

# Multiple-Subunit Genes of the Aromatic-Ring-Hydroxylating Dioxygenase Play an Active Role in Biphenyl and Polychlorinated Biphenyl Degradation in *Rhodococcus* sp. Strain RHA1

Takumi Iwasaki, Keisuke Miyauchi, Eiji Masai, and Masao Fukuda\*

Department of Bioengineering, Nagaoka University of Technology, Nagaoka, Niigata 940-2118, Japan

Received 6 February 2006/Accepted 2 June 2006

**A gram-positive strong polychlorinated biphenyl (PCB) degrader, *Rhodococcus* sp. strain RHA1, can degrade PCBs by cometabolism with biphenyl or ethylbenzene. In RHA1, three sets of aromatic-ring-hydroxylating dioxygenase genes are induced by biphenyl. The large and small subunits of their terminal dioxygenase components are encoded by *bphA1* and *bphA2*, *etbA1* and *etbA2*, and *ebdA1* and *ebdA2*, respectively, and the deduced amino acid sequences of *etbA1* and *etbA2* are identical to those of *ebdA1* and *ebdA2*, respectively. In this study, we examined the involvement of the respective subunit genes in biphenyl/PCB degradation by RHA1. Reverse transcription-PCR and two-dimensional polyacrylamide gel electrophoresis analyses indicated the induction of RNA and protein products of *etbA1* and *ebdA1* by biphenyl. Single- and double-disruption mutants of *etbA1*, *ebdA1*, and *bphA1* were constructed by insertional inactivation. The 4-chlorobiphenyl (4-CB) degradation activities of all the mutants were lower than that of RHA1. The results indicated that all of these genes are involved in biphenyl/PCB degradation. Furthermore, we constructed disruption mutants of *ebdA3* and *bphA3*, encoding ferredoxin, and *etbA4*, encoding ferredoxin reductase components. The 4-CB degradation activities of these mutants were also lower than that of RHA1, suggesting that all of these genes play a role in biphenyl/PCB degradation. The substrate preferences of *etbA1A2/ebdA1A2*- and *bphA1A2*-encoded dioxygenases for PCB congeners were examined using the corresponding mutants. The results indicated that these dioxygenase isozymes have different substrate preferences and that the *etbA1A2/ebdA1A2*-encoded isozyme is more active on highly chlorinated congeners than the *bphA1A2*-encoded one.**

Polychlorinated biphenyls (PCBs) are synthetic compounds that have excellent chemical stability, insulation properties, and resistance to combustion, and they have been used in a wide range of industrial applications. However, since the revelation that PCBs have strong toxicity and are recalcitrant in the environment, environmental contamination by PCBs has become a serious worldwide problem. Degradation of PCBs by microorganisms has been regarded as an effective tool for removal of PCBs from the environment, and many kinds of bacteria which degrade PCBs aerobically have been isolated and characterized (2, 4, 7, 11, 14). These bacteria degrade PCBs by cometabolism, with PCBs being degraded via the biphenyl catabolic pathway in the presence of biphenyl (5, 10). In the initial step of the biphenyl catabolic pathway, biphenyl is transformed to 2,3-dihydroxy-1-phenylcyclohexa-4,6-diene (dihydrodiol compound) by an aromatic-ring-hydroxylating dioxygenase (RHDO), biphenyl 2,3-dioxygenase (BDO) (Fig. 1A), which consists of the large and small subunits of terminal oxygenase, ferredoxin, and ferredoxin reductase. BDO is a key enzyme that primarily determines the substrate specificity of PCB degradation.

*Rhodococcus* sp. strain RHA1 is a gram-positive strong PCB degrader. RHA1 can degrade mono- to octachlorobiphenyls by cometabolism with biphenyl (17). Various genes responsible for biphenyl and PCB degradation in RHA1 have been iso-

lated and characterized (8, 12, 13, 15, 19, 20). The *bphA1A2A3A4* genes encoding BDO were initially isolated from and characterized for RHA1 (13). Based on results from PCR amplification of RHDO genes using consensus primers and Northern hybridization analysis with the amplified fragments as probes, Kitagawa et al. indicated that three RHDO large-subunit genes are induced by biphenyl in RHA1 (9). These three genes are *bphA1* and two other genes. The latter two genes share an identical nucleotide sequence except for a single nucleotide, and their deduced amino acid sequences are identical. These genes were expected to be involved in the PCB degradation induced by ethylbenzene in RHA1 and RDA1, which is a *bphA1* insertion mutant of RHA1 (16), and they were designated *etbA1* and *ebdA1* (18). The nucleotide sequences of the flanking regions of these genes were determined, and it was revealed that the genes are included in the gene clusters shown in Fig. 1B. Open reading frames (ORFs) downstream of *etbA1* and *ebdA1*, which encode RHDO small subunits, were designated *etbA2* and *ebdA2*, respectively. Their nucleotide sequences are completely identical. Furthermore, an ORF which showed similarity to that for ferredoxin was found downstream from *ebdA1A2* and was designated *ebdA3*. The *ebdA1A2A3* cluster is located 6 kb downstream from *etbA1A2*. In the region located 6 kb upstream from *etbA1A2*, an ORF whose gene product exhibited 34.2% identity with that of *bphA4* was found and was designated *etbA4*. RHA1 has three linear plasmids, pRHL1 (1,100 kb), pRHL2 (450 kb), and pRHL3 (330 kb). The *bphA1A2A3A4* gene cluster is located on pRHL1, and the *etbA4*, *etbA1A2*, and *ebdA1A2A3* genes are located on pRHL2 (12, 18). Recently, Takeda et al.

\* Corresponding author. Mailing address: Department of Bioengineering, Nagaoka University of Technology, Kamitomioka, Nagaoka, Niigata 940-2188, Japan. Phone: 81-258-47-9405. Fax: 81-258-47-9450. E-mail: masao@vos.nagaokaut.ac.jp.

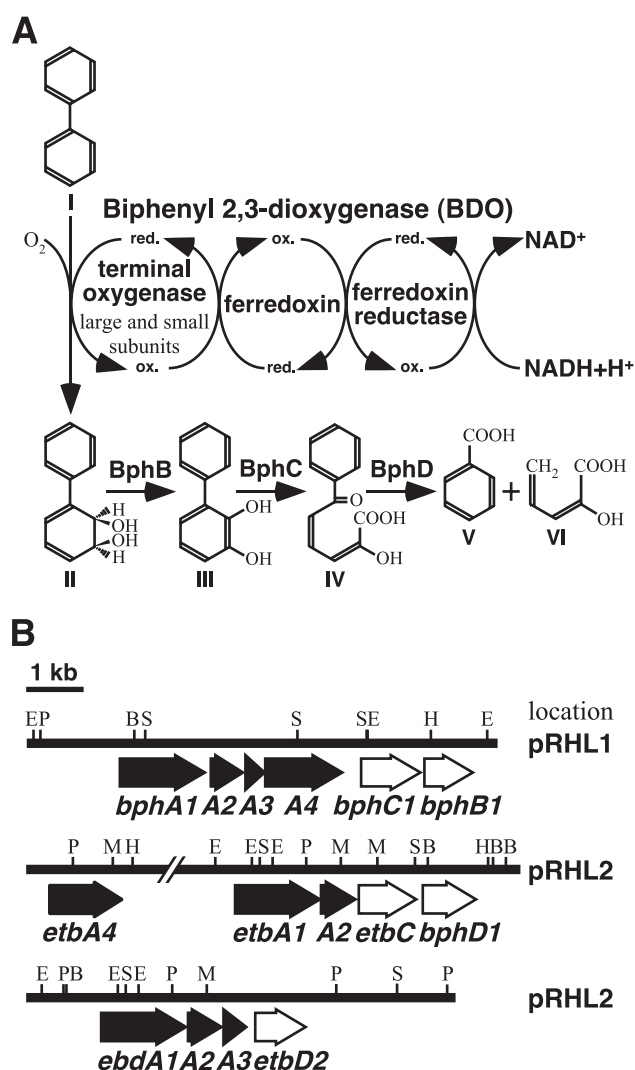


FIG. 1. Components and functions of BDO and proposed pathway for aerobic degradation of biphenyl (A) and organization of the *bph*, *etb*, and *ebd* genes (B) in *Rhodococcus* sp. strain RHA1. (A) The proposed electron transfer reactions and the conversion of biphenyl to benzoate and 2-hydroxypenta-2,4-dienoate are indicated. The BDO consists of three components, including a terminal oxygenase complex of large and small subunits, a ferredoxin, and a ferredoxin reductase. The complex catalyzes the dihydroxylation of biphenyl and is preferentially involved in the determination of substrate specificity. Ferredoxin and ferredoxin reductase promote electron transfer from NADH to the large- and small-subunit complex. Compounds: I, biphenyl; II, *cis*-(2*R*,3*S*)-dihydroxy-1-phenylcyclohexa-4,6-diene (dihydrodiol); III, 2,3-dihydroxybiphenyl (2,3-DHBP); IV, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate (*meta*-cleavage compound [HOPD]); V, benzoate; VI, 2-hydroxypenta-2,4-dienoate. Enzymes: BphB, dihydrodiol dehydrogenase; BphC, 2,3-DHBP 1,2-dioxygenase; BphD, HOPD hydrolase. (B) Arrows indicate ORFs. The filled arrows represent the ORFs encoding BDO subunits. The names of the genes are indicated below the arrows. The *etbA4* gene is located 6.0 kb upstream from *etbA1*. The *bphA1*, *ebdA1*, and *etbA1* genes encode the large subunit of BDO, the *bphA2*, *ebdA2*, and *etbA2* genes encode the small subunit of BDO, *bphA3* and *ebdA3* encode the ferredoxin, and *bphA4* and *etbA4* encode the ferredoxin reductase. The plasmid location of each gene segment is indicated on the right. Abbreviations: B, BamHI; E, EcoRI; H, HindIII; M, MluI; P, PstI; S, StuI.

indicated that the gene clusters including *bphA1*, *etbA1*, *ebdA1*, and *etbA4* are under the control of the *bphST*-encoded two-component regulatory system and are inducibly transcribed in the presence of biphenyl (18). The BphST system is also responsible for transcriptional induction by ethylbenzene, toluene, and other aromatic compounds (19).

In this paper, we examine the involvement of the RHDO genes in the biphenyl/PCB degradation activity of RHA1. We constructed single- and double-disruption mutants of RHDO large-subunit genes and investigated the 4-chlorobiphenyl (4-CB) degradation activities of these mutants. In addition, we constructed insertion mutants of electron transfer component genes. Our results revealed that all of these genes are involved in biphenyl/PCB degradation activity in RHA1.

#### MATERIALS AND METHODS

**Bacterial strains, plasmids, and culture conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. Vector plasmids pFAJ2574 (3), pK4 (6), and pBsRG6 (8) were used as sources for plasmid construction. *Rhodococcus* strains were grown in Luria-Bertani (LB) medium, 1/5 LB medium (2 g of Bacto tryptone, 1 g of Bacto yeast extract, 5 g of NaCl per liter), 1/3 LB medium (3.33 g of Bacto tryptone, 1.66 g of Bacto yeast extract, 5 g of NaCl per liter), or W minimal medium (13) with biphenyl or vapor of ethylbenzene at 30°C. *Escherichia coli* JM109 was used as a host strain and was grown in LB medium at 37°C.

**RT-PCR.** Total RNA of RHA1 was prepared from cells grown on biphenyl, ethylbenzene, or LB medium as described by Ausubel et al. (1). Reverse transcription-PCR (RT-PCR) was carried out using a BcaBest RNA PCR kit, version 1.1 (Takara Shuzo, Kyoto, Japan), according to the manufacturer's instructions. Forward primers were designed using the sequences 32 to 55 bp and 29 to 46 bp upstream from the *etbA1* and *ebdA1* start codons, respectively. These sequences are located downstream from the transcriptional start site of each gene (18). The *etbA1* forward, *ebdA1* forward, and common reverse primer sequences were 5'-CGTTCACAAATAGTCGCAATTACG-3', 5'-GACCATTTGCGGTGCTG G-3', and 5'-CGATGCGGACAAGAATTTCG-3', respectively. The expected sizes of the PCR products for *etbA1* and *ebdA1* were 330 bp and 321 bp, respectively. To check that the primer pair for *etbA1* or *ebdA1* did not amplify the other gene, pETQ1 and pHX11, carrying *etbA1* and *ebdA1*, respectively, were used as template DNAs.

**2-D PAGE.** RHA1 was grown in 500 ml of 1/5 LB medium to an optical density at 600 nm (OD<sub>600</sub>) of approximately 1.5. The cells were harvested by centrifugation, washed twice with 5 ml of W minimal medium, and resuspended in W minimal medium to give an OD<sub>600</sub> of 1.5. The cell suspension was incubated for 16 h with biphenyl at 30°C. The cells were then harvested by centrifugation, washed twice with 5 ml of 25 mM potassium phosphate buffer (pH 7.0), and resuspended in 10 ml of 25 mM potassium phosphate buffer (pH 7.0). They were disrupted by a single passage through a French press (SLM-Aminco, Rochester, NY) at an operating pressure of 20,000 lb/in<sup>2</sup>. Cell debris was removed by centrifugation (30,000 × g, 4°C, 15 min). The clear supernatant fluid was used for two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) as a protein sample. The concentration of protein in a sample was determined by means of a protein assay kit (Bio-Rad Laboratories, Hercules, Calif.), using bovine serum albumin as a standard. 2-D PAGE was carried out using a Mini-PROTEAN II 2-D cell (Bio-Rad Laboratories) according to the instructions of the manufacturer. Proteins (0.06 to 0.255 mg) were separated in the first dimension by isoelectric focusing on a pI gradient ranging from 4 to 7 and then further separated in the second dimension by sodium dodecyl sulfate-PAGE with a 12% polyacrylamide gel. After the second-dimension gel electrophoresis, the proteins were stained with a Coomassie brilliant blue R250 or silver stain kit (ATTO, Tokyo, Japan).

**N-terminal amino acid sequencing.** The gel pieces containing target proteins were excised from the Coomassie brilliant blue-stained second-dimension gel and subjected to sodium dodecyl sulfate-PAGE analysis. After electrophoresis, the target proteins were blotted electrically onto a polyvinylidene difluoride membrane at 20 V for 1 h using a Trans-Blot SD cell (Bio-Rad). The part of the polyvinylidene difluoride membrane containing the target protein was excised and applied directly to a PPSQ-23 protein sequencer (Shimadzu, Kyoto, Japan).

**Gene disruption.** The strategy for gene disruption in RHA1 was described previously (8). To construct single-insertion mutants, the 761-bp StuI-PvuII,

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s) <sup>a</sup>	Reference
<b>Strains</b>		
<i>Rhodococcus</i> sp. strain RHA1	Wild type; BPH <sup>+</sup> ETB <sup>+</sup>	17
<i>Rhodococcus</i> sp. strain HDT1	Mutant derivative of RHA1; <i>aphII</i> gene insertion mutant of <i>etbA1</i> ; Km <sup>r</sup>	This study
<i>Rhodococcus</i> sp. strain HDB1	Mutant derivative of RHA1; <i>aphII</i> gene insertion mutant of <i>ebdA1</i> ; Km <sup>r</sup>	This study
<i>Rhodococcus</i> sp. strain HDA1	Mutant derivative of RHA1; <i>aphII</i> gene insertion mutant of <i>bphA1</i> ; Km <sup>r</sup>	This study
<i>Rhodococcus</i> sp. strain HDB3	Mutant derivative of RHA1; <i>aphII</i> gene insertion mutant of <i>ebdA3</i> ; Km <sup>r</sup>	This study
<i>Rhodococcus</i> sp. strain HDA3	Mutant derivative of RHA1; <i>aphII</i> gene insertion mutant of <i>bphA3</i> ; Km <sup>r</sup>	This study
<i>Rhodococcus</i> sp. strain HDT4	Mutant derivative of RHA1; <i>aphII</i> gene insertion mutant of <i>etbA4</i> ; Km <sup>r</sup>	This study
<i>Rhodococcus</i> sp. strain HDAT1	Mutant derivative of HDA1; <i>cmrA</i> gene insertion mutant of <i>etbA1</i> ; Km <sup>r</sup> Cm <sup>r</sup>	This study
<i>Rhodococcus</i> sp. strain HDAB1	Mutant derivative of HDA1; <i>cmrA</i> gene insertion mutant of <i>ebdA1</i> ; Km <sup>r</sup> Cm <sup>r</sup>	This study
<i>Rhodococcus</i> sp. strain HDBT1	Mutant derivative of HDB1; <i>cmrA</i> gene insertion mutant of <i>etbA1</i> ; Km <sup>r</sup> Cm <sup>r</sup>	This study
<i>Rhodococcus</i> sp. strain HDAB33	Mutant derivative of HDA3; <i>cmrA</i> gene insertion mutant of <i>ebdA3</i> ; Km <sup>r</sup> Cm <sup>r</sup>	This study
<i>Escherichia coli</i> JM109	<i>recA1 endA1 gyrA96 thi-1 hsdR17</i> (r <sub>K</sub> <sup>-</sup> m <sub>K</sub> <sup>-</sup> ) <i>e14 (mcrA) supE44 relA1 Δ(lac-proAB)</i> [F' <i>traD36 proAB lacI<sup>q</sup>ZΔM15</i> ]	
<b>Plasmids</b>		
pBluescript II SK(+)	Cloning vector; Ap <sup>r</sup>	Stratagene
pETQ1	pUC119 with 10.0-kb HindIII fragment of RHA1 carrying <i>etbA1A2C</i> and <i>bphD1</i>	This study
pHX11	pUC119 with 3.8-kb XhoI fragment of RHA1 carrying <i>ebdA1A2A3</i>	This study
pK4tsr	pK4 with insertion of <i>tsr</i> gene from pBsRG6; Km <sup>r</sup> Ts <sup>r</sup>	This study
pBlueKm	pBluescript II SK(+) with insertion of <i>aphII</i> gene; Ap <sup>r</sup> Km <sup>r</sup>	This study
pBKE2	pBlueKm containing a 761-bp StuI-PvuII <i>etbA1</i> internal fragment	This study
pBAK1	pBlueKm containing a 1,099-bp Eco52I <i>bphA1</i> internal fragment	This study
pBKEA3	pBlueKm containing a 330-bp AatII-NarI <i>ebdA3</i> internal fragment	This study
pBKBA3	pBlueKm containing a 302-bp PCR-amplified <i>bphA3</i> internal fragment	This study
pBKA4-2	pBlueKm containing a 731-bp MluI-SmaI <i>etbA4</i> internal fragment	This study
pBlueCm	pBluescript II SK(+) with insertion of <i>cmrA</i> gene; Ap <sup>r</sup> Cm <sup>r</sup>	This study
pCEA1	pBlueCm containing a 761-bp StuI-PvuII <i>etbA1</i> internal fragment	This study
pCEB3	pBlueCm containing a 330-bp AatII-NarI <i>ebdA3</i> internal fragment	This study
pK4TEB1	pK4tsr with 2.3-kb BamHI-MluI fragment of RHA1 carrying <i>ebdA1</i>	This study
pK4TEB3	pK4tsr with 1.0-kb EcoRV-KpnI fragment of RHA1 carrying <i>ebdA3</i>	This study
pK4TBA21	pK4tsr with 2.7-kb EcoRI-NspV fragment of RHA1 carrying <i>bphA1</i>	This study
pK4TBA3	pK4tsr with 0.5-kb NspV-StuI fragment of RHA1 carrying <i>bphA3</i>	This study
pK4TBA4-2	pK4tsr with 2.0-kb BglII-HindIII fragment of RHA1 carrying <i>etbA4</i>	This study

<sup>a</sup> BPH<sup>+</sup>, growth on biphenyl; ETB<sup>+</sup>, growth on ethylbenzene; Ap<sup>r</sup>, ampicillin resistance; Cm<sup>r</sup>, chloramphenicol resistance; Km<sup>r</sup>, kanamycin resistance; Ts<sup>r</sup>, thiostrepton resistance.

1,099-bp Eco52I, 330-bp AatII-NarI, and 731-bp MluI-SmaI fragments containing the internal segments of *etbA1*, *bphA1*, *ebdA3*, and *etbA4*, respectively, were inserted into pBlueKm, which is a pBluescript II SK(+) derivative harboring the *aphII* gene. The internal segment of *bphA3* was amplified using the *bphA3*-F (5'-CCTCACAAAGATATGCTCCGG-3') and *bphA3*-Rs (5'-CCTCTGCTGCAAGAGCACCTGGAGATC-3') primer pair and was inserted into pBlueKm. The resulting plasmids, pBKE2, pBAK1, pBKEA3, pBKA4-2, and pBKBA3, respectively, were introduced independently into RHA1 cells by electroporation. Transformants were selected on 1/3 LB agar plates containing 50 mg of kanamycin per liter and were subjected to Southern hybridization analysis in order to examine the insertion of pBKE2, pBAK1, pBKEA3, pBKA4-2, and pBKBA3 into *etbA1*, *bphA1*, *ebdA3*, *etbA4*, and *bphA3*, respectively, by single crossovers. Because *ebdA1* is identical to *etbA1*, an *ebdA1* insertion was selected along with an *etbA1* insertion. In an insertion mutant, a pBlueKm segment is expected to be sandwiched between a pair of genes inactivated by terminal deletions.

To construct double-insertion mutants, the 761-bp StuI-PvuII and 330-bp AatII-NarI fragments containing the internal segments of *etbA1* and *ebdA3*, respectively, were inserted into pBlueCm, which is a pBluescript II SK(+) derivative harboring the *cmrA* gene (3). The resulting plasmids, pCEA1 and pCEB3, respectively, were introduced independently into the cells of a single-insertion mutant by electroporation. Transformants were selected on 1/3 LB agar plates containing 50 mg of kanamycin and 15 mg of chloramphenicol per liter and were subjected to Southern hybridization analysis to examine the insertion of pCEA1 and pCEB3 into *etbA1* or *ebdA1* and *ebdA3* by single crossover.

**Resting cell assay.** RHA1 and its insertion mutants were grown on 1/5 LB medium at 30°C to give an OD<sub>600</sub> of 0.2, and then the degradation genes were induced by incubation with vapor of ethylbenzene for another 16 h. Cells were collected by centrifugation, washed twice with 2 ml of W minimal medium, and resuspended in W minimal medium to give an OD<sub>600</sub> of 1.0. One milliliter of cell

suspension was preincubated for 5 min at 30°C and was incubated in a sealed 4.5-ml glass vial with shaking at 30°C after the addition of 50 nmol of 4-CB or 1 ppm each of PCB congeners. The cell suspensions of single- and double-disruption mutants were incubated for 60 and 120 min, respectively, with 4-CB and for 24 h with PCB congeners. Control cells were inactivated by being autoclaved at 121°C for 15 min prior to the addition of substrate. To stop the reaction, 0.1 ml of 6 N HCl was added to the mix, and 50 nmol of phenanthrene or 5 nmol of 4-chlorobiphenyl was added as an internal standard. After the addition of NaCl to saturation, 3 ml of ethyl acetate was added to the mixture, which was then mixed on a vortex mixer for 1 min. The supernatant was recovered, dehydrated with sodium sulfate, evaporated, and dissolved in 0.1 ml of ethyl acetate. One microliter of extract was analyzed by gas chromatography-mass spectrometry (GC-MS) (model 5971A; Agilent Technologies Co., Palo Alto, Calif.), using an Ultra-2 capillary column (50 m by 0.2 mm; Agilent Technologies Co.) as described previously (17).

## RESULTS AND DISCUSSION

**Expression of *etbA1* and *ebdA1* in RHA1.** To discriminate between the *etbA1* and *ebdA1* transcripts, RT-PCR analysis was performed with RNAs from RHA1 cells grown on biphenyl, ethylbenzene, or LB, using the sequences upstream from *etbA1* and *ebdA1* as one of the primers, since the sequences upstream from these two genes are different. The *etbA1* and *ebdA1* forward primers were designed using the sequences downstream from the transcriptional start site of each gene, as determined by Takeda et al. (18). The primer pair for *etbA1*

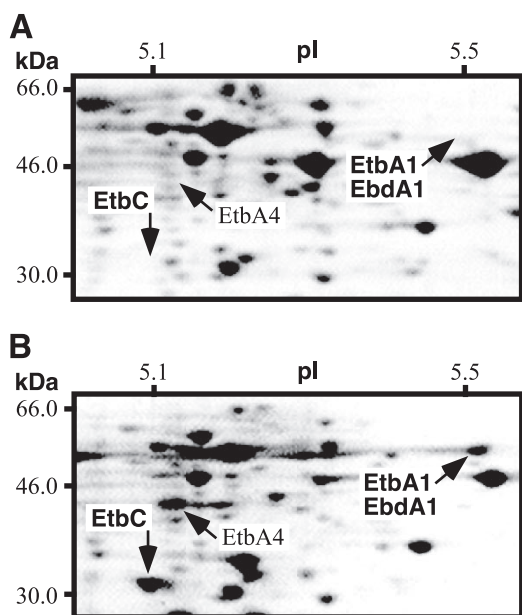


FIG. 2. Two-dimensional polyacrylamide gel electrophoresis analysis of soluble proteins from RHA1 cells before (A) and after (B) incubation in the presence of biphenyl for 16 h. Proteins were separated in an isoelectric point (pI) gradient from 4 to 7 and then in a 12% polyacrylamide gel, after which they were stained with Coomassie brilliant blue R250. The area of the pI gradient from 5.1 to 5.5 and of molecular mass from 30.0 to 66.0 kDa is shown. The pI and molecular mass scales are indicated at the top and left of each panel, respectively.

did not produce any PCR product with *ebdA1* plasmid DNA as the template, and that for *ebdA1* produced no PCR product with *etbA1* plasmid DNA as the template (data not shown). When total RNAs of the cells grown on biphenyl or ethylbenzene were used as templates, PCR products with the expected sizes for *etbA1* and *ebdA1* were obtained. No PCR product was obtained from the cells grown in LB medium (data not shown). Takeda et al. indicated the induction of *bphA1* gene transcription in the presence of biphenyl or ethylbenzene by RT-PCR (19) and suggested that transcription from five adjacent promoters upstream from the degradation gene clusters containing *etbA1*, *ebdA1*, and *bphA1* is coordinately controlled by the BphST system (18). The BphST system induced transcription beginning with each of the five promoters in response to ethylbenzene and other aromatic compounds as well as biphenyl (19). Our results indicated that *etbA1* and *ebdA1* as well as *bphA1* are inducibly transcribed in the presence of biphenyl or ethylbenzene.

To verify the protein products of *etbA1* and *ebdA1* in RHA1 induced during incubation with biphenyl, we performed 2-D PAGE (Fig. 2). The results for protein samples from the cells grown on biphenyl revealed the identity of a protein spot whose molecular mass and isoelectric point (pI) were 51 kDa and 5.5, respectively (Fig. 2B). These values were very close to those of 51.7 kDa and 5.2, respectively, which were estimated from the amino acid sequence of EtbA1/EbdA1. The N-terminal amino acid sequence of the protein from this spot was determined, and the obtained sequence, MLXSEXFSPG (X is an unknown), was correspondent with the sequence of EtbA1 and EbdA1 (MLRSERFSPG). These results indicated that

either *etbA1*, *ebdA1*, or both are inducibly expressed as a protein product in RHA1 grown on biphenyl. In addition to EtbA1/EbdA1, 15 protein spots were unique to the cells grown on biphenyl in comparison to those grown in LB medium. One spot with a molecular mass of 34 kDa and a pI of 4.8 was assigned as the EtbC dioxygenase protein based on its deduced molecular mass, pI, and N-terminal amino acid sequence, AKVTELGYL. Another spot with a molecular mass of 45 kDa and a pI of 5.1 was estimated to be EtbA4, whose deduced molecular mass and pI are 44 kDa and 4.9, respectively (Fig. 2B).

**Disruption of genes encoding RHDO large subunits.** To examine whether the *etbA* and *ebdA* genes are really involved in biphenyl/PCB degradation, *etbA1* and *ebdA1* were independently disrupted by insertional inactivation using homologous recombination as described in Materials and Methods. The *bphA1* gene, which was proved to be involved in biphenyl/PCB degradation in RHA1 (13, 16), was also disrupted by the same method. The *etbA1*, *ebdA1*, and *bphA1* insertion mutants were designated HDT1, HDB1, and HDA1, respectively.

The degradation activity toward 4-CB of each insertion mutant was investigated by GC-MS. Autoclaved cells were used as a negative control. The degradation activities of HDB1 and HDA1 were diminished, and the reduction of 4-CB by either of these insertion mutants was half that by RHA1 after 30 min (Fig. 3A). The activity of HDT1 was only slightly lower than that of RHA1 (Fig. 3A). Taking into account the polar effect exerted on the activities of genes downstream from the inactivated gene, these results suggested that the *etbA1*, *ebdA1*, and *bphA1* genes and/or the genes downstream from them are involved in biphenyl/PCB degradation.

To examine whether the reduction of 4-CB degradation activity was caused by the inactivated gene, a wild-type gene was introduced into the mutant to complement each mutation. A plasmid, pK4TEB1, containing *ebdA1* and its promoter region was introduced into the *etbA1* mutant, HDT1, and the *ebdA1* mutant, HDB1, by electroporation. pK4TBA21, containing *bphA1* and its promoter region, was introduced into the *bphA1* mutant, HDA1. The degradation activity of each recombinant was then examined. However, none of the 4-CB degradation activities of the insertion mutants were restored (Fig. 3A). In the case of HDA1, the introduction of pK4TBA21 resulted in a further reduction of degradation activity. Excluding the case of HDA1, these results suggested that the expression of RHDO subunit genes located downstream from *etbA1* and *ebdA1* was suppressed by a polar effect. In the case of HDA1, containing the *bphA1* plasmid, the impaired high-level expression of a terminal dioxygenase subunit appears to have resulted in the formation of an inactive heterologous complex between BphA1 and EtbA2/EbdA2.

**Double disruption of three RHDO large-subunit genes.** Because the insertion mutants mentioned above did not provide sufficient evidence that RHDO terminal oxygenase component genes play a role in biphenyl/PCB degradation in RHA1, we performed double-disruption experiments. The double-insertion *etbA1 bphA1* and *ebdA1 bphA1* mutants were constructed by using the *bphA1* mutant, HDA1, as the parent strain and were designated HDAT1 and HDAB1, respectively. The double-insertion *etbA1 ebdA1* mutant, designated HDBT1, was constructed from the *ebdA1* mutant, HDB1.

After examining the ability of the single-disruption mutants

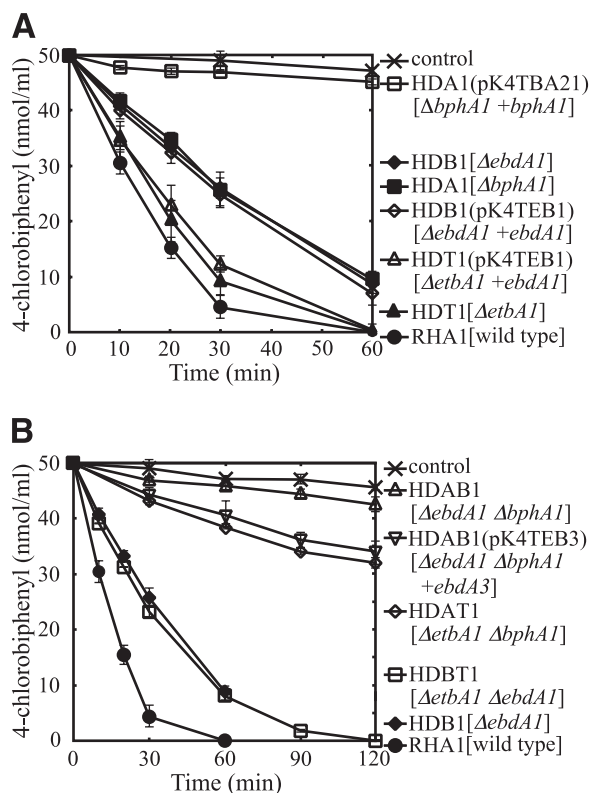


FIG. 3. 4-Chlorobiphenyl degradation activities of RHA1 and RHDO large-subunit mutants. The results for single-insertion mutants (A) and double-insertion mutants (B) are presented. The remaining amount of 4-chlorobiphenyl was determined by gas chromatography-mass spectrometry. The degradation activities of the derivatives harboring plasmids containing wild-type genes are also shown. Each value is the mean of triplicate independent experiments, with the standard deviation indicated by error bars. Symbols are explained to the right of the panels. Plasmids are indicated in parentheses, and the mutation genotype of a strain and the wild-type gene in a plasmid are indicated in square brackets.

to degrade 4-CB, we investigated the 4-CB degradation activity of each double-insertion mutant. The degradation activity of HDAT1 was lower than that of its parent strain, HDA1, and was one-quarter that of RHA1 (Fig. 3B). This degradation activity was estimated to depend solely on the *ebdA1A2*-encoded terminal oxygenase component (EbdA1A2 component), suggesting that the EbdA1A2 component plays a part in biphenyl/PCB degradation in RHA1. Because HDAT1 was constructed with the *etbA1* inactivation of HDA1, the reduction of degradation activity appears to have originated from the inactivation of *etbA1*. Thus, the terminal oxygenase component encoded by *etbA1A2* (EtbA1A2 component) appears to play an important role in biphenyl/PCB degradation in RHA1. On the other hand, the 4-CB degradation activity of HDAB1 was approximately equal to that of the negative control, irrespective of the presence of intact *etbA1A2* (Fig. 3B). For HDAB1, it was suggested that the expression of both *bphA3* and *ebdA3* was inhibited by the polar effect caused by the respective insertions into *bphA1* and *ebdA1*, respectively. Because of the absence of the electron transfer component, the EtbA1A2 component, which is intact in HDAB1, appears to have lost its

activity. This notion was supported by the introduction of plasmid pK4TEB3, containing *ebdA3*, into HDAB1. The degradation activity of HDAB1 was restored by the introduction of pK4TEB3 to a level approximately equal to that of HDAT1 (Fig. 3B). These results again indicated that the EtbA1A2 component is involved in biphenyl/PCB degradation in RHA1. For HDAB1, we conjectured that not only the *bphA3* gene but also the adjacent *bphA4* gene is inhibited by the polar effect. However, the introduction of the sole *ebdA3* gene restored the activity of HDAB1. These results suggested that another ferredoxin reductase gene, probably *etbA4*, plays a role in biphenyl/PCB degradation.

The degradation activity of HDBT1 was estimated to depend solely on *bphA1A2*, which confirmed that the *bphA1A2*-encoded terminal oxygenase component is functional in biphenyl/PCB degradation in RHA1. HDBT1 exhibited almost the same activity as its parent strain, HDB1 (Fig. 3B), although intact *etbA1A2* existed in HDB1. These results may suggest that the sole electron transfer components in HDB1 encoded by *bphA3* and *bphA4* are not sufficient to account for the obvious activity of the *etbA1A2*-encoded dioxygenase.

**Disruption of two RHDO ferredoxin genes and a ferredoxin reductase gene.** As previously mentioned, *ebdA3* and *etbA4*, which encode a ferredoxin and a ferredoxin reductase, respectively, are thought to be involved in biphenyl/PCB degradation in RHA1. To confirm this involvement, we disrupted these genes by the same method used for the disruption of the RHDO large subunits. The *bphA3* gene was also disrupted by the same method. The *ebdA3*, *etbA4*, and *bphA3* insertion mutants were designated HDB3, HDT4, and HDA3, respectively. We also made repeated attempts to construct a corresponding insertion mutant of *bphA4*, but these failed.

The ability of each insertion mutant to degrade 4-CB was investigated by GC-MS. The degradation activities of the ferredoxin gene mutants were diminished, with HDB3 and HDA3 realizing one-third and one-half of the 4-CB reduction achieved by RHA1, respectively (Fig. 4). To examine whether the reduction of 4-CB degradation activity was caused by the inactivated gene, a wild-type gene was inserted next to the *bphA1* promoter in vector pK4tsr, which was introduced into the mutant to complement each mutation. Plasmid pK4TEB3, containing *ebdA3*, and pK4TBA3, containing *bphA3*, were independently introduced into HDB3 and HDA3, respectively, by electroporation. The 4-CB degradation activities of both HDB3 and HDA3 were restored by the introduction of either *ebdA3* or *bphA3* (Fig. 4A and B). These results indicated that both ferredoxin genes, *bphA3* and *ebdA3*, are involved in biphenyl/PCB degradation. A double-insertion *ebdA3 bphA3* mutant was also constructed by using HDA3 as the parent strain and was designated HDAB33. The 4-CB degradation activity of HDAB33 was approximately equal to that of the negative control (Fig. 4B), suggesting that EbdA3 and BphA3 are the only dominant ferredoxin components involved in biphenyl/PCB degradation in RHA1.

The degradation activity of the ferredoxin reductase mutant HDT4 was diminished to about half that of RHA1 (Fig. 4C). To complement the *etbA4* gene deficiency, pK4TBA4-2, containing *etbA4* and its promoter region, was introduced into HDT4 by electroporation. The 4-CB degradation activity of HDT4 was restored by introduction of the *etbA4* gene (Fig.

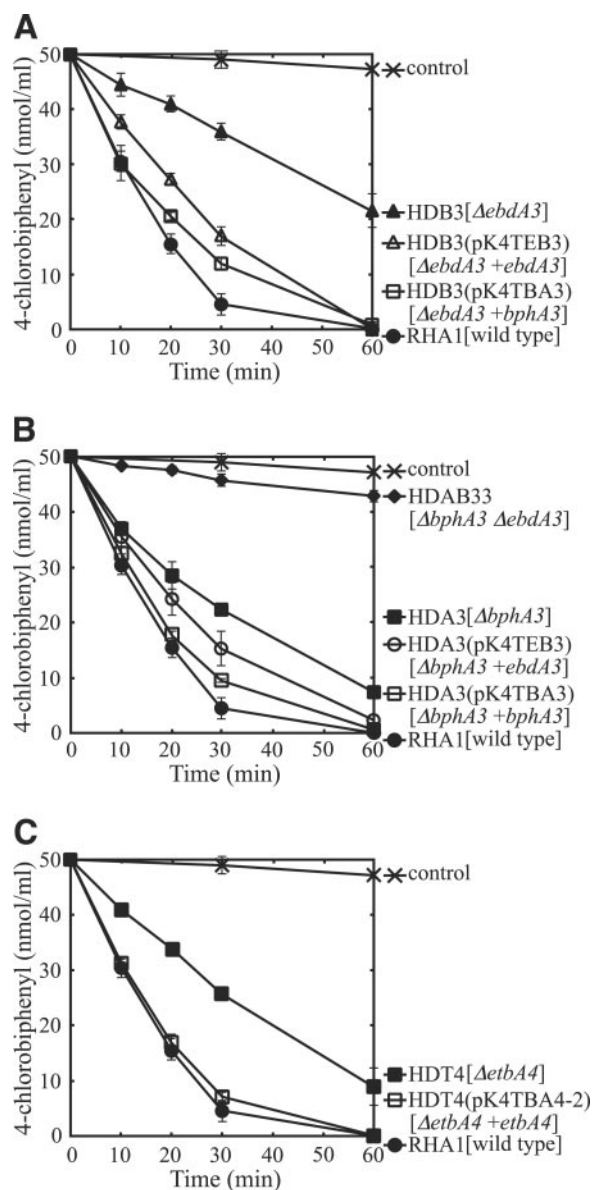


FIG. 4. 4-Chlorobiphenyl degradation activities of mutant strains with insertion mutations in RHDO electron transfer components. The results for HDB3 (A), HDA3 (B), and HDT4 (C), with insertion mutations in *ebdA3*, *bphA3*, and *etbA4*, respectively, are presented. The remaining amount of 4-chlorobiphenyl was determined by gas chromatography-mass spectrometry. The degradation activities of the strains harboring plasmids for complementation are also shown. Each value is the mean of triplicate independent experiments, with the standard deviation indicated by error bars. Symbols are explained to the right of the panels. Plasmids are indicated in parentheses, and the mutation genotype of a strain and the wild-type gene in a plasmid are indicated in square brackets.

4C). These results indicated that a ferredoxin reductase gene of RHDO, *etbA4*, is involved in biphenyl/PCB degradation in RHA1.

**Transformation of PCB congeners by insertion mutants.** It is known that the large subunits of terminal oxygenase components are major determinants of the substrate preference of the RHDO. In consideration of the low sequence similarity

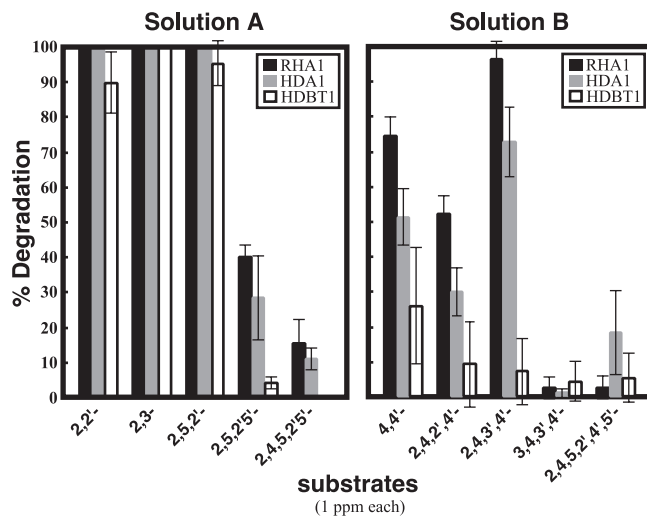


FIG. 5. Degradation of PCB congeners by RHA1 (black bars), by the *bphA1* mutant, HDA1 (gray bars), and by the *etbA1 ebdA1* double knockout, HDBT1 (white bars). Solution A consisted of five mainly *ortho*-substituted PCB congeners, 2,2'-dichlorobiphenyl, 2,3-dichlorobiphenyl, 2,5,2'-trichlorobiphenyl, 2,5,2',5'-tetrachlorobiphenyl, and 2,4,5,2',5'-pentachlorobiphenyl. Solution B consisted of five mainly *para*-substituted PCB congeners, 4,4'-dichlorobiphenyl, 2,4,2',4'-tetrachlorobiphenyl, 2,4,3',4'-tetrachlorobiphenyl, 3,4,3',4'-tetrachlorobiphenyl, and 2,4,5,2',4',5'-hexachlorobiphenyl. Each value is the mean of triplicate independent experiments, with the standard deviation indicated by error bars.

between *EtbA1A2/EbdA1A2* and *BphA1A2*, it was expected that the substrate preferences would differ between *EtbA/EbdA* and *BphA* dioxygenase species. Therefore, the substrate preferences of *EtbA/EbdA* and *BphA* for PCB congeners were investigated by using the insertion mutants. The *bphA1* mutant, HDA1, and the *etbA1 ebdA1* mutant, HDBT1, were employed as *EtbA/EbdA*- and *BphA*-expressing strains, respectively. A mixture of five *ortho*-substituted PCB congeners that consisted of 2,2'-, 2,3-, 2,5,2'-, 2,5,2',5'-, and 2,4,5,2',5'-chlorobiphenyl (solution A) and a mixture of five *para*-substituted PCB congeners that consisted of 4,4'-, 2,4,2',4'-, 2,4,3',4'-, 3,4,3',4'-, and 2,4,5,2',4',5'-chlorobiphenyl (solution B) were used as substrates. The results are shown in Fig. 5. Although the degradation activity of HDA1 was lower than that of RHA1, the substrate preference of *EtbA/EbdA*-expressing HDA1 was almost identical to that of RHA1. On the other hand, *BphA*-expressing HDBT1 showed extremely low degradation activities toward 2,4,2',4'-, 2,4,3',4'-, 2,5,2',5'-, and 2,4,5,2',5'-chlorobiphenyls. The results for HDBT1 were almost correspondent with those obtained by using *Rhodococcus erythropolis* IAM1399 expressing *bphA1A2A3A4* (16). These results indicated that *EtbA/EbdA* and *BphA* have different substrate preferences for PCB congeners and suggested that *EtbA/EbdA* plays an important role in the degradation of highly chlorinated biphenyls. *BphA* appears to play a role in the degradation of PCBs to some extent, because HDBT1 showed good activities toward 2,2'-, 2,3-, and 2,5,2'-chlorobiphenyls in addition to 4-CB (Fig. 3 shows the data for 4-CB).

Multiple isozyme genes for biphenyl/PCB degradation in *Rhodococcus* strains have been reported (9, 11, 18). However, the potential involvement of these isozyme genes in degrada-

tion has not been investigated. To our knowledge, this is the first report to demonstrate the cooperative involvement of multiple RHDO isozymes encoded by the *bphA*, *etbA*, and *ebdA* genes in a single PCB-degrading bacterium. These genes were indicated to be under the control of the *bphST* regulatory system, which has a broad substrate spectrum for transcription induction (18, 19). Thus, these isozymes might be simultaneously involved in the catabolism of substrates other than biphenyl.

#### ACKNOWLEDGMENTS

We thank René De Mot for the kind gift of plasmid pFAJ2574. We are grateful to R. van der Geize for his helpful suggestions regarding the insertional inactivation of rhodococcal genes.

This study was supported in part by a grant-in-aid for the study of hazardous chemicals from the Ministry of Agriculture, Forestry, and Fisheries of Japan (HC-05-2322-1).

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