APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1996, p. 3967–3977 Vol. 62, No. 11
0099-2240/96/$04.00+0

Cloning, Sequencing, and Expression of Isopropylbenzene Degradation Genes from *Pseudomonas* sp. Strain JR1: Identification of Isopropylbenzene Dioxygenase That Mediates Trichloroethene Oxidation

ULRICH PFLUGMACHER, BEATE AVERHOFF,* AND GERHARD GOTTSCHALK

Institut für Mikrobiologie, Georg-August-Universität Göttingen, 37077 Göttingen, Germany

Received 20 May 1996/Accepted 26 August 1996

***Pseudomonas* sp. strain JR1, recently isolated with isopropylbenzene (IPB) as the inducer substrate for trichloroethene (TCE) oxidation (B. Dabrock, J. Riedel, J. Bertram, and G. Gottschalk, Arch. Microbiol. 158:9–13, 1992), is able to degrade IPB via the *meta*-cleavage pathway. The genes encoding the first three enzymes in the catabolism of isopropylbenzene were isolated from a genomic library with the broad-host-range cosmid vector pWE15. A 7.6-kb fragment from a 37.7-kb primary cosmid clone was subcloned and sequenced. It contained seven complete open reading frames, designated *ipbA1A2orf3A3A4BC*. *ipbA* codes for the three subunits of a multicomponent IPB dioxygenase, *ipbB* codes for 2,3-dihydro-2,3-dihydroxy-IPB dehydrogenase, and *ipbC* codes for 3-isopropylcatechol 2,3-dioxygenase. The deduced amino acid sequences of *ipbA1A2A3A4BC* exhibited the highest homologies with the corresponding proteins of biphenyl-degradative pathways in gram-negative and gram-positive bacteria. The gene products of the *ipb* genes were identified by an in vitro transcription-translation system on the basis of their expected molecular masses. IPB dioxygenase and 3-isopropylcatechol 2,3-dioxygenase expressed in *E. coli* oxidized a wide range of alkyl aromatic compounds. Incubation of *E. coli* cells carrying *ipbA1A2A3A4* with IPB and $^{18}O_2$ yielded reaction products containing both atoms of molecular oxygen, which is in accordance with a dioxygenation reaction. *E. coli* recombinants harboring and expressing the IPB dioxygenase exhibited the ability to degrade TCE. The *ipbA1A2A3A4*-carrying *E. coli* strain required neither IPB nor isopropyl-β-D-thiogalactopyranoside for induction; the rate of TCE degradation was comparable to that by fully induced *Pseudomonas* strain JR1.**

Trichloroethene (TCE), a widely used organic solvent and degreasing agent, is one of the most frequently detected groundwater contaminants and is a potential health hazard (40). Therefore, extensive efforts have been made to document the biodegradation of TCE by bacteria. Although no aerobic growth on TCE as the sole carbon source has been reported yet, TCE was found to be cooxidized by some ammonia-oxidizing bacterial species and by some bacterial species able to grow on hydrocarbons such as methane (32), propane (41), and isoprene (10). It was demonstrated that TCE oxygenation by monooxygenase reactions implicates the production of epoxide intermediates (11, 24, 29). In the case of aromatic inducer substrates (17, 37), initial mono- or dioxygenases of the degradative pathways may be responsible for the aerobic TCE degradation. Several aromatic monooxygenase systems, such as a toluene-4-monooxygenase in *Pseudomonas mendocina* KR-1 (44), a toluene-*ortho*-monooxygenase in *P. cepacia* G4 (37), and, recently, a phenol hydroxylase in *P. putida* (12), have been identified to cooxidize TCE. To our knowledge, only one aromatic dioxygenase system, the toluene dioxygenase in *P. putida* F1, has so far been shown to be involved in TCE degradation. Products generated by the toluene dioxygenase from TCE include formic and glyoxylic acids (30, 42). The toluene dioxygenase in *P. putida* F1 was identified as class IIB multicomponent dioxygenase comprising the large subunit of the terminal dioxygenase (TodC1), which is most important for TCE cooxidation (15), the small subunit of the terminal dioxygenase (TodC2), a Rieske-type ferredoxin (TodB), and a ferredoxin reductase (TodA) (45).

Recently, we reported the isolation and characterization of *Rhodococcus erythropolis* BD1 and *Pseudomonas* sp. strain JR1, both of which exhibit TCE oxidation activity with isopropylbenzene (IPB) as a novel aromatic inducer substrate (6). In accordance with the results of Eaton and Timmis on the IPB degradation pathway in *P. putida* RE204 (7), we found that IPB degradation in strain JR1 is initiated by a IPB 2,3-dioxygenase. The IPB 2,3-dioxygenase reaction product 2,3-dihydro-2,3-dihydroxy-IPB is subsequently dehydrogenated to give 3-isopropylcatechol (3-IPC).

In this study, we report on the cloning and sequencing of a 7.6-kb *Eco*RI-*Xba*I fragment of JR1 containing the genes for the conversion of isopropylbenzene to 3-IPC. TCE degradation experiments with *Escherichia coli* recombinant strains expressing IPB dioxygenase activity revealed that the IPB dioxygenase of JR1 is responsible for TCE oxidation.

MATERIALS AND METHODS

Strains. *Pseudomonas* strain JR1 was isolated in our laboratory (6). The *E. coli* strains used in this study were DH5α [F^- *lacZ*Δ*M15 recA1 hsdR17 supE44* Δ(*lacZYA*, *argF*)] as a host for recombinant cosmids and plasmids and XL1-Blue [*recA1 lac endA1 gyrA96 thi hsdR17 supE44 relA1* (F' *proA*$^+$*B*$^+$*lacI*^{q}*Z*Δ*M15* Tn*10*]) as a host for bacteriophage M13K07 and recombinant plasmids.

Growth of organisms. *Pseudomonas* strain JR1 was grown on minimal medium MC′, which consists of (per liter of distilled water) 100 ml of stock solution A ($Na_2HPO_4 \cdot 2H_2O$, 8.9 g; KH_2PO_4, 6.8 g; H_2O, to 1,000 ml [pH 7.0]), 100 ml of stock solution B ($MgSO_4 \cdot 7H_2O$, 1 g; KNO_3, 10 g; yeast extract, 500 mg; H_2O to 1000 ml), 1 ml of trace element solution SL9 (39) and 1 ml of a vitamin solution (4). The stock solutions were autoclaved separately. IPB as the source of carbon was supplied in the gas phase. *E. coli* strains were grown on standard Luria

* Corresponding author. Mailing address: Institut für Mikrobiologie, Georg-August-Universität, Grisebachstrasse 8, D-37077 Göttingen, Germany. Phone: 49-551-394041. Fax: 49-551-393793. Electronic mail address: BAVERHO@gwdg.de.

Bertani (LB) medium or mineral medium M9 or in Terrific Broth (TB) (33) as stated below. Incubation was carried out at 30°C.

Nucleic acid isolation and manipulation. Plasmids were isolated from *E. coli* with the Midi kit as specified by the manufacturer (Qiagen GmbH, Hilden, Germany). DNA manipulations were done by standard methods. Restriction enzymes and T4 DNA ligase were obtained from GIBCO/BRL GmbH (Eggenstein, Germany).

Cloning and screening strategies. A cosmid library was prepared by ligating partially *Sau*3A1-digested chromosomal DNA of strain JR1 into the single *Bam*HI site of the broad-host-range cosmid vector pWE15. After in vitro packaging into bacteriophage λ and infection, approximately 1,500 recombinant *E. coli* clones were tested for IPB dioxygenase-catalyzed formation of indigo on LB plates containing 100 μg of ampicillin per ml and 1 mM indole as well as for 3-IPC *meta*-cleavage activity by spraying plates with 10 mM catechol, yielding the yellow compound 2-hydroxy-6-oxo-7-methylocta-2,4-dienoate (HOMODA). Recombinant cosmids from positive clones were isolated, analyzed, and used for subcloning the *ipb* genes into the broad-host-range vector pBluescriptII SK+/−, KS +/− (Stratagene GmbH, Heidelberg, Germany).

Nucleotide sequencing and computer analysis. Nucleotide sequence determination was carried out by the chain termination method of Sanger et al. (34) with double- or single-stranded DNA templates. Double-stranded DNA was prepared with the Qiagen Midi kit as specified by the manufacturer and denatured with NaOH. For single-stranded DNA preparation, cultures of *E. coli* XL1-Blue harboring pBluescript phagemid subclones of interest were cultivated in Terrific Broth and infected with M13K07 at an optical density at 600 nm (OD_{600}) of 1 prior to an overnight incubation at 30°C. The recombinant phages were precipitated from the supernatant and extracted with phenol and phenol-chloroform until no protein debris was visible. DNA was precipitated with 2.5 volumes of ethanol and resuspended in water or Tris-EDTA (TE) buffer, respectively.

For radioactive sequencing, a Sequenase version 2.0 DNA sequencing kit (U.S. Biochemicals, Braunschweig, Germany) and [α-^{35}S]dATP (DuPont, NEN Research Products, Bad Homburg, Germany) were used as recommended by the manufacturers. The dideoxy-terminated fragments were separated on 6% polyacrylamide gradient gels with a Macrophor sequencing unit (Pharmacia LKB GmbH, Freiburg, Germany). The nonradioactive sequencing reactions were performed on an Automated Laser Fluorescent Sequencer (A.L.F.; Pharmacia LKB GmbH) after carrying out the sequencing reactions with an AutoRead sequencing kit (Pharmacia LKB GmbH) with fluorescein-labeled primers or by internal labeling with fluorescein-dATP (Boehringer, Mannheim, Germany) as specified by the manufacturer.

From the DNA fragments of interest, a series of small subclones were constructed in pBluescriptII SK+, SK−, KS+ and KS− (Stratagene GmbH) and self-designed oligonucleotide primers and standard primers were used in combination. Oligonucleotides were synthesized on a Gene Assembler Plus (Pharmacia LKB GmbH). Thus, both the forward and reverse strands were sequenced in their entirety to yield the nucleotide sequence of a 7,578-bp DNA fragment.

The DNA sequence data and deduced amino acid sequences were analyzed with the GCG sequence analysis software package, version 8.0 (Genetics Computer Group, Madison, Wis.) and the programs DNA-Strider (25) and DNAid for the Apple Macintosh.

Identification of *ipb* gene products. In vitro expression of the cloned *ipb* genes was carried out with the *E. coli* S30 extract system (Promega, Madison, Wis.) as specified by the manufacturer. The polypeptides were labeled with L-[^{35}S]methionine (Hartmann, Braunschweig, Germany). Samples were loaded onto a sodium dodecyl sulfate–14% polyacrylamide gel, electrophoresed, dried at 80°C, and exposed to X-ray film (X-OMAT AR5; Eastman Kodak, Rochester, N.Y.) for 18 h.

Determination of IPB dioxygenase and 3-IPC 2,3-dioxygenase activities in recombinant *E. coli* cells. *E. coli* clones expressing different fragments of cloned *ipb* genes were tested for their ability to oxidize different *n*-alkylbenzenes and *n*-alkylcatechols in a Clarke-type oxygen electrode (Rank Bros., Cambridge, England). For this purpose, resting-cell suspensions in 50 mM potassium phosphate buffer (pH 7.2) were stirred in the reaction chamber of the electrode at 30°C, 3-μl portions of stock solutions of the aromatic substrates in dimethyl formamide (200 mM) were added, and the substrate-dependent oxygen uptake of the cells was recorded. Protein was determined by the method of Schmidt et al. (35).

Incorporation of ^{18}O into IPB. Recombinant *E. coli* cells expressing the initial IPB dioxygenase were grown in 150 ml of TB (33) medium in the presence of ampillin (100 μg/ml) until the mid-log phase (OD_{600} = 7), harvested by centrifugation at 4°C, and washed twice with ice-cold potassium phosphate buffer (50 mM; pH 7.2). The cells were concentrated in 50 ml of the same buffer to an OD_{600} = 20 and incubated in a gastight 1-liter Müller-Krempel bottle under an O_2 atmosphere containing approximately 10% $^{18}O_2$ and traces of nitrogen. After the addition of 60 μl of IPB, the bottle was incubated overnight on a rotary shaker at 30°C. Products were extracted twice from the cell-free supernatant with equal volumes of ethyl acetate. The solvent was evaporated, and after an additional extraction with methanol and evaporation, the residual products were analyzed by mass spectrometry.

TCE degradation assays. Recombinant *E. coli* DH5α cells were grown in 80 ml of TB/Amp medium in 1-liter gastight Erlenmeyer flasks sealed with Mininert valves (Dynatech, Baton Rouge, Fla.) in the presence of 100 μM TCE. The cells were incubated at 30°C with reciprocal shaking at 150 rpm. Samples (150 μl) of the gas phase were removed after 24 h and injected into a CP 9000 gas chromatograph (Chrompack, Frankfurt, Germany) equipped with a flame ionization detector and a Carbopack B/1% SP-1000 column (Supelco, Bad Homburg, Germany). The operating conditions of the gas chromatograph were as follows: column temperature, 200°C; injector and detector temperature, 250°C; carrier gas, nitrogen (10 ml/min). The concentration of TCE was calculated as if all the remaining TCE were dissolved in the aqueous phase.

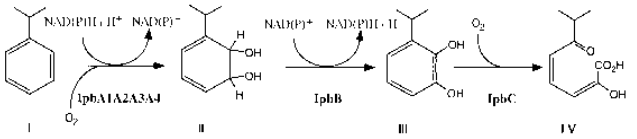

FIG. 1. Pathway for oxidation of IPB to HOMODA by *Pseudomonas* strain JR1. Compound I, IPB; compound II, 2,3-dihydro-2,3-dihydroxy-IPB; compound III, 3-IPC; compound IV, HOMODA; IpbA1A2A3A4, IPB dioxygenase; IpbB, 2,3-dihydro-2,3-dihydroxy-IPB dehydrogenase; IpbC, 3-IPC 2,3-dioxygenase.

Nucleotide sequence accession number. The nucleotide sequence of the 7,578-bp region of *Pseudomonas* strain JR1 containing the genes *ipbA1A2A3A4BC(E)* has been deposited in GenBank under accession number U53507.

RESULTS

Cloning *ipb* genes after screening a cosmid library. The pathway of IPB degradation was studied by monitoring the substrate-dependent oxygen uptake rates and by performing spectral analysis of IPB pathway intermediates. The results obtained plus those from additional inhibitor studies were consistent with the degradation of IPB in *P. putida* RE204 (7) and *R. erythropolis* BD2 (5) via 3-IPC and the *meta*-fission product HOMODA. The pathway is depicted in Fig. 1. Approximately 1,500 recombinant *E. coli* strains carrying the wide-host-range cosmid pWE15 were tested for IPB dioxygenase and 3-IPC 2,3-dioxygenase activity after growth in LB medium. The former activity was indicated by the accumulation of indigo in the presence of indole, and the latter was indicated by the formation of the yellow *meta*-cleavage product by a catechol spray assay. One strain with IPB and 3-IPC 2,3-dioxygenase activities was isolated. The recombinant cosmid conferring these activities and designated pUP1 was found to contain a 38-kb chromosomal fragment of strain JR1 (Fig. 2). Restriction mapping of pUP1, subcloning into the broad-host-range plasmid pBluescriptII (pBII) SK+, and subsequent activity testing of the generated clones yielded a 7.6-kb *Eco*RI-*Xba*I fragment (pUP3). *E. coli*(pUP3) turned blue in the presence of indole and yellow in the presence of IPB as well as IPC after growth in LB medium. This suggested the presence of IPB dioxygenase (*ipbA*), 2,3-dihydro-2,3-dihydroxy-IPB dehydrogenase (*ipbB*) and 3-IPC 2,3-dioxygenase (*ipbC*) activities encoded by the inserted JR1 DNA fragments.

Mapping of the IPB dioxygenase and 3-IPC 2,3-dioxygenase genes. To localize the structural genes of the IPB degradative pathway and to identify the enzyme responsible for TCE oxidation, seven deletion subclones of pUP3 were generated in pBIISK+: pUP4 (*Hin*dIII deletion), pUP5 (*Hin*dIII-*Nhe*I deletion) pUP6 (*Xba*I-*Bam*HI deletion), pUP7 (*Xba*I-*Cla*I deletion), pUP8 (*Xba*I-*Eco*RV deletion), pUP9 (*Bam*HI-*Eco*RI deletion), and pUP10 (second *Hin*dIII deletion). The recombinant *E. coli* clones were grown in succinate minimal medium (M9) and assayed for IPB- and 3-IPC-dependent oxygen consumption and additionally analyzed by indole and catechol spray assays. pUP4, pUP5, and pUP6 enabled *E. coli* DH5α to transform indole to indigo and to oxidize IPB (Fig. 2). None of these three deletion derivatives transformed catechol or 3-IPC to the yellow 2-hydroxymuconic acid semialdehydes. These results suggest that at least that part of the multicomponent

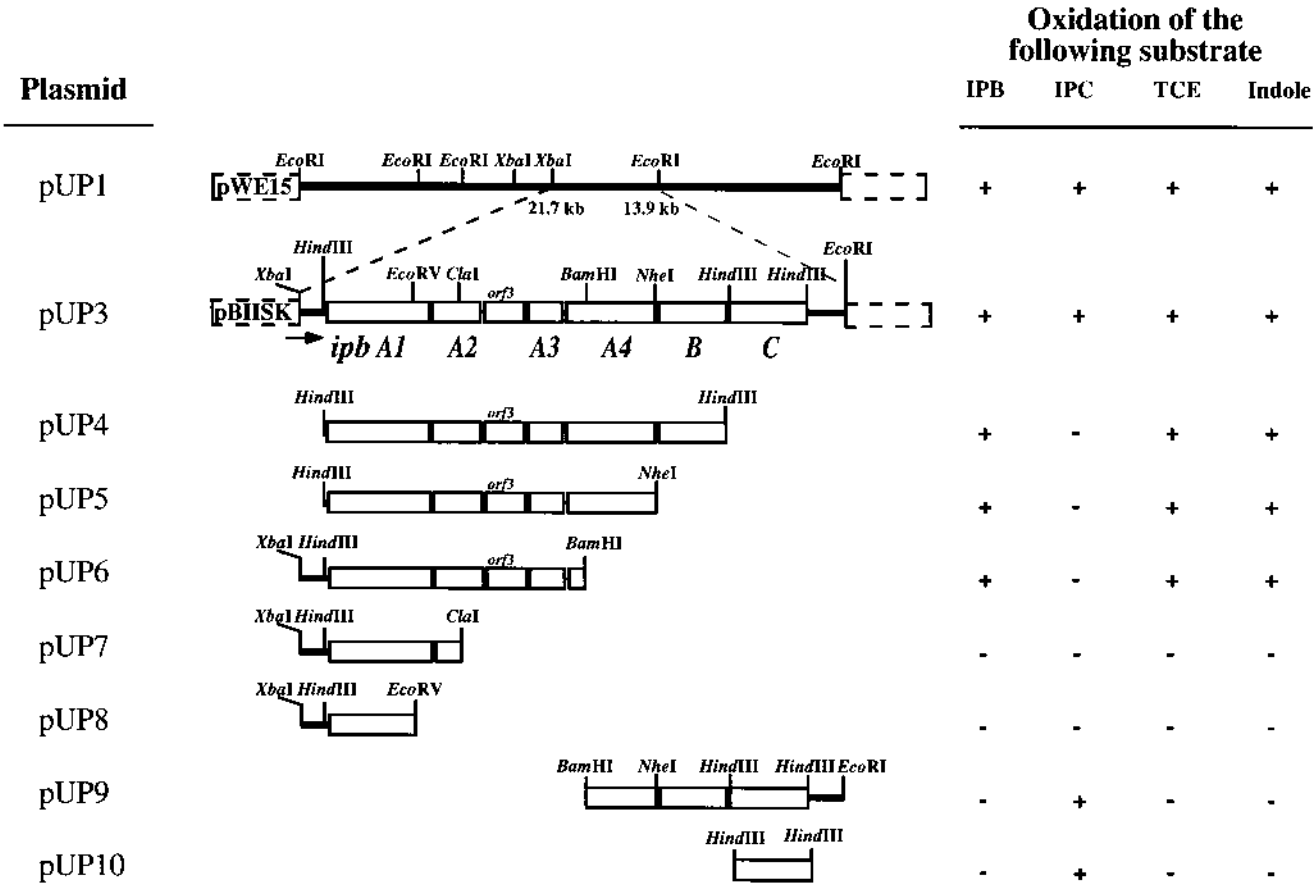

FIG. 2. Restriction maps of cosmid pUP1 and subclones in pBluescriptII derived from pUP1 and corresponding IPB dioxygenase, 3-IPC 2,3-dioxygenase, and TCE-degrading activities. IPB, IPC, and TCE-oxidizing activities were transcribed independently from the *lac* promoter on the vector. When a significant decrease in TCE concentration (30 to 100%) was observed in the degradation test by *E. coli* DH5α harboring the indicated plasmids, the TCE-degrading capability was determined to be positive (+).

IPB dioxygenase mediating the oxidation of indole is encoded on pUP4, pUP5, and pUP6. When *E. coli* DH5α cells harboring pUP7, pUP8, pUP9, or pUP10 were tested for *meta*-cleavage activity by spraying with catechol, only *E. coli* DH5α (pUP9) and DH5α(pUP10) revealed the typical yellow color due to the formation of the aromatic ring cleavage product. These results indicate that a 3-IPC 2,3-dioxygenase gene conferring catechol *meta*-cleavage activity to *E. coli* DH5α is harbored by the 1-kb *Hin*dIII fragment in pUP10.

Nucleotide sequence analysis and organization of *ipb* genes. The precise location of the *ipb* genes was unravelled by sequencing the 7.6-kb *Xba*I-*Eco*RI DNA fragment of pUP3. The entire sequence is shown in Fig. 3. Seven closely clustered open reading frames (ORFs) followed by one incomplete ORF and the presumptive Shine-Dalgarno sequences were identified on this DNA fragment. The organization and features of the complete ORFs are presented in Table 1. Translation of seven successive ORFs and sequence comparisons revealed significant homologies to genes for biphenyl, toluene, and benzene degradation (Table 2). Very recently, cumene (IPB)-degrading enzymes were cloned from *P. fluorescens* IP01 (1). Interestingly, the analogous genes from strain JR1 were almost identical to the corresponding genes in strain IP01 (Table 2). Despite the identities of the analogous *ipb* and *cum* genes, the two strains are different; for example, fluorescent pigments were identified in *P. fluorescens* IP01 (1) but not in *Pseudomonas* strain JR1 (6). In combination with the foregoing results on enzyme activities of *E. coli* DH5α recombinant clones, these seven ORFs were assigned to the following genes: *ipbA1*, large subunit of the IPB terminal dioxygenase; *ipbA2*, small subunit of the IPB terminal dioxygenase; *orf3*, ORF very similar to those occurring in association with biphenyl-degradative genes but apparently not encoding a protein directly involved in IPB degradation; *ipbA3*, ferredoxin$_{IPB}$; *ipbA4*, ferredoxin reductase component; *ipbB*, IPB dihydrodiol dehydrogenase; *ipbC*, 3-IPC 2,3-dioxygenase (Fig. 1), and *orfE*, incomplete ORF of a 2-hydroxy-2,4-dienoate-hydratase.

Analysis of the *ipbA1A2A3A4*, *ipbB*, and *ipbC* gene products. The molecular masses of five different polypeptides identified by an in vitro transcription-translation assay corresponded to sizes of 54, 24, 43.5, 28.5, and 36 kDa and approximately matched those determined on the basis of the deduced sequences of *ipbA1*, *ipbA2*, *ipbA4*, *ipbB*, and *ipbC* (Table 1). Although the sequence data predicted the presence of *orf3* and of *ipbA3* encoding the ferredoxin component, polypeptides of the predicted size could not be detected (data not shown).

Substrate specificity of IPB dioxygenase and 3-IPC 2,3-dioxygenase. LB medium-grown recombinant *E. coli* strains harboring pUP1, pUP3, pUP4, pUP5, pUP9, or pUP10 were assayed for dioxygenase activities by determining substrate-dependent oxygen uptake rates with an oxygen electrode. Interestingly, oxygen consumption rates of *E. coli* DH5α (pUP1) and DH5α(pUP3) were remarkably higher than those of *E. coli* DH5α(pUP4) and *E. coli* DH5α(pUP5) (Table 3). The highest activities were recorded with IPB and 3-IPC, but


1 CACTAGTTCTAGAGTTTAGCTACTAGGGTTTGGCTATTGCGAGCGCATACTGAATATTCGCTTCATGTGTTCCAGGAAGGCGGCTTGTCACGGTGGTTCG 100
101 TCGGGCGCTGTATTGAAGCTTCGGGTCAGCAAGAGAAAGAAGCTTGTTTTTTATTCTAACAAGAGCATCTTGAAGGAGAGTATAGGTCATGAGTTCAATA 200
iphA1 M S S I
201 ATAAATAAAGAAGTGCAGGAAGCCCCTTTGAAATGGGTGAAAAACTGGTCTGACGAGGAGATTAAAGCGCTCGTTGATGAGGAAAAGGGGTTGCTTGATC 300
I N K E V Q E A P L K W V K N W S D E E I K A L V D E E K G L L D P
301 CACGTATTTTCTCTGATCAGGATTTGTATGAGATCGAGCTTGAGAGGGTGTTTGCTCGATCCTGGCTGCTGCTTGGGCACGAGGGGCACATTCCCAAAGC 400
R I F S D Q D L Y E I E L E R V F A R S W L L L G H E G H I P K A
401 CGGGGATTATCTGACCACCTACATGGGTGAAGACCCAGTAATTGTAGTGAGGCAGAAAGACCGGAGCATTAAAGTCTTTTTAAACCAATGTCGGCATCGC 500
G D Y L T T Y M G E D P V I V V R Q K D R S I K V F L N Q C R H R
501 GGTATGCGTATTGAGCGATCGAATTTTGGCAACGCAAAGTCATTTACCTGCACTTATCACGGGTGGGCCTATGACACCGCCGGTAATCTGGTCAATGTAC 600
G M R I E R S N F G N A K S F T C T Y H G W A Y D T A G N L V N V P
601 CCTACGAGAAAGAGGCTTTTGTGACAAAAAAAGAGGGTGACTGCGGGTTCGACAAGGCCGACTGGGGGCCGCTGCAAGCGCGGGTGGATACTTACAAGGG 700
Y E K E A F V T K K E G D C G F D K A D W G P L Q A R V D T Y K G
701 GCTGATTTTTGCCAACTGGGATACCGAAGCCCCTGATTTGAAGACCTATCTGAGCGATGCAACACCCTATATGGACGTGATGCTCGATCGGACCGAGGCA 800
L I F A N W D T E A P D L K T Y L S D A T P Y M D V M L D R T E A
801 GGTACTGCAGTCATCACCGGTATGCAAAAGACGGTAATCCCCTGTAACTGGAAATTCGCCGCCGAGCAATTCTGTAGCGATATGTACCATGCGGGAACGA 900
G T A V I T G M Q K T V I P C N W K F A A E Q F C S D M Y H A G T M
901 TGGCGCATCTTTCAGGTGTATTGTCCAGCCTCCCGCCTGAAATGGATTTGTCCCAAGTAAAGTTACCGTCAAGTGGGAATCAGTTCCGGGCTAAGTGGGG 1000
A H L S G V L S S L P P E M D L S Q V K L P S S G N Q F R A K W G
1001 TGGACATGGGACCGGCTGGTTCAATGACGATTTCGCACTTCTGCAAGCCATCATGGGTCCTAAGGTTGTCGATTACTGGACCAAAGGTCCAGCTGCTGAG 1100
G H G T G W F N D D F A L L Q A I M G P K V V D Y W T K G P A A E
1101 CGTGCAAAAGAGCGTCTGGGTAAAGTTCTTCCGGCTGATCGCATGGTTGCTCAGCATATGACCATTTTTCCGACATGCTCATTTCTTCCTGGCATCAATA 1200
R A K E R L G K V L P A D R M V A Q H M T I F P T C S F L P G I N T
1201 CAGTCCGTACTTGGCACCCACGTGGCCCTAATGAGATCGAAGTTTGGTCTTTCATCGTAGTGGATGCTGATGCACCTGCCGATATCAAGGAAGAATATCG 1300
V R T W H P R G P N E I E V W S F I V V D A D A P A D I K E E Y R
1301 TCGGAAAAACATCTTCACCTTCAATCAAGGGGGAACCTACGAGCAGGACGATGGCGAAAACTGGGTGGAGGTTCAGCGGGGATTGCGCGGCTACAAGGCT 1400
R K N I F T F N Q G G T Y E Q D D G E N W V E V Q R G L R G Y K A
1401 AGAAGTAGACCTCTTTGTGCCCAGATGGGGGCGGGTGTGCCAAACAAGAACAACCCGGAGTTTCCTGGAAAGACCAGCTACGTTTATAGCGAAGAAGCTG 1500
R S R P L C A Q M G A G V P N K N N P E F P G K T S Y V Y S E E A A
1501 CGCGAGGGTTCTACCACCACTGGAGCCGCATGATGTCCGAGCCGAGTTGGGACACGCTAAAGTCTTGACAGATAAAGTGACCGAAAAAAGCAATCACTTT 1600
R G F Y H H W S R M M S E P S W D T L K S *
1601 CATCGGGTTTCTACCGTGGTAGACAAGGGTTTAGCCTGTTTTTTGGTTGCTGGAAGTGCCTAAGTGAATTGATTAACTTGGGTAAACCCCTGGCTTTGTC 1700
1701 GGGGGTATTTACTCGGGTGCATTCCAAAATGTACAGCTGTGCGTTTGGTGATAATCGTCATGCTATGGATTTGCTATTTGCATGAGCCGAGTGCAGGTCG 1800
1801 CCCAACATATATACAGGAAACTAATTATGACATCCGCTGATTTGACAAAACCCATCGAGTGGCCAGAAATGCCCGTCAGTCTTGAATTGCAAAATGCCGT 1900
iphA2 M T S A D L T K P I E W P E M P V S L E L Q N A V
1901 TGAGCAATTCTACTATCGCGAAGCACAGTTGCTTGATTATCAAAACTATGAGGCCTGGCTGGCTTTACTGACCCAAGACATCCAATATTGGATGCCAATT 2000
E Q F Y Y R E A Q L L D Y Q N Y E A W L A L L T Q D I Q Y W M P I
2001 CGTACTACTCATACATCCCGGAATAAGGCGATGGAGTACGTGCCCCCCGGCGGTAATGCCCATTTTGACGAGACGTATGAGAGCATGCGTGCGCGCATTC 2100
R T T H T S R N K A M E Y V P P G G N A H F D E T Y E S M R A R I R
2101 GGGCGAGGGTTTCGGGGCTTAACTGGACTGAAGATCCACCGTCGCGCAGCCGGCACATTGTAAGCAACGTTATCGTCCGCGAAACTGAGAGTGCTGGTAC 2200
A R V S G L N W T E D P P S R S R H I V S N V I V R E T E S A G T
2201 TTTGGAAGTTAGTTCTGCGTTCCTTTGTTACCGTAATCGATTGGAGCGTATGACGGACATCTATGTCGGTGAGCGTCGAGATATTTTGCTCCGTGTAAGT 2300
L E V S S A F L C Y R N R L E R M T D I Y V G E R R D I L L R V S
2301 GACGGGCTGGGATTCAAAATTGCCAAGCGAACGATCTTGCTCGACCAGAGCACGATTACAGCGAATAATCTCAGCCAGTTTTTCTAACTAGGGAATGCTG 2400
D G L G F K I A K R T I L L D Q S T I T A N N L S Q F F *
2401 GCCACTTACCCTATACCCAGCCTATTCATGAGACGGCCTGAAATGAAGAGGAGCTACCCGATAGCTACGCAAACTAATCGCGCTCGCCCTTTCCTGATC 2500
orf3 M K R S Y P I A T Q T N R A R P F L I
2501 GCGATCGGTATCTTTTACTTGGCCAATCTCCTCGGGACTTTGCATTTCAGCAGCCTGCGCTGTTTCGGCATGATGTATTCGGGTGTGGATTTGCAGGTCG 2600
A I G I F Y L A N L L G T L H F S S L R C F G M M Y S G V D L Q V G
2601 GCGCTCCGGTATTCACCCTGCTGCAGGATGCCTGGGCCCTAGTCGGGCTGCAGCTGGGGGGCACTGGGCTGGTTGCGTTGTGGGGCGCACGTCAGCCCGT 2700
A P V F T L L Q D A W A L V G L Q L G G T G L V A L W G A R Q P V

```
2701 GCCGTTCATGGCGGTTGTCCCCGTGGTCATCGTCACGGAAGTGCTCGACGGTATCTGGGACTTGTACAGCATCGTTTGGAGTCACGAAGCCATGTGGTTC 2800
      P  F  M  A  V  V  P  V  V  I  V  T  E  V  L  D  G  I  W  D  L  Y  S  I  V  W  S  H  E  A  M  W  F

2801 GGGCTCCTGACGTTCGCCATCCACGTGGTGTGGATCGTCTGGGGGTTACAGGTATGGCGCGTGTCGTCTCGCCGGTCATCTGGCTTAACCGTCCCAACCT 2900
      G  L  L  T  F  A  I  H  V  V  W  I  V  W  G  L  Q  V  W  R  V  S  S  R  R  S  S  G  L  T  V  P  T  S

2901 CCTGAATCTGTGGCCTGAATTGAACTCTGTCATTTCCCATGCGCGTCGACGTTATCCGGCCGCTTGGCCCAAGCTGGCTGGCTGCCGGATTTAATGAGTA 3000
      *

3001 GTTAAAGTTAGCTATAGAAACTCTGAAAAGGCTTGACCTCATGAGATATCCAGTCTGCAGTCCTATAGAAACTCTGAAAAAGGCTTGACCTCATGAGATA 3100

3101 TCCCAGTCTGCAGTCCGCGTGGTTACTGGCGTGCATTTTCCGAGTGCGTACTTTTTCAGACCAACTCTATAATAAGAGACAAAAAAGAATGACTTTTTCC 3200
                                                                                ipbA3  M  T  F  S

3201 AAAGTTTGTGAAGTATCTGATGTGCCCGTCGGTGACGCCTTGCAGGTTGAAAGTAAGGGCGAAGCCGTCGCGATTTTCAACGTCGATGGAGAGTTGTTCG 3300
      K  V  C  E  V  S  D  V  P  V  G  D  A  L  Q  V  E  S  K  G  E  A  V  A  I  F  N  V  D  G  E  L  F  A

3301 CAACACAGGAGCGTTGCACTCATGGTGACTGGTCCTTGTCCGAAGGCGGCTACCTAGAGGGTGACATTGTCGAATGCTCGCTGCACATGGGTAGGTTCTG 3400
      T  Q  E  R  C  T  H  G  D  W  S  L  S  E  G  G  Y  L  E  G  D  I  V  E  C  S  L  H  M  G  R  F  C

3401 TGTCCGCACGGGCAAGGTAAAAGCAGCACCGCCCTGTGAGCCGCTGAAGATATATCCGATTCGAATAGATGGCAGCGATGTGTTCGTAGACTTTGATGCC 3500
      V  R  T  G  K  V  K  A  A  P  P  C  E  P  L  K  I  Y  P  I  R  I  D  G  S  D  V  F  V  D  F  D  A

3501 GGGTATCTAGCGCCATGATTAAATCAATCGTCATTATTGGTGCTGGCTTGGCTGGCGCAACTGCCACTCGCTATCTTCGCGCCCAAGGATATCAGGGAAA 3600
      G  Y  L  A  P  *
               ipbA4  M  I  K  S  I  V  I  I  G  A  G  L  A  G  A  T  A  T  R  Y  L  R  A  Q  G  Y  Q  G  K

3601 GATCCATCTGGTCGGGGAGGAGTTGCATGTGGCTTACGATCGCCCCTCCTTGTCCAAGGTCGCCCTGTCAGGAAAAGTGGTCGAACCACCCGCGATCCTG 3700
      I  H  L  V  G  E  E  L  H  V  A  Y  D  R  P  S  L  S  K  V  A  L  S  G  K  V  V  E  P  P  A  I  L

3701 GATCCTTGTTGGTATGCATCGGCCGATATAGATCTCCATTTAGGTGTACGCGTGACCGGTATTGATGTGGTAAACCACCAGGTACTTTTCGAATCCGGTG 3800
      D  P  C  W  Y  A  S  A  D  I  D  L  H  L  G  V  R  V  T  G  I  D  V  V  N  H  Q  V  L  F  E  S  G  D

3801 ACATTCTAGCCTACGACCGACTGCTATTAGCCACCGGCGCTCGCGCGCGGCGTATGGCTATTACGGGAAGCGAGTTGGCCGGCATTCACACCTTGCGTGA 3900
      I  L  A  Y  D  R  L  L  L  A  T  G  A  R  A  R  R  M  A  I  T  G  S  E  L  A  G  I  H  T  L  R  D

3901 CCGCGCCGACAGCCAGGCGCTGAGGCAGGCGCTTGAGCCGGGCCAGTCTCTGGTAATTGTCGGCGGTGGCCTGATCGGTTGCGAAGTGGCGACCACTGCT 4000
      R  A  D  S  Q  A  L  R  Q  A  L  E  P  G  Q  S  L  V  I  V  G  G  G  L  I  G  C  E  V  A  T  T  A

4001 ATTAATGCCGGTGCCCACGTCACTGTTCTGGAGGCCGGGGACGAACTGCTGTTGCGAGTGCTAGGCCGATCAACCGGGGCCTGGTGTCGCAACGAGTTGG 4100
      I  N  A  G  A  H  V  T  V  L  E  A  G  D  E  L  L  L  R  V  L  G  R  S  T  G  A  W  C  R  N  E  L  E

4101 AGCGTTTGGGTGTCCGGGTTGAACTGAACGCACAGGCAGCGCATTTCGAGGGCGAGGGACACGTGCATGCCGTCGTTTGTGCCGATGGACGTCGGATAGC 4200
      R  L  G  V  R  V  E  L  N  A  Q  A  A  H  F  E  G  E  G  H  V  H  A  V  V  C  A  D  G  R  R  I  A

4201 AGCTGGCACAGTTTTGGTGAGCATCGGTGTAGAACCAGCCGACGAACTGGCACGTGCGGCCGGCTTCGCATGTGAGCGCGGCGTGGTAGTTGACGCTACG 4300
      A  G  T  V  L  V  S  I  G  V  E  P  A  D  E  L  A  R  A  A  G  F  A  C  E  R  G  V  V  V  D  A  T

4301 GGTGCCAGCTCATGTCCTGCAGTATTCGCGGCAGGTGACGTAGCAGCCTGGCCCCTGAGGTCCGGTGAACTGCGCTCGCTGGAGACCTACCTGAACAGCC 4400
      G  A  S  S  C  P  A  V  F  A  A  G  D  V  A  A  W  P  L  R  S  G  E  L  R  S  L  E  T  Y  L  N  S  H

4401 ACATGCAGGCTGAAACTGCCGCCGCGGCCATGTTAGGCAAGTCTATCCCGGCTCTTCAGGTGCCAACCTCTTGGACGGAGATTGCAGGGCATCGGATACA 4500
      M  Q  A  E  T  A  A  A  A  M  L  G  K  S  I  P  A  L  Q  V  P  T  S  W  T  E  I  A  G  H  R  I  Q
4501 GATGGTTGGCGACATCGAAGGCCCCGGAGAAGTTGTCTTGCGCGGTAACGTCGAGAATGGTCAGCCGCTGGTGCAGTTCAGGGTTCTTGATGGTCGCGTT 4600
      M  V  G  D  I  E  G  P  G  E  V  V  L  R  G  N  V  E  N  G  Q  P  L  V  Q  F  R  V  L  D  G  R  V

4601 GAAGCCGCAACGGCTATCAATGCCCCGAAGGATTTTTCCGTTGCAACCCGATTGGTGGCTGACCACATTCCTGTATCGGCCACAAAATTGCAGGACGCTA 4700
      E  A  A  T  A  I  N  A  P  K  D  F  S  V  A  T  R  L  V  A  D  H  I  P  V  S  A  T  K  L  Q  D  A  S

4701 GCTCTAACTTGCGGGATTTTATGAAAGCTAAAGCTGAGCGATGCGAGTGAACGTCGACTTATTCTTAATAACTAGAAGAGCTTAAAAGTGAAACTAAAAG 4800
      S  N  L  R  D  F  M  K  A  K  A  E  R  C  E  *                                ipbB  V  K  L  K  E

4801 AAGAAGTTATATTAATCACGGGGGGCGCATCTGGGACTGGGCATGCCCTTGTGGAACGGTTCGTGGCCGAAGGTGCCAAGGTGGCTGTCCTCGATAAGTG 4900
      E  V  I  L  I  T  G  G  A  S  G  T  G  H  A  L  V  E  R  F  V  A  E  G  A  K  V  A  V  L  D  K  C

4901 TGCCGAGCGACTTCAACAGTTGGAGTCTGATCACGGCGAAGATGTGGTATGCATTGTCGGTGATGTGCGTTCAATGGAAGACCAGAAACTGGCTGCCAGT 5000
      A  E  R  L  Q  Q  L  E  S  D  H  G  E  D  V  V  C  I  V  G  D  V  R  S  M  E  D  Q  K  L  A  A  S

5001 CGTTGTATTGCCAAGTTCGGGAGGATTGACACTCTGATCCCCAACGCCGCTATCTGGGATTACAACACGGCACTTGTCGATCTACCTGAAGACAGTATCG 5100
      R  C  I  A  K  F  G  R  I  D  T  L  I  P  N  A  A  I  W  D  Y  N  T  A  L  V  D  L  P  E  D  S  I  D

5101 ACAAAGCATTTGATGAAGTATTTCAGATTAATGTTAAGGGCTATATTCTCGCCGTCAAGGCATGCCTGCCAGCGCTGGTTGCCAGCCGAGGCAGCGTGAT 5200
      K  A  F  D  E  V  F  Q  I  N  V  K  G  Y  I  L  A  V  K  A  C  L  P  A  L  V  A  S  R  G  S  V  I

5201 CTGCACGATCTCGAATGCAGGTTTTTACCCCAATGGCGGTGGTCCTCTTTACACGGCGACGAAGCACGCAGTGGTGGGGCTGGTGCGCGAGCTGGCATTC 5300
      C  T  I  S  N  A  G  F  Y  P  N  G  G  G  P  L  Y  T  A  T  K  H  A  V  V  G  L  V  R  E  L  A  F

5301 GAGTTGGCACCATACGTCCGGGTCAATGGTGTGGGGGTTGGCGGTATCAATACGGATTTGAGAGGACCTTGCTCGTTGGGAATGAGTGAGCAGTCGATAT 5400
      E  L  A  P  Y  V  R  V  N  G  V  G  V  G  G  I  N  T  D  L  R  G  P  C  S  L  G  M  S  E  Q  S  I  S
```

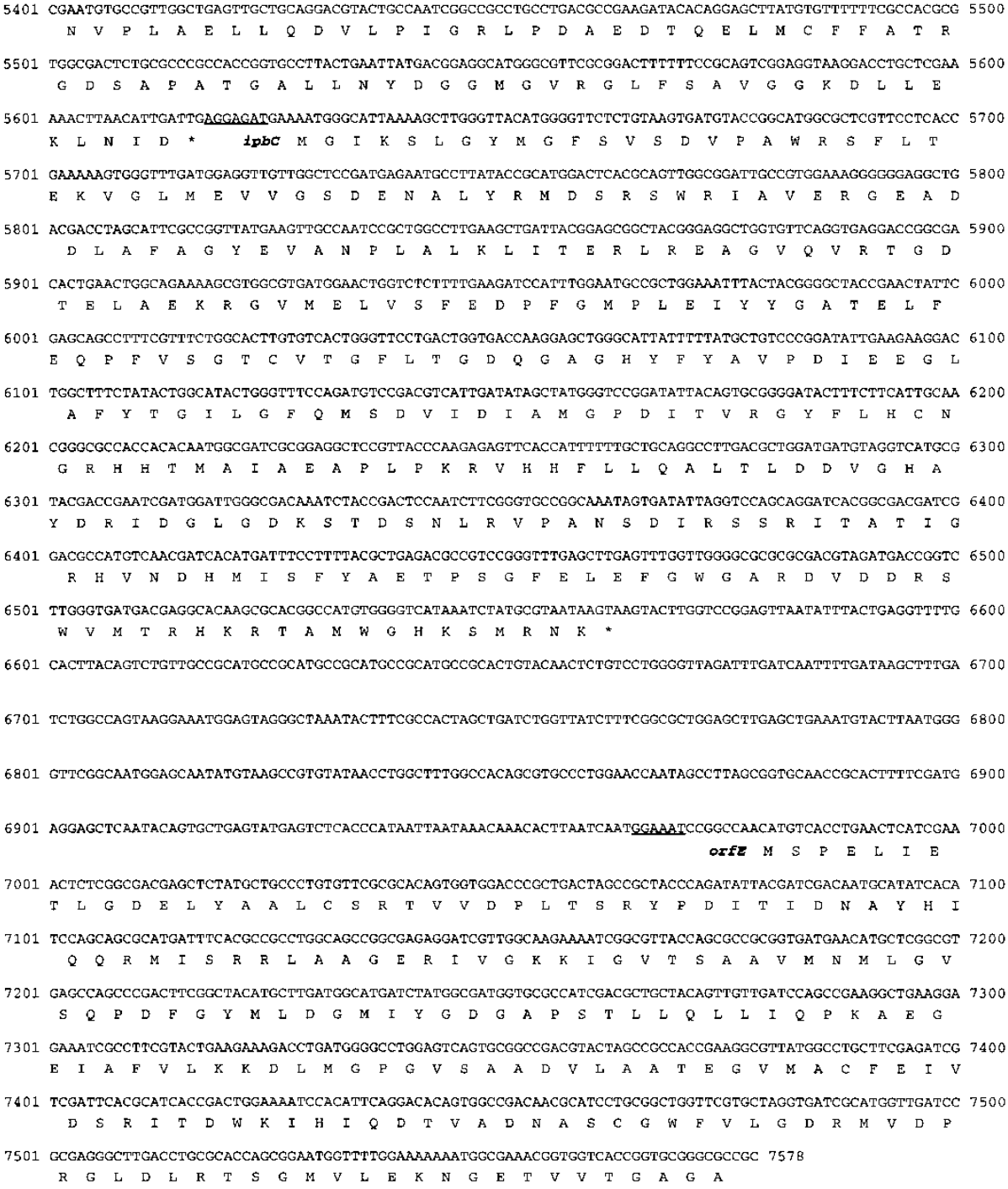

FIG. 3. Nucleotide sequence and deduced amino acid sequences of *ipbA1A2A3A4*, *orf3*, *ipbB*, and *ipbC*. The sequence region spans the coordinates (kb 21.7 to 13.9) shown in Fig. 2. The deduced amino acid sequences are shown in the one-letter code, and asteriks indicate stop codons. The putative Shine-Dalgarno sequences are underlined.

various *n*-alkylbenzenes and *n*-alkylcatechols, benzene, biphenyl, and indole were also oxygenated.

Incorporation of ^{18}O into IPB by *E. coli* DH5α(pUP4). *E. coli* DH5α(pUP4) carrying the initial IPB dioxygenase and the 2,3-dihydro-2,3-dihydroxy IPB dehydrogenase genes converted IPB to 3-IPC as shown by thin-layer chromatography and spectrophotometry (data not shown). The mass spectrum of 3-IPC produced by *E. coli* DH5α(pUP4) oxidizing IPB in the presence of $^{18}O_2$ revealed a parent peak at $m/e = 152$ and an additional small peak at $m/e = 156$, whereas in a control experiment performed without $^{18}O_2$, the $m/e = 156$ peak was missing (data not shown). The absence of an $m/e = 154$ peak in the mass spectrum of the 3-IPC synthesized in the presence of $^{18}O_2$ demonstrates that two atoms of oxygen are

TABLE 1. Organization and features of the *ipb* catabolic genes of *Pseudomonas* strain JR1

Gene	Region of nucleotide sequence	G+C content (%)	Function	No. of amino acid residues	Subunit mol wt[a]
ipbA1	189–1569	51.0	IPB dioxygenase (ISP[b] large subunit)	460	52,100
ipbA2	1829–2390	49.6	IPB dioxygenase (ISP small subunit)	187	21,600
orf3	2444–2905	59.3	Unknown	153	17,000
ipbA3	3194–3524	52.0	Ferredoxin	109	11,800
ipbA4	3521–4757	58.3	Ferredoxin reductase	412	43,300
ipbB	4788–5618	53.8	*cis*-IPB dihydrodiol dehydrogenase	276	29,200
ipbC	5629–6564	52.8	3-IPC 2,3-dioxygenase	311	34,700

[a] Determined from the nucleotide sequence.
[b] ISP, iron-sulfur protein

incorporated into the aromatic nucleus from a single O_2 molecule.

TCE degradation by recombinant *E. coli* DH5α clones. To identify the enzyme of the IPB-degradative pathway in strain JR1 which catalyzes the cooxidation of TCE, the TCE-degrading capability of *E. coli* DH5α recombinant strains carrying and expressing different *ipb* genes was investigated in the presence of 100 μM TCE. Because only pUP1 and pUP3 to pUP6 conferred TCE oxidation activity to *E. coli* DH5α, it is apparent that the initial dioxygenase is responsible for this activity (Fig. 2 and 4). Growing cells of *E. coli* DH5α(pUP3) and DH5α(pUP5) degraded up to 90% of the TCE, whereas *E. coli* DH5α(pUP6), lacking part of the reductase gene, was less efficient (Fig. 4). The degradation started in mid-logarithmic growth and slowed in the stationary phase. In the experiment depicted, the specific TCE degradation rate of *E. coli* DH5α(pUP3) and DH5α(pUP5) was 0.1 nmol of TCE per min per mg of protein, whereas with suspensions of resting cells harvested in the mid-log phase, a 10-fold-higher specific rate was achieved (data not shown).

DISCUSSION

We cloned and characterized from *Pseudomonas* strain JR1 six genes corresponding to *ipbA1A2A3A4*, *ipbB*, and *ipbC* of the IPB *meta*-cleavage pathway and to one unknown ORF (*orf3*). Furthermore, an incomplete ORF (*orfE*) similar to genes of 2-hydroxy-2,4-dienoate-hydratases was detected. Sequence analysis revealed an almost 100% identity of *ipbA1A2A3A4* (IPB dioxygenase) to the cumene dioxygenase in *P. fluorescens* IP01 (1) and strong homologies to the biphenyl dioxygenases of *Pseudomonas* strain KKS102 (13, 21, 22), *Pseudomonas* strain LB400 (9, 18, 19), *P. pseudoalcaligenes* KF707 (38), *Rhodococcus erythropolis* RHA1 (13, 26), *R. erythropolis* BD2 (20), and *R. globerulus* P6 (2, 3) and to the toluene dioxygenase of *P. putida* F1 (45). *E. coli* harboring pUP1, pUP3, pUP4, or pUP5 exhibited IPB dioxygenase activity.

Analysis of the deduced protein sequence of *ipbA4* (reductase$_{IPB}$) revealed two conserved regions with a consensus sequence of Xaa-Gly-Xaa$_2$-Gly-Xaa$_3$-Ala (or a homologous amino acid)-Xaa$_6$-Gly (where Xaa is any amino acid) predicted to bind NAD^+ or FAD (27). The consensus sequence CXHX$_{16–17}$CXXH of Rieske iron-sulfur proteins for the binding of a (2Fe2S) cluster (27) was present in IpbA1 and IpbA3. These observations suggest that the IPB dioxygenase in strain JR1 consists of three protein components, the terminal oxygenase (α subunit IpbA1 and β subunit IpbA2), the intermediate Rieske iron-sulfur ferredoxin (IpbA3), and a NADH-(Rieske iron-sulfur) ferredoxin oxidoreductase (protein IpbA4). These characteristics in the electron transport chain, the consistency of the *ipbA1A2A3A4* gene products with analogous dioxygenase proteins, and the expression in *E. coli* clearly show that *ipbA1A2A3A4* encode the subunits of the IPB dioxygenase of *Pseudomonas* strain JR1, an aromatic dioxygenase belonging to the class IIB ring-activating dioxygenases (27).

The production of the characteristic yellow color of the *meta*-cleavage product during incubation of *E. coli* harboring pUP1 or pUP3 with IPB, the enzyme assays, and the sequence analysis of the downstream region of *ipbA1A2A3A4* show that *ipbB* encodes 2,3-dihydro-2,3-dihydroxy-IPB dehydrogenase and *ipbC* 3-IPC 2,3-dioxygenase. Within the deduced amino acid sequence of IpbB, the conserved tyrosine (amino acid 155) and lysine residues (amino acid 159) suggest that IpbB is a member of the short-chain alcohol dehydrogenase superfamily. The conserved tyrosine residue has been shown to be important for the catalytic activity and/or for subunit binding (8).

The deduced amino acid sequence of *ipbC* is characteristic of extradiol dioxygenases, and the high homologies to *bphC* from *P. pseudoalcaligenes* KF707 (38) and to *todE* from *P. putida* F1 (45) suggest that the 3-IPC 2,3-dioxygenase (IpbC) belongs to the same extradiol dioxygenase superfamily (16) comprising multimeric ferrous iron-containing enzymes that mediate aromatic ring cleavage in many aromatic catabolic pathways. High homologies of IpbC to BphC1 in *R. globerulus* P6 (2) and BphC in *Rhodococcus* strain RHA1 were also found (26).

The predicted amino acid sequence of ORF3 showed significant homology to ORF3 from *P. pseudoalcaligenes* KF707 (36), ORF1 from *Pseudomonas* strain LB400 (9), and ORF4 from *Pseudomonas* strain KKS102 (14), whose functions are unknown. The deduced amino acid sequence of the ORF3 carboxy terminus exhibits considerable homology to the c(F_0) subunit of the ATPase, whereas the predicted amino acid sequence of the amino terminus of ORF3 reveals extensive homology to the DNA-binding domain of transcriptional activators of the LysR family. Recently, a truncated LysR-type regulator has been identified upstream of the toluene degradation genes in *P. putida* F1 (43), but no role in regulation of the *tod* genes was found. To identify the function of ORF3 in *Pseudomonas* strain JR1, further work is needed.

Comparison of the organization of the *ipb* genes with that of analogous genes of biphenyl-degradative pathways in *Pseudomonas* strains KKS102 and LB400, *P. pseudoalcaligenes* KF707, *Rhodococcus* strain RHA1, and *R. globerulus* P6 and with the *tod* genes in *P. putida* F1 (Fig. 5) and the *ipb* genes in *P. putida* RE204 mapped by Tn*5* insertions (7) revealed an analogous organization. However, *orf3*, which is located between *ipbA2* and *ipbA3* in *Pseudomonas* strain JR1, is absent between the analogous subunits of the biphenyl dioxygenase in *Pseudomonas* strain KKS102, *Rhodococcus* strain RHA1, and *R. globerulus* P6, as well as between the small subunit of the terminal

TABLE 2. Homology of IpbA1, IpbA2, IpbA3, IpbA4, IpbB, and IpbC in *Pseudomonas* strain JR1 and corresponding proteins in other bacteria which degrade aromatic compounds[a]

Pseudomonas strain JR1 enzyme	*P. fluorescens* IP01 (1)		*Pseudomonas* strain KKS102 (21, 22)		*Pseudomonas* strain LB400 (9, 18, 19)		*P. pseudoalcaligenes* KF707 (38)		*Rhodococcus* RHA1 (26)		*R. erythropolis* BD2 (20)		*R. globerulus* P6 (2, 3)		*P. putida* F1 (43, 45)	
	Protein	Homology	Protein	Homology	Protein	Homology	Protein	Homology	Protein	Homology	Protein	Homology	Protein	Homology	Protein	Homology
IpbA1	CumA1	99/99	BphA1	75/85	BphA	74/84	BphA1	75/85	BphA1	66/79	IpbA1	65/78	BphA1	63/77	TodC1	66/79
IpbA2	CumA2	100/100	BphA2	67/79	BphA2/E	59/74	BphA2	59/74	BphA2	48/68	IpbA2	47/68	BphA2	49/68	TodC2	51/68
IpbA3	CumA3	99/100	BphA3	71/84	BphA3/G	76/89	BphA3	77/90	BphA3	48/74	IpbA3	47/74	BphA3	49/72	TodB	55/77
IpbA4	CumA4	99/100	BphA4	33/53	BphA4/G	73/86	BphA4	73/86	BphA4	48/68	IpbA4	49/69	BphA4	53/67	TodA	50/69
IpbB	CumB	96/98	BphB	67/81	BphB	78/88	BphB	77/88	BphB	54/75			BphB	54/76	TodD	55/75
IpbC	CumC	94/97			BphC	64/81	BphC	64/81	BphC	53/73	IpbC	53/73	BphC1	51/68	TodE	53/69

[a] Homology is given as the percent identity/percent similarity. The percent identities and similarities to *ipb* ORFs were obtained from overall pairwise comparisons of amino acid sequences by using the GCG program gap with a gap weight of 3 and gap length weight of 0.1.

oxygenase$_{TOD}$ (TodC2) and the ferredoxin$_{TOD}$ (TodB). The order of the *ipb*, *bph*, and *tod* genes encoding analogous dehydrogenases and *meta*-cleavage dioxygenases is identical except for the biphenyl operon of *Rhodococcus* strain RHA1, in which an inverse order was observed (*bphB* downstream of *bphC*).

The significant homologies of the *ipb*, *cum*, *tod*, and *bph* genes on the one hand and the differences in organization of the *ipb* and *bph* genes in *Pseudomonas* and *Rhodococcus* species on the other indicate that recombination and rearrangement events have occurred during the formation of *ipb*, *cum*, *bph*, and *tod* gene clusters from a possibly common phylogenetic origin.

The tight clustering, the head-to-tail organization of *ipbA1A2A3A4BC*, and the coexpression of IPB dioxygenase and 3-IPC 2,3-dioxygenase in *E. coli* suggest that *ipbA1A2A3A4*, *ipbB*, and *ipbC* are cotranscribed. The expression of *ipbA1A2A3A4* was found to be independent of the *lac* promoter in the vector pBII, indicating that the cloned *ipbA1A2A3A4BC* genes were expressed in *E. coli* from their own promoter.

In *Pseudomonas* strain JR1, the IPB dioxygenase and 3-IPC 2,3-dioxygenase activity was strictly dependent on the presence of the inducer substrate IPB or analogous alkylbenzenes such as ethylbenzene and toluene (data not shown). Although an IPB-induced TCE degradation has not been reported for the IPB-degrading *P. putida* RE204, intensive studies of the *ipb* operon in strain RE204 revealed that in addition to aromatic inducer substrates, the *ipb* genes in this strain are induced by nonaromatic inducer substrates including TCE (36). Whether TCE serves as an inducer substrate of the IPB dioxygenase-mediated TCE oxidation in strain JR1 remains to be tested. Interestingly, IPB did not affect the level of expression of the *ipb* genes in *E. coli* harboring pUP1 to pUP6. The inducer-

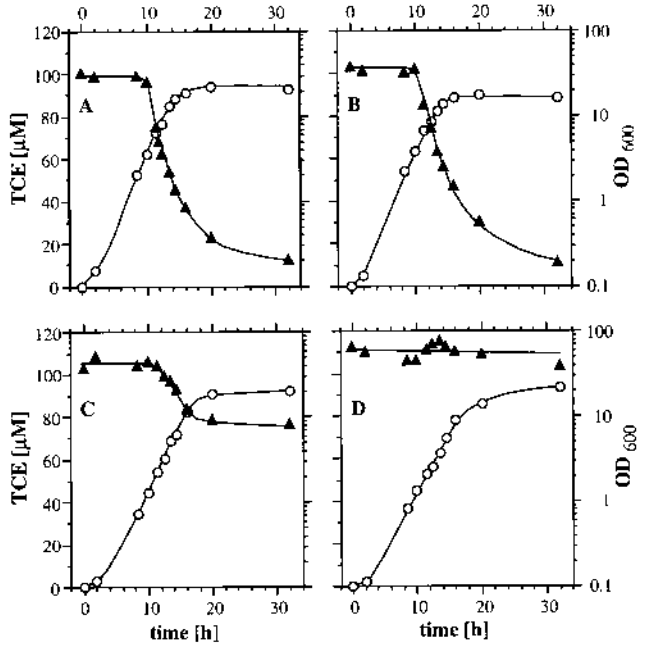

FIG. 4. TCE degradation by recombinant *E. coli* clones under growth conditions. The recombinant strains *E. coli* DH5α(pUP3) (A), *E. coli* DH5α(pUP5) (B), *E. coli* DH5α(pUP6) (C), and *E. coli* DH5α(pUP9) (D) were grown in gastight 1-liter Erlenmeyer flasks containing 80 ml of TB/Amp medium under an oxygen atmosphere with reciprocal shaking at 30°C. The TCE concentration (▲) was monitored by gas chromatography. ○, OD_{600}.

TABLE 3. Oxidation of aromatic compounds by resting cells of recombinant *E. coli* strains containing plasmids with *ipb* genes of *Pseudomonas* strain JR1[a]

Substrate	Specific oxygen uptake rates (nmol of O_2/min/mg of protein) of recombinant *E. coli* strain[b]:					
	DH5α(pUP1)	DH5α(pUP3)	DH5α(pUP4)	DH5α(pUP5)	DH5α(pUP9)	DH5α(pUP10)
IPB	454	615	30	30	0	0
Ethylbenzene	338	475	26	24	0	0
Propylbenzene	372	495	19	16	0	0
sec-Butylbenzene	246	337	ND	ND	0	0
Butylbenzene	248	369	ND	ND	0	0
tert-Butylbenzene	187	297	ND	ND	0	0
Isobutylbenzene	215	343	ND	ND	0	0
Toluene	200	335	ND	ND	0	0
Benzene	76	103	ND	ND	0	0
Biphenyl	+	+	ND	ND	0	0
Indole	+	+	+	+	0	0
3-IPC	984	1,089	0	0	844	643
3-Methylcatechol	948	900	0	0	812	589
Catechol	516	488	0	0	479	271

[a] Cells were grown on LB medium with 100 μg of ampicillin per ml at 30°C to an OD_{600} of 1.3 and washed twice with 50 mM potassium phosphate buffer (pH 7.2). The pellets were resuspended in the same buffer, and the protein concentration in the reaction chamber of the O_2 electrode was set to 50 to 360 μg/ml. Stock solutions of different *n*-alkylbenzenes and *n*-alkylcatechols (200 mM) were prepared in dimethyl formamide, and 3-μl portions were added to 2 ml of cell suspensions to a final concentration of 300 μM. The rates presented were corrected for endogenous respiration. The indole and biphenyl oxidation proceeded very slow and therefore could not be recorded.

[b] +, formation of indigo from indole or a yellow ring-cleavage compound from biphenyl; ND, not detectable.

independent expression of the *ipb* genes from strain JR1 in the recombinant *E. coli* strains might be due to the absence of a putative negative regulator protein. Another explanation could be alteration or absence of a putative multiple promoter controlling the IPB dioxygenase synthesis on the cloned fragments in combination with a multicopy gene effect. Transcription of an aromatic dioxygenase from multiple promoters showing activities in *E. coli* has been suggested for the biphenyl dioxygenase in *Pseudomonas* strain LB400. The data of Erickson and Mondello (9) indicate that transcription from one promoter site (p3), located far upstream of the *bphA* start codon, is significantly increased in biphenyl-grown cells, whereas the level of transcription from two additional promoter sites (p1 and p2), both mapping very close to the *bphA* start codon and only weakly matching the consensus sequences for *E. coli* promoters, was not decreased in the absence of biphenyl.

Sequence analysis of the DNA region in pUP3 located upstream of *ipbA1* did not lead to the identification of any sites matching the consensus sequences for *E. coli* promoters, and future studies will be directed toward identification and exam-

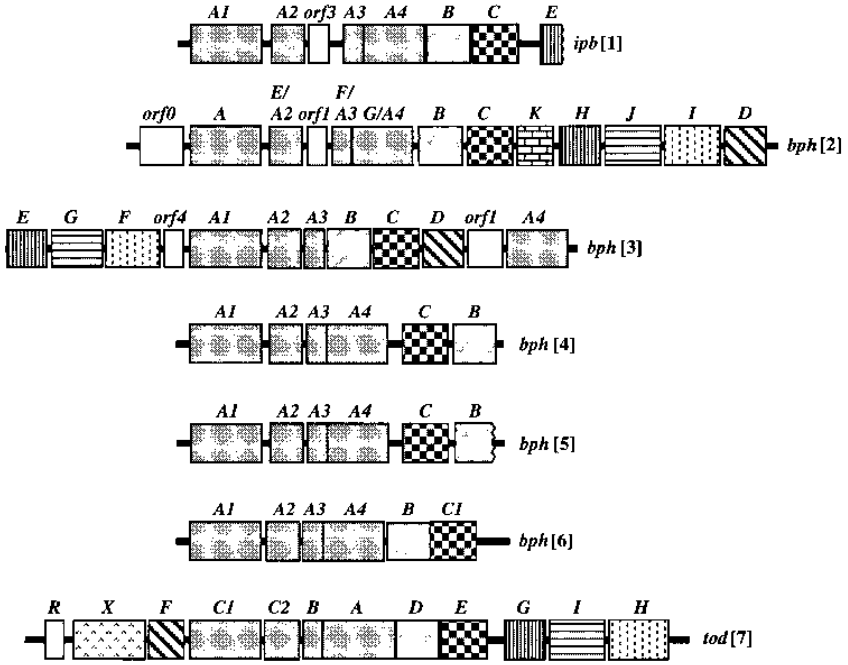

FIG. 5. Comparison of the organization of the *ipb* genes in *Pseudomonas* strain JR1 [1] with the operon structures of highly homologous biphenyl or toluene degradative pathways. [2] *Pseudomonas* strain LB400 (9, 18, 19), [3] *Pseudomonas* strain KKS102 (14, 21, 22), [4] *Rhodococcus* strain RHA1 (13, 26), [5] *R. erythropolis* BD2 (20), [6] *R. globerulus* P6 (2, 3), and [7] *P. putida* F1 (23, 28, 29, 43, 45). Analogous genes are marked by identical hatching.

ination the transcription start sites of the *ipb* genes and the regulation of the degradative genes.

E. coli DH5α(pUP1) and DH5α(pUP3) to DH5α(pUP6) expressed IPB dioxygenase activity and were able to degrade TCE. This correlation of IPB dioxygenase and TCE degradation activity in recombinant *E. coli* cells carrying the IPB dioxygenase genes of JR1 clearly shows that the IPB dioxygenase in *Pseudomonas* strain JR1 is involved in the IPB-induced oxidation of TCE. Besides the toluene dioxygenase in *P. putida* F1 (41, 46), the IPB dioxygenase in strain JR1 is the second aromatic dioxygenase shown to cooxidize TCE. *E. coli* DH5α(pUP6) cells carrying *ipbA1A2A3* and a truncated *ipbA4* gene still showed the ability to degrade TCE. This result indicates that the reductase component is not essential for the IPB dioxygenase-mediated TCE oxidation in *Pseudomonas* strain JR1. Further studies of the TCE degradation capabilities with *E. coli* clones carrying IPB dioxygenase deletion derivatives are under way, and their results may contribute to a better knowledge of the reaction mechanism of TCE oxidation by the IPB dioxygenase.

ACKNOWLEDGMENTS

We thank C. Ratledge for supplying 3-isopropylcatechol and J. Fuchser and A. Zeeck for helpful discussions, for providing the $^{18}O_2$, and for preparing the mass spectra.

This work was supported by grant 0319482 A of the Bundesminister für Forschung und Technologie and by the Deutsche Forschungsgemeinschaft.

REFERENCES

1. **Aoki, H., T. Kimura, H. Habe, H. Yamane, T. Kodama, and T. Omori.** 1996. Cloning, nucleotide sequence, and characterization of the genes encoding enzymes involved in the degradation of cumene to 2-hydroxy-6-oxo-methylocta-2,4-dienoic acid in *Pseudomonas fluorescens* IP01. J. Ferment. Bioeng. **81:**187–196.
2. **Asturias, J. A., E. Diaz, and K. N. Timmis.** 1995. Evolutionary relationship of the biphenyl dioxygenase of the gram-positive bacterium *Rhodococcus globerulus* P6 to multicomponent dioxygenases of gram-negative bacteria. Gene **156:**11–18.
3. **Asturias, J. A., and K. N. Timmis.** 1993. Three different 2,3-dihydroxybiphenyl-1,2-dioxygenase genes in the gram-positive polychlorobiphenyl-degrading bacterium *Rhodococcus globerulus* P6. J. Bacteriol. **175:**4631–4640.
4. **Claus, D., B. Lack, and B. Neu.** 1983. Deutsche Sammlung von Mikroorganismen, catalogue of strains, 3. Aufl. Gesellschaft für Biotechnologische Forschung mbH, Braunschweig, Germany.
5. **Dabrock, B., M. Keßeler, B. Averhoff, and G. Gottschalk.** 1994. Identification and characterization of a transmissible linear plasmid from *Rhodococcus erythropolis* BD2 that encodes isopropylbenzene and trichloroethene catabolism. Appl. Environ. Microbiol. **60:**853–860.
6. **Dabrock, B., J. Riedel, J. Bertram, and G. Gottschalk.** 1992. Isopropylbenzene (cumene)—a new substrate for the isolation of trichloroethene-degrading bacteria. Arch. Microbiol. **158:**9–13.
7. **Eaton, R. W., and K. N. Timmis.** 1986. Characterization of a plasmid-specified pathway for catabolism of isopropylbenzene in *Pseudomonas putida* RE204. J. Bacteriol. **168:**123–131.
8. **Ensor, C. M., and H. H. Tai.** 1991. Site-directed mutagenesis of the conserved tyrosine 151 of human placental NAD(+)-dependent 15-hydroxyprostaglandin dehydrogenase yields a catalytically inactive enzyme. Biochem. Biophys. Res. Commun. **176:**840–845.
9. **Erickson, B. D., and F. J. Mondello.** 1992. Nucleotide sequencing and transcriptional mapping of the genes encoding biphenyl dioxygenase, a multicomponent polychlorinated-biphenyl-degrading enzyme in *Pseudomonas* strain LB400. J. Bacteriol. **174:**2903–2912.
10. **Ewers, J., D. Freier-Schröder, and H.-J. Knackmus.** 1990. Selection of trichloroethene (TCE) degrading bacteria that resist inactivation by TCE. Arch. Microbiol. **154:**410–413.
11. **Fox, B. G., J. G. Borneman, L. P. Wackett, and J. D. Lipscomb.** 1990. Haloalkene oxidation by the soluble methane monooxygenase from *Methylosinus trichosporium* OB3b: mechanistic and environmental implications. Biochemistry **29:**6419–6427.
12. **Fujita, M., I. Michihiko, J.-I. Hioki, K. Kataoka, and M. Takeo.** 1995. Trichloroethylene degradation by genetically engineered bacteria carrying cloned phenol catabolic genes. J. Ferment. Bioeng. **79:**100–106.
13. **Fukuda, M., and K. Yano.** 1995. Characterization of biphenyl catabolic genes of gram-positive polychlorinated biphenyl degrader *Rhodococcus* sp. strain RHA1. Appl. Environ. Microbiol. **61:**2079–2085.
14. **Fukuda, M., Y. Yasukouchi, Y. Kikuchi, Y. Nagata, K. Kimbara, H. Horiuchi, M. Takagi, and K. Yano.** 1994. Identification of the *bphA* and *bphB* genes of *Pseudomonas* spec. strain KKS102 involved in degradation of biphenyl and polychlorinated biphenyls. Biochem. Biophys. Res. Commun. **202:**850–856.
15. **Furukawa, K., J. Hirose, S. Hayashida, and K. Nakamura.** 1994. Efficient degradation of trichloroethylene by a hybrid aromatic ring dioxygenase. J. Bacteriol. **176:**2121–2123.
16. **Harayama, S., and M. Rekik.** 1989. Bacterial aromatic ring-cleavage enzymes are classified into two different gene families. J. Biol. Chem. **264:** 15328–15333.
17. **Harker, A. R., and Y. Kim.** 1990. Trichloroethylene degradation by two independent aromatic-degrading pathways in *Alcaligenes eutrophus* JMP134. Appl. Environ. Microbiol. **56:**1179–1181.
18. **Hofer, B., S. Backhaus, and K. N. Timmis.** 1994. The biphenyl/polychlorinated biphenyl degradation locus (*bph*) of *Pseudomonas* spec. LB400 encodes four additional metabolic enzymes. Gene **144:**9–16.
19. **Hofer, B., L. D. Eltis, D. N. Dowling, and K. N. Timmis.** 1993. Genetic analysis of a Pseudomonas locus encoding a pathway for biphenyl/polychlorinated biphenyl degradation. Gene **130:**47–55.
20. **Keßeler, M., E. R. Dabbs, B. Averhoff, and Gottschalk, G.** Studies on the isopropylbenzene 2,3-dioxygenase and the 3-isopropylcatechol 2,3-dioxygenase genes encoded by the linear plasmid of *Rhodococcus erythropolis* BD2. Microbiology, in press.
21. **Kikuchi, Y., Y. Nagata, M. Hinata, K. Kimbara, M. Fukuda, K. Yano, and M. Takagi.** 1994. Identification of the *bphA4* gene encoding ferredoxin reductase involved in biphenyl and polychlorinated biphenyl degradation in *Pseudomonas* sp. strain KKS102. J. Bacteriol. **176:**1689–1694.
22. **Kimbara, K., T. Hashimoto, M. Fukuda, T. Koana, M. Takagi, M. Oishi, and K. Yano.** 1989. Cloning and sequencing of two tandem genes involved in degradation of 2,3-dihydroxybiphenyl to benzoic acid in the polychlorinated biphenyl-degrading soil bacterium *Pseudomonas* sp. strain KKS102. J. Bacteriol. **171:**2740–2747.
23. **Lau, P. C., H. Bergeron, D. Labbe, Y. Wang, R. Brousseau, and D. T. Gibson.** 1994. Sequence and expression of the *todGIH* genes involved in the last three steps of toluene degradation by *Pseudomonas putida* F1. Gene **146:**7–13.
24. **Little, C. D. Palumbo, A. V., Herbes, S. E. Lidstrom, M. E., R. L. Tyndall, and P. J. Gilmer.** 1988. Trichloroethylene biodegradation by a methane-oxidizing bacterium. Appl. Environ. Microbiol. **54:**951–956.
25. **Marck, C.** 1988. DNA-Strider: a C-program for the fast analysis of DNA and protein sequences on the Apple Macintosh family of computers. Nucleic Acids Res. **16:**1829–1836.
26. **Masai, E., A. Yamada, J. M. Healy, T. Hatta, K. Kimbara, M. Fukuda, and Yano K.** 1995. Characterization of biphenyl catabolic genes of gram-positive polychlorinated biphenyl degrader *Rhodococcus* sp. strain RHA1. Appl. Environ. Microbiol. **61:**2079–2085.
27. **Mason, J. R., and R. Cammack.** 1992. The electron-transport proteins of hydroxylating bacterial dioxygenases. Annu. Rev. Microbiol. **46:**277–305.
28. **Menn, F. M., G. J. Zylstra, and D. T. Gibson.** 1991. Location and sequence of the *todF* gene encoding 2-hydroxy-6-oxohepta-2,4-dienoate hydrolase in *Pseudomonas putida* F1. Gene **104:**91–94.
29. **Miller, R. E., and F. P. Guengerich.** 1982. Oxidation of trichloroethylene by liver microsomal cytochrome P-450: evidence of chlorine migration in a transition state not involving trichloroethylene oxide. Biochemistry **21:**1090–1097.
30. **Nelson, M. J. K., S. O. Montgomery, W. R. Mahaffey, and P. H. Pritchard.** 1987. Biodegradation of trichloroethylene and involvement of an aromatic biodegradative pathway. Appl. Environ. Microbiol. **53:**949–954.
31. **Nelson, M. J. K., S. O. Montgomery, and P. H. Pritchard.** 1988. Trichloroethylene metabolism by microorganisms that degrade aromatic compounds. Appl. Environ. Microbiol. **54:**604–606.
32. **Oldenhuis, R., R. L. J. M. Vink, D. B. Janssen, and B. Witholt.** 1989. Degradation of chlorinated aliphatic hydrocarbons by *Methylosinus trichosporium* OB3b expressing soluble methane monooxygenase. Appl. Environ. Microbiol. **55:**2819–2826.
33. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
34. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **85:**8934–8938.
35. **Schmidt, K., S. Liaanen-Jensen, and H. G. Schlegel.** 1963. Die Carotinoide der *Thiorhodaceae*. Arch. Microbiol. **46:**117–126.
36. **Selifonova, O. V., and R. W. Eaton.** 1996. Use of an *ipb-lux* fusion to study regulation of the isopropylbenzene catabolism operon of *Pseudomonas putida* RE204 and to detect hydrophobic pollutants in the environment. Appl. Environ. Microbiol. **62:**778–783.
37. **Shields, M. S., S. O. Montgomery, P. J. Chapman, S. M. Cuskey, and P. H. Pritchard.** 1989. Novel pathway of toluene catabolism in the trichloroethylene-degrading bacterium G4. Appl. Environ. Microbiol. **55:**1624–1629.
38. **Taira, K., J. Hirose, S. Hayashida, and K. Furukawa.** 1992. Analysis of *bph* operon from the polychlorinated biphenyl-degrading strain of *Pseudomonas*

pseudoalcaligenes KF707. J. Biol. Chem. **267:**4844–4853.
39. **Tschech, A., and N. Pfennig.** 1984. Growth yield increase linked to caffeate reduction in *Acetobacterium woodii*. Arch. Microbiol. **137:**163–167.
40. **Tsien, H.-C., G. A. Brusseau, R. S. Hanson, and L. P. Wackett.** 1989. Biodegradation of trichloroethylene by *Methylosinus trichosporium* OB3b. Appl. Environ. Microbiol. **55:**3155–3161.
41. **Wackett, L. P., G. A. Brusseau, S. R. Householder, and R. S. Hanson.** 1989. Survey of microbial oxygenases: trichloroethylene degradation by propane-oxidizing bacteria. Appl. Environ. Microbiol. **55:**2960–2964.
42. **Wackett, L. P., and D. T. Gibson.** 1988. Degradation of trichloroethylene by toluene dioxygenase in whole-cell studies with *Pseudomonas putida* F1. Appl. Environ. Microbiol. **54:**1703–1708.
43. **Wang, Y., M. Rawlings, D. T. Gibson, D. Labbe, H. Bergeron, R. Brousseau, and P. C. K. Lau.** 1995. Identification of a membrane protein and a truncated LysR-type regulator associated with the toluene degradation pathway in *Pseudomonas putida* F1. Mol. Gen. Genet. **246:**570–579.
44. **Winter, R. B., K.-M. Yen, and B. D. Ensley.** 1989. Efficient degradation of trichloroethylene by a recombinant *Escherichia coli.* Bio/Technology **7:**282–285.
45. **Zylstra, G. J., and D. T. Gibson.** 1989. Toluene degradation by *Pseudomonas putida* F1. Nucleotide sequence of the *todC1C2BADE* genes and their expression in *Escherichia coli*. J. Biol. Chem. **264:**14940–14946.
46. **Zylstra, G. J., P. L. Wackett, and D. T. Gibson.** 1989. Trichloroethylene degradation by *Escherichia coli* containing the cloned *Pseudomonas putida* F1 toluene dioxygenase genes. Appl. Environ. Microbiol. **55:**3162–3166.