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Characterization of Biphenyl Catabolic Genes of Gram-Positive Polychlorinated Biphenyl Degrader *Rhodococcus* sp. Strain RHA1

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***Rhodococcus* sp. strain RHA1 is a gram-positive polychlorinated biphenyl (PCB) degrader which can degrade 10 ppm of PCB48 (equivalent to Aroclor1248), including tri-, tetra-, and pentachlorobiphenyls, in a few days. We isolated the 7.6-kb *Eco*RI-*Bam*HI fragment carrying the biphenyl catabolic genes of RHA1 and determined their nucleotide sequence. On the basis of deduced amino acid sequence homology, we identified six *bph* genes, *bphA1A2A3A4*, *bphB*, and *bphC*, that are responsible for the initial three steps of biphenyl degradation. The order of *bph* genes in RHA1 is *bphA1A2A3A4-bphC-bphB*. This gene order differs from that of other PCB degraders reported previously. The amino acid sequences deduced from the RHA1 *bph* genes have a higher degree of homology with the *tod* genes from *Pseudomonas putida* F1 (49 to 79%) than with the *bph* genes of *Pseudomonas* sp. strains KF707 and KKS102 (30 to 65%). In *Escherichia coli*, *bphA* gene activity was not observed even when expression vectors were used. The activities of *bphB* and *bphC*, however, were confirmed by observing the transformation of biphenyl to a *meta*-cleavage compound with the aid of benzene dioxygenase activity that complemented the *bphA* gene activity (S. Irie, S. Doi, T. Yorifuji, M. Takagi, and K. Yano, J. Bacteriol. 169:5174–5179, 1987). The expected products of the cloned *bph* genes, except *bphA3*, were observed in *E. coli* in an in vitro transcription-translation system. Insertion mutations of *bphA1* and *bphC* of *Rhodococcus* sp. strain RHA1 were constructed by gene replacement with cloned gene fragments. The *bphA1* and *bphC* insertion mutants lost the ability to grow on biphenyl, demonstrating that the cloned *bph* genes are essential for biphenyl catabolism in this strain.**

Polychlorinated biphenyls (PCBs) were synthesized and widely used for industrial materials because of their high levels of chemical stability, highly insulating properties, and resistance to burning. A large amount of PCBs were released into the environment during their early usage, and they are now one of the most serious recalcitrant pollutants. Many PCB-degrading bacteria have been isolated for the purpose of bioremediation (1), and a number of genes encoding enzymes for the degradation of biphenyl and PCB have been characterized (1, 8, 20, 27). In the first step of a typical biphenyl/PCB metabolic pathway, biphenyl is converted to 2,3-dihydroxy-1-phenylcyclohexa-4,6-diene (dihydrodiol) by a multicomponent biphenyl dioxygenase (BphA). Dihydrodiol is oxidized to 2,3-dihydroxybiphenyl (23DHBP) by dihydrodiol dehydrogenase (BphB). 23DHBP is cleaved at the 1,2 position by 23DHBP dioxygenase (BphC), and the resulting *meta*-cleavage product (2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid) is hydrolyzed to benzoic acid and 2-hydroxypenta-2,4-dienoic acid by hydrolase (BphD) (Fig. 1).

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The catabolic genes for biphenyl (*bph* genes) were shown to be conserved in many gram-negative biphenyl-degrading bacteria (1, 8, 12, 20, 27). Interestingly, it was also reported that the genes responsible for toluene degradation (*tod* genes) were similar to those for biphenyl degradation (31). This observation suggests that these two catabolic genes have the same ancestral origin. Evolutionary relationships among degradation genes for aromatic compounds have been described (9, 14). Recently, Asturias et al. isolated three *bphC* genes and a *bphB* gene from *Rhodococcus globerulus* P6 and determined their nucleotide sequences (3, 4). Until recently, most research on the biphenyl catabolic pathway and genes has been done on gram-negative bacteria, especially in the genus *Pseudomonas*. Biphenyl catabolic genes from gram-positive bacteria are thus poorly understood.

We isolated gram-positive *Rhodococcus* sp. strain RHA1 from γ-hexachlorocyclohexane-contaminated soil (23). RHA1 degraded PCB efficiently and completely transformed PCB48, which consists mainly of tetrachlorobiphenyl (23). Di- and trichlorobenzoic acids were identified among the intermediate metabolites of PCB degradation. These chlorobenzoic acids were gradually degraded by this strain. RHA1 exhibited high transformation activity on both *ortho*- and *para*-substituted PCB congeners. This is a superior characteristic of PCB degradation by RHA1 compared with other PCB degraders, including *Pseudomonas pseudoalcaligenes* KF707 and *Pseudomonas* sp. strain LB400 (13). In this study, we isolated the 7.6-kb *Eco*RI-*Bam*HI fragment containing the *bphA1A2A3A4CB* genes and determined their nucleotide sequences. Disruption of the *bph* genes in strain RHA1 indicated that the cloned *bph*

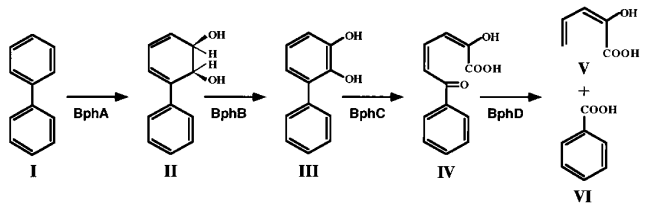


FIG. 1. Proposed degradation pathway of biphenyl. Biphenyl dioxygenase (BphA) converts biphenyl (I) to 2,3-dihydroxy-1-phenylcyclohexa-4,6-diene (dihydrodiol compound) (II). Compound II is converted to 23DHBP (III) by dihydrodiol dehydrogenase (BphB). Compound III is cleaved at the 1,2 position by 23DHBP dioxygenase (BphC) to generate *meta*-cleavage compound IV (2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid). Compound IV is hydrolyzed by *meta*-cleavage compound hydrolase (BphD) and yields 2-hydroxypenta-2,4-dienoic acid (V) and benzoic acid (VI).

genes were essential for biphenyl catabolism in this strain. This is the first report on the structure and function of the *bphACB* genes from a gram-positive bacterium.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and plasmids. *Rhodococcus* sp. strain RHA1 is a PCB degrader which was isolated from γ-hexachlorocyclohexane-contaminated soil in Japan. It was grown at 30°C in W minimal salt medium (20) containing 0.5% biphenyl, LB (Bacto-tryptone 10 g/liter; yeast extract, 5 g/liter; NaCl, 5 g/liter), and diluted LB (Bacto-tryptone, 3.3 g/liter; yeast extract, 1.7 g/liter; NaCl, 5 g/liter). *Escherichia coli* JM109 [*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ(*lac-proAB*) F′(*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15)] was employed as a host strain. The plasmids used in this study are listed in Table 1.

DNA manipulations and nucleotide sequence. The *bph* gene DNA fragments were subcloned into pUC119, pUC118, or pBluescript II SK(+). A Kilosequence kit (Takara Shuzo Co., Ltd., Kyoto, Japan) was used to construct a series of deletion derivatives, whose nucleotide sequences were determined by the dideoxy termination method (22) with a 373A DNA sequencer (Applied Biosystems, Inc., Foster City, Calif.). Sequence analysis and homology search were done with the programs GeneWorks (IntelliGenetics, Inc., Mountain View, Calif.) and SDC-GENETYX (Software Development Corp., Tokyo, Japan). Total DNA from *Rhodococcus* sp. strain RHA1 and its *bphA1* and *bphC* insertion mutants was isolated by using CTAB (hexadecyltrimethyl ammonium bromide) for selective precipitation of cell wall debris, polysaccharides, and remaining protein (5). Southern blot and colony hybridization analyses (26) were done with the DIG-System (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) according to the procedure recommended by the manufacturer.

Assay of biphenyl-transforming activity. *E. coli* JM109 carrying *bph* gene recombinants was cultured in LB containing ampicillin (50 mg/liter) at 30°C. When the optical density at 620 nm (OD_{620}) of the 10-ml culture reached 0.6, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. After 4 h, the cells were washed with 50 mM potassium phosphate buffer (pH 7.5) and resuspended in 1 ml of the same buffer. Cell suspensions of transformants were mixed and incubated with 100 mg of biphenyl per liter. The yellow color of the *meta*-cleavage compound (2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid) (molar extinction coefficient is 13,200 cm^{-1} M^{-1} at 434 nm) produced from biphenyl was monitored. *E. coli* JM109 harboring pBZ1, which is pUC19 carrying the 4.2-kb *Pvu*II fragment including the benzene dioxygenase gene (*bnzA*) of *Pseudomonas putida* BE-81 (17) (an equivalent of *P. putida* F1), was used to complement the BphA activity.

In vitro expression of *bph* genes. In vitro expression of the cloned genes was carried out with the *E. coli* S30 extract system (Promega, Madison, Wis.) according to the manufacturer's protocol. The polypeptides were labeled with L-[^{35}S]methionine (Amersham Corp., Little Chalfont, United Kingdom). Samples were loaded onto a sodium dodecyl sulfate (SDS)–15% polyacrylamide gel (21). After electrophoresis, the gel was exposed to X-ray film.

***bph* gene disruption.** The 1.3-kb kanamycin resistance gene of pUC4K was inserted into the coding region of a *bph* gene in recombinant plasmids. The resulting plasmids were linearized with restriction enzyme and introduced into RHA1 cells by electroporation.

E. coli-Rhodococcus shuttle vector pK4 (15) was used to optimize the conditions of electroporation. We modified the methods of Hashimoto et al. (15) and Desomer et al. (7). When the cell density of RHA1 cultured in 10 ml of LB reached 0.6 to 0.7 OD_{620} units, the cells were harvested and washed with 1 ml of ice-cold water twice and resuspended with 500 μl of ice-cold 30% polyethylene glycol 1000. One to three micrograms of DNA was mixed with 100 μl of cells. Electroporation was performed under high-voltage conditions (25 kV/cm, 800 Ω, and 25-μF capacitor) with a Genepulser (Bio-Rad, Richmond, Calif.).

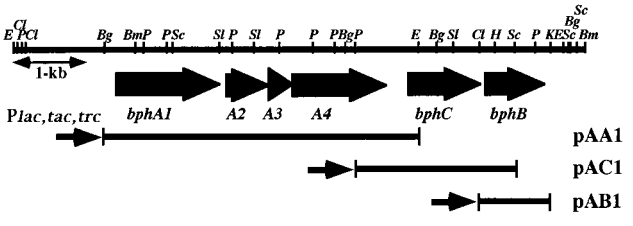
Nucleotide sequence accession number. The nucleotide sequence in this report has been submitted to the GSDB, DDBJ, EMBL, and NCBI DNA databases under accession number D32142.

RESULTS

Cloning and sequencing of *bph* genes. In Southern blot analysis, a 6.0-kb *Bam*HI fragment of *Rhodococcus* sp. strain RHA1 had homology with the *bphAB* genes of *P. pseudoalcaligenes* KF707 (27) and *Pseudomonas* sp. strain KKS102 (10, 16, 20). This fragment was cloned into pUC119 by the colony hybridization technique with the KKS102 *bphAB* probe, and the resulting plasmid was designated pHA101 (16). The *bphC* gene region in this clone was established by screening *E. coli* transformants containing deletion derivatives of pHA101 that have 23DHBP dioxygenase activity, and its nucleotide sequence was determined (16). Nucleotide sequencing of the terminus of this fragment revealed a partial sequence of the *bphA1* gene. The entire *bphA* gene of RHA1 was cloned by colony hybridization of the 0.5-kb *Bam*HI-*Sac*I fragment probe (Fig. 2); the resulting construct, pUH3, carries a 5.4-kb *Eco*RI fragment that includes the 1.6-kb DNA segment upstream of the 6.0-kb *Bam*HI fragment. A restriction map of the 7.6-kb

TABLE 1. Plasmids used in this study

Plasmid	Relevant characteristic(s)	Reference or source
pUC118, pUC119	Cloning vectors, Ap^r	29
pUC19	Cloning vector, Ap^r	30
pBluescript II SK(+)	Cloning vector, Ap^r	25
pKK223-3	Expression vector, Ap^r, p*tac*	6
pTrc99A	Expression vector, Ap^r, p*trc lacI*^{q}	2
pUC4K	Ap^r Km^r, source of Km^r cartridge	28
pK4	*Rhodococcus-E. coli* shuttle vector, Km^r	15
pHA101	pUC119 with 6.0-kb *Bam*HI fragment of RHA1 carrying *bphA2A3A4CB*	16
pUH3	pUC119 with 5.4-kb *Eco*RI fragment of RHA1 carrying *bphA1A2A3A4*	This study
pAT301	pUC119 with 6.0-kb *Bgl*II-*Kpn*I fragment of RHA1 carrying *bphA1A2A3A4CB*	This study
pAA1	pUC19 carrying 4.2-kb *Bgl*II-*Eco*RI fragment of pUH3	This study
pAB1	pUC19 carrying 0.9-kb *Cla*I-*Kpn*I fragment of pHA101	This study
pAC1	pUC19 carrying 2.1-kb *Pst*I-*Sac*I fragment of pHA101	This study
pBZ1	pUC19 carrying 4.2-kb *Pvu*II fragment encoding *bnzA* of *P. putida* BE-81	This study
pH122	pUC119 with 2.1-kb *Pst*I-*Sac*I fragment carrying *bphC*	This study
pH122K	pH122 with insertion of Km^r gene of pUC4K into *Sal*I site	This study
pUA1	pUC118 with 2.8-kb *Eco*RI-*Sal*I fragment carrying a part of *bphA1*	This study
pDA1	pUA1 with insertion of Km^r gene of pUC4K into *Bam*HI site	This study



genotype (plasmid)			production of *meta*-cleavage compound
bphA (pAA1)	*bphB* (pAB1)	*bphC* (pAC1)	–
bnzA (pBZ1)	*bphB* (pAB1)	*bphC* (pAC1)	+
bnzA (pBZ1)	*bphB* (pAB1)	-	–
bnzA (pBZ1)	-	*bphC* (pAC1)	–

FIG. 2. Organization of *bph* genes in *Rhodococcus* sp. strain RHA1 and transformation of biphenyl by RHA1 *bph* genes in *E. coli*. Large arrows indicate ORFs encoding *bphA1*, *bphA2*, *bphA3*, *bphA4*, *bphC*, and *bphB*, as deduced from the nucleotide sequence presented in Fig. 3. *Bg*, *Bgl*II; *Bm*, *Bam*HI; *Cl*, *Cla*I; *E*, *Eco*RI; *H*, *Hin*dIII; *K*, *Kpn*I; *P*, *Pst*I; *Sc*, *Sac*I; *Sl*, *Sal*I. Plasmids pAA1, pAB1, and pAC1 were introduced into *E. coli* JM109 individually, the *E. coli* transformant cells were mixed, and the conversion of biphenyl to the *meta*-cleavage compound was monitored. pBZ1 carrying the *bnzA* gene of *P. putida* BE-81 was used to complement BphA activity.

DNA region bounded by the *Eco*RI and *Bam*HI sites is shown in Fig. 2. The nucleotide sequences of these clones were determined by the dideoxy termination method (22).

The complete nucleotide sequence of the 5,957-bp *Bgl*II-*Kpn*I fragment and the deduced amino acid sequence of these open reading frames (ORFs) are presented in Fig. 3. Among the ORFs found, six ORFs showed significant homology with the previously characterized *bph* genes. One of these ORFs, spanning nucleotide positions 4090 to 5043, was formerly identified as the *bphC* gene on the basis of the results of deletion analysis and sequence similarity with other *bphC* genes reported previously (16). The deduced amino acid sequence of the RHA1 *bphC* gene exhibited 45% similarity to that of *Pseudomonas* sp. strain KKS102 *bphC*. Based on the homology, these six ORFs are presumed to be *bphA1* (nucleotide positions 180 to 1562), *bphA2* (1646 to 2209), *bphA3* (2218 to 2541), *bphA4* (2538 to 3779), and *bphC* and *bphB* (5106 to 5897). Each of these ORFs had a possible ribosome-binding (Shine-Dalgarno) sequence (24) preceding the start codon. A homology search showed that all the *bph* genes of RHA1 except *bphB* have higher degrees of similarity with the *tod* genes for toluene degradation of *P. putida* F1 (31) (49 to 79%) than with the *bph* genes of *P. pseudoalcaligenes* KF707 (27) (50 to 65%) and *Pseudomonas* sp. strain KKS102 (10, 18, 20) (30 to 62%) at the amino acid level (Table 2).

Expression of *Rhodococcus bph* genes in *E. coli*. Expression of the cloned genes in *E. coli* was examined to determine the function encoded on the DNA fragment. *E. coli* cells containing pUC119 carrying the *bphACB* genes (pAT301) were incubated with biphenyl, but no accumulation of the yellow *meta*-cleavage compound was observed. Then *bphA1A2A3A4*, *bphB*, and *bphC* were cloned separately into pUC19; the resulting plasmids were designated pAA1, pAB1, and pAC1, respectively, and were then introduced into *E. coli* JM109. The transformation of biphenyl to the *meta*-cleavage compound was not observed when *E. coli* cells containing pAB1 and pAC1 were mixed with those harboring pAA1 (Fig. 2). The cells carrying pAB1 and pAC1 were then combined with cells carrying the *bnzA*-carrying plasmid (pBZ1), which encodes the benzene dioxygenase of *P. putida* BE-81 (17) (Fig. 2). This combination produced the *meta*-cleavage compound from biphenyl. We tried to express *bphA1A2A3A4*, *bphB*, and *bphC* of RHA1 in *E. coli* by using the expression vectors pKK223-3, carrying the *tac* promoter, and pTrc99A, containing the *trc* promoter, but conversion of biphenyl to the *meta*-cleavage product was still not detected (data not shown).

Identification of *bph* gene products. The products of the cloned genes were identified by an in vitro transcription-translation assay. Plasmids pAA1, pAB1, and pAC1 were used as DNA templates for the *E. coli* extract system (Promega); the results are shown in Fig. 4. Gene products of 27 and 35 kDa were observed from pAB1 and pAC1, respectively. On the basis of the molecular mass expected from nucleotide sequence analysis, the 35-kDa product was identified as the *bphC* product (BphC) (lane 1), and the 27-kDa product was identified as the *bphB* product (BphB) (lane 2). Polypeptides of 52, 22, and 44 kDa were produced from pAA1 (lane 3) and assigned as the gene products of *bphA1*, *bphA2*, and *bphA4*, respectively. However, a polypeptide corresponding to the *bphA3* product was not detected.

Disruption of *bph* genes. To demonstrate that these genes are essential for biphenyl degradation in *Rhodococcus* sp. strain RHA1, the *bph* genes were disrupted and introduced into RHA1 by homologous recombination. The optimal electrotransformation conditions for RHA1 were 25 kV/cm, 800 Ω, and 25-μF capacitor with the Genepulser (Bio-Rad). Under these conditions, more than 1,000 transformants per μg of pK4 DNA were obtained.

For disruption of the *bphC* gene, which encodes the 23DHBP dioxygenase, the 2.1-kb *Pst*I-*Sac*I fragment containing *bphC* was cloned into pUC119 to form pH122. A 1.3-kb kanamycin resistance gene from pUC4K was inserted into the *Sal*I site within *bphC* in pH122 to disrupt the gene. The resulting plasmid, pH122K, was linearized and introduced into *Rhodococcus* sp. strain RHA1 (Fig. 5A). Kanamycin-resistant transformants were collected, and hybridization analysis was

TABLE 2. Comparison between *bph* gene products of *Rhodococcus* sp. strain RHA1 and *Pseudomonas*[a] *tod* and *bph* gene products

Gene product	No. of residues				% Homology with RHA1 product		
	RHA1	KF707	KKS	F1	KF707	KKS	F1
BphA1/TodC1	460	458	458	450	65	62	75
BphA2/TodC2	187	213	193	187	52	56	79
BphA3/TodB	107	109	109	107	51	50	65
BphA4/TodA	413	408	410	410	50	30	58
BphC/TodE	317	298	293	291	52	45	64
BphB/TodD	263	277	276	275	52	53	49

[a] KF707, *P. pseudoalcaligenes*; KKS, *Pseudomonas* sp. strain KKS102; F1, *P. putida*.

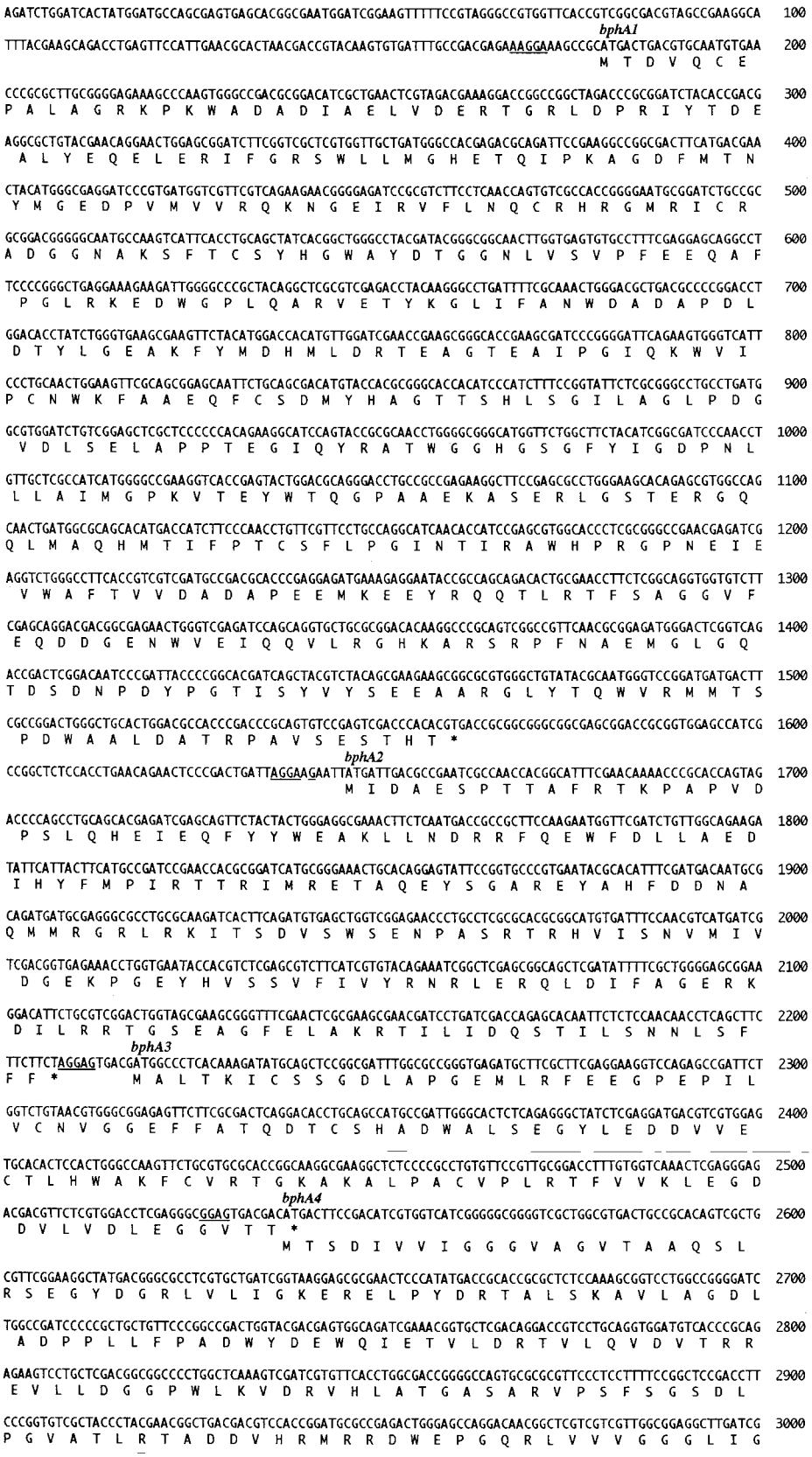


FIG. 3. Nucleotide sequence and deduced amino acid sequence of the *bph* genes in *Rhodococcus* sp. strain RHA1. The coding regions for *bphA1*, *bphA2*, *bphA3*, *bphA4*, *bphC*, and *bphB* span nucleotides 180 to 1562, 1646 to 2209, 2218 to 2541, 2538 to 3779, 4090 to 5043, and 5106 to 5897, respectively. Putative Shine-Dalgarno sequences are indicated by an underline.

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GCTGTGAGGTCGCCACCACCGCCCGGAAGCTCGGTCTCGAGGTCAGCATCCTGGAGGCCTCAGACGAGTTGCTGCAGCGCGTTCTGGGCCGCCGGATCGG  3100
  C  E  V  A  T  T  A  R  K  L  G  L  E  V  S  I  L  E  A  S  D  E  L  L  Q  R  V  L  G  R  R  I  G

CGGCTGGTGCCGGGCGCGCCTCATGGAACTCGGGATCTCGGTCGTGTTGAACACAGGAGTCGCCGAGTTCAAGGGGGTGGACCGGATCACCACTGTAATC  3200
  G  W  C  R  A  R  L  M  E  L  G  I  S  V  V  L  N  T  G  V  A  E  F  K  G  V  D  R  I  T  T  V  I

GGCACCGACGGAAGATCTTTCGTGGCAGATCGTGCAATCGTGTGCGTCGGCGCGGAACCAGAGACGGCGATAGCCGAACAGTCCGGGCTTGCGTGCAACC  3300
G  T  D  G  R  S  F  V  A  D  R  A  I  V  C  V  G  A  E  P  E  T  A  I  A  E  Q  S  G  L  A  C  N  R

GCGGCATCCTCGTGAACGATTCCGGCGGCACCGCCGCTGAGGGGGTCTTCGCTGCAGGCGACGTGGCATCGTGGCCGCTCCTGACGGGCGGGCGGCGGTC  3400
  G  I  L  V  N  D  S  G  G  T  A  A  E  G  V  F  A  A  G  D  V  A  S  W  P  L  L  T  G  G  R  R  S

GCTCGAGACCTACATCAACAGTCAGAGAGAGGCGACCGCCGTTGCGTCGGCGATGTTAGGGAAAGCCGTTCATGGACCGCAACTCCCGCTGTCGTGGACC  3500
L  E  T  Y  I  N  S  Q  R  E  A  T  A  V  A  S  A  M  L  G  K  A  V  H  G  P  Q  L  P  L  S  W  T

GAGATGGCCGGACATCGCATACAGATGATCGGCGACATCGAAGGTTCAGGGGAGTACGTCATGCGAGGCGATCCCGACGACGGCCCGGCTCTGCTCTTCA  3600
E  M  A  G  H  R  I  Q  M  I  G  D  I  E  G  S  G  E  Y  V  M  R  G  D  P  D  D  G  P  A  L  L  F  R

GGCTCAGCGACGGCAGAGTGACTGCGGCAGTTTCGGTGGATGCTCCGCGCGATTTCGCCATGGCGACGCGCCTTGTCGAGAGGGGTGCTCAGGTGGGCCG  3700
  L  S  D  G  R  V  T  A  A  V  S  V  D  A  P  R  D  F  A  M  A  T  R  L  V  E  R  G  A  Q  V  G  R

GGAGGTTCTCGGCGACACCAGCATGGAGTTGCGCGAACTGAATCGTGCGGCACGCGAGCGAGCCCTGATCGCGGAGTGATCACCGAGGACCGCTAGGGAC  3800
E  V  L  G  D  T  S  M  E  L  R  E  L  N  R  A  A  R  E  R  A  L  I  A  E  *

GATCACTCATCCGCTCTGCCGGGCCACCGGTCCGGCGACGACGTGCACCCCACGGTGGCCGGCTCCGCTGGACCGAGCGCAGGTCGAAAGATGGGGTAGG  3900
AAGCCAACGCAGGCACCGCGATCGAGTCGCATCTCCCAAACCAGCGGTCGAGTCGGCTCGGGTCGCCGGGTAGCCAATCTCCTTCGGGCCGTCGAGCACG  4000
                                                                                                  bphC
GCAACAACAGGAAAGCCATACCCGAGGTCCCTGGGTGATTTAACAACCTACGGCCGACGGCGCAAATCACACTCCAAGAAAGGTAAGAAATGAGCGTTCA  4100
                                                                                                  M  S  V  Q

GAGACTCGGTTACATGGGCTTCGAGGTTGCGGATGTGCCCGCGTGGCGCGCCTTCATGACGGAAAAACTGGGGGCCATGGAGGCCTCGAGCAGCGAGAAT  4200
R  L  G  Y  M  G  F  E  V  A  D  V  P  A  W  R  A  F  M  T  E  K  L  G  A  M  E  A  S  S  S  E  N

TCGGCGCGGTTCAGGGTTGACTCCCGCAGTTGGCGCCTGATGGTCGAAAAGGGCCCGTCGGATGACATCTCGCTCTCGGGCTACGAGGTCGATAGCGCGG  4300
S  A  R  F  R  V  D  S  R  S  W  R  L  M  V  E  K  G  P  S  D  D  I  S  L  S  G  Y  E  V  D  S  A  D

ACTCGCTACTCGCGATCAAGAAGCGTCTCGAAGCGCATGGCATCGAGGTGACAACAGAGAGCGGCGAGTTGGCGGCCGATCGCGGTGTCCTCGGACTGAT  4400
  S  L  L  A  I  K  K  R  L  E  A  H  G  I  E  V  T  T  E  S  G  E  L  A  A  D  R  G  V  L  G  L  I

TTCGTGCACGGACACGGCAAACACCCGGGTCGAGATCTACTATGGCGCGACGGAGCTGTTCGAGAAGCCGTTCATCTCGCCGACCGGGGTCTCGGGCTTC  4500
S  C  T  D  T  A  N  T  R  V  E  I  Y  Y  G  A  T  E  L  F  E  K  P  F  I  S  P  T  G  V  S  G  F

ACAACCGGCGACCAGGGGTTCGGCCACTACGTGCTGGCTGTGCCGGATATAGATGCTGCCCTGGATTTCTATGTCAAGGGCCTGGGCTTCCATCTCTCCG  4600
T  T  G  D  Q  G  F  G  H  Y  V  L  A  V  P  D  I  D  A  A  L  D  F  Y  V  K  G  L  G  F  H  L  S  D

ACATCATCGACTGGAAGCTCAACTATGAACTGACGGTCAAACTCCACTTCCTGCACTGCAACGGTCGACACCACACCCTGGCGCTCGCCGGTCTCCCGGG  4700
  I  I  D  W  K  L  N  Y  E  L  T  V  K  L  H  F  L  H  C  N  G  R  H  H  T  L  A  L  A  G  L  P  G

AGCAAAGAAGACTCACCACTTCATGCTCGAGACCAAGCATATGGATGACGTGGGCCTTGCCTACGACAAGTTCGATGCGGACGGGACAGTGGTCATGACG  4800
A  K  K  T  H  H  F  M  L  E  T  K  H  M  D  D  V  G  L  A  Y  D  K  F  D  A  D  G  T  V  V  M  T

CTTGGCCGCCACACCAATGACCACATGCTCTCATTCTACGGCGCGACCCCCTCCGGATTTGCCGTCGAATACGGCTGGGGCGCTCGCCAGGTCGAACCAG  4900
L  G  R  H  T  N  D  H  M  L  S  F  Y  G  A  T  P  S  G  F  A  V  E  Y  G  W  G  A  R  Q  V  E  P  G

GCTGGTCCGTCGTGCGCTACGACAAGATCAGCATCTGGGGTCACAAATTCGTTGCCGAGCGCGACAGGCAAGTGAGCAGCAACGCAATCGAGGATGAATT  5000
  W  S  V  V  R  Y  D  K  I  S  I  W  G  H  K  F  V  A  E  R  D  R  Q  V  S  S  N  A  I  E  D  E  L

GATTGACATCGATGCCACGCTGAGCGCCCCGGCGCAAGCGTAAGACGAACCGAACTCACAAGACGAAGAAAGGGATTCTGATGGGTTGGCTGGAAGACTA  5100
I  D  I  D  A  T  L  S  A  P  A  Q  A  *
   bphB
ACGTCATGATCGTCACCGGCGGAGGGTCGGGCCTAGGCCGGGCGCTGGTCGAGCGATTCCTGGGAGAGGGAGCCCGGGTGGGTGTCCTGGAGAAGTCGGC  5200
    M  I  V  T  G  G  G  S  G  L  G  R  A  L  V  E  R  F  L  G  E  G  A  R  V  G  V  L  E  K  S  A

GGAGAAGGCTGAGAAGCTTGCCAACGACTTCGGCGAGGATGTCCTCGTGGTCGAAGGCGACGTGCGCAAGTACGACGACAATGCGCGCGTCGTCCAGGAA  5300
E  K  A  E  K  L  A  N  D  F  G  E  D  V  L  V  V  E  G  D  V  R  K  Y  D  D  N  A  R  V  V  Q  E

ACGGTTCGCCAGTTCGGTCGCCTCGACACGTTCGTCGCGAACGCCGCGATCTGGGACTTCTCGACGAAGATGGTCGATATCCCGGTGGACCGACTGGACG  5400
T  V  R  Q  F  G  R  L  D  T  F  V  A  N  A  A  I  W  D  F  S  T  K  M  V  D  I  P  V  D  R  L  D  A

CCCTCTTCGACGAAATGTTCCACATCAACGTCAAGGGCTACTTGCACGGTGCACGTGCGGCGGTCGAGGAGCTCGCCGCCACCGGAGGCTCGATCATCTA  5500
  L  F  D  E  M  F  H  I  N  V  K  G  Y  L  H  G  A  R  A  A  V  E  E  L  A  A  T  G  G  S  I  I  Y

CACCGTGTCGAACGCCGGCTTCTACCCCGGCGGCGGCGGCCCGCTCTACACCGCGTCGAAGCACGCGGTAGTCGGGCTGATCAAGGAACTGGCCTTCGAA  5600
T  V  S  N  A  G  F  Y  P  G  G  G  G  P  L  Y  T  A  S  K  H  A  V  V  G  L  I  K  E  L  A  F  E

CTCGGCCCCAAGATCCGCGTCAACGGGGTGGCTCCGGGCGCAATGCCCACCGATCTGAGGGGACCTGGTTCGCTCGGAATGGGCGAAACCACCATCACCT  5700
L  G  P  K  I  R  V  N  G  V  A  P  G  A  M  P  T  D  L  R  G  P  G  S  L  G  M  G  E  T  T  I  T  S

CAGCGGTATCCCTCGGTGATCTGGTCAAACAGTGCACAGTGCTGCAGGAGCTGCCCGAGGCTGCGGACTACACCGGGCACTACGTTCTGCTGGCTTCGAA  5800
  A  V  S  L  G  D  L  V  K  Q  C  T  V  L  Q  E  L  P  E  A  A  D  Y  T  G  H  Y  V  L  L  A  S  K

GGCGAACTCGAGGACGGCAACCGGCGCGATCATCAACTGTGACGGCGGGATGGGTGTGCGCGGCTTGGCCGAGACGGCCGGCGGAAACGACCTGTAAGAC  5900
A  N  S  R  T  A  T  G  A  I  I  N  C  D  G  G  M  G  V  R  G  L  A  E  T  A  G  G  N  D  L  *

GCTGCCCTCGGACAGGAAGAAACAACAGCGAACGACACCAGGCCCACTTTCGGTACC                                          5957
```

FIG. 3—*Continued.*

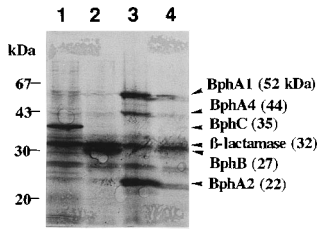

FIG. 4. In vitro expression of *bph* genes of *Rhodococcus* sp. strain RHA1. pAA1, pAB1, pAC1, and pUC19 were used for the *E. coli* S30 extract system (Promega) as DNA templates. Shown is an autoradiogram of an SDS-polyacrylamide gel of [^{35}S]methionine-labeled gene products from pAC1 (lane 1), pAB1 (lane 2), PAA1 (lane 3), and pUC19 (lane 4). The positions of molecular size markers are shown on the left. The expected *bph* gene products are indicated, with their deduced molecular masses in parentheses. Gene products of more than 20 kDa were focused in this photograph. The *bphA3* product, which is expected to be about 12 kDa, was obscure in triplicate experiments.

performed with both the *bphC* and the kanamycin resistance gene as probes in order to ascertain the presence of a disrupted *bphC*. In the *bphC* insertion mutant RDC1, the size of *bphC* gene was increased by the insertion of the 1.3-kb kanamycin resistance gene (Fig. 5C).

To construct an insertion mutation in *bphA1*, the 1.3-kb kanamycin resistance gene was inserted into the *Bam*HI site within *bphA1* in pUA1, and the resulting plasmid was designated pDA1 (Fig. 5B). Transformation of RHA1 was carried out by using linearized molecules of pDA1. The *bphA1* insertion mutant RDA1 was isolated and confirmed to have an expected *bphA1* disruption by hybridization analysis (Fig. 5D).

RDA1, RDC1, and RHA1 cells harboring pK4 were grown in LB containing kanamycin (50 mg/liter) and inoculated into 10 ml of W minimal salt medium containing 0.5% biphenyl and kanamycin (50 mg/liter). *Rhodococcus* sp. strain RHA1 containing pK4 started to grow after 50 h and reached on OD_{620} of 5.0 after incubation at 30°C for 100 h. No growth was observed in the RDC1 and RDA1 cultures. These results indicate that the cloned *bph* genes are essential for growth of this strain on biphenyl.

DISCUSSION

The *bphACB* genes from *Rhodococcus* sp. strain RHA1 were isolated and their nucleotide sequence was determined. The order of the *bph* genes in RHA1 is *bphA1A2A3A4-bphC-bphB*. The presence of the *bphC* gene between *bphA4* and *bphB* differs from the order of other known biphenyl pathway genes, for which the order is *bphA-bphB-bphC* (10, 18, 20, 27). The deduced amino acid sequence identities between the RHA1 *bph* genes and the *tod* genes of *P. putida* F1 were considerably significant. A close evolutionary relationship between these two genes is indicated by this sequence homology. Alternatively, the degrees of homology between the *bph* genes from RHA1 and *Pseudomonas* sp. strains KF707 and KKS102 are not as high, 30 to 65%. Recently, the nucleotide sequences of three *bphC* genes and one *bphB* gene in *R. globerulus* P6 were reported (3, 4). The P6 *bphC1* nucleotide sequence showed a high degree of similarity (64%) to that of RHA1 *bphC*. The *bphC* nucleotide sequence is also more similar to that of the F1 *todE* gene than to that of the *bphC* genes of *Pseudomonas* strains KF707 and KKS102. Comparison of the *bph* genes of gram-positive *Rhodococcus* PCB degraders with those from gram-negative *Pseudomonas* PCB degraders suggests that they are all derived from the same ancestral origin. The divergence between the *Rhodococcus* and *Pseudomonas* strains KF707 and KKS102 *bph* genes suggests the evolutionary divergence of *bph* genes between gram-positive and gram-negative bacteria.

E. coli strains carrying the RHA1 *bphACB* genes were not able to transform biphenyl to the *meta*-cleavage compound even when induced by strong promoters. The RHA1 *bphB* and *bphC* genes were expressed in *E. coli* and were able to convert biphenyl to the *meta*-cleavage compound in the presence of the benzene dioxygenase gene (*bnzA*). It appears that *bphA* from RHA1 is poorly expressed in *E. coli*. It is possible that post-translational events such as the assembly and interaction of subunits caused the poor expression of RHA1 *bphA*. Evidence supporting this hypothesis is that (i) the *bphA3* gene appears to be cotranscribed with *bphA1A2A3A4*, *bphB*, and *bphC*, judging from the tandem organization of these genes, and (ii) the *bphA3* gene has a possible ribosome-binding sequence at the appropriate position. The *bphA3* product contains few methionine residues per molecule. This would result in a poorly labeled product which would escape detection in the in vitro expression system. Asturias et al. also mentioned the poor expression of the *R. globerulus* P6 *bphA* gene in *E. coli* (4). The

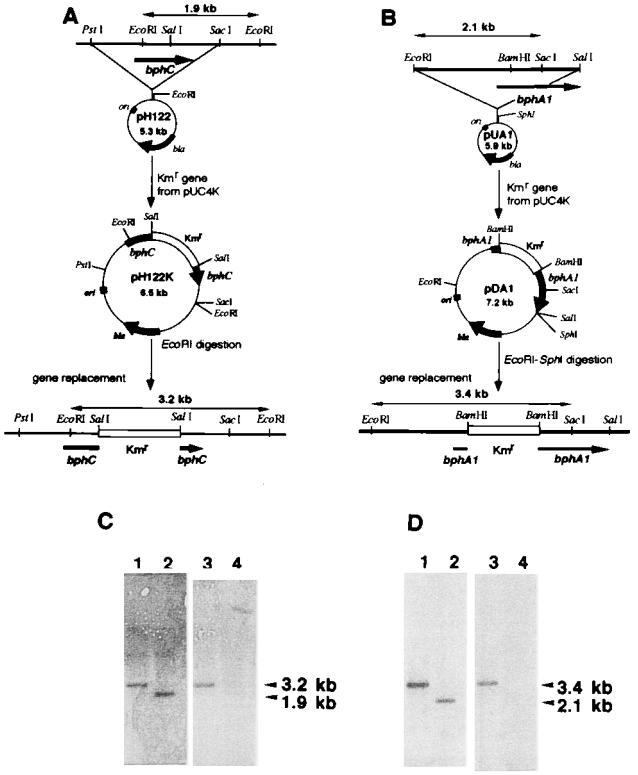

FIG. 5. *bphC* and *bphA1* disruption in *Rhodococcus* sp. strain RHA1. (A) Construction of pH122K. The 2.1-kb *Pst*I-*Sac*I fragment containing *bphC* was cloned in pUC119 to form pH122. The 1.3-kb kanamycin resistance gene from pUC4K was inserted into the *Sal*I site within *bphC* in pH122. The resulting plasmid, pH122K, was digested with *Eco*RI and introduced into RHA1. (B) Construction of pDA1. For disruption of *bphA1*, pDA1 was constructed by inserting the pUC4K kanamycin resistance gene into the *Bam*HI site within *bphA1* in pUA1. pDA1 was digested with *Eco*RI and *Sph*I and introduced into RHA1. Southern blot analyses of the *bphC* (RDC1) and *bphA1* (RDA1) insertion mutants are shown in panels C and D, respectively. (C) Lanes: 1 and 3, total DNA of RDC1 digested with *Eco*RI; 2 and 4, total DNA of RHA1 digested with *Eco*RI. The 1.9-kb *Eco*RI fragment carrying partial *bphC* (lanes 1 and 2) and 1.3-kb *Sal*I fragment carrying the kanamycin resistance gene (lanes 3 and 4) were used as probes. (D) Lanes: 1 and 3, total DNA of RDA1 digested with *Eco*RI and *Sac*I; 2 and 4, total DNA of RHA1 digested with *Eco*RI and *Sac*I. The 2.1-kb *Eco*RI-*Sac*I fragment (lanes 1 and 2) and the kanamycin resistance gene (lanes 3 and 4) were used as probes.

Rhodococcus bphA genes might have some difficulties in expression in *E. coli.*

The *bphA1* and *bphC* genes in RHA1 were disrupted by the insertion of a kanamycin resistance gene, using the gene replacement technique. The electroporation procedure was optimized for the transformation of RHA1 after some modifications of the protocols reported for *Rhodococcus* strains (7, 15). Neither the *bphA1* nor *bphC* insertion mutant grew on biphenyl. In the case of the *bphC* insertion mutant, the *bphB* gene appeared to be inactivated by the polar effect caused by the *bphC* insertional inactivation, because the expected metabolic intermediate of biphenyl, 2,3-dihydroxybiphenyl, was not accumulated and the transformation activity of *cis*-toluene dihydrodiol conferred by the *bphB* gene product was hardly detected (data not shown). The results obtained with these insertion mutants indicated that *bphA* and probably *bphB* play an essential role in biphenyl metabolism in RHA1.

R. globerulus P6 was reported to have three BphC isozyme genes. *bphC2* and *bphC3* have no homology to *bphC1* (3, 4). Preliminary examination by PCR amplification with specific oligonucleotide primers suggested the presence of a *bphC2* homolog in RHA1 (data not shown). It will be interesting to investigate whether these BphC isozymes are involved in biphenyl/PCB degradation.

The head-to-tail tandem distribution of the RHA1 *bph* genes suggests that they are organized in an operon. The cloned *bphA1A2A3A4CB* gene cluster is required for biphenyl metabolism in RHA1. Preliminary nucleotide sequencing of the *bphA1* upstream region and the *bphB* downstream region suggests the disconnected distribution of *bphD* from the *bphACB* cluster in RHA1. The separation of *bphD* from the *bphABC* cluster also occurs in *P. pseudoalcaligenes* KF707 (11). In KF707, the *bphD* gene is separated from the *bphABC* genes by genes that are homologous to the *bphEGF* genes of *Pseudomonas* sp. strain KKS102 (19). Presently, we are cloning the RHA1 *bphD* gene to compare the organization of the *bph* genes in RHA1 and KF707.

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