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Identification of a Novel Dioxygenase Involved in Metabolism of *o*-Xylene, Toluene, and Ethylbenzene by *Rhodococcus* sp. Strain DK17

Dockyu Kim,[1]† Jong-Chan Chae,[2] Gerben J. Zylstra,[2] Young-Soo Kim,[3] Seong-Ki Kim,[3] Myung Hee Nam,[4] Young Min Kim,[1] and Eungbin Kim[1]*

Department of Biology and Institute of Life Science and Biotechnology, Yonsei University,[1] Department of Life Science, Chung-Ang University,[3] and Korea Basic Science Institute,[4] Seoul, Korea, and Biotechnology Center for Agriculture and the Environment, Cook College, Rutgers University, New Brunswick, New Jersey[2]

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Rhodococcus **sp. strain DK17 is able to grow on *o*-xylene, benzene, toluene, and ethylbenzene. DK17 harbors at least two megaplasmids, and the genes encoding the initial steps in alkylbenzene metabolism are present on the 330-kb pDK2. The genes encoding alkylbenzene degradation were cloned in a cosmid clone and sequenced completely to reveal 35 open reading frames (ORFs). Among the ORFs, we identified two nearly exact copies (one base difference) of genes encoding large and small subunits of an iron sulfur protein terminal oxygenase that are 6 kb apart from each other. Immediately downstream of one copy of the dioxygenase genes (*$akbA1_a$* and *$akbA2_a$*) is a gene encoding a dioxygenase ferredoxin component (*akbA3*), and downstream of the other copy (*$akbA1_b$* and *$akbA2_b$*) are genes putatively encoding a *meta*-cleavage pathway. RT-PCR experiments show that the two copies of the dioxygenase genes are operonic with the downstream putative catabolic genes and that both operons are induced by *o*-xylene. When expressed in *Escherichia coli*, $AkbA1_a$-$AkbA2_a$-AkbA3 transformed *o*-xylene into 2,3- and 3,4-dimethylphenol. These were apparently derived from an unstable *o*-xylene *cis*-3,4-dihydrodiol, which readily dehydrates. This indicates a single point of attack of the dioxygenase on the aromatic ring. In contrast, attack of $AkbA1_a$-$AkbA2_a$-AkbA3 on ethylbenzene resulted in the formation of two different *cis*-dihydrodiols resulting from an oxidation at the 2,3 and the 3,4 positions on the aromatic ring, respectively.**

Members of the genus *Rhodococcus* demonstrate a remarkable ability to utilize a wide variety of natural organic and xenobiotic compounds, including aliphatic, aromatic, and alicyclic hydrocarbons (references 7 and 28; see also the special issue of Antonie Van Leeuwenhoek, volume 74). Besides the ability to degrade a broad spectrum of chemical compounds, many rhodococcal strains are known to catalyze the stereoselective oxidation of structurally different compounds such as indene (25), monoterpene (26), aliphatic alkenes (23), and phenylpropionitrile (10). Accordingly, rhodococci have the great potential to synthesize valuable chemical synthons, and of particular interest is the incorporation of molecular oxygen into the aromatic nucleus to form vicinal arene *cis*-diols (5, 19). For example, an aromatic dioxygenase from *Rhodococcus* sp. strain I24 was used for the oxidation of indene to *cis*-(1*S*,2*R*)-dihydroxyindan, which could serve as a precursor for indinavir, a new anti-human immunodeficiency virus drug (25).

To date, several gene clusters involved in the degradation of aromatic compounds have been cloned from *Rhodococcus* spp. These include degradative genes for biphenyl from *Rhodococcus* sp. strain M5 (27), *Rhodococcus globerulus* P6 (3), and *Rhodococcus* sp. strain RHA1 (21); isopropylbenzene-degrading genes from *Rhodococcus erythropolis* BD2 (14); and benzoate dioxygenase genes from *Rhodococcus* sp. strain 19070 (11). However, no in-depth genetic work has been reported regarding the abilities of *Rhodococcus* strains to degrade *o*-xylene.

Rhodococcus sp. strain DK17 was originally isolated in Yeochon, Korea, for the ability to grow on *o*-xylene and was found to also have the capability to grow on benzene, toluene, ethylbenzene, isopropylbenzene, and *n*-propyl- to *n*-hexylbenzenes (16). The degradation of *o*-xylene and toluene in *Rhodococcus* sp. strain DK17 is initiated by a ring-oxidizing dioxygenase pathway through 3,4-dimethylcatechol and 3- and 4-methylcatechol, respectively (16). This indicates that the *o*-xylene dioxygenase in strain DK17 can perform unique regioselective hydroxylations depending on the position of the substituent groups on the aromatic ring. This hypothesis is further supported by the finding that the cells of DK17 grown on *o*-xylene have the ability to oxidize *m*- and *p*-xylene to 2,4-dimethylresorcinol and 2,5-dimethylhydroquinone, respectively, although DK17 does not have the capability to grow on the other two xylene isomers (15). We also reported that the genes encoding the initial steps in this alkylbenzene pathway are present on the 330-kb megaplasmid pDK2 (15, 16). Thus, the present work was initiated to identify the genes for the initial ring hydroxylation dioxygenase enzyme and determine whether a single oxygenase is capable of the different regio-

* Corresponding author. Mailing address: Department of Biology, Yonsei University, Seoul 120-749, Korea. Phone: 82-2-2123-2651. Fax: 82-2-312-5657. E-mail: eungbin@yonsei.ac.kr.

† Present address: Microbial Resources Bank, Microbial Genomics and Applications Center, Korea Research Institute of Bioscience and Biotechnology, Taejon 305-333, Korea.

specific hydroxylation reactions observed with *Rhodococcus* sp. strain DK17.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Rhodococcus* sp. strain DK17 is the wild-type strain capable of growth on alkylbenzenes. *Rhodococcus* sp. strains DK176 and DK180, derived from DK17, are unable to grow on alkylbenzenes due to the loss of a 330-kb plasmid (pDK2) and *meta*-cleavage enzyme activity, respectively (16). *Escherichia coli* EPI100 [F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) f80d*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ(*ara*, *leu*)7697 *galU galK* λ$^-$ *rpsL nupG*] (Epicentre, Madison, Wis.) was used as the host strain for the cosmid library construction. *Escherichia coli* TOP10 [F$^-$ *mcr*A Δ(*mrr-hsdRMS-mcrBC*) Φ80*lacZ*ΔM15 Δ*lacX74 recA1 deoR araD139* Δ(*ara-leu*)7697*galU galK rpsL* (Str^r) *endA1 nupG*] (Invitrogen, Carlsbad, Calif.) was used as the host for the small-insert library construction.

Rhodococcus sp. strains were grown on mineral salts basal medium (24) containing 20 mM glucose at 30°C. *E. coli* strains for library construction or cloning were grown on Luria-Bertani (LB) medium at 37°C.

Preparation of cell extracts and SDS-PAGE. Bacterial cells reaching the exponential phase on 20 mM glucose were harvested and resuspended in 200 ml of fresh mineral salts basal medium containing 5 mM glucose. To induce the alkylbenzene degradative genes, *o*-xylene was added directly to the suspension at a final concentration of 0.1% (vol/vol) and further incubated at 30°C for 12 h. Cell extracts for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were prepared as described previously (19). SDS-PAGE was performed on 12.5% acrylamide gel in a Hoefer Mighty Small SE245 electrophoresis cell (Amersham Biosciences, Little Chalfont, England). The separated proteins were transferred to a polyvinylidene difluoride membrane. The appropriate Coomassie-stained protein band was excised from the polyvinylidene difluoride membrane and installed into the blot cartridge of a Procise cLC 492 protein sequencer (Applied Biosystems, Foster City, Calif.) for N-terminal sequence analysis.

DNA manipulation. Total DNA from *Rhodococcus* sp. strain DK17 was prepared according to the method of Asturias and Timmis (3). Plasmid DNA was purified by a plasmid spin kit (Genenmed, Daejeon, Korea). Agarose gel electrophoresis was performed in Tris-acetate-EDTA buffer. Transfer of DNA from agarose gels to Hybond-N+ membranes (Amersham Biosciences) was carried out using a TurboBlotter transfer system as recommended by the supplier (Schleicher & Schuell, Dassel, Germany). PCR DNA products to be used as probes in colony or Southern blotting experiments were separated by gel electrophoresis and eluted from agarose gels with a gel extraction kit (Genenmed). Colony and Southern hybridizations were performed as recommended by the supplier of a DIG nonradioactive nucleic acid labeling and detection system (Boehringer Mannheim, Mannheim, Germany).

Genomic and small insert DNA library construction and nucleotide sequencing. A total genomic DNA library was constructed using a pWEB::TNC cosmid cloning kit (Epicentre), as described by the manufacturer. The selected cosmid clone was mechanically sheared with a HydroShear DNA shearing device (Genemachines, San Carlos, Calif.) for the generation of random DNA fragments. Insert fragments were converted to blunt-end DNA with an End-It DNA-Repair kit (Epicentre) and ligated into the vector pCR-Blunt (Invitrogen). The ligation mixture was transformed into TOP10 chemically competent cells (Invitrogen).

An ABI PRISM BigDye Terminator cycle sequencing kit was used to carry out cycle sequencing reactions as recommended by the manufacturer (Applied Biosystems). The cycle sequencing reactions were analyzed using a model 3100 automated DNA sequencer (Applied Biosystems). Custom primers were used for filling in gaps in the assembled sequence. DNA fragments were completely sequenced on both strands. Nucleotide sequences were assembled with DNAstar Lasergene software (DNAstar, Madison, Wis.) and analyzed using BLAST software against the GenBank database (1).

PCR and cloning procedures. PCR amplification was carried out in a PTC-150 MiniCycler (MJ Research, Watertown, Mass.). Custom primers were supplied by Cosmo Genetech (Seoul, Korea). The PCR was performed in 20 μl of a reaction mixture containing approximately 100 ng of template DNA and 10 pmol of each primer with ReadyMix *Taq* PCR mix (Sigma, St. Louis, Mo.) according to instructions of the manufacturer. The *akbA1*$_a$, *akbA2*$_a$, and *akbA3* genes were amplified by PCR (forward primer, 5′-ATGGAGTGGAGCATGTTG-3′; reverse primer, 5′-TCATTGAGACTCGGCGCC-3′) and cloned using a pCR T7 TOPO TA expression kit (Invitrogen) according to the manufacturer's instructions. The thermal cycling program was a 10-min hot start (95°C), 30 cycles of 30 s of denaturation (95°C), 30 s of annealing (55°C), and 1 min of extension (72°C), and a final 10 min of extension (72°C).

The reverse transcription-PCRs (RT-PCRs) were performed in 25 μl of a reaction mixture with 70 ng of total RNA and 25 pmol of each primer with OneStep RT-PCR enzyme mix (QIAGEN, Hilden, Germany). The thermocycler program used for the RT-PCRs was as follows: 50°C for 30 min, 95°C for 15 min, 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, and 72°C for 10 min. The following primers were designed: to amplify two adjacent *akb* genes, *akbA1*$_b$-*akbA2*$_b$ and *akbA1*$_a$-*akbA2*$_a$, 5′-ATGGAGTGGAGCATGTTG-3′ (forward) and 5′-TCAGAGGAAGATGTTGAG-3′(reverse); to amplify *akbA2*$_b$ and *akbC*, 5′-ATGACATCGACCGCGGCG-3′ (forward) and 5′-TTATGCGGGGATGTCG AG-3′ (reverse); to amplify *akbCD*, 5′-ATGGCAAAAGTGACCGAA-3′ (forward) and 5′-CTATGCCGCGCGGAAATG-3′ (reverse); to amplify *akbDE*, 5′-ATGGCGAAGACTGTCGAA-3′ (forward) and 5′-CTAACCGAAACGAA ATGA-3′ (reverse); to amplify *akbEF*, 5′-ATGCTTGACGAACAGACG-3′ (forward) and 5′-TCACGAATACGCCACCTG-3′ (reverse); and to amplify *akbA2aA3*, 5′-ATGACATCGACCGCGGCG-3′ (forward) and 5′-TCATTGAG ACTCGGCGCC-3′ (reverse).

Analysis and identification of *o*-xylene/ethylbenzene metabolites. A preculture of *E. coli* BL21(DE3) containing the *akbA1*$_a$, *akbA2*$_a$, and *akbA3* genes was prepared by inoculating one colony into 50 ml of LB medium supplemented with ampicillin (100 μg/ml) and incubating overnight at 37°C. A total of 4 ml of the overnight culture was transferred to 200 ml of LB medium and incubated under the same conditions. The culture was induced by adding IPTG (isopropyl-β-D-thiogalactopyranoside) to achieve a concentration of 1 mM, when bacterial cells reached an optical density at 600 nm of 0.5 to 0.7, and further incubated for 2 h. Subsequently, the culture was harvested by centrifugation at 10,000 × *g* for 15 min, washed with 50 mM potassium phosphate buffer (pH 7.4), and resuspended in 30 ml of the same solution supplemented with 20 mM glucose and ampicillin. *o*-Xylene and ethylbenzene were individually provided in the vapor phase, and the cells were incubated at 30°C for 13 h. The supernatant was extracted with ethyl acetate and dried by a rotary evaporator, and the dried residues were acetylated for stabilization as described previously (16). *o*-Xylene or ethylbenzene metabolites formed by the *meta*-cleavage dioxygenase mutant strain DK180 were prepared as described previously (16).

Gas chromatography-mass spectrometry (GC-MS) analysis of metabolites was carried out with a Hewlett-Packard 5973 mass spectrometer (electron impact ionization, 70 eV) connected to a 6890 gas chromatogram fitted with a fused silica capillary column (HP-5) (0.25 by 30 m; 0.25 μm film thickness). The following conditions were used for the GC: 1 ml of He/min; on-column injection mode; oven temperature, 60°C for 2 min; thermal gradient, 5°C/min to 220°C and then held at 220°C.

Chemicals. Aromatic compounds used in this study were obtained from Sigma-Aldrich Korea (Seoul, Korea). (+)-*cis*-3-Ethyl-3,5-cyclohexadiene-1,2-diol (*cis*-2,3-ethylbenzenedihydrodiol) and 3-ethylcatechol were prepared from ethylbenzene by use of *Pseudomonas putida* 39/D (8) and *P. putida* PpF107 (30), respectively, following published procedures. All solvents were purchased from Mallinckrodt Baker, Inc. (Phillipsburg, New Jersey). All chemicals were analytical-grade purity or above.

Nucleotide sequence accession numbers. The nucleotide sequences determined in this study have been deposited in the GenBank database under accession number AY502075.

RESULTS

Cloning the genes for *o*-xylene degradation. *Rhodococcus* sp. strain DK17 harbors a large catabolic megaplasmid, designated pDK2, carrying the genes for *o*-xylene degradation (16). Plasmid-cured derivatives such as DK176 lose the ability to grow not only on *o*-xylene but also on benzene, toluene, and ethylbenzene. To identify enzymes involved in *o*-xylene degradation, the total cellular protein patterns were compared between DK17 and DK176 following growth on glucose and *o*-xylene. Several new and very obvious protein bands were present in the DK17 extract that were not present in the DK176 extract when the soluble cell extracts were separated on one-dimensional SDS-PAGE (data not shown). These proteins appear to be encoded by the plasmid pDK2 and are specifically induced upon exposure of DK17 to *o*-xylene. Many oxygenase components of ring hydroxylation dioxygenase enzymes are comprised of a large and small subunit of approximately 50 and

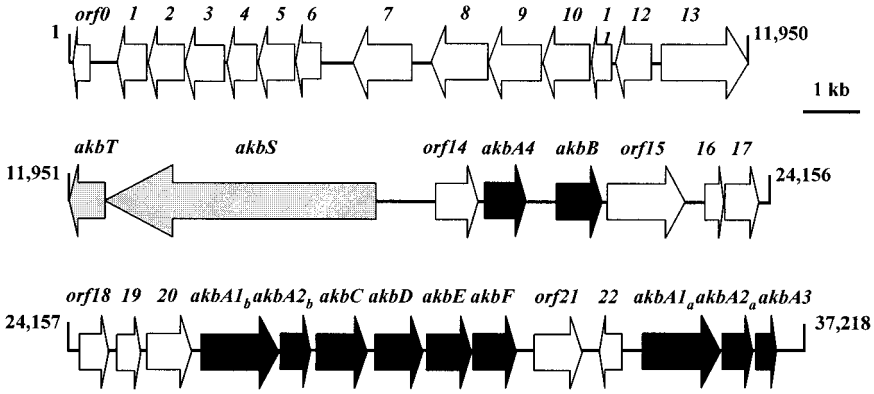


FIG. 1. Gene organization of a 37,218-bp region from *Rhodococcus* sp. strain DK17. Black arrows indicate structural genes for alkylbenzene metabolism. Two-component regulatory genes are shown as gray-shaded arrows. Genes for miscellaneous or unknown functions are shown as white arrows. The directions of transcription are indicated by arrowheads. The nucleotide numbers are marked on the beginning and the end of each line.

25 kDa (12). The N-terminal amino acid sequence of a protein band with an approximate size of 50 kDa, which is differentially produced by DK17 in comparison to DK176, was determined to be MLRSERFSPGEDFGQ. A search of the GenBank database revealed that this 15-amino-acid N-terminal sequence is identical to the N-terminal sequence of the large subunit of a ring hydroxylation dioxygenase from the polychlorinated biphenyl-degrading *Rhodococcus* sp. strain RHA1 (17).

Since the N-terminal sequence of the DK17 *o*-xylene oxygenase matched that of an oxygenase in RHA1 it is likely that the genes share significant identity as well. In their initial analysis of genes encoding oxygenases in RHA1, Kitagawa et al. (17) described a set of slightly degenerate primers designed to amplify oxygenase genes. Application of the primers (forward, 5′-TGCASSTWTCACGGSTGG-3′; reverse, 5′-CTCG ACTCCGAGCTTCCAGTT-3′) in a PCR with total genomic DNA from DK17 amplified a ~300-bp fragment, as expected from a potential ring hydroxylation oxygenase gene target. With the PCR fragment used as a probe, 1,000 cosmid colonies were screened, with only one clone, designated pKEB2002, showing a strong hybridization signal.

A Southern hybridization experiment was performed with the cosmid clone pKEB2002 as the probe for PFGE-separated total genomic DNA from the wild-type strain DK17 and the pDK2-minus strain DK176. The probe hybridized to pDK2 in the DK17 lane, while no hybridization was seen for the plasmid-minus strain DK176. This observation confirms that the insert in pKEB2002 is derived from the 330-kb megaplasmid pDK2 as expected, since the genes for *o*-xylene degradation were previously located on pDK2 (16).

Identification and *o*-xylene-mediated induction of the degradative genes. The entire pKEB2002 cosmid clone containing the gene encoding an *o*-xylene dioxygenase oxygenase component was sequenced as described in the Materials and Methods. Analysis of the 37,218-bp sequenced region (Fig. 1) identified two genes (designated *akbA1*$_a$ and *akbA1*$_b$, approximately 6 kb apart from each other) encoding an oxygenase component large subunit with a deduced N-terminal sequence identical to the sequence of 15 N-terminal amino acids determined for the putative *o*-xylene oxygenase component large subunit described above. Immediately following each of the two genes encoding a large subunit are genes (designated *akbA2*$_a$ and *akbA2*$_b$) encoding a small subunit for an oxygenase component. It is interesting that the 1,963-bp *akbA1*$_a$-*akbA2*$_a$ and *akbA1*$_b$-*akbA2*$_b$ gene regions are identical to each other except for one base pair. The region of identity starts 18 bp in front of the two *akbA1* genes (just in front of the ribosome binding site) and continues to 3 bp after the two *akbA2* stop codons. The single base difference (T versus C) between the two regions does not affect the amino acid sequence of the encoded AkbA1$_a$ and AkbA2$_a$ proteins, as *akbA1*$_a$ has an ATT Ile codon for the 32nd amino acid in the protein whereas *akbA1*$_b$ has an ATC Ile codon. Also, deduced amino acid sequence alignment of AkbA1$_a$ and AkbA1$_b$ with the large subunits of different Rieske dioxygenases reveals the presence of motifs for a Rieske-type (2Fe-2S) iron-sulfur center and one mononuclear nonheme iron (12, 20).

In addition to the duplicated *akbA1A2* genes, other genes putatively involved in *o*-xylene degradation can be identified in the sequence of the pKEB2002 cosmid clone. The *akbA1*$_a$ and *akbA1*$_b$ genes on the 3′ edge of the cosmid clone are followed by a gene, designated *akbA3*, encoding a ferredoxin component of a multicomponent dioxygenase. The 5′ end of a gene encoding a putative *meta*-cleavage product hydrolase is at the end of the sequenced region. On the other hand, the *akbA1*$_b$ and *akbA2*$_b$ genes are followed by genes, designated *akbCDEF*, putatively encoding the proteins for a complete *meta*-cleavage pathway, namely, a *meta*-cleavage dioxygenase (AkbC), a *meta*-cleavage hydrolase product (AkbD), a hydratase (AkbE), and an aldolase (AkbF). More than 5 kb upstream of the *akbA1*$_a$ and *akbA1*$_b$ genes are two genes, designated *akbA4* and *akbB*, encoding a putative reductase component of a dioxygenase and a *cis*-dihydrodiol dehydrogenase. Similarities between the open reading frames (ORFs) and representative homologs are summarized in Table 1.

To confirm that the identified *akb* genes are actually expressed in response to *o*-xylene and to confirm the operonic nature of the *akb* genes, RT-PCR experiments were performed with total RNA extracted from the *o*-xylene-induced cells of DK17 as described in Materials and Methods. The PCR primers were designed to generate PCR products as follows:

TABLE 1. Predicted ORFs identified on a 37,218 base pair region from the 330-kb megaplasmid pDK2

ORF	Start-stop codons	Representative homolog (gene)	Identity (%)[a]	Organism	NCBI protein database access no.[b]
orf0	312–7	Hypothetical protein	No match		
orf1	1,285–761	Outer-membrane protein (*orf6*)	44	*Rhodococcus* sp. NCIMB12038	AAQ98851
orf2	1,985–1,338	Transmembrane protein (*orf5*)	87	*Rhodococcus* sp. NCIMB12038	AAQ98850
orf3	2,689–1,982	Transmembrane protein (*orf4*)	86	*Rhodococcus* sp. NCIMB12038	AAQ98849
orf4	3,271–2,738	Unknown protein (*orf3*)	76	*Rhodococcus* sp. NCIMB12038	AAQ98848
orf5	3,935–3,282	Unknown protein (*orf2*)	83	*Rhodococcus* sp. NCIMB12038	AAQ98847
orf6	4,387–3,932	Unknown protein (*orf1*)	88	*Rhodococcus* sp. NCIMB12038	AAQ98846
orf7	5,982–4,936	Hypothetical protein	No match		
orf8	7,313–6,303	4-Hydroxy-2-oxovalerate aldolase (*xylK*)	61	*Novosphingobium aromaticivorans* F199	AAD03993
orf9	8,257–7,310	Acetaldehyde dehydrogenase (*cmtH*)	67	*Pseudomonas putida* F1	2209341L
orf10	9,102–8,254	2-Hydroxypenta-2,4-dienoate hydratase(*tobG*)	54	*Pseudomonas putida* PB4071	AAG09414
orf11	9,475–9,110	Hypothetical protein	No match		
orf12	10,171–9,527	Putative enoyl-CoA-hydratase	92	*Rhodococcus erythropolis* BD2	AAP74050
orf13	10,385–11,923	Putative medium-chain acyl-CoA ligase (*alkK*)	99	*Rhodococcus erythropolis* BD2	AAP74049
akbT	12,580–11,951	Response regulator (*bphT*)	96	*Rhodococcus* sp. RHA1	BAC75412
akbS	17,367–12,577	Sensor kinase (*bphS*)	83	*Rhodococcus* sp. RHA1	BAC75411
orf14	18,396–19,154	Hypothetical protein	No match		
akbA4	19,271–20,500	Ferredoxin reductase (*etbA4*)	100	*Rhodococcus* sp. RHA1	BAD03966
akbB	20,533–21,345	Dihydrodiol dehydrogenase (*bphB2*)	100	*Rhodococcus* sp. RHA1	BAD03967
orf15	21,453–22,826	6-Oxohexanoate dehydrogenase (*chnE*)	62	*Rhodococcus* sp. Phi2	AAN37492
orf16	23,213–23,548	Hypothetical protein	No match		
orf17	23,566–24,156	Hypothetical protein	No match		
orf18	24,348–24,860	Hypothetical protein	No match		
orf19	24,995–25,414	Hypothetical protein	No match		
orf20	26,307–25,510	Hypothetical protein	No match		
$akbA1_b$	26,466–27,848	Ethylbenzene dioxygenase large subunit (*etbA1*)	100	*Rhodococcus* sp. RHA1	BAC92712
$akbA2_b$	27,871–28,419	Ethylbenzene dioxygenase small subunit (*etbA2*)	100	*Rhodococcus* sp. RHA1	BAC92713
akbC	28,479–29,396	2,3-Dihydroxybiphenyl 1,2-dioxygenase (*etbC*)	100	*Rhodococcus* sp. RHA1	BAC92714
akbD	29,517–30,374	2-Hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase (*bphD*)	100	*Rhodococcus* sp. RHA1	BAC92715
akbE	30,425–31,228	2-Hydroxypenta-2,4-dienoate hydratase (*bphE2*)	100	*Rhodococcus* sp. RHA1	BAC92716
akbF	31,240–32,016	4-Hydroxy-2-oxovalerate aldolase (*bphF2*)	100	*Rhodococcus* sp. RHA1	BAC92717
orf21	32,562–33,413	Putative N5,N10-methylenetetrahydromethanopterin reductase-related protein	55	*Streptomyces avermitilis* MA-4680	BAC75195
orf22	34,129–33,722	Hypothetical protein	No match		
akbA1a	34,461–35,843	Ethylbenzene dioxygenase large subunit (*ebdA1*)	100	*Rhodococcus* sp. RHA1	BAC92718
akbA2a	35,866–36,414	Ethylbenzene dioxygenase small subunit (*ebdA2*)	100	*Rhodococcus* sp. RHA1	BAC92719
akbA3	36,444–36,809	Ethylbenzene dioxygenase ferredoxin (*ebdA3*)	100	*Rhodococcus* sp. RHA1	BAC92720

[a] Percentages of identity were obtained by aligning the deduced amino acid sequences by use of Blastp.
[b] NCBI, National Center for Biotechnology Information.

$akbA1_a$ to $akbA2_a$ and $akbA1_b$ to $akbA2_b$, 1,954 bp; $akbA2_b$ to *akbC*, 1,526 bp; *akbC* to *akbD,* 1,896 bp; *akbD* to *akbE,* 1,712 bp; *akbE* to *akbF,* 1,592 bp; and $akbA2_a$ to *akbA3,* 944 bp. Whereas PCR without RT did not show any PCR product, the RT-PCR showed PCR products of the expected size (Fig. 2). These data show that the $akbA1_a$, $akbA2_a$, and *akbA3* genes and $akbA2_a$, $akbA2_b$, and *akbCDEF* genes are transcribed as operons. In addition, since no RT-PCR product was detectable for any of the *akb* genes following growth of DK17 on glucose (data not shown), identified *akb* genes are specifically induced by growth on *o*-xylene.

Heterologous expression of $akbA1_a$, $akbA2_a$, and *akbA3*. The protein production and gene expression data indicate that the *akbA1A2* genes encode a corresponding putative oxygenase large subunit produced in response to growth of *Rhodococcus* sp. strain DK17 on *o*-xylene. To functionally confirm the role of this putative oxygenase in alkylbenzene degradation, the $akbA1_a$, $akbA2_a$, and *akbA3* genes were cloned and expressed in *E. coli*. Dioxygenase enzymes often require a short electron transfer chain to shuttle electrons from NAD(P)H to the oxygenase component that performs the catalytic reaction (20, 29). In many cases of heterologous expression of genes for dioxygenases in *E. coli*, the native reductase component may be left out, as *E. coli* reductases may substitute for them (11, 18). This being the case, the contiguous $akbA1_a$, $akbA2_a$, and *akbA3* genes encoding the oxygenase and ferredoxin components were PCR amplified and cloned into the expression vector pCRT7/CT-TOPO to construct the recombinant plasmid pKEB051.

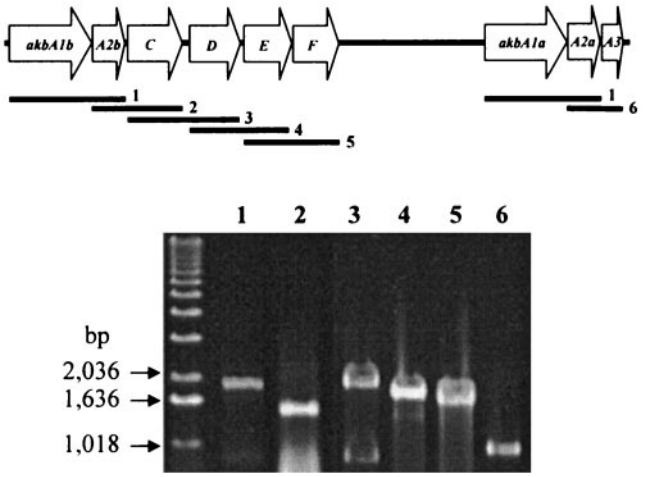


FIG. 2. Operonic nature of the *akb* genes in *Rhodococcus* sp. strain DK17 and agarose gel electrophoresis of RT-PCR products. The expected PCR products for each well are indicated in the gene map. The first lane was loaded with a molecular weight marker.

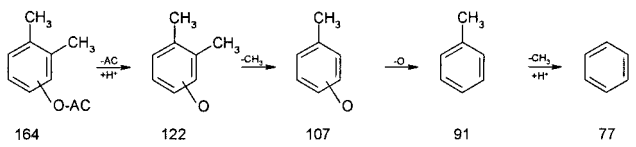
The resting induced cells of *E. coli* BL21(DE3) harboring pKEB051 were incubated in the presence of *o*-xylene to allow for conversion of *o*-xylene to an oxidized product by the expressed *o*-xylene oxygenase. The potential oxidized products were extracted and stabilized by acetylation for GC-MS analysis. Two peaks were detected at 14.02 min (*o*-xylene metabolite I) and 14.71 min (*o*-xylene metabolite II) on the total ion chromatogram. Both metabolites have the same molecular ion at *m*/*z* 164 and a prominent ion due to fission of acetate at *m*/*z* 122 (Table 2). Since this result indicates that the original metabolites are monohydroxylated *o*-xylenes, the acetylated derivatives of authentic 2,3- and 3,4-dimethylphenol were analyzed for comparison and found to have the same mass spectra and GC retention times as those of *o*-xylene metabolites I and II, respectively. As determined on the basis of previous work with DK17 (16), the expected product of the *o*-xylene dioxygenase biotransformation is *o*-xylene *cis*-3,4-dihydrodiol. However, this compound is likely to be unstable due to the electron-donating nature of the two adjacent methyl groups and would readily dehydrate to 2,3- and 3,4-dimethylphenol (3- and 4-hydroxy-*o*-xylene, respectively).

Previous work with DK17 implicated the *o*-xylene catabolic pathway in the degradation of a number of other alkylbenzenes (15, 16). This being the case, the ability of *o*-xylene dioxygenase to oxidize other alkyl-substituted benzenes was tested. Ethylbenzene was chosen as an alternative substrate due to its representative nature of alkylbenzenes in general, the availability of *cis*-dihydrodiol standards, and the fact that growth of DK17 on ethylbenzene is comparable to that on *o*-xylene. Following incubation of induced BL21(DE3)(pKEB051) resting cells in the presence of ethylbenzene, the extracted metabolites were acetylated and analyzed by GC-MS. Two peaks for ethylbenzene metabolites are seen at 18.77 min (major) and 19.00 min (minor) on the total ion chromatogram which have the same molecular ion at *m*/*z* 224 (Table 3). This suggests that the metabolites have two additional hydroxyls compared to ethylbenzene and thus are most likely *cis*-dihydrodiols. Two possible *cis*-dihydrodiols could be synthesized from ethylbenzene: *cis*-2,3 and *cis*-3,4. Authentic acetylated *cis*-2,3-ethylbenzene dihydrodiol shows an retention time (18.77 min) and mass spectrum identical to those of the major metabolite formed from ethylbenzene by DK17 *o*-xylene dioxygenase. Since the minor product at 19.00 min has a molecular weight the same as and a mass spectrum similar to those of *cis*-2,3-ethylbenzene dihydrodiol, it is most likely *cis*-3,4-ethylbenzene dihydrodiol.

To corroborate the above assessment, the mutant strain *Rhodococcus* sp. strain DK180 was chosen for additional biotransformation experiments. This mutant is blocked in the *meta*-cleavage step and accumulates 3,4-dimethylcatechol from *o*-xylene and both 3- and 4-methylcatechol from toluene (16). DK180 was grown on glucose in the presence of ethylbenzene, the culture supernatant extracted with ethyl acetate and potential metabolites derivatized with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide. Analysis by capillary GC-MS revealed two peaks for ethylbenzene metabolites at 19.80 min (major) and 19.92 min (minor) on the total ion chromatogram. GC-MS comparison with authentic standards of 3- and 4-ethylcatechol reveal that the major metabolite at 19.80 min is 3-ethylcatechol whereas the minor metabolite at 19.92 min is 4-ethylcatechol. These data suggest that the minor *cis*-ethylbenzene dihydrodiol metabolite produced from ethylbenzene by the *o*-xylene oxygenase expressed in *E. coli* is most likely *cis*-3,4-ethylbenzene dihydrodiol.

TABLE 2. GC-MS data for 2,3- and 3,4-dimethylphenol and *o*-xylene metabolites formed by *E. coli* carrying pKEB051 and for the fragmentation patterns of each metabolite

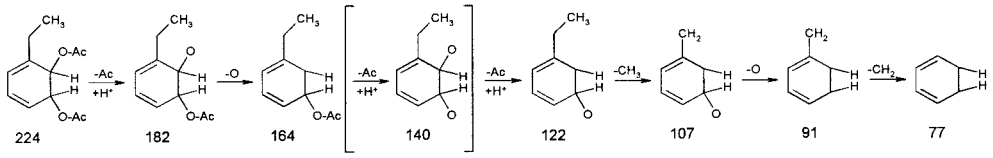
Compound[a]	Retention time on GC (min)	Prominent ions (*m*/*z*, % relative intensity)
o-Xylene metabolite I	14.02	164 (M^+, 17), 122 (100), 107 (59), 91 (18), 77 (21)
2,3-Dimethylphenol	14.02	164 (M^+, 19), 122 (100), 107 (61), 91 (13), 77 (14)
o-Xylene metabolite II	14.71	164 (M^+, 13), 122 (100), 107 (70), 91 (12), 77 (14)
3,4-Dimethylphenol	14.71	164 (M^+, 13), 122 (100), 107 (69), 91 (12), 77 (14)



[a] Each sample was analyzed as an acetatylated derivative.

TABLE 3. GC-MS data for *cis*-2,3-ethylbenzene dihydrodiol and ethylbenzene metabolites formed by *E. coli* carrying pKEB051 and for the fragmentation patterns of each metabolite

Compound[a]	Retention time on GC (min)	Prominent ions (*m/z*, % relative intensity)
cis-2,3-Ethylbenzene dihydrodiol	18.77	224 (M^+, 0.6), 182 (0.6), 164 (15), 140 (9), 122 (100), 107 (74), 91 (14), 77 (17)
Metabolite I	18.77	224 (M^+, 0.2), 182 (0.2), 164 (12), 140 (2), 122 (100), 107 (87), 91 (10), 77 (16)
Metabolite II	19.00	224 (M^+, 0.1), 182 (0.1), 164 (5), 140 (15), 122 (100), 107 (48), 91 (17), 77 (25)



[a] Each sample was analyzed as an acetatylated derivative.

DISCUSSION

The present study utilized a combination of whole-cell protein analysis, gene cloning and sequencing, and heterologous gene expression to identify the genes encoding a three- component (sulfur protein terminal oxygenase, ferredoxin, and reductase) *o*-xylene dioxygenase. Using the N-terminal sequence of an *o*-xylene-induced iron sulfur protein large subunit, we identified a cosmid clone containing two nearly exact copies (one base difference) of genes encoding large and small subunits of an iron sulfur protein terminal oxygenase 6 kb apart from each other. The *Rhodococcus* sp. strain DK17 genes encoding an *o*-xylene dioxygenase iron sulfur protein large subunit show a remarkable degree of identity with genes encoding a large subunit identified in *Rhodococcus* sp. strain RHA1, which are genes proposed to be involved in the degradation of biphenyl or ethylbenzene (17). Although no definitive function was assigned to the genes identified in strain RHA1, the high (over 99%) degree of identity to the genes encoding an *o*-xylene/alkylbenzene dioxygenase large subunit in DK17 suggests that the RHA1 oxygenase genes are involved in the degradation of alkylbenzenes. This hypothesis is also in good agreement with the fact that RHA1 grows on *o*-xylene as well as on toluene, isopropylbenzene, and ethylbenzene (17). In fact, inspection of the unfinished RHA1 genome sequence (http://www.bcgsc.ca/gc/rhodococcus) reveals a high degree of identity along the entire sequenced cosmid clone from DK17. One end of RHA1 contig 520 (107,903 bp) is over 99% identical to bases 1 to 11,401 of the DK17 sequence presented here, with the exception of a possible insertion sequence in the RHA1 sequence at position 7,486 in the DK17 sequence. RHA1 contig 399 (11,892 bp) is over 99% identical to bases 15,418 to 27,286 of the DK17 sequence. One end of RHA1 contig 479 (21,198 bp) is over 99% identical to bases 28,026 to 37,218 (the end of the cloned DK17 region) of the DK17 sequence. Since the cloned DK17 genes encoding the *o*-xylene catabolic pathway are located on a large catabolic plasmid (16), it is quite possible that this has promoted spread of the catabolic genes throughout the *Rhodococcus* population and thus shows up in the RHA1 genome sequence.

Since the focus of the present paper is on the identification of the genes for the initial dioxygenase iron sulfur protein, the genes (*akbA1*$_a$, *akbA2*$_a$, and *akbA3*) encoding the iron sulfur protein and ferredoxin components of *o*-xylene dioxygenase were expressed in *E. coli* to determine whether this enzyme is able to perform regioselective hydroxylations depending on the position of the substituent groups on the aromatic ring. With *o*-xylene as the substrate, two phenolic compounds were detected as products: 2,3- and 3,4-dimethylphenol (3- and 4-hydroxy-*o*-xylene). These mostly likely were derived from an initial *o*-xylene *cis*-3,4-dihydrodiol, a relatively unstable compound that would dehydrate readily. Also, a mutation in the *akbC* gene causing a nonsense codon at the 41st amino acid in the deduced protein was identified in the mutant strain DK180 by PCR amplification and sequencing (data not shown). DK180 was previously shown to be blocked at the *meta*-cleavage dioxygenase step for the degradation of a wide variety of alkyl-substituted hydrocarbons, including *o*-xylene, toluene, ethylbenzene, isopropylbenzene, and *n*-propyl- through *n*-hexylbenzene (16), implicating *akbC* as an essential enzyme in the degradation of alkylbenzenes by DK17. These results led us to postulate a dioxygenase-initiated pathway for *o*-xylene degradation in DK17 (Fig. 3). In fact, previous to the present

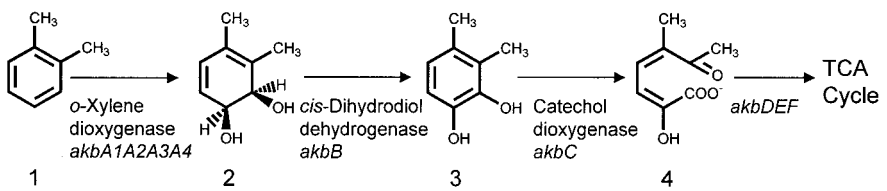


FIG. 3. Proposed pathway for early steps in the catabolism of *o*-xylene by *Rhodococcus* sp. strain DK17. The enzyme and gene designations for each step in the pathway are shown. The pathway intermediates are indicated as follows: compound 1, *o*-xylene; compound 2, 1,2-dihydroxy-3,4-dimethylcyclohexa-3,5-diene; compound 3, 3,4-dimethylcatechol; compound 4, 2-hydroxy-5-methyl-6-oxo-hepta-2,4-dienoate.

work, a monooxygenase-initiated pathway was predominantly known for *o*-xylene (2, 4, 6). Although it has been proposed that a *Nocardia* sp. strain (9) and *Rhodococcus* sp. strain C125 (22) metabolize *o*-xylene through an initial aromatic dioxygenase to form a *cis*-dihydrodiol, there is no direct evidence for the presence of a dioxygenase in either strain.

We previously observed that DK17 oxidized toluene at the 2,3- and 3,4 positions on the aromatic ring, resulting in two different *cis*-dihydrodiols and 3- and 4-methylcatechol, respectively (16). In this work we showed that the cloned *o*-xylene dioxygenase expressed in *E. coli* is capable of oxidizing ethylbenzene to 2,3- and 3,4-*cis*-dihydrodiols. Similar results were obtained with toluene as the substrate (data not shown). This confirms that a single dioxygenase is capable of hydroxylating aromatic compounds such as toluene and ethylbenzene at two different positions on the aromatic ring. These data suggest that *o*-xylene, with two alkyl side chains, fits into the active site of the enzyme only one way whereas substrates with only a single alkyl side chain can fit into the active site of the enzyme two different ways, allowing oxidation of the aromatic ring at two possible positions. The novel ability of the *o*-xylene dioxygenase to oxidize unusual positions on the aromatic ring of alkyl-substituted benzenes is also seen in the oxidization of *m*-xylene to 2,4-dimethylresorcinol and *p*-xylene to 2,5-dimethylhydroquinone (15). *Rhodococcus* sp. strain DK17 *o*-xylene dioxygenase thus catalyzes unique oxidations of aromatic compounds, producing products that are not seen in oxidations by other dioxygenases (13).

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