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Characterization of a Plasmid-Specified Pathway for Catabolism of Isopropylbenzene in *Pseudomonas putida* RE204

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A *Pseudomonas putida* strain designated RE204, able to utilize isopropylbenzene as the sole carbon and energy source, was isolated. Tn*5* transposon mutagenesis by means of the suicide transposon donor plasmid pLG221 yielded mutant derivatives defective in isopropylbenzene metabolism. These were characterized by the identification of the products which they accumulated when grown in the presence of isopropylbenzene and by the assay of enzyme activities in cell extracts. Based on the results obtained, the following metabolic pathway is proposed: isopropylbenzene → 2,3-dihydro-2,3-dihydroxyisopropylbenzene → 3-isopropylcatechol → 2-hydroxy-6-oxo-7-methylocta-2,4-dienoate → isobutyrate + 2-oxopent-4-enoate → amphibolic intermediates. Plasmid DNA was isolated from strain RE204 and mutant derivatives and characterized by restriction enzyme cleavage analysis. Isopropylbenzene-negative isolates carried a Tn*5* insert within a 15-kilobase region of a 105-kilobase plasmid designated pRE4. DNA fragments of pRE4 carrying genes encoding isopropylbenzene catabolic enzymes were cloned in *Escherichia coli* with various plasmid vectors; clones were identified by (i) selection for Tn*5*-encoded kanamycin resistance in the case of Tn*5* mutant plasmids, (ii) screening for isopropylbenzene dioxygenase-catalyzed oxidation of indole to indigo, and (iii) use of a Tn*5*-carrying restriction fragment, derived from a pRE4::Tn*5* mutant plasmid, as a probe for clones carrying wild-type restriction fragments. These clones were subsequently used to generate a transposon insertion and restriction enzyme cleavage map of the isopropylbenzene metabolic region of pRE4.

The monoalkylbenzenes isopropylbenzene (cumene), ethylbenzene, and toluene are important industrially as synthetic intermediates and solvents (32). As a consequence of their widespread use and subsequent introduction into the environment, the latter two compounds were included on the U.S. Environmental Protection Agency's list of priority pollutants (39).

The bacterial metabolism of toluene has been shown to occur by two different pathways. One, exemplified by that encoded by the well-studied TOL plasmid (54), is initiated by a multistep oxidation of the methyl group to give benzoate (40); the other, which is apparently chromosome encoded (26), is initiated by a dioxygenase-catalyzed oxidation of the aromatic ring to give 2,3-dihydro-2,3-dihydroxytoluene which is subsequently dehydrogenated to give 3-methylcatechol (28, 29). Similarly, ethylbenzene has been shown to be primarily metabolized through 2,3-dihydro-2,3-dihydroxyethylbenzene to 3-ethylcatechol (27). A general pathway for the complete metabolism of these and other monoalkylbenzenes in which the side chain is not oxidized before ring cleavage has been described (Fig. 1), and evidence for this is summarized in two recent reviews (31, 47). Some evidence has been presented for the metabolism of isopropylbenzene through the ring-cleavage product IV depicted in Fig. 1 (29) and for the metabolism of this compound to isobutyrate, pyruvate, and acetaldehyde (16), but the complete metabolism of isopropylbenzene in a single organism has not thus far been elucidated.

We report here the isolation and characterization of *Pseudomonas putida* RE204 (formerly ipba [22]) which is able to grow with isopropylbenzene, toluene, ethylbenzene, and various other aromatic compounds as the sole sources of carbon and energy. The generation and analysis of Tn*5* mutants of this strain permitted elucidation of a pathway for isopropylbenzene metabolism and the cloning and mapping of genes encoding isopropylbenzene-metabolizing enzymes in this organism.

(Some preliminary data from this work were presented at the 3rd International Symposium on Microbial Ecology, Lansing, Mich. [22].)

MATERIALS AND METHODS

The minimal medium used for cultivation of strain RE204 and its derivatives was R medium (21) to which water-soluble carbon sources were added to 0.1%. Hydrocarbons were usually provided as vapor unless otherwise indicated. Media were solidified by the addition of 1.5% Bacto-Agar (Difco Laboratories, Detroit, Mich.).

Isolation and identification of strain RE204. Strain RE204 was isolated from the Rhone River in Geneva, Switzerland, by using selective enrichment in R medium with isopropylbenzene as the sole carbon and energy source. It was identified by the criteria of Stanier et al. (51).

***Escherichia coli* strains and plasmids.** *Escherichia coli* strains and plasmids used are listed in Table 1.

Isolation of transposon mutants. Tn*5* mutants were isolated by using the Tn*5* donor *E. coli* ED2196(pLG221) or LE392(pLG221). Equal volumes of overnight cultures (LB medium [14]) of a donor strain and the recipient, strain RE204, were mixed, and 0.2 ml was placed on a 25-mm nitrocellulose filter on LB agar. After incubation at 30°C for 4 to 6 h, filters were removed from the plates and washed in 1 ml of 50 mM KH_2PO_4-NaOH buffer, pH 6.8. Samples of this bacterial suspension were then spread on plates containing R medium supplemented with 0.1% fumarate and 100 μg of kanamycin sulfate per ml. After incubation of these plates for 2 days at 30°C, colonies were picked off onto gridded R medium-fumarate-kanamycin plates which were then incu-

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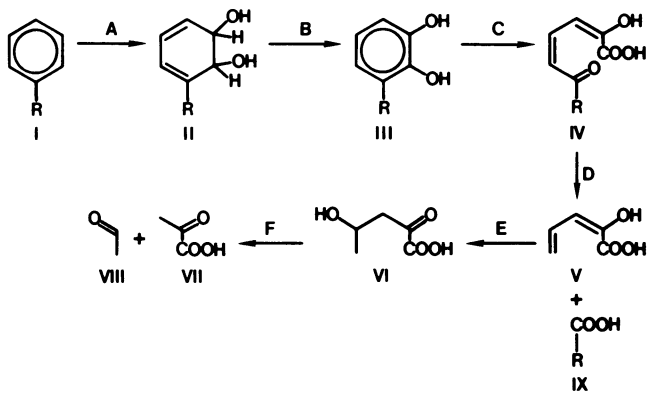

FIG. 1. General pathway for the bacterial metabolism of monoalkylbenzenes. If compound I is toluene, R = $-CH_3$; if ethylbenzene, R = $-CH_2CH_3$; and if *n*-butylbenzene, R = $-CH_2CH_2CH_2CH_3$. For isopropylbenzene and its metabolites, R = $-CH(CH_3)_2$; compound II, 2,3-dihydro-2,3-dihydroxyisopropylbenzene; compound III, 3-isopropylcatechol; compound IV, HOMODA; compound V, 2-oxopent-4-enoate; compound VI, 4-hydroxy-2-oxopentanoate; compound VII, pyruvate; compound VIII, acetaldehyde; and compound IX, isobutyrate. Enzyme A, Isopropylbenzene dioxygenase; B, 2,3-dihydro-2,3-dihydroxyisopropylbenzene dehydrogenase; C, 3-isopropylcatechol dioxygenase; D, HOMODA hydrolase; E, 2-oxopent-4-enoate hydratase; and F, 4-hydroxy-2-oxopentanoate aldolase.

bated overnight. Colonies that developed were replicated to R medium plates containing no carbon source, and after tubes containing isopropylbenzene were placed in the lids, the plates were incubated at 30°C for 2 days, after which they were scored for mutants unable to grow on isopropylbenzene.

Isopropylbenzene-negative mutants were initially analyzed for the accumulation of isopropylbenzene catabolic intermediates. After overnight growth at 30°C in 5 ml of R medium plus succinate in 20-ml McCartney bottles in which a small glass tube containing isopropylbenzene was placed, cells were removed by centrifugation, and UV-visible spectra of culture supernatant fluids diluted in 50 mM KH_2PO_4-NaOH buffer (pH 6.8) were recorded.

Assay of enzyme activities in cell extracts. Strains were inoculated into 50 ml of R medium supplemented with 0.1% succinate and 0.02% yeast extract. After incubation at 30°C overnight, the cultures were used to inoculate 500 ml of R medium supplemented with 0.2% succinate and 0.02% yeast extract. These cultures were incubated at 30°C for 5 h with or without the addition of isopropylbenzene to the medium (to 1 mM) after 2 h. Cells were harvested by centrifugation, washed once with 30 ml of KH_2PO_4-NaOH buffer (pH 7.5), and resuspended in 8 ml of this buffer. Extracts were prepared by sonication (Branson sonifier; Branson Sonic Power Co., Danbury, Conn.) for a total of 1 min on ice, followed by centrifugation at 20,000 rpm (Sorvall SM-24 rotor) for 40 min. The upper 5 ml of supernatant fluid was carefully decanted and used for enzyme assays.

Dihydrodiol dehydrogenase assay. A 3.5-ml cuvette contained 2.7 ml of 50 mM KH_2PO_4-NaOH buffer (pH 7.5), 20 μl of 25 mM NAD, 20 to 200 μl of cell extract, and as the substrate, 200 μl of the supernatant fluid from a culture of mutant RE213 grown on succinate-R medium in the presence of isopropylbenzene. Formation of NADH was recorded at 340 nm (ϵ_{340} of NADH at pH 7.5 is 6,200).

3-Isopropylcatechol dioxygenase assay. A 3.5-ml cuvette contained 2.9 ml of KH_2PO_4-NaOH buffer (pH 7.5), 10 μl of 3-isopropylcatechol, and 5 to 200 μl of cell extract. Formation of the ring cleavage product 2-hydroxy-6-oxo-7-methylocta-2,4-dienoate (HOMODA) was recorded at 393 nm (ϵ_{393} of this compound at pH 7.5 is 9,700 [K. A. Lampel, Ph.D. thesis, University of Miami, Coral Gables, Fla., 1979).

HOMODA hydrolase assay. A 3.5-ml cuvette contained 2.9 ml of KH_2PO_4 buffer (pH 7.5) and 70 μl of the supernatant from a 5-ml culture of mutant RE225 grown on R medium plus succinate in the presence of isopropylbenzene. Disappearance of the ring cleavage product was recorded at 393 nm.

Protein concentrations were determined with the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories AG, Glattbrug, Switzerland [6]) with bovine serum albumin as the standard.

Plasmid DNA preparation. Plasmid DNA was isolated from strain RE204 and mutant derivatives by the method of Hansen and Olsen (34), followed by centrifugation to equilibrium in a cesium chloride-ethidium bromide gradient. Plasmid DNA was isolated from *E. coli* strains by the procedure of Holmes and Quigley (35) when rapidly screening many recombinants and by the procedure of Clewell and Helsinki for large-scale preparations (10).

Restriction enzyme cleavage analysis and cloning of DNA. Restriction endonucleases and DNA ligase were obtained from Genofit S.A., Geneva, Switzerland, and New England BioLabs, Schwalbach, Germany, and were used according to the instructions of the manufacturers. *Rsr*II (46) was a gift from C. D. O'Connor. Restriction enzyme-digested DNA was analyzed by electrophoresis through 0.5 to 0.7% agarose gels in Tris borate-EDTA buffer (14). Ligated DNA was used to transform *E. coli* recipients (41). Transformants harboring Tn*5*-carrying plasmids were selected by plating the transformation mixture on antibiotic medium 3 plates (Difco Laboratories) supplemented with 100 μg of kanamycin sulfate per ml. Otherwise, plasmid vector-encoded antibiotic resistances were selected: for pBR322, 25 μg of ampicillin per ml or 10 μg of tetracycline per ml; for pACYC184, 30 μg of chloramphenicol per ml or 10 μg of tetracycline per ml; and for pLV59, 30 μg of chloramphenicol per ml. Clones carrying the isopropylbenzene dioxygenase genes were readily screened by plating them on media containing 1 mM indole which is converted by isopropylbenzene dioxygenase (see below) and other dioxygenases (25) to the water-insoluble blue dye indigo. Dioxygenase-containing colonies therefore develop a dark green-to-blue color.

Analysis of plasmid-encoded products in minicells. Plasmids pRE47, pRE48, pRE49, pRE50, pBR322, and pACYC184 were transformed into the *E. coli* minicell-producing strain DS410. Plasmid-carrying minicells were purified and labeled with [^{35}S]methionine at 30°C as described by Dougan and Kehoe (18). After electrophoresis of labeled proteins on a sodium dodecyl sulfate-12.5% polyacrylamide gel, the gel was treated with En^{3}Hance (New England Nuclear Chemicals, Dreiech, Federal Republic of Germany), dried, and exposed to X-ray film with intensifier screens at −70°C.

RESULTS

Characterization of strain RE204. Strain RE204 is a motile, oxidase-positive, catalase-positive, gram-negative rod. It produces a fluorescent pigment on King medium B but no pigment on King medium A. It does not produce extracellular hydrolases for starch, gelatin, or poly-β-hydroxybutyrate. Strain RE204 is nondenitrifying, grows at 4°C but not at 37°C, and oxidizes protocatechuate by the *ortho*-cleavage pathway after growth with *p*-hydroxybenzoate. It utilizes as

TABLE 1. Strains of *E. coli* and plasmids used

Strains and plasmids	Characteristics[a]	Reference
Strains		
LE392	F⁻, *hsdR514 supE44 supF58 lacY1 (lacI24) galK2 galT22 metB1 trpR55*, λ⁻	42
ED2196	*his trp nal*	53
DS410	*minA minB rpsL sup*	19
Plasmids		
pBR322	Ap^r Tc^r	4
pACYC184	Cm^r Tc^r	9
pLV59	Cm^r, encodes *Eco*RI restriction enzyme and temperature-sensitive modification enzyme	45
pLG221	ColIb *drd-1 cib*::Tn*5* Km^r	5
pRE4	Encodes isopropylbenzene degradation, Ipb$^+$	This paper
pRE5	pRE4::Tn*5*, from mutant RE205, Ipb$^-$ Km^r	This paper
pRE6–pRE23	pRE4::Tn*5*, from 18 mutants, RE206 to RE223, Ipb$^-$ Km^r	This paper
pRE24	pRE4::Tn*5*, from mutant RE224, Ipb$^-$ Km^r	This paper
pRE25	pRE4::Tn*5*, from mutant RE225, Ipb$^-$ Km^r	This paper
pRE26	Tn*5*-carrying *Eco*RI fragment of pRE5 inserted into pBR322, Km^r Ap^r Tc^r	This paper
pRE27–pRE45	Tn*5*-carrying *Eco*RI fragments of pRE6 through pRE24 inserted into pBR322, Km^r Ap^r Tc^r	This paper
pRE46	Tn*5*-carrying *Eco*RI fragment of pRE25 inserted into pBR322, Km^r Ap^r Tc^r	This paper
pRE47	3.3-kb *Cla*I fragment (coordinates 13 to 16.3, Fig. 3) of pRE4 inserted into pBR322, Ind$^+$ Ap^r	This paper
pRE48	3.3-kb *Cla*I fragment (coordinates 13 to 16.3, Fig. 3) of pRE4 inserted into pACYC184, Ind$^+$ Cm^r	This paper
pRE49	13.2- and 2.7-kb *Eco*RI fragments (coordinates 6.3 to 22.2) of pRE4 inserted into pBR322, Ind$^+$ Ap^r Tc^r	This paper
pRE50	13.2- and 2.7-kb *Eco*RI fragments (coordinates 6.3 to 22.2) of pRE4 inserted into pACYC184, Ind$^+$ Tc^r	This paper
pRE51	13.2-kb *Eco*RI fragment (coordinates 6.3 to 19.5) of pRE50 inserted into pACYC184, Tc^r	This paper
pRE52	2.7-kb *Eco*RI fragment (coordinates 19.5 to 22.2) of pRE50 inserted into pACYC184, Tc^r	This paper
pRE58	11.0-kb *Bgl*II fragment (coordinates 0 to 11) of pRE4 hybridizing to Tn*5*-carrying *Eco*RI fragment of pRE46 inserted into pLV59, Cm^r	This paper
pRE60	10-kb *Bgl*II fragment (coordinates 11 to 21) of pRE4 inserted into pLV59, Ind$^+$ Cm^r	This paper
pRE61	9.8-kb *Bam*HI fragment (coordinates 12.9 to 22.7) of pRE4 inserted into pBR322, Ind$^+$ Ap^r	This paper

[a] Phenotype symbols: Ap^r, Tc^r, Cm^r, and Km^r, resistance to ampicillin, tetracycline, chloramphenicol, and kanamycin, respectively; Ipb$^+$, isopropylbenzene utilization; Ind$^+$, indigo production from indole (see text).

sole carbon and energy sources δ-aminovalerate, D-glucose, 2-ketogluconate, DL-lactate, L-arginine, L-valine, betaine, putrescine, pelargonate, L-tryptophan, L-kynurenine, anthranilate, and galactose but not D-fucose, trehalose, cellobiose, maltose, starch, *myo*-inositol, mannitol, geraniol, maleate, glycolate, adipate, *m*-hydroxybenzoate, testosterone, acetamide, norleucine, D-tryptophan, or nicotinate. These characteristics identify strain RE204 as *P. putida* biotype B (51). In addition to isopropylbenzene, strain RE204 grows with a number of other aromatic compounds including toluene, ethylbenzene, *n*-butylbenzene, phenol, benzoate, protocatechuate, terephthalate, and phenylacetate but not benzene, *n*-hexylbenzene, biphenyl, or chlorobenzene.

Isolation and characterization of Tn*5* mutants. After Tn*5* mutagenesis of strain RE204, some 14,000 mutants were analyzed, and 21 were identified as being unable to grow with isopropylbenzene. These were initially classified into four groups according to the compounds which accumulated during growth in the presence of isopropylbenzene (Table 2). One group consisting of eight isolates did not appear to metabolize isopropylbenzene. The second group, consisting of mutants RE213 and RE214, accumulated a compound having a UV absorbance maximum at 266 nm. After the addition of dilute HCl to a culture supernatant containing this compound, the UV absorbance maximum was irreversibly changed to 269 nm with a decrease in extinction to about one-third of the original. The acidified solution, neutralized and treated with the phenol reagent *N*,2,6-trichlorobenzoquinone imine, gave a positive blue color, although the nonacidified culture supernatant did not. This is consistent with the dehydration of a dihydrodiol in acid to give phenolic products (15, 28). These products were isolated in a separate large-scale experiment by acidifying a culture supernatant fluid with HCl to pH 2 and then extracting twice with equal volumes of diethyl ether which was subsequently dried over sodium sulfate and evaporated. The products were identified by thin-layer chromatography on silica gel plates (Chromagram Sheets; Eastman Kodak Co., Rochester, N.Y.). The major product comigrated with 2-hydroxyisopropylbenzene and the minor product comigrated with 3-hydroxyisopropylbenzene in three solvent systems (Table 3). These results suggest that the compound formed by mutant strain RE213 is 2,3-dihydro-2,3-dihydroxyisopropylbenzene (dihydrodiol).

A third group of mutants (isolates RE215 and RE216) accumulated a brown compound having a UV absorbance maximum at 272 nm (pH 6.8) and giving a purple color with *N*,2,6-trichlorobenzoquinone imine. This compound was isolated by acid-ether extraction of a culture supernatant fluid of mutant RE215 grown in the presence of isopropylbenzene and analyzed by thin-layer chromatography as above. Its mobility was identical to that of authentic 3-isopropylcatechol (Table 3) which would be the expected product of the dehydrogenation of the dihydrodiol accumulated by mutant RE213.

The fourth group of mutants (nine isolates) accumulated a compound which had absorbance maxima at 393 nm (alka-

TABLE 2. Enzyme activities in cell extracts of strain RE204 and Tn*5*-generated mutant strains[a]

Strain	Sp act (nmol min^{-1} mg of protein^{-1})						Compound accumulated from isopropylbenzene[d]
	Dihydrodiol dehydrogenase		3-Isopropylcatechol dioxygenase		HOMODA hydrolase		
	− ipb[b]	+ ipb[c]	− ipb	+ ipb	− ipb	+ ipb	
RE204	4.0	233	13	740	12.2	54	—
RE205	2.3	2.3	12	6.1	14.7	11.7	—
RE206	1.7	1.2	4.0	2.2	5.7	4.4	—
RE207	5.3	4.5	22	12	11.1	11	—
RE208	11	11	90	31	26	15	—
RE209	9.5	8.4	83	40	21.2	16.4	—
RE210	11.7	9	53	27	15.5	14.4	—
RE211	12.5	11	114	41	27	20.2	—
RE212	34	29	313	174	45	35	—
RE213	0	0	75	44	29.6	23.6	DHD
RE214	0	0	204	164	40.9	39	DHD
RE215	1.7	87	0	0	9.0	7.4	IPCAT
RE216	2.8	96	0	0	13.6	5.3	IPCAT
RE217	1.2	141	1.3	329	53	45	HOMODA
RE218	1.5	93	1.3	108	25	20	HOMODA
RE219	1.7	122	1.2	164	37	21	HOMODA
RE220	1.8	143	1.8	176	19.3	11.4	HOMODA
RE221	2.2	234	3.5	234	58.6	23.3	HOMODA
RE222	2.3	154	4.4	170	36.4	27.8	HOMODA
RE223	2.8	170	9.1	340	8.8	6.1	HOMODA
RE224	4.2	164	12	229	6.3	9.0	HOMODA
RE225	6.7	236	18	315	0	0	HOMODA

[a] Extracts were prepared and enzyme assays were carried out as described in Materials and Methods.
[b] Extracts of cells grown in the absence of isopropylbenzene.
[c] Extracts of cells grown in the presence of isopropylbenzene.
[d] —, No UV-absorbing compounds accumulated; DHD, 2,3-dihydro-2,3-dihydroxyisopropylbenzene; IPCAT, 3-isopropylcatechol.

line) and 323 nm (acidic). These maxima are identical to those reported for the product of oxygenative ring cleavage of 3-isopropylcatechol between carbons 2 and 3, HOMODA (16).

The wild-type and mutant strains were further examined by measuring the activities of several enzymes in extracts of induced and uninduced cells (Table 2). The dihydrodiol dehydrogenase and 3-isopropylcatechol dioxygenase were both inducible to similar levels (57 to 58-fold) by growth in the presence of isopropylbenzene. The ring cleavage product (HOMODA) hydrolase was only slightly inducible (about 4.5-fold). As expected, mutants RE213 and RE214 which accumulated the dihydrodiol failed to metabolize this compound and thus carry Tn*5* elements inserted in the structural gene for the dihydrodiol dehydrogenase; similarly, mutants RE215 and RE216 failed to metabolize 3-isopropylcatechol. However, all the mutants which accumulated the ring cleavage product except one, RE225, were capable of metabolizing that compound, although their activities were generally lower than that in the wild-type strain.

The ring cleavage product was further metabolized in extracts of induced wild-type cells and of all the mutants which accumulated it, except for strain RE225 which did not metabolize it and strain RE221, through 2-oxopent-4-enoate (vinylpyruvate; λ_{max} = 265 nm), which transiently accumulated, to products lacking UV or visible light absorbance (Fig. 2a). Extracts of strain RE221 formed 2-oxopent-4-enoate from HOMODA; however, 2-oxopent-4-enoate was not subsequently rapidly metabolized as in the wild-type extracts but slowly converted to a compound with a λ_{max} at 229 nm (Fig. 2b). In a separate experiment, 2-oxopent-4-enoate prepared enzymatically from allylglycine (11) was shown spectrophotometrically to undergo the same transformation in phosphate buffer in the absence of cell extract (data not shown). 4-Ethyl-2-oxobutyrolactone, a compound analogous to the lactone shown in Fig. 2b, has been shown

TABLE 3. Identification of compounds accumulated by isopropylbenzene-negative strains RE213 and RE215 growing on succinate in the presence of isopropylbenzene by thin-layer chromatography on silica gel plates

Solvent	R_f					
	4-Hydroxyisopropylbenzene	3-Hydroxyisopropylbenzene	RE213 product + acid	2-Hydroxyisopropylbenzene	RE215 product	3-Isopropylcatechol
Benzene	0.20	0.22	0.22–0.41	0.38	0.06	0.06
Diethyl ether-petroleum ether, 1:1 (vol/vol)	0.65	0.65	0.65–0.77	0.74	0.20	0.21
Chloroform-acetone, 8:2 (vol/vol)	0.57	0.59	0.59–0.62	0.62	0.38	0.36
Color with *N*,2,6-trichlorobenzoquinone imine	Tan	Light blue	Light blue/violet	Violet	Brown	Brown

previously to be formed from 2-oxohex-4-enoate (12). The lactone shown in Fig. 2b (4-methyl-2-oxobutyrolactone) has been reported to have an absorbance maximum at 229 nm (13). These data suggest that mutant RE221 is defective in the 2-oxopent-4-enoate hydratase and accumulates 2-oxopent-4-enoate which spontaneously converts to the corresponding lactone.

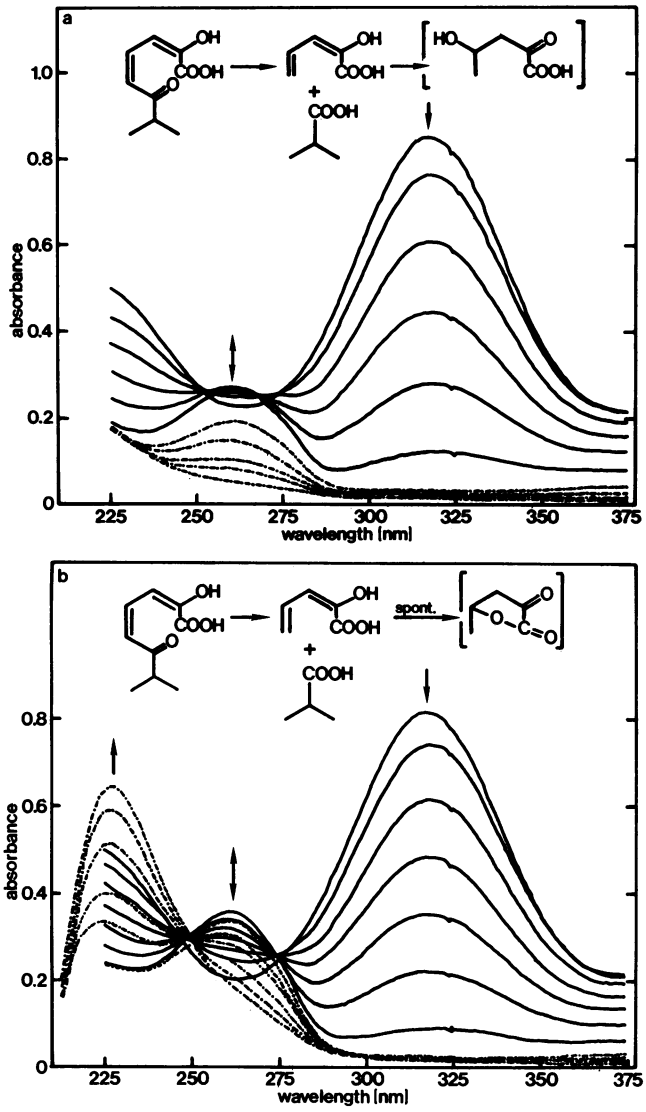

FIG. 2. Transformation of HOMODA by cell extracts of wild-type strain RE204 and mutant RE221. (a) The quartz sample cuvette initially contained 3 ml of KH_2PO_4-NaOH buffer (pH 6.8), 3 μl of 3-isopropylcatechol, and 2 μl of cell extract of the wild-type strain grown on succinate plus isopropylbenzene. The reference cuvette was identical but lacked the 3-isopropylcatechol. When the A_{323} reached a maximum, the first spectrum was recorded, and 8 μl of wild-type extract was added. Spectra were subsequently recorded after 0.17, 3.5, 7, 10.5, 14, 17.5, 21, 31.5, 45.5, 66.5, and 94.5 min. (b) The cuvettes were identical to those used in panel a except initially they contained 6 μl of cell extract of mutant strain RE221 grown on succinate in the presence of isopropylbenzene. When the A_{323} reached a maximum, the first spectrum was recorded. Subsequently, 24 μl of strain RE221 extract was added, and spectra were recorded after 0.17, 3.5, 7, 10.5, 14, 17.5, 21, 52.5, 80.5, 143.5, 206.5, and 269.5 min. Solid lines indicate spectra leading to the formation of 2-oxopent-4-enoate, and dashed lines indicate spectra leading to the removal of that compound.

Several isopropylbenzene-negative mutants were tested for growth on other analogous monoalkylbenzenes: toluene, ethylbenzene, and *n*-butylbenzene, but none was able to grow with these compounds. Moreover, when grown on succinate in the presence of these compounds, they accumulated the intermediates analogous to those accumulated from isopropylbenzene.

Localization of Tn*5* insertion sites in catabolic mutants. Wild-type strain RE204 carries a plasmid, pRE4, which was isolated and determined by analysis of the products of a number of restriction enzyme digests to have a molecular size of about 105 kilobases (kb). Plasmid DNA was similarly isolated from all the mutant strains and shown by digestion with restriction endonucleases *Eco*RI and *Xho*I to carry Tn*5*. *Eco*RI does not cut within Tn*5*, and *Eco*RI restriction fragments carrying Tn*5* are about 5.7 kb larger than nonmutant fragments; *Xho*I cuts three times in Tn*5* and yields a characteristic pair of fragments of 2.5 and 2.3 kb (38). In all the mutants except RE225 and RE205, the Tn*5* element was located in the largest (13.2 kb) of the more than 30 *Eco*RI fragments of pRE4.

*Eco*RI fragments of mutant pRE4 plasmids were ligated to the vector pBR322, and hybrid plasmids were transformed into *E. coli* LE392 followed by selection of Tn*5*-carrying clones on antibiotic medium 3-kanamycin plates. Plasmid DNA isolated from such clones was digested with *Xho*I and various other enzymes to locate precisely the sites of insertion of the Tn*5* elements in the hybrid plasmids. From this information, a transposon insertion map of the isopropylbenzene catabolic gene region of pRE4 was constructed (Fig. 3). The location of the Tn*5* insertion in pRE25 (from mutant RE225) was determined by digesting pRE25 and pRE24 (from mutant RE224) with *Xho*I. This enzyme cuts three times in pRE4, one site of which is about 4.1 kb from the Tn*5* element in pRE25 and 6.6 kb from the Tn*5* element in pRE24. The location of Tn*5* in pRE5 (from mutant RE205) was determined by digesting pRE5 with *Not*I and *Xho*I. *Not*I cuts twice in pRE4 and its Tn*5* derivatives, and one of these sites was found to be located about 2.1 kb from the Tn*5* element in pRE5.

A number of other restriction enzyme fragments were subsequently cloned from the wild-type plasmid, pRE4. Most of the hybrid plasmids containing these fragments were selected on the basis of the observation of Ensley et al. (25) that various bacterial dioxygenases catalyze the oxidation of indole to indigo; colonies of bacteria producing such a dioxygenase thus develop a green-to-blue color on indole-containing media. Hybrid plasmids containing DNA fragments generated by *Eco*RI, *Cla*I, *Bgl*II, and *Bam*HI (kilobase coordinates 6.3 to 22.2, 13.0 to 16.3, 11.0 to 21.0, and 12.9 to 22.7, respectively; Fig. 3) were constructs that resulted in the formation of blue clones. Clones generated with *Eco*RI always contained both the 13.2- and 2.7-kb *Eco*RI fragments (coordinates 6.3 to 19.5 and 19.5 to 22.2, respectively, Fig. 3). No *E. coli* transformants containing only the 13.2-kb *Eco*RI fragment developed this blue color. Blue-green clones could be obtained from the wild-type strain and most of the Tn*5* mutants tried, including mutants RE208, RE209, RE210, RE211, and RE212, but not mutants RE205, RE206, and RE207.

The final clone was a *Bgl*II fragment (coordinates 0 to 11.0, Fig. 3) inserted into the positive selection cloning vector pLV59 (45). Bacteria carrying the desired cloned DNA fragment were identified by hybridization (33) of transformant colonies on a nitrocellulose filter to a ^{32}P-labeled DNA probe consisting of the Tn*5*-carrying *Eco*RI

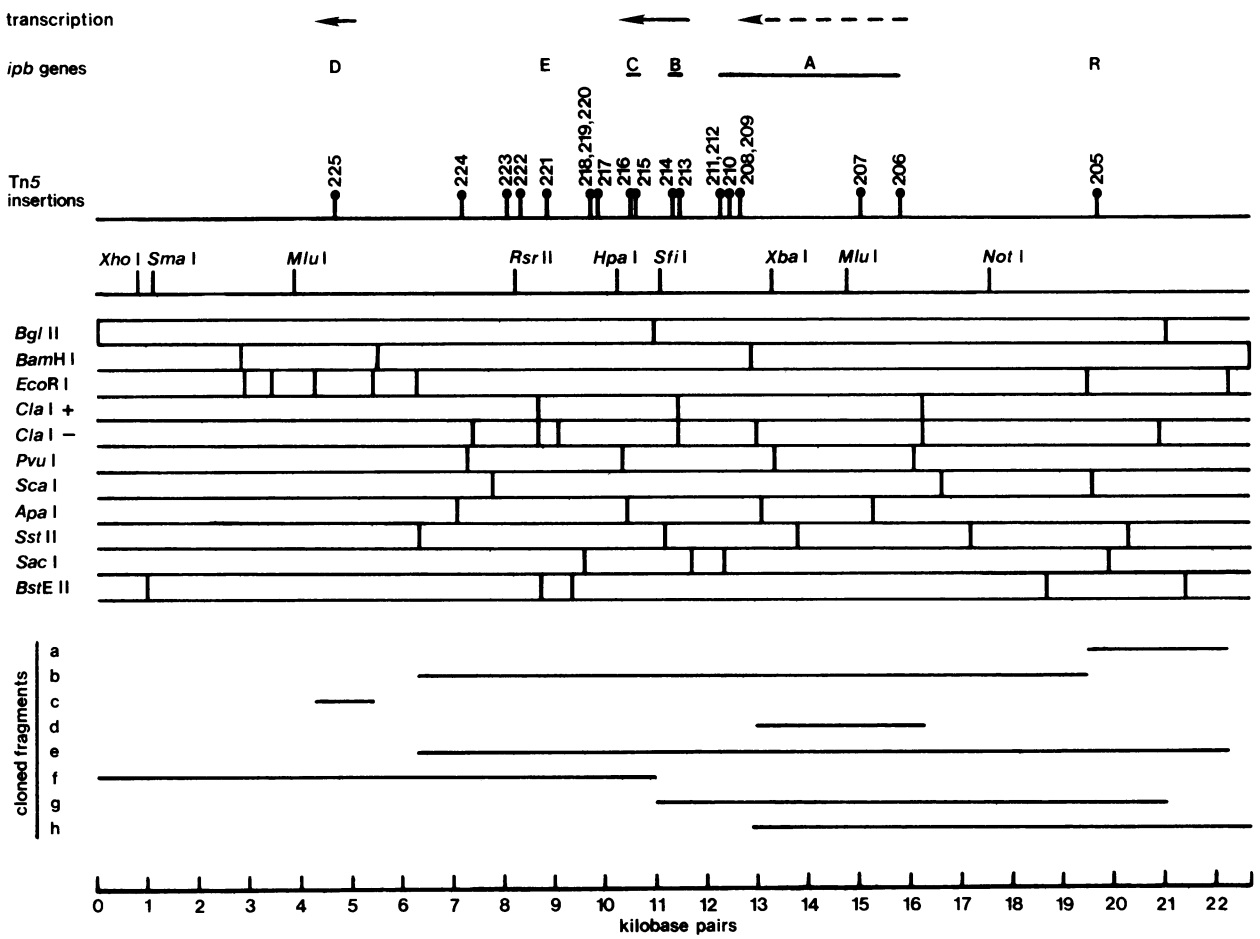

FIG. 3. Map of the region of plasmid pRE4 encoding isopropylbenzene metabolism. Shown is a DNA segment 22.7 kb in length, bordered on the left by a *Bgl*II site and on the right by a *Bam*HI site, which carries the genes (*ipbA*, *ipbB*, *ipbC*, *ipbD*, *ipbE*, and *ipbR*) encoding five of the enzymes and a putative regulatory protein of the isopropylbenzene metabolic pathway. The map includes the locations of these genes, sites of Tn*5* insertions, direction of transcription of genes *ipbA*, *ipbB*, *ipbC*, and *ipbD*, and restriction endonuclease cleavage sites. *Cla*I + and *Cla*I − indicate the restriction sites for this enzyme in DNA prepared from *dam*$^+$ and *dam*$^-$ (or *P. putida* RE204) hosts, respectively. At the bottom of the figure are indicated the DNA fragments which were cloned from pRE4 or its derivatives to yield the plasmids listed in Table 1: a, pRE26 and pRE52; b, pRE27 through pRE45 and pRE51; c, pRE25; d, pRE47 and pRE48; e, pRE49 and pRE50; f, pRE58; g, pRE60; and h, pRE61.

fragment (coordinates 4.3 to 5.5, Fig. 3) from pRE46 which had been purified by electrophoresis on an agarose gel, electroeluted (20), and labeled by nick translation (37).

Two additional groups of mutants were isolated during the Tn*5* mutagenesis experiments. The first group did not metabolize isopropylbenzene, and all its members have an apparently identical deletion of about 20 kb which extends to either side of the isopropylbenzene metabolic region in pRE4 (22); characterization of these mutants is described elsewhere (23). The second group consisted of isolates RE226, RE227, RE228, and RE229 which were identified by their inability to grow on isobutyrate (compound IX, Fig. 1), one of the products of HOMODA hydrolysis. Although all these derivatives were initially thought to exhibit wild-type growth with isopropylbenzene, they were later shown to grow only very slowly on that substrate. The isobutyrate-negative mutants were subsequently found to grow normally on succinate-R medium but poorly on this medium supplemented with 0.1% isobutyrate; it is thus likely that isobutyrate or one of its metabolites inhibits growth of the isobutyrate-negative mutants on isopropylbenzene. The genes encoding isobutyrate metabolism are apparently not located on pRE4 since plasmid DNA isolated from these Tn*5* mutants did not contain the Tn*5* element.

Expression of plasmid-encoded proteins in minicells. A fluorogram of proteins synthesized in minicells containing pRE47, pRE48, pRE49, and pRE50 is shown in Fig. 4. At least seven major proteins of apparent molecular weights of 51,000, 45,000, 39,000, 36,000, 33,000, 25,000, and 13,500 were seen in minicells carrying pRE49 and pRE50, both of which contain the 13.2- and 2.7-kb *Eco*RI fragments of pRE4 (coordinates 6.3 to 22.2, Fig. 3). Of these seven, only the 51,000- and 25,000-molecular-weight species were seen in minicells carrying pRE47 or pRE48 which contain the 3.3-kb *Cla*I fragment (coordinates 13.0 to 16.3, Fig. 3). These latter proteins have sizes similar to those of the terminal components of the toluene (52,500 and 20,800 molecular weight [31]), benzoate (50,000 and 20,000 molecular weight [55]), naphthalene (55,000 and 20,000 molecular weight [24]), and toluate (57,000 and 20,000 molecular weight [34a]) dioxygenases and are likely to be the two terminal components of the isopropylbenzene dioxygenase. This conclusion is also supported by mutagenesis data and the fact that *E. coli* bacteria carrying pRE47 or pRE48 produce indigo from

indole and the dihydrodiol from isopropylbenzene. These results suggest that the components of the isopropylbenzene dioxygenase which are required for the transfer of electrons from NADH to the terminal protein, and which are equivalent to ferredoxin$_{TOL}$ and ferredoxin$_{TOL}$ reductase in the analogous toluene dioxygenase system (31), may be provided by the *E. coli* host.

DISCUSSION

In this study, Tn*5* mutagenesis and gene cloning were used to characterize a pathway for the oxidative catabolism of isopropylbenzene by *P. putida* RE204 (Fig. 1). When grown in the presence of isopropylbenzene, Tn*5* mutant bacteria accumulated various metabolic intermediates which were subsequently identified. That these compounds were metabolic intermediates was confirmed by enzyme assays with cell extracts.

Degradation of isopropylbenzene is initiated by a dioxygenase which yields 2,3-dihydro-2,3-dihydroxyisopropylbenzene (dihydrodiol; compound II of Fig. 1). The dihydrodiol is metabolized by a dehydrogenase to give 3-isopropylcatechol (compound III, Fig. 1), which is a substrate for a dioxygenase-catalyzed ring cleavage between carbons 2 and 3 to give HOMODA (IV, Fig. 1). HOMODA is hydrolyzed to isobutyrate (IX) and 2-oxopent-4-enoate (V). 2-Oxopent-4-enoate is presumed to be further metabolized by hydration to give 4-hydroxy-2-oxopentanoate (VI), which is cleaved to give pyruvate (VII) and acetaldehyde (VIII [2, 13]). Isobutyrate metabolism has not been studied in this organism but is expected to follow a pathway similar to that described earlier for isobutyryl coenzyme A metabolism (43). The isopropylbenzene pathway defined here is analogous to pathways previously proposed for the degradation of toluene (29), 2-phenylbutane (1), *t*-butylbenzene (8), and biphenyl (7, 30), some steps of which had previously been suggested to be implicated in isopropylbenzene catabolism (16, 29). The study described here, however, represents the first systematic analysis of an isopropylbenzene pathway in a single organism.

From the enzyme assay data presented here, it can be seen that in *P. putida*, Tn*5* does not always completely inactivate downstream genes and appears to carry a constitutive promoter. Such a promoter has also been shown to be active in *Alcaligenes eutrophus* (17) and sometimes, at low levels, in *E. coli* (3). This promoter is useful because it allows us to identify the direction of transcription as going from right to left, at least for the genes encoding the three enzymes assayed, since genes located downstream from the transposon are constitutively expressed. For example, the activities of the dihydrodiol dehydrogenase and the 3-isopropylcatechol dioxygenase are 8.5- and 24-fold greater in extracts of uninduced cells of mutant 9c, whose mutation is located to the right of the genes of these enzymes (Fig. 3), than those found in extracts of uninduced wild-type cells. Similarly, insertions of the Tn*5* element to the right of the HOMODA hydrolase gene resulted, in almost all of the mutants studied, in an increase in uninduced levels of HOMODA hydrolase. Whereas in wild-type cells the 2,3-dihydro-2,3-dihydroxyisopropylbenzene dehydrogenase and 3-isopropylcatechol dioxygenase are induced to similar levels during growth with isopropylbenzene and may therefore be coordinately regulated, the increase in HOMODA hydrolase activity in such cells is about 10-fold less; this enzyme therefore appears to be regulated separately. However, definitive conclusions must await the isolation and characterization of mutants defective in the regulation of isopropylbenzene metabolism.

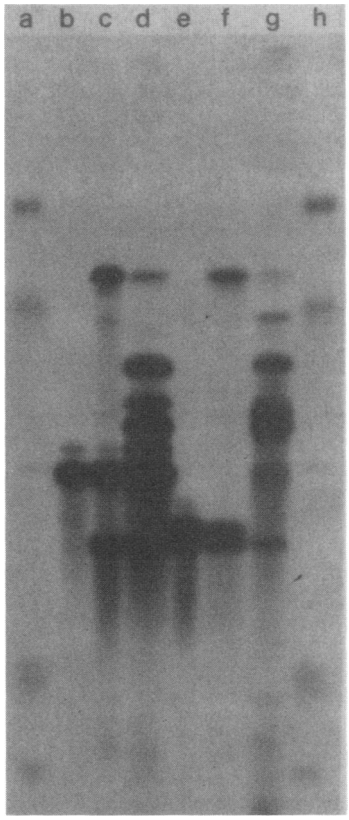

FIG. 4. Expression of cloned isopropylbenzene metabolic genes in *E. coli* minicells. The fluorogram of a sodium dodecyl sulfate-polyacrylamide gel of ^{35}S-labeled plasmid-encoded proteins (see Materials and Methods) is shown. Lanes a and h contained ^{14}C-labeled protein standards: bovine serum albumin (molecular weight, 69,000), ovalbumin (46,000), carbonic anhydrase (30,000), lactoglobulin A (18,367), and cytochrome *c* (12,300). Lanes b to g contained proteins from *E. coli* DS410 carrying the following plasmids: b, pBR322 (molecular weight of TEM β-lactamase, 28,000, and its precursor, 30,000 [19]); c, pRE47; d, pRE49; e, pACYC184 (molecular weight of chloramphenicol acetyl transferase, 25,668 [49]); f, pRE48; and g, pRE50.

One apparent paradox of our results is that the 3.3-kb *Cla*I fragment (coordinates 13.0 to 16.3, Fig. 3) alone is sufficient to permit host bacteria to form indigo from indole and must therefore encode the isopropylbenzene dioxygenase (or the essential components thereof); Tn*5* insertions in strains RE206 and RE207 are within this fragment. On the other hand, Tn*5* insertions in strains RE208, RE209, RE210, RE211, and RE212 which prevent dioxygenase expression in *P. putida* RE204 are located to the left of this *Cla*I fragment (between coordinates 12.3 and 12.7, Fig. 3), and *Eco*RI fragments which have isopropylbenzene dioxygenase activity in *E. coli* were cloned from these mutants. One explanation for the lack of dioxygenase activity in these mutants is that the constitutive Tn*5* promoter in these mutants is initiating transcription events from left to right which diminish expression of the dioxygenase which is transcribed from right to left. In *E. coli* this Tn*5*-encoded promoter is not active, so that transcription of the dioxygenase gene is not inhibited in the same way.

The 2.7-kb *Eco*RI fragment required in addition to the

13.2-kb *Eco*RI fragment for indigo production in *E. coli* clones presumably does not carry isopropylbenzene dioxygenase genes as these are present on the *Cla*I fragment and hence also on the 13.2-kb *Eco*RI fragment; it may, however, carry a regulatory gene required for expression of the oxygenase genes on the 13.2-kb *Eco*RI fragment. Consistent with this possibility is the fact that it is not necessary for the two *Eco*RI fragments to be contiguous or even on the same plasmid for indigo production in *E. coli* (R. Eaton, unpublished data); the 2.7-kb *Eco*RI fragment seems not therefore to carry a *cis*-acting element such as a promoter which is required for oxygenase gene expression but more probably encodes a *trans*-acting positive regulatory protein. In those bacterial pathways for the catabolism of aromatics that have so far been analyzed in detail, positive regulation of the expression of structural genes is the rule (36, 44, 48, 56 [but see also reference 52]). That expression of the dioxygenase gene in hybrid plasmids carrying the 3.3-kb *Cla*I fragment does not require the presence of sequences located on the 2.7-kb *Eco*RI fragment almost certainly reflects the fact that its orientation in the pBR322 and pACYC184 vectors is such that its transcription could be initiated at the antitetracycline (P1) promoter (50). In these hybrids, therefore, the dioxygenase gene may not require its own promoter for expression, even though it may in *Eco*RI clones.

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