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Characterization of a Second 2,3-Dihydro-2,3-Dihydroxybiphenyl Dehydrogenase Gene of the Polychlorinated Biphenyl Degrader *Rhodococcus* sp. Strain RHA1

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In a previous study, we characterized the *bphAC1B* genes of *Rhodococcus* sp. RHA1, which participate in the conversion of biphenyl to the *meta*-cleavage product and are on the 1,100-kb linear plasmid. In this study, we analyzed the 4.4-kb *Sph*I fragment of RHA1, which hybridized to the 1.5-kb *Eco*RI fragment carrying *bphC1* and *bphB*. The nucleotide sequence of this fragment showed the presence of a second 2,3-dihydro-2,3-dihydroxybiphenyl dehydrogenase gene, *bphB2*. The *bphB2* gene consists of an 810-bp open reading frame encoding a polypeptide of molecular mass 28,234 Da. The nucleotide and deduced amino acid sequences of *bphB2* showed 76% and 81% identity, respectively, with those of the RHA1 *bphB* gene. The *etbA4* gene and *orf3* whose deduced amino acid sequences are similar to ferredoxin reductase and aldehyde dehydrogenase, respectively, were identified as near to *bphB2*. The *etbA4-bphB2-orf3* gene cluster was approximately 5-kb upstream of the previously characterized *etbA1-etbA2-etbC-bphD1-bphE2-bphF2* gene cluster on the 450-kb linear plasmid. Reverse transcription-PCR analysis showed that the *bphB2* and *bphB* genes were transcribed in biphenyl- and ethylbenzene-growing cells. The *bphB2* gene was expressed in *Escherichia coli* to find the substrate preference, and BphB2 showed a higher activity toward the dihydrodiol compound of toluene than that of biphenyl. The insertional inactivation of the *bphB* gene in RHA1 resulted in reduced growth on biphenyl. These results suggest that both *bphB* and *bphB2* genes participate in biphenyl degradation in this strain.

Key words: PCB degradation, *Rhodococcus*, 2,3-dihydro-2,3-dihydroxybiphenyl dehydrogenase, *bphB*

1. Introduction

Polychlorinated biphenyls (PCBs) are compounds of considerable environmental concern because of their toxicity and recalcitrance. Microbial degradation is one of the most effective procedures to remove PCBs from the environment. Many PCB-degrading bacteria have been isolated, and they commonly cometabolize PCBs through the biphenyl catabolic pathway. In this pathway, biphenyl is transformed to 2,3-dihydro-2,3-dihydroxybiphenyl (23DDB) by a multicomponent biphenyl dioxygenase (BphA). 23DDB is converted to 2,3-dihydroxybiphenyl (23DHBP) by 23DDB dehydrogenase (BphB), and 23DHBP is cleaved at the 1,2 position (*meta*-ring cleavage) by 23DHBP dioxygenase (BphC). The ring cleavage product, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate (HOPD), is hydrolyzed to benzoate and 2-hydroxypenta-2,4-dienoate (HPD) by HOPD hydrolase (BphD) (Fig. 1A), and the resulting HPD is further converted to tricarboxylic acid cycle intermediates by HPD hydratase, 4-hydroxy-2-oxovalerate aldolase, and acetaldehyde dehydrogenase (BphE, BphF, and BphG, respectively).

Rhodococcus sp. RHA1 is a strong Gram-positive PCB degrader, which efficiently transforms the mixture of Kanechlors 200, 300, 400, and 500, including mono- to octachlorobiphenyls, for three days. To clarify why this strain shows the strong PCB degradation ability and to obtain insight into evolutional aspects of the degradation genes for aromatic compounds, we isolated and characterized the *bphAC1B*[13] and *etbCbphD1*[12] genes, which are on the 1,100- and 450-kb linear plasmids, respectively. These genes have roles in the transformation of biphenyl to benzoate and HPD. Benzoate[10] and HPD[19] degradation genes have also been characterized. In addition to *bphC1* and *etbC*, we found previously four other extradiol dioxygenase genes in RHA1[18], and two sets of the *bphA1A2* homologs were found just upstream of *etbC* (*etbA1A2*; Fig. 1B) and downstream of *bphF2* (*ebdA1A2A3*)[22,26]. Asturias et al.[3,4] and Maeda et al.[11] have reported on multiple *bphC* in *Rhodococcus globerulus* P6 and *Rhodococcus erythropolis* TA421, respectively. Two 23DDB dehydrogenase genes

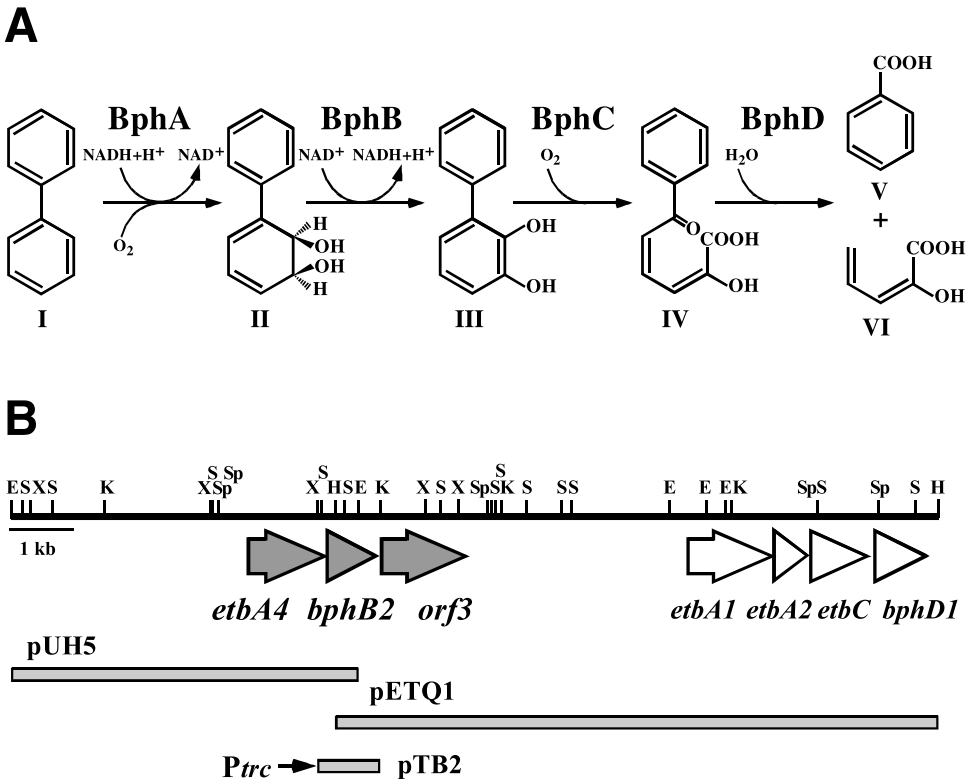

Fig. 1. Proposed transformation pathway of biphenyl to benzoate and HPD in *Rhodococcus* sp. RHA1 (A) and genetic map of the 15-kb DNA fragment carrying *etbA4-bphB2-orf3* (B). (A) Compounds: I, biphenyl; II, 23DDB; III, 23DHBP; IV, HOPD; V, benzoate; VI, HPD. The gene product responsible for each reaction step is indicated above an arrow and is described in the text. (B) The 3' end of the DNA fragment includes *etbA1-etbA2-etbC-bphD1* whose nucleotide sequences were found in a previous studies[12,26]. Abbreviations: E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; S, *Sal*I; Sp, *Sph*I; X, *Xho*I.

(*bphB*) have also been found in TA421[2]. Therefore the existence of multiple *bph* genes seems to be a marked characteristic of rhodococcal biphenyl and PCB degraders.

In this study, the *bph* gene homologs in RHA1 were screened by using Southern hybridization analysis using a RHA1 gene probe containing *bphC1* and *bphB*. We found another 23DDB dehydrogenase gene (*bphB2*), and its gene product expressed in *Escherichia coli* was characterized. The participation by two *bphB* genes of RHA1 in biphenyl degradation was examined.

2. Materials and Methods

Bacterial strains and plasmids. A PCB degrader, *Rhodococcus* sp. RHA1, was grown in Luria-Bertani medium (LB), diluted LB, and W-minimal medium (W)[13] supplied with 0.2% biphenyl, vapor of ethylbenzene, or 0.2% succinate. *R. erythropolis* IAM1399, *E. coli* MV1190, and *E. coli* DH5 were used as host strains. A *Rhodococcus-E. coli* shuttle vector pK4[9], charomid 9–36[16], pUC119[23], pTrc99A[1], and pKK223-3[7] were used as cloning vehicles.

Preparation of substrates. To prepare 23DDB from biphenyl, *R. erythropolis* IAM1399 cells containing pKRA17 carrying *bphA1A2A3A4* in pK4[21] were used. IAM1399 cells containing pKRA17 were grown in LB containing 100 mg/l of kanamycin. The cells were collected and were incubated with 0.2% biphenyl in a 200 ml W medium for 6 h at 30°C. The supernatant of the culture was collected by centrifugation and was saturated by NaCl. The metabolites were extracted by ethyl acetate and were dried *in vacuo*. This extract was separated by using silica gel chromatography (Wakogel C-200, Wako), using a solvent consisted of chloroform and acetone (4:1). The 23DDB fraction was trimethylsilylated and analyzed by gas chromatography-mass spectrometry (GC-MS) (model 5971A, Hewlett-Packard Co.). The GC-MS condition was the same as described previously[12].

Cloning and DNA sequencing. Total DNA of RHA1 was prepared as described previously[13]. A gene library containing the *Hin*dIII fragments of RHA1 total DNA in charomid 9–36 was constructed as described previously[12]. Southern and colony hybridization were done by using the DIG-system (Roche). The 5.7-kb *Eco*RI and 9.9-kb *Hin*dIII fragments carrying the partial *bphB2* gene in charomid 9–36 were cloned into pUC119, and the resulting plasmids were designated pUH5 and pETQ1, respectively. Subclones and deletion clones of these fragments were produced by using restriction enzymes and exonuclease III. Nucleotide sequencing was done by using the dideoxy termination method[20] with an ALFred DNA sequencer (Pharmacia). The sequences were analyzed by using the GeneWorks programs (Intelligenetics, Inc). Nucleotide and amino acid se-

quences were aligned by using the CLUSTAL W program. Phylogenetic analysis was done by using the neighbor-joining method[17], and graphics for phylogenetic trees were produced by using the TreeView program[14]. To find the expression of *bphB2*, the 1.0-kb *Xho*I-*Kpn*I fragment carrying *bphB2* was cloned into pTrc99A to produce pTB2.

Enzyme assay. 2,3-dihydro-2,3-dihydroxytoluene (23DDT; Fluka) or 23DDB (100 μM) were used as substrates and were incubated with the cell extract (43 μg) of *E. coli* MV1190 cells containing pTB2 and 100 μM NAD^+ or $NADP^+$ in 50 mM KH_2PO_4-NaOH buffer (pH 7.4). The dihydrodiol dehydrogenase activity was assayed by measuring the increase in the absorbance at 340 nm derived from the formation of NADH (ε_{340}=6,000 $M^{-1}cm^{-1}$; pH 7.4) or NADPH (ε_{340}=5,800 $M^{-1}cm^{-1}$; pH 7.4) by using a spectrophotometer (Biospec 1600, Shimadzu).

Preparation of cell extracts. *E. coli* MV1190 cells containing pTB2 were grown in LB containing 100 mg/l of ampicillin and 1 mM isopropyl-β-D-thiogalactopyranoside as described previously[12], and the cells were disrupted by sonication. The supernatant after centrifugation (10,000×*g* for 20 min at 4°C) was used as the cell extracts.

Reverse transcription-PCR (RT-PCR) analysis. RHA1 cells were grown in LB or W medium containing each of biphenyl, ethylbenzene, and succinate, and the total RNA was prepared by using the method described by Ausubel et al.[5]. RT-PCR was done by using a *Bca*Best RNA PCR kit ver.1.1 according to the instructions of the manufacturer (Takara Shuzo). The following primers were used to amplify the internal regions of *bphB* and *bphB2*:
bphB forward, 5'-AACGACTTCGGCGAGGATGTCC-3';
bphB reverse, 5'-GGTTGCCGTCCTCGAGTTCG-3';
bphB2 forward, 5'-TTGCGGTTATCACCGGTGGC-3';
bphB2 reverse, 5'-TTGATCAGATCTGCGAGCGG-3'.

Disruption of *bphB* in RHA1. The 0.6-kb *Hin*dIII-*Xho*I fragment carrying the truncated *bphB* gene whose 5' and 3' termini were deleted was cloned into pUCKmD, which contains the *aphII* gene in pUC19[19]. The resulting plasmid pDB1 was introduced into RHA1 cells by electroporation. Transformants were selected on a diluted LB agar plate containing 50 mg/l of kanamycin, and underwent colony PCR analysis to examine the insertion of pDB1 into the *bphB* gene in RHA1 by a single crossover. The primers used for the colony PCR were:
bphB forward 2,5'-CGCTGGTCGAGCGATTCC-3';
aphII reverse, 5'-TCCATCTTGTTCAATCATGC-3'.

Nucleotide sequence accession number. The nucleotide sequence of this study was deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession no. AB127606.

3. Results and Discussion

Isolation and sequencing of the *bphB2* gene. Southern hybridization analysis of the RHA1 total DNA showed that the 1.9-kb *Eco*RI fragment probe carrying the RHA1 *bphC1* and *bphB* genes hybridized to the 5.7-kb *Eco*RI fragment in addition to the 1.9-kb fragment (data not shown). This result suggested the presence of another *bphC* or *bphB* homolog in the RHA1 genome. To isolate the *bph* gene homolog, the 5.7-kb *Eco*RI fragment was isolated from the charomid library containing the *Eco*RI fragments of RHA1 total DNA by using a colony hybridization technique. This fragment was cloned into pUC119, and the resulting plasmid was designated pUH5. The nucleotide sequence of the 3' end of the fragment suggested the presence of a *bphB* homolog, but this gene seemed to be truncated. To isolate the entire gene, the adjacent 9.9-kb *Hin*dIII fragment was isolated from the charomid library containing the *Hin*dIII digests of RHA1 total DNA. Fig. 1B shows the restriction map of the 15-kb DNA fragment containing the overlapping 5.7-kb *Eco*RI and 9.9-kb *Hin*dIII fragments.

The nucleotides of the 4.4-kb *Sph*I fragment in the cloned fragments were sequenced. Three open reading frames (ORFs) preceded by the putative ribosome binding sites were found. The second ORF of 810 bp encoding 270 amino acid residues showed 76% and 81% identity with the RHA1 *bphB* gene at the nucleotide and amino acid level, respectively. We therefore designated this gene as *bphB2*. The deduced amino acid sequences at positions 9–37 showed a high sequence similarity with the region thought to participate in the NAD^+ binding[25] in BphB and TodD[3].

Fig. 2 shows the phylogenetic tree of dehydrogenases for dihydrodiol of monoaryl, biaryl, and polycyclic aromatic hydrocarbons. The biphenyl dihydrodiol dehydrogenases of Gram-positive bacteria including *R. globerulus* P6[3] and *Rhodococcus* sp. M5[24] together with one of *R. erythropolis* TA421 BphB are almost identical (99% identity) and were relatively similar to BphB of Gram-negative aromatic compound-degrading bacteria (approximately 60% identity). However, RHA1 BphB and BphB2 showed moderate similarity with biphenyl dihydrodiol dehydrogenases of Gram-positive and -negative bacteria (approximately 50% identity), and they are not grouped with other biphenyl dihydrodiol dehydrogenases in the phylogenetic tree. This fact suggests that RHA1 *bphB*s evolved separately from other biphenyl dihydrodiol dehydrogenases, and that one *bphB* gene might have been duplicated in the RHA1 genome. Recently, the rhodococcal dihydrodiol dehydrogenase gene, *akbB* of *Rhodococcus* sp. DK17, that is identical to RHA1 *bphB2*, has appeared in databases (AAR90124).

An ORF of 1,272 bp was found upstream of *bphB2* (Fig. 1B). From the amino acid sequence similarity of this ORF with a ferredoxin reductase component (PhnD) of phenanthrene dioxygenase of *Nocardioides* sp. KP7[15] (37% identity), this ORF was predicted to encode a ferredoxin reductase and was designated as *etbA4*. As indicated for PhnD, EtbA4 is distantly related to other ferredoxin reductases. The genes that encode iron-sulfur proteins and ferredoxin were not found proximal to *etbA4*, but *etbA4* might encode a functional ferredoxin reductase, which participates in hydroxylation of aromatic compounds together with other

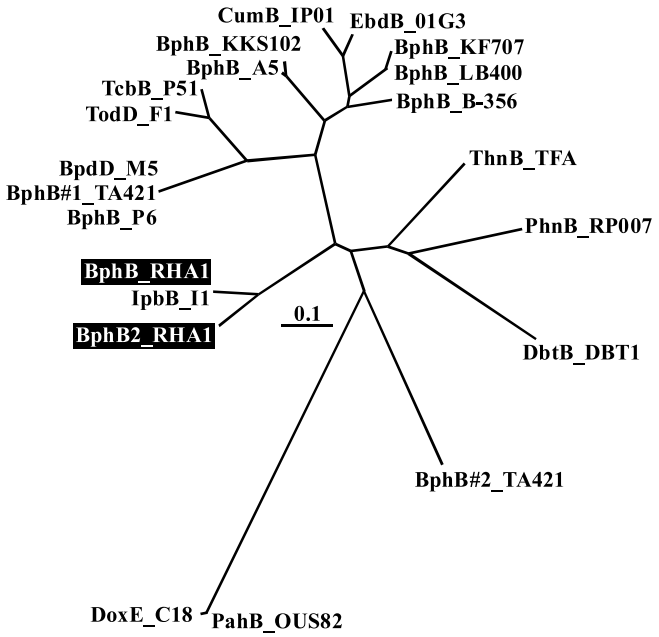

Fig. 2. Phylogenetic tree of RHA1 BphB and BphB2 with the aryl dihydrodiol dehydrogenases. The scale corresponds to a genetic distance of 0.1 substitution at each position (10% difference). Accession numbers for the sequences are as follows: BphB_KF707, 23DDB dehydrogenase of *Pseudomonas pseudoalcaligenes* KF707 (P08694); BphB_LB400, 23DDB dehydrogenase of *Burkholderia fungorum* LB400 (CAA46909); BphB_B-356, 23DDB dehydrogenase of *Comamonas testosteroni* B-356 (AAB18304); BphB_A5, 23DDB dehydrogenase of *Ralstonia oxalatica* A5 (CAD61143); BphB_KKS102, 23DDB dehydrogenase of *Pseudomonas* sp. KKS102 (BAA04140); CumB_IP01, 2,3-dihydro-2,3-dihydroxycumene dehydrogenase of *Pseudomonas fluorescens* IP01 (BAA07079); EbdB_01G3, *cis*-2,3-dihydroxy-2,3-hydroalkylbenzene dehydrogenase of *Pseudomonas putida* 01G3 (CAB99200); TcbB_P51, chlorobenzene glycol dehydrogenase of *Pseudomonas* sp. P51 (AAC43636); TodD_F1, 23DDT dehydrogenase of *P. putida* F1 (P13859); BpdD_M5, 23DDB dehydrogenase of *Rhodococcus* sp. M5 (AAB07754); BphB#1_TA421, 23DDB dehydrogenase of *R. erythropolis* TA421 (BAA25607); BphB_P6, 23DDB dehydrogenase of *R. globerulus* P6 (CAA53296); BphB_RHA1, 23DDB dehydrogenase of *Rhodococcus* sp. RHA1 (BAA06873); IpbB_I1, isopropylbenzene dihydrodiol dehydrogenase of *Rhodococcus* sp. I1 (CAA06877); BphB2_RHA1, 23DDB dehydrogenase of RHA1 (BAD03967); DoxE_C18, *cis*-1,2-dihydro-1,2-dihydroxynaphthalene dehydrogenase of *Pseudomonas* sp. C18 (D49343); PahB_OUS82, naphthalene/phenanthrene dihydrodiol dehydrogenase of *P. putida* OUS82 (BAA20393); BphB#2_TA421, 23DDB dehydrogenase of TA421 (BAA25626); ThnB_TFA, 1,2-dihydroxy-1,2,5,6,7,8-hexahydronaphthalene dehydrogenase of *Sphingopyxis macrogoltabida* TFA (AAN26445); PhnB_RP007, naphthalene/phenanthrene dihydrodiol dehydrogenase of *Burkholderia* sp. RP007 (AAD09874); DbtB_DBT1, dihydrodiol dehydrogenase of *Burkholderia* sp. DBT1 (AAK62355).

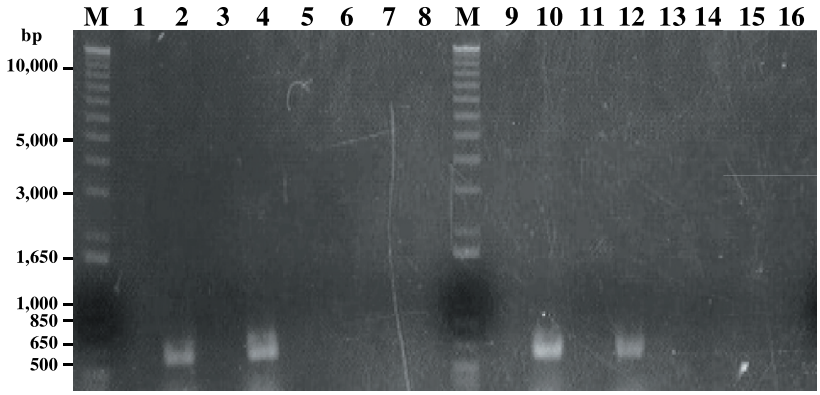

Fig. 3. Agarose gel electrophoresis of RT-PCR products of *bphB* and *bphB2*. Total RNAs isolated from the RHA1 cells grown on biphenyl (lanes 1, 2, 9, and 10), ethylbenzene (lanes 3, 4, 11, and 12), LB (lanes 5, 6, 13, and 14), and succinate (lanes 7, 8, 15, and 16) were used for the templates for RT-PCR. Lanes 1 to 8 and 9 to 16 were amplifications with the *bphB* and *bphB2* primer sets, respectively. The sizes of molecular weight markers in lane M are indicated on the left side of the gel. Odd-numbered lanes are controls without reverse transcriptase.

bphA components, including *bphA1A2A3*, *etbA1A2*, and *ebdA1A2A3*. The functional analysis of *etbA4* is currently underway in our laboratory.

The third ORF of 1,371 bp showed 64% and 36% amino acid sequence identities with 6-oxohexanoate dehydrogenase (ChnE) of *Rhodococcus* sp. Phi2[8] and succinic semialdehyde dehydrogenase (GabD) of *E. coli*[6], respectively. This result suggests that *orf3* encodes a semialdehyde dehydroge-

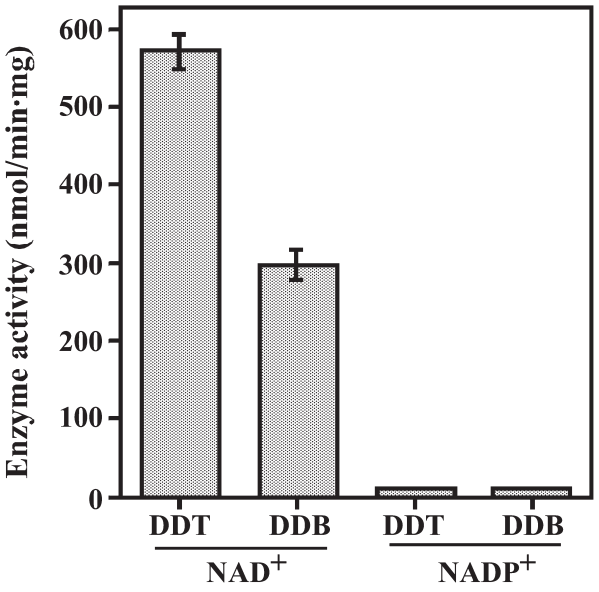

Fig. 4. Substrate preference of BphB2. 23DDT (DDT) and 23DDB (DDB) were used as substrates. NAD^+ or $NADP^+$ was added to the reaction mixture, and the activity was assayed by measuring the increase in the amount of NADH or NADPH formed from NAD^+ or $NADP^+$, respectively. Each value is the average±standard deviation (error bar) of at least three measurements.

nase, but the function of *orf3* remains to be clarified.

Nucleotide sequencing of the 3' terminus of the 9.9-kb *Hin*dIII fragment showed that this region includes the *bphD1* and partial *bphE2* genes, which we characterized in a previous study (Fig. 1B)[12]. This result indicates that the *bphB2* gene cluster is approximately 5-kb upstream of *etbA1* on the RHA1 450-kb linear plasmid[22].

RT-PCR analysis of the *bphB* and *bphB2* genes in RHA1. To obtain an insight into the roles of *bphB* and *bphB2* in the degradation of aromatic compounds, we examined each of the *bphB* transcripts in RHA1 cells grown on various carbon sources. Total RNAs were extracted from RHA1 cells grown in biphenyl, ethylbenzene, succinate, and LB and then underwent RT-PCR analysis using each *bphB* internal primer. The PCR products were analyzed by using agarose gel electrophoresis (Fig. 3). Both RT-PCR products of *bphB* and *bphB2* of the expected sizes (600 bp) were obtained from the total RNAs of the cells grown in biphenyl and ethylbenzene. However, no PCR product was obtained from the total RNAs of the cells grown in LB and succinate. These results indicate that the transcription of *bphB* and *bphB2* were induced during the growth in biphenyl and ethylbenzene, suggesting these genes participate in the degradation of biphenyl and ethylbenzene.

Substrate preference of BphB2. The 1.0-kb *Xho*I-*Kpn*I fragment carrying *bphB2* was cloned into pTrc99A to produce pTB2 (Fig. 1B), which was used for the *bphB2* expression in *E. coli* MV1190. The gene product of *bphB2* was observed by using SDS-PAGE (data not shown), and the molecular mass was estimated as 33 kDa, which is close to the molecular mass calculated from the deduced amino acid sequence of *bphB2* (28,234 Da). The cell extract of *E. coli* containing pTB2 was used for the enzyme assay with

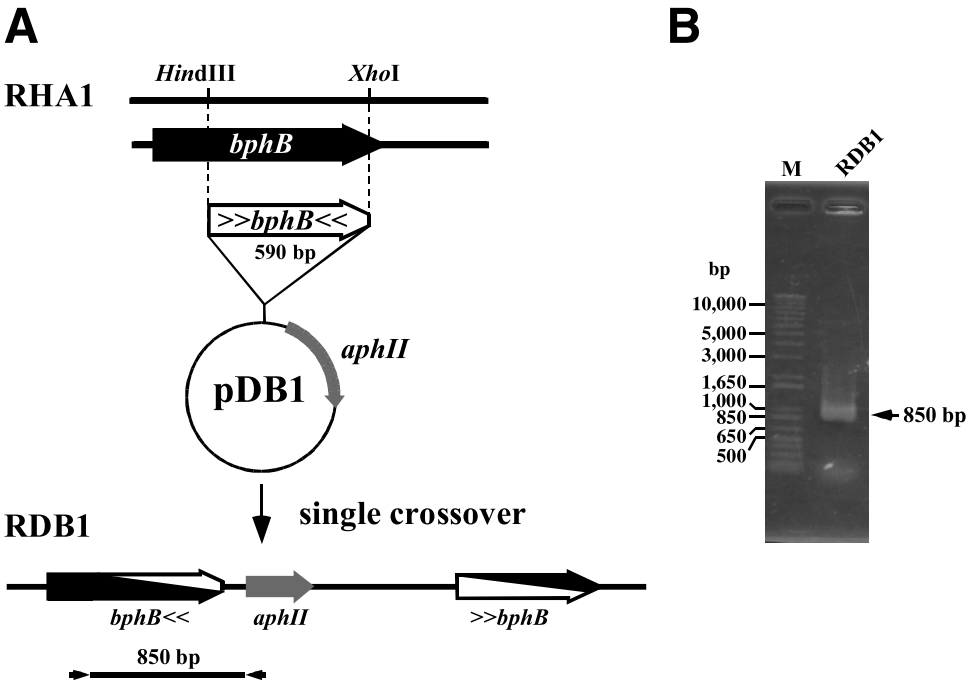

Fig. 5. Disruption of *bphB* in *Rhodococcus* sp. RHA1. (A) Schematic overview of the disruption of *bphB* by integration of pDB1. The *bphB* disruption was accomplished by a single crossover between *bphB* on the 1,100-kb linear plasmid and the truncated *bphB* in pDB1. ≫ and ≪ represent 5' and 3' deletions, respectively. (B) PCR analysis of the *bphB* disruption mutant (RDB1) using the *bphB* forward 2 and *aphII* reverse primers. The *bphB* forward 2 primer was complementary to the 5' region of *bphB*, which is not included in the truncated *bphB* of pDB1. The sizes of molecular weight markers in lane M are indicated on the left side of the gel.

the dihydrodiol compounds of biphenyl and toluene, 23DDB and 23DDT, as substrates (Fig. 4). The BphB2 enzyme showed NAD^+-dependent activities toward both substrates, but 23DDT was preferable to 23DDB as a substrate. Comparing the substrate preferences between BphB2 and BphB was interesting, but we failed to express *bphB* in *E. coli* using *tac* or *trc* promoters.

Disruption of *bphB* in RHA1. To examine the participation of *bphB* in the degradation of biphenyl and ethylbenzene, the *bphB* gene in RHA1 was disrupted by homologous recombination using pUC19-based plasmid pDB1, which carries *aphII* and the truncated *bphB* gene whose 5' and 3' termini were deleted (Fig. 5A). The single crossover between *bphB* on the 1,100-kb linear plasmid and the truncated *bphB* in pDB1 was expected to result in an insertion of the entire pDB1 plasmid into the linear plasmid, in which the region of pDB1 containing *aphII* and the vector was sandwiched between the 3'- and 5'-truncated *bphB* genes. pDB1 was introduced into RHA1 by electroporation, and a kanamycin-resistant transformant, RDB1, was obtained. The colony PCR analysis of RDB1 indicated the expected disruption of *bphB* (Fig. 5B). We tried to disrupt *bphB2* by using the same strategy, but the mutant was not obtained.

To examine the growth of RDB1 on biphenyl and ethylbenzene, RDB1 and RHA1 containing a vector, pK4 (Km^r), were streaked on W medium containing biphenyl or ethylbenzene with kanamycin. RDB1 showed reduced growth on biphenyl compared with RHA1. This result strongly suggests that *bphB* participates in biphenyl degradation. The enzyme activity of the *bphB2* gene product seemed to support the growth of RDB1 on biphenyl. Therefore, most likely is that RHA1 needs both *bphB* and *bphB2* for maximum growth on biphenyl. However, RDB1 grew normally on ethylbenzene, suggesting that *bphB2* has a major role in ethylbenzene degradation. However, further research is needed to clarify the contribution of *bphB* and *bphB2* to degradation of biphenyl and ethylbenzene.

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