*RI-cut pIB1362 donor DNA. If the *A. calcoaceticus* chromosome is roughly the same size as that of *E. coli*, however, there should be about 1,000 more copies of the

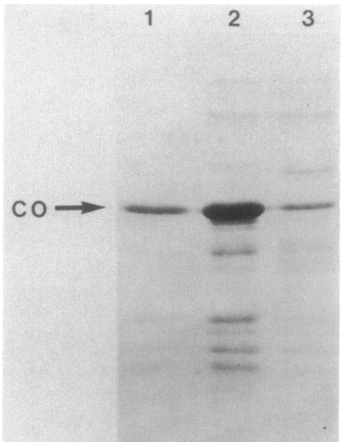

FIG. 4. Comparison of partially purified catechol dioxygenase (CO) from *A. calcoaceticus* (lane 1), JM101(pIB1343) (lane 2), and JM101(pIB1345) (lane 3). After fractionation of crude extracts by high-performance liquid chromatography anion exchange, 50-μl samples of the fractions having peak catechol dioxygenase activity were electrophoresed on a 12% SDS-polyacrylamide gel. The arrow points to the 40-kilodalton proteins.

6.7-kbp *Eco*RI fragment carrying the *catA* gene per μg of DNA in the plasmid versus the chromosomal donor DNA. Thus, transformation frequencies are comparable for donor DNA purified from *E. coli* and from *A. calcoaceticus*.

DISCUSSION

Several lines of evidence indicate that the cloned *catA* gene is from *A. calcoaceticus*. Restriction fragments carrying the gene hybridize to *Acinetobacter* chromosomal fragments of corresponding size, and mutants from which the *catA* gene has been deleted lack this hybridizing DNA. Furthermore, the cloned DNA effectively transforms *Acinetobacter catA* mutants to the wild type, and Juni (9) has shown that the capacity to transform *Acinetobacter* mutants is restricted to DNA from members of this genus.

The organisms used in this study were derived from Juni's strain BD413 which he showed to be extraordinarily competent for genetic transformation (9). This quality was exploited in the present investigation to demonstrate the presence of the *catA* gene in recombinant *E. coli* strains in which the gene was not expressed. In principle, the procedure can be applied to identify *E. coli* strains carrying recombinant plasmids carrying any gene for which there exists a corresponding *Acinetobacter* mutant: transformation of the mutant to the wild type by crude lysates of the recombinant *E. coli* strain would serve as presumptive evidence for the presence of at least part of the gene. The procedure may prove to be valuable in exploring the genetic basis for the nutritional versatility of *Acinetobacter* species. The complex catabolic pathways of this genus are catalyzed by many enzymes that, even if expressed in *E. coli*, may be difficult to detect.

Detection of cloned DNA by colony hybridization was greatly facilitated by determining the size of the desired DNA fragments and by size fractionating the chromosomal DNA before ligation with the cloning vector. Combining these techniques, as opposed to using a total genomic library, increases the chances of isolating a desired clone. In one case we found the desired insert in 1 of 100 screened colonies, and in the second case we found four desired clones after screening 220 recombinant colonies. Since only several hundred colonies need to be screened by this method, individual colonies can be patched onto gridded nitrocellulose filters and master plates. Few filters need be used, and identification of colonies corresponding to positive hybridizations by this method is quite direct.

Catechol dioxygenase is produced in *E. coli* when the *A. calcoaceticus catA* gene is under control of the *lac* promoter of pUC19. The 100-fold difference in expression between pIB1362 and pIB1343 suggests that most transcripts originating from the *lac* promoter do not extend through the region between the *Eco*RI and *Sal*I sites of pIB1362. The reason for the differential expression of pIB1343 and pIB1345 is not evident from this study. Poor expression of the *catA* gene of pIB1361 may result from an inability of *E. coli* RNA polymerase to recognize and to bind efficiently to the *A. calcoaceticus* promoter region.

The *catA* gene is transcribed in the same direction as the *catBCDE* gene cluster, but the two genetic regions lie more than 3 kbp apart (Fig. 2 and 3). Perhaps investigation of the intervening region will provide some clues about the basis for the physical linkage of the independently controlled genes. The inability of *cis,cis*-muconate to regulate the *catA* gene in *E. coli* may be due to either the absence of necessary regulatory regions on the clone or the inability of these regions to be recognized in *E. coli*.

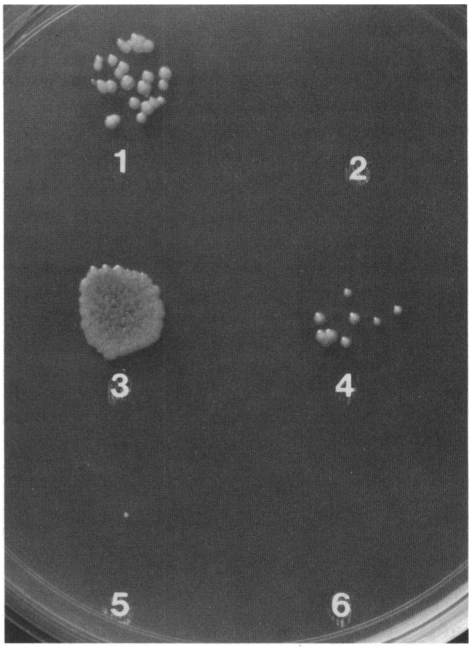

FIG. 5. *Acinetobacter* transformation assay. Growth of *catA* mutant strain ADP134 on a benzoate plate occurs only in spots where DNA has transformed the mutant to wild type. ADP1 DNA (10^{-3} μg) was dropped on spot 1; pPAN4 DNA (10^{-3} μg) was dropped on spot 2. Different dilutions of pIB1361 DNA were dropped on spots 3 to 6: 3, 10^{-5} μg; 4, 10^{-6} μg; 5, 10^{-7} μg; 6, 10^{-8} μg.

The *Acinetobacter catA* gene is the first chromosomal catechol 1,2-dioxygenase structural gene to be cloned. A plasmidborne *Pseudomonas* gene encoding a catechol 1,2-dioxygenase of relatively broad specificity has been isolated on a 4.2-kbp DNA fragment (5). Comparison of the structures of the two dioxygenase genes may indicate whether they share a common ancestor and, if so, may provide evidence about the mechanisms underlying their evolutionary divergence.

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