JOURNAL OF BACTERIOLOGY, Dec. 1995, p. 6983–6988 Vol. 177, No. 23
0021-9193/95/$04.00+0

Formation of Indigo and Related Compounds from Indolecarboxylic Acids by Aromatic Acid-Degrading Bacteria: Chromogenic Reactions for Cloning Genes Encoding Dioxygenases That Act on Aromatic Acids†

RICHARD W. EATON* AND PETER J. CHAPMAN

Gulf Ecology Division, National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, Gulf Breeze, Florida 32561

Received 12 June 1995/Accepted 15 September 1995

The *p*-cumate-degrading strain *Pseudomonas putida* F1 and the *m*- and *p*-toluate-degrading strain *P. putida* mt-2 transform indole-2-carboxylate and indole-3-carboxylate to colored products identified here as indigo, indirubin, and isatin. A mechanism by which these products could be formed spontaneously following dioxygenase-catalyzed dihydroxylation of the indolecarboxylates is proposed. Indolecarboxylates were employed as chromogenic substrates for identifying recombinant bacteria carrying genes encoding *p*-cumate dioxygenase and toluate dioxygenase. Dioxygenase gene-carrying bacteria could be readily distinguished as dark green-blue colonies among other colorless recombinant *Escherichia coli* colonies on selective agar plates containing either indole-2-carboxylate or indole-3-carboxylate.

Dioxygenases are well recognized as important bacterial enzymes initiating aerobic catabolism of aromatic hydrocarbons (20, 37). The actions of these enzymes furnish vicinal *cis*-dihydrodiols, precursors of *o*-diphenols which serve as substrates for ring cleavage enzymes. Of the many aromatic hydrocarbons shown to undergo reactions of this type, examples of a range of mono- and multinuclear substrates can be cited. Thus, benzene, toluene, ethylbenzene, cumene (isopropylbenzene), naphthalene, biphenyl, and phenanthrene have all been shown to yield *cis*-dihydrodiols, in many instances with isotopic evidence of incorporation of both atoms of molecular oxygen. A useful chromogenic property of several of these enzyme systems is the ability to oxygenate the heterocyclic ring of indole, facilitating indigo formation (17). This property has been valuable in the cloning of aromatic hydrocarbon dioxygenase genes (10, 11, 16, 29, 30, 45) and in the development of processes for the industrial production of indigo (32).

Dioxygenase reactions leading to 1,2-diphenol formation and ring cleavage are also employed by bacteria in the catabolism of a number of aromatic carboxylic acids. This is the case for compounds such as benzoic acid (35, 36), phthalic acid (2, 15, 38), phenylpropionic (hydrocinnamic) acid (4, 42), *m*- and *p*-toluic acids (1, 43), and *p*-cumic acid (8). These are utilizable carbon sources in their own right but are also established intermediates in the degradation of, respectively, mandelic acid (18), phenanthrene (27, 28), 1-phenylalkanes with odd-carbon-number side chains (39), *m*- and *p*-xylene (1, 44), and *p*-cymene (8).

To learn more about *p*-cumic acid, 2,3-dioxygenase, a study of the range of carboxylic acid substrates acted on by cells of the *p*-cymene-utilizing strain *Pseudomonas putida* F1 was undertaken. In the course of this work, it was noted that blue reaction products, reminiscent of indigo, were formed from certain indolecarboxylate isomers. Similar reactions were subsequently found with cells of *P. putida* mt-2 induced for *m*- and *p*-toluate 1,2-dioxygenase activity. The results reported here show that indigo and related products are, in fact, formed from indole-2-carboxylic acid and indole-3-carboxylic acid, and a reaction scheme is proposed to account for these observations. These chromogenic reactions have been used to identify cloned genes encoding the respective *p*-cumate and toluate dioxygenases.

(A preliminary account of this work has appeared elsewhere [12].)

Materials. The bacterial strains and plasmids used in this study are listed in Table 1. *P. putida* F1, which degrades *p*-cymene and *p*-cumate, and the *m*- and *p*-toluate-degrading strain *P. putida* mt-2 and its pWW0-cured derivative strain PPO200 were examined for their ability to transform indolecarboxylates. Strain F1-BPC$^+$ was isolated as a spontaneous constitutive mutant derivative of wild-type strain F1 (F1-WT) by selection for growth with biphenyl-4-carboxylate, a metabolizable substrate that does not induce the cumate catabolic pathway operon, as previously described by DeFrank and Ribbons (8). Indole-2-carboxylic acid and indole-3-carboxylic acid were obtained from Sigma Chemical Co., St. Louis, Mo., while indigo, isatin, *p*-cumic acid, and *m*-toluic acid were from Aldrich Chemical Co., Milwaukee, Wis. Other chemicals were of the highest purity commercially available. Restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs, Beverly, Mass., and used according to the instructions of the manufacturer.

Testing strains for ability to form colored products from indolecarboxylates. Bacterial strains *P. putida* F1 and *P. putida* mt-2 and, for comparison, *P. putida* PPO200 were streaked onto minimal agar plates containing 1 mM indolecarboxylate and 0.1% *p*-cumate, *m*-toluate, or benzoate. Plates were incubated for several days at 30°C and checked daily for color formation. Strains F1 and mt-2 but not strain PPO200 produced colored colonies on both indole-2-carboxylate- and indole-3-carboxylate-containing media. The colors of these colonies ranged from blue through olive green to red. Strain PPO200 failed to produce color, although it grows with ben-

* Corresponding author. Phone: (904) 934-9345. Fax: (904) 934-9201. Electronic mail address: reaton@gulfbr.gbr.epa.gov.

† Contribution 920 from the Gulf Ecology Division, NHEERL, U.S. Environmental Protection Agency, Gulf Breeze, Fla.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference(s)
Pseudomonas putida		
F1 (F1-WT)	Grows with *p*-cumate and *p*-cymene	5, 19
F1-BPC$^+$	Derivative of strain F1 constitutive for metabolism of *p*-cumate and biphenyl-4-carboxylate	5
mt-2	Grows with *m*-toluate and *p*-toluate; carries plasmid pWW0 (TOL)	1
PPO200	*P. putida* mt-2 cured of pWW0; *m*- and *p*-toluate$^-$, benzoate$^+$	25
Escherichia coli LE392	F$^-$ *hsdR514 metB1 trpR55*	16
Plasmids		
pLV59	Encodes *Eco*RI restriction endonuclease and temperature-sensitive *Eco*RI methylase; Cm^r; positive-selection cloning vector	33
pRE611	7.45-kb *Hin*dIII fragment from the chromosome of strain F1 inserted into pLV59; indolecarboxylate$^+$	This study
pRE619	25-kb *Hin*dIII fragment from pWW0 (TOL) inserted into pLV59; indolecarboxylate$^+$	This study
pRE792	3.6-kb *Xba*I-*Bam*HI fragment from pRE619 inserted into pUCBM21 (Boehringer Mannheim) (11); indolecarboxylate$^+$	This study

zoate by using a chromosome-encoded pathway; apparently, the benzoate-1,2-dioxygenase does not act on indolecarboxylates.

Identification of colored products. To identify the products of the transformation of indole-2-carboxylate and indole-3-carboxylate by strains F1 and mt-2, those strains were grown in 1 liter of minimal medium (R medium [14]) containing 0.02% yeast extract, 0.1% lactate, and 0.1% *p*-cumate for strain F1 or 0.1% *m*-toluate for strain mt-2. Cells were harvested, washed, and then resuspended in 250 ml of minimal medium supplemented with 0.2% lactate and 2 mM indole-2-carboxylate or indole-3-carboxylate in a 1-liter flask. After overnight incubation at 30°C, entire cultures were extracted three times with equal volumes of chloroform over a steam bath. Extraction of the entire culture was necessary because of the association of colored products with cell material. Aqueous and chloroform layers were separated by centrifugation. The chloroform layer was dried with anhydrous sodium sulfate and then removed in the rotary evaporator under reduced pressure. Chloroform-extracted products were analyzed by thin-layer chromatography on activated silica gel plates (EM Science, Gibbstown, N.J.), which were developed with either toluene-acetone (4:1) (solvent A) or chloroform-acetone (97:3) (solvent B) (24). Incubations of strains mt-2 and F1 with indole-2-carboxylate and indole-3-carboxylate gave similar mixtures of three reaction products. With solvent A, the mobilities of these compounds were: blue, R_f 0.58; red, R_f 0.42; and yellow, R_f 0.31. With solvent B, the mobilities were: blue, R_f 0.33; red, R_f 0.15; and yellow, R_f 0.06.

A preparative-scale separation of these products formed from indole-2-carboxylate and indole-3-carboxylate by strain F1 was achieved by column chromatography on silica gel (60-200 mesh; J. T. Baker, Inc., Phillipsburg, N.J.) (23 by 1.5 cm) with chloroform-acetone (97:3) as the solvent. Separations were evaluated visually, and fractions were collected as different-colored products emerged from the column; the solvent was removed in the rotary evaporator under reduced pressure. Purified products were then characterized by mass spectrometry with a Hewlett-Packard model 5988A spectrometer operating in the electron impact mode. Samples were introduced either with the direct probe, heating from 60 to 210°C (for indigo and indirubin), or with a coupled Hewlett-Packard 5890 gas chromatograph (for isatin). Samples were injected at 300°C onto a type HP-5 capillary column (cross-linked 5% phenylmethylsilicone; 25 m by 0.2 mm, with a film thickness of 0.11 μm) with helium as a carrier gas at an average linear velocity of 37 cm s^{-1} and a temperature program which retained the oven temperature at 55°C for 0.5 min and then increased the temperature to 105°C over 3 min and to 250°C over the next 14.5 min.

The blue products (λ_{max}, 597 nm in CH_2Cl_2) of the transformations of indole-2-carboxylate and indole-3-carboxylate by strain F1 had identical mass spectra with major ions of *m*/*z* (intensity for the indole-2-carboxylate product, proposed composition of ions) 263 (19%), 262 (100%, $[M]^+$), 234 (16%, $[M - CO]^+$), 205 (19%, $[M - CO - CHO]^+$), 179 (3%, $[M - CO - CO - C_2H_2]^+$), 157 (7%), 131 (14%, $[M/2]^+$), 104 (24%, $[M/2 - HCN]^+$), 103 (15%, $[M/2 - CO]^+$), and 76 (10%, $[M/2 - CO - HCN]^+$). These mass spectra are identical to that of authentic indigo and are similar to a mass spectrum published for indigo (24).

The red products (λ_{max}, 548 nm in CH_2Cl_2) of the transformations of indole-2-carboxylate and indole-3-carboxylate by strain F1 had identical mass spectra, with major ions of *m*/*z* (intensity for the indole-2-carboxylate product, proposed composition of ions): 263 (18%), 262 (100%, $[M]^+$), 234 (56%, $[M - CO]^+$), 205 (31%, $[M - CO - CHO]^+$), 179 (4%, $[M - CO - CO - C_2H_2]^+$), 151 (4%), 131 (13%, $[M/2]^+$), 103 (14%, $[M/2 - CO]^+$), and 76 (10%, $[M/2 - CO - HCN]^+$). These mass spectra are very similar to a mass spectrum published for indirubin (24).

The yellow products of the transformation of indole-2-carboxylate and indole-3-carboxylate by strain F1 both emerged from the gas chromatograph at 12.69 min and had mass spectra with major ions of *m*/*z* (intensity for the indole-2-carboxylate product, proposed composition of ions): 147 (64%, $[M]^+$), 119 (100%, $[M - CO]^+$), 92 (73%, $[M - CO - HCN]^+$), 91 (14%, $[M - CO - CO]^+$), 76 (7%, $[M - CO - CONH]^+$), 64 (23%, $[M - CO - HCN - CO]^+$), and 63 (16%). The gas chromatography retention times and mass spectra are identical to those obtained with authentic isatin.

Assay for the production of indigo. The availability of a constitutive strain provided the opportunity to confirm the relationship of indigo production to the expression of enzymes involved in *p*-cumate metabolism in *P. putida* F1. Assays were carried out in which washed cells of the constitutive strain

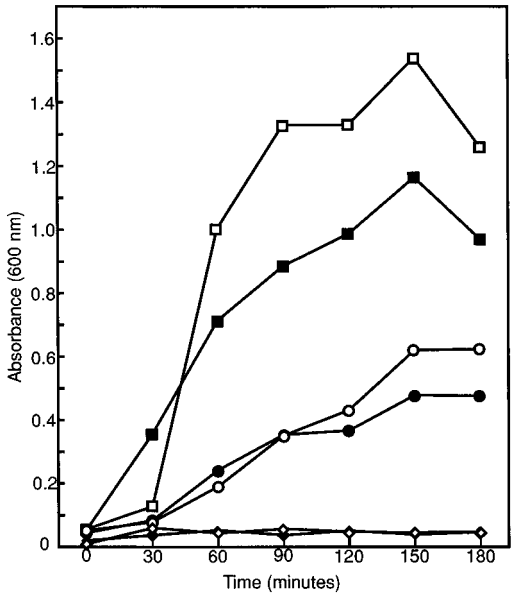

FIG. 1. Biotransformation of indole-2-carboxylate and indole-3-carboxylate to indigo by *P. putida* F1 (F1-WT) and its constitutive derivative, F1-BPC$^+$. Symbols: open, indole-2-carboxylate; solid, indole-3-carboxylate; squares, cumate-induced strain F1-WT; circles, lactate-grown strain F1-BPC$^+$; diamonds, lactate-grown strain F1-WT.

F1-BPC$^+$, grown without exposure to *p*-cumate, and wild-type strain F1-WT, grown with or without *p*-cumate, were incubated with indole-2-carboxylate or indole-3-carboxylate (Fig. 1). Overnight cultures of strains F1-WT and F1-BPC$^+$ in 50 ml of minimal medium containing 0.02% yeast extract and 0.2% lactate and of strain F1-WT in 100 ml of the same medium but with 0.1% *p*-cumate and only 0.1% lactate were used to inoculate 1 liter of the same media. Cultures were incubated with shaking for 4 h at 30°C, at which time they were harvested, washed with 30 ml of carbon-free minimal medium, and resuspended in the same medium with chloramphenicol at 200 μg ml^{-1} to give a cell density corresponding to an A_{600} of 100 (by measuring cell density after dilution). The cell suspensions were added to an equal volume of minimal medium containing 0.2% lactate and 4 mM indole-2-carboxylate or indole-3-carboxylate. These were mixed and immediately dispensed (1 ml) in 12-ml screw-cap, round-bottom polypropylene tubes (Sarstedt, Inc., Newton, N.C.). The tubes were then shaken at a 45° angle at 300 rpm and 30°C. Tubes were removed from the shaker at time zero, and at half-hour intervals thereafter, 4 ml of chloroform was added to each tube, and the tubes were vortexed before they were placed in a boiling water bath. Over the next 2 min, tubes were removed twice for additional vortexing and then centrifuged at 4°C and 4,300 × *g* (6,000 rpm in the Sorvall SS-34 rotor) for 15 min. Afterwards, 1.5 ml of the lower, chloroform layer was carefully removed to a 1.5-ml microcentrifuge tube. Before the A_{600} of the contents of the tubes was measured, it was often necessary to centrifuge for 5 min to remove small particulate material. The A_{600} was measured with a Hewlett-Packard 8452A diode array spectrophotometer.

Production of indigo from both indolecarboxylates began immediately in incubations with F1-BPC$^+$ and with F1-WT induced with *p*-cumate, while no indigo production was detected in incubations with uninduced F1-WT (Fig. 1).

Cloning catabolic genes. The attribution of color production from indolecarboxylates to enzymes that catalyze the metabolism of *p*-cumate in *P. putida* F1 and *m*-toluate in *P. putida* mt-2 suggested that it should be possible to use indolecarboxylates as chromogenic substrates to identify recombinant bacteria carrying cloned genes encoding these enzymes. This possibility was examined. Chromosomal DNA was purified from strain F1 as previously described (13), and the plasmid pWW0 (TOL) was isolated from strain mt-2 by the method of Hansen and Olsen (21), followed by centrifugation to equilibrium in a cesium chloride-ethidium bromide gradient. DNA was digested with *Hin*dIII, *Bgl*II, or *Pst*I, and after the enzyme was inactivated by phenol and ether extractions followed by an ethanol precipitation, the DNA was ligated to the similarly treated plasmid cloning vector pLV59 (33). Ligated DNA was used to transform *E. coli* LE392 (16), which was subsequently spread on LB (6) agar plates containing 30 μg of chloramphenicol per ml, 1 mM indolecarboxylate, and 0.5 mM *p*-cumate (F1) or 0.5 mM *m*-toluate (pWW0). Plates were incubated at 37°C and then checked for several days for the appearance of colored colonies.

The positive-selection cloning vector pLV59 allows the cloning of DNA fragments into one of three restriction sites. In cloning experiments in which DNA from strain F1 was ligated to pLV59, 400 to 500 colonies carrying recombinant plasmids generated with *Hin*dIII, *Bgl*II, or *Pst*I were obtained on selective medium containing chloramphenicol, indole-2-carboxylate, and *p*-cumate. Of these, two *Hin*dIII-generated recombinant colonies were blue. Both of these carried pLV59 into which a 7.4-kb *Hin*dIII fragment had been inserted (pRE611). No blue colonies were obtained with *Bgl*II or *Pst*I; it might therefore be concluded that insufficient recombinants generated with these enzymes had been screened. However, restriction and functional mapping of the 7.4-kb *Hin*dIII fragment and subcloned fragments demonstrated that both of these enzymes cut within the genes encoding the *p*-cumate dioxygenase (data not shown). Thus, the probability that these enzymes can be used to obtain clones having an intact set of genes encoding the *p*-cumate dioxygenase is not high. Bacteria carrying pRE611 transform *p*-cumate to a compound having a UV-visible spectrum identical to that of 2-hydroxy-6-oxo-7-methylocta-2,4-dienoate (λ_{max}, 323 and 393 nm at pH 7). This transformation requires four enzyme-catalyzed steps in the organism in which it was initially studied, *P. putida* PL (8, 9). Bacteria carrying plasmid derivatives of pRE611, which carry *p*-cumate 2,3-dioxygenase genes but lack the dihydrodiol dehydrogenase gene, transform indolecarboxylates to colored products and *p*-cumate to *cis*-2,3-dihydroxy-2,3-dihydro-*p*-cumate, while bacteria carrying plasmids that lack genes encoding any of the four components of *p*-cumate 2,3-dioxygenase fail to catalyze either conversion (10).

In cloning experiments in which *Hin*dIII-digested pWW0 DNA from *P. putida* mt-2 was ligated to pLV59, 15,000 transformant colonies were scored on medium containing chloramphenicol, indole-2-carboxylate, indole-3-carboxylate, and *m*-toluate. Of these colonies, only five were blue. All carried the same approximately 25-kb *Hin*dIII fragment inserted in pLV59; one was selected for further study (pRE619). Bacteria carrying this plasmid or a derivative carrying the 25-kb *Hin*dIII fragment are capable of degrading *m*-toluate with the transient appearance of 2-hydroxy-6-oxohepta-2,4-dienoate (λ_{max}, 388 nm), the product of *meta*-cleavage of 3-methylcatechol (3). These bacteria also degrade *p*-toluate to non-UV-absorbing products, with the transient appearance of an intermediate having a spectrum with an absorbance maximum at 382 nm; this is the spectrum of the product of *meta*-cleavage of 4-methylcatechol, 2-hydroxy-5-methyl-6-oxohexa-2,4-dienoate (data

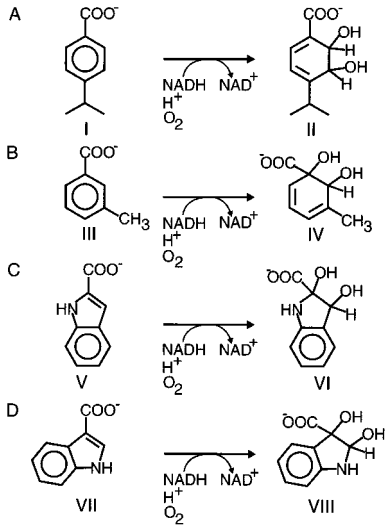

FIG. 2. Reactions catalyzed by *p*-cumate dioxygenase and *m*-toluate dioxygenase with their respective substrates, *p*-cumate (A) and *m*-toluate (B), and with indole-2-carboxylate (C) and indole-3-carboxylate (D).

not shown) (3). This DNA fragment was previously shown to carry genes encoding all of the proteins required for the degradation of *m*- and *p*-toluates (22). The toluate 1,2-dioxygenase genes have been subcloned on a 3.6-kb *Xba*I-*Bam*HI fragment (pRE792) (see restriction map in reference 1) by using indolecarboxylates to screen for recombinants. Bacteria carrying pRE792 catalyze the conversion of *m*- and *p*-toluates to *cis*-carboxyhydrodiols (10).

Two bacterial strains that grow with aromatic carboxylic acids have been shown to convert indole-2-carboxylate and indole-3-carboxylate to colored products. These products are identified here as indigo, indirubin, and isatin. Although it is possible that initial oxidation may have occurred at the benzene ring, no attempt was made to isolate and identify products other than those responsible for color formation. Indigo is usually the major product; however, the relative amounts of these products can vary, with the result that indolecarboxylate-transforming bacterial colonies range from blue through olive green to red. A similar range of colors has been observed for the conversion of indole to indigo and may be due to a similar mixture of products (10, 23, 40). Hart et al. (23) have identified both indigo and indirubin as products of the transformation of indole by *E. coli* carrying a DNA fragment cloned from a *Rhodococcus* sp.

A mechanism by which indole-2-carboxylate may be converted to these products is proposed (Fig. 2 and 3). This conversion is initiated by a reductive dioxygenase (Fig. 2), an enzyme that catalyzes the incorporation of both atoms of molecular oxygen together with a pair of hydrogens, leading to the formation of a *cis*-dihydrodiol (II) (7, 8), in the case of *p*-cumate-2,3-dioxygenase, or a *cis*-carboxyhydrodiol (IV) (1, 43), in the case of *m*- and *p*-toluate-1,2-dioxygenases. Both of these reductive dioxygenases are shown as catalyzing the formation of 2,3-dihydroxy-3-hydroindole-2-carboxylate (VI) from indole-2-carboxylate (V) (Fig. 2 and 3). This hydrated cyclic ketimine opens spontaneously (Fig. 3), and the product (IX) undergoes successive keto-enol tautomerizations to produce the β-keto acid XI, which readily undergoes decarboxylation. Subsequent tautomerization and ring closure yield 2,3-dihydroxy-2,3-dihydroindole (XIV), which is spontaneously dehydrated to give 2-hydroxyindole (XVI, oxindole) and 3-hydroxyindole (XV, indoxyl). Condensation of two molecules of indoxyl followed by air oxidation leads to the production of indigo, while the condensation of indoxyl and oxindole yields indirubin. Isatin may be formed by the decomposition of either indirubin or indigo or by the oxidation of XIV. Another mechanism which has been considered for indigo formation is the direct elimination of the tertiary hydroxyl of VI as water, yielding a β-keto acid which would spontaneously decarboxylate to form indoxyl (XV). However, the formation of products other than indigo indicates that this is not the sole mechanism, although it may make a contribution. A similar series of enzyme-catalyzed and spontaneous reactions can be proposed for the conversion of indole-3-carboxylate to these colored products.

Alternatively, enzyme-catalyzed reactions beyond dioxygenation may have a role in the conversion of indolecarboxylate diols (e.g., VI) to oxindole (XVI) and indoxyl (XV). However, recombinant bacteria carrying *m*-toluate dioxygenase genes or *p*-cumate dioxygenase genes but lacking the diol dehydrogenase gene still make colored products from indolecarboxylates (10).

The construction of recombinant gene libraries from bacteria has become a relatively simple laboratory procedure; the

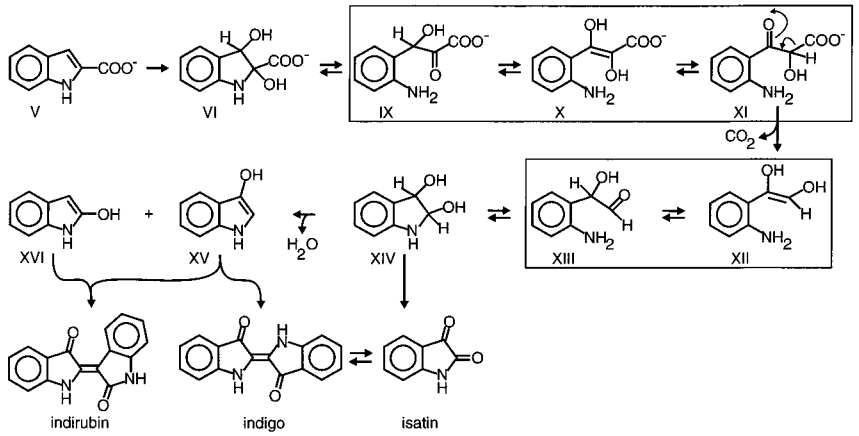

FIG. 3. Proposed pathway for the formation of the identified products from indole-2-carboxylate. The initial reaction is catalyzed by *p*-cumate-2,3-dioxygenase or *m*- or *p*-toluate-1,2-dioxygenase; subsequent reactions are spontaneous. Similar spontaneous reactions can be proposed to occur with indole-3-carboxylate. Individual products are discussed in the text.

most difficult part of a cloning experiment is often the identification of recombinant bacteria that carry the genes of interest. Since the discovery that *E. coli* carrying cloned naphthalene dioxygenase genes produces the water-insoluble blue dye indigo from indole (17), that transformation has been the basis for the identification of recombinant bacteria carrying not only naphthalene dioxygenase genes (11, 29, 30, 45) but also genes encoding other aromatic hydrocarbon dioxygenases, including toluene dioxygenase (40) and isopropylbenzene dioxygenase (10, 16), as well as 2,4-dinitrotoluene dioxygenase (41). The transformation of indole to indigo also has potential for use in the identification of recombinant bacteria carrying xylene monooxygenase genes (26, 31).

A chromogenic substrate like indole has not existed previously for cloning genes encoding dioxygenases that act on aromatic acids. Parke (34) has described the use of *p*-toluidine with ferric chloride in media designed to detect the formation of catechol and protocatechuate from benzoate and *p*-hydroxybenzoate, respectively; this method has the potential to detect a variety of diphenolics formed by dihydroxylation of other aromatics. However, color formation requires that the diphenol accumulate and the color can diffuse into the medium. The bacterial conversion of the novel chromogenic substrates indole-2-carboxylate and indole-3-carboxylate to the water-insoluble blue dye indigo has been used here to identify recombinant bacteria carrying *p*-cumate dioxygenase genes from *P. putida* F1 and toluate dioxygenase genes from *P. putida* mt-2. The DNA fragments cloned in these experiments may contain not only dioxygenase genes but also neighboring genes. Thus, the cloned 25-kb toluate dioxygenase gene-carrying *Hin*dIII fragment from pWW0 (TOL) also carries the complete operon that encodes the degradation of *m*- and *p*-toluate (22), while the 7.4-kb *Hin*dIII fragment cloned from the chromosome of strain F1 encodes the metabolism of *p*-cumate through four steps to 2-hydroxy-6-oxo-7-methylocta-2,4-dienoate.

It is possible that indolecarboxylates can be used in cloning genes encoding other metabolic pathways; other strains that form colored products from indolecarboxylates include an *o*-xylene-degrading strain, TRP2, and its *o*-toluate-degrading derivative, TRP2-OTA$^+$ (5); a 2-naphthoate-degrading strain (38); and the *p*-cymene/*p*-cumate-degrading strains *Pseudomonas aureofaciens* cym4 (5) and *P. putida* PL-pT11 (8, 9).

We thank W. Gilliam of this laboratory for mass spectrometry analyses, P. Webster and S. Resnick of Technical Resources, Inc., Gulf Breeze, Fla., for technical assistance in the early stages of this work, and S. Selifonov of the University of Minnesota, St. Paul, for valuable discussions.

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