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Identification of the *bphA4* Gene Encoding Ferredoxin Reductase Involved in Biphenyl and Polychlorinated Biphenyl Degradation in *Pseudomonas* sp. Strain KKS102

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The nucleotide sequence of the downstream region of the *bph* operon from *Pseudomonas* sp. strain KKS102 was determined. Two open reading frames (ORF1 and ORF2) were found in this region, and the deduced amino acid sequence of ORF2 showed homology with the sequences of four ferredoxin reductases of dioxygenase systems. When this region was inserted just upstream of the *bph* operon, which does not contain a gene encoding ferredoxin reductase, biphenyl dioxygenase activity was detected. The 24- and 44-kDa polypeptides predicted from the two open reading frames were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Crude extract which contained the products of ORF2 and *bphA1A2A3* showed cytochrome *c* reduction activity. These data clearly suggest that ORF2 encodes ferredoxin reductase. The deduced amino acid sequence of ORF1 does not show significant homology with the sequences of any other proteins in the SWISS-PROT data bank, and the function of ORF1 is unknown.

PCBs (polychlorinated biphenyls) are widespread environmental pollutants, and contamination with them causes a serious problem. Since the first report on the biodegradation of PCBs appeared in 1973, many studies of their biodegradation, catabolic pathway, and degrading genes have been reported (1, 3–5, 8, 9, 22). A PCB degrader, *Pseudomonas* sp. strain KKS102, was isolated by Kimbara et al. from a mixed culture (13). This mixed culture exhibited a capability to degrade a mixture of highly chlorinated PCBs such as PCB48, which mainly consists of tetrachlorobiphenyls. It was shown that the two strains in the mixed culture, *Pseudomonas fluorescens* KKL101 and *Pseudomonas* sp. strain KKS102, had a symbiotic relationship and that KKS102 played a major role in the degradation of PCBs.

The major catabolic pathway of PCBs has been elucidated to be the oxidative route (Fig. 1) (8, 9). In the first catabolic step, two atoms of oxygen are inserted at carbon positions 2 and 3 by biphenyl dioxygenase (encoded by the *bphA* region) to produce a dihydrodiol. The biphenyl dioxygenase is a multicomponent enzyme which consists of large and small subunits of iron-sulfur protein (products of *bphA1* and *bphA2*, respectively), ferredoxin (product of *bphA3*), and ferredoxin reductase (product of *bphA4*). The 2,3-dihydrodiol is converted to a 2,3-dihydroxybiphenyl by 2,3-dihydrodiol dehydrogenase (product of *bphB*). The 2,3-dihydroxybiphenyl is cleaved at the *meta* position by 2,3-dihydroxybiphenyl dioxygenase (product of *bphC*) to yield 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid, which is transformed into corresponding chlorobenzoic acid and 2-hydroxypenta-2,4-dienoate by 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid hydrolase (product of *bphD*).

We have already cloned and sequenced six genes from KKS102 (*bphA1A2A3B* [7] and *bphCD* [14]) for biphenyl and PCB degradation. From these data, we found that the *bph* operon of KKS102 does not contain a gene encoding ferredoxin reductase (*bphA4*). When the ferredoxin reductase gene from a benzene degrader, *Pseudomonas putida* BE-81 (11), was put together with this operon, biphenyl dioxygenase activity was detected (7). This result suggests that the products of *bphA1A2A3* have biphenyl dioxygenase activity when they are complemented by ferredoxin reductase. (The nucleotide sequence data of *bphA1A2A3B* have been submitted to the DDBJ, EMBL, and GenBank nucleotide sequence data bases under accession number D17319.)

In this study, we sequenced the downstream region of *bphD* and found a gene for ferredoxin reductase. The reductase activity of the gene product was detected as cytochrome *c* reduction. These data clearly show that the gene encodes ferredoxin reductase involved in biphenyl and PCB degradation in KKS102.

MATERIALS AND METHODS

Bacterial strains and medium. *Escherichia coli* MV1190 (Δ*lac-proAB thi supE* Δ*srl-recA306*::Tn*10* F′ *traD36 proAB lacI*^{q}ZΔM15) (27) was utilized as the host of all recombinant plasmids.

L broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) was used for the growth of all cultures. Agar (1.5%) was added to obtain solid medium and ampicillin (50 μg/ml) was added when necessary. *E. coli* MV1190 was cultivated at 37°C except where indicated otherwise.

Construction of plasmids. DNA manipulation and transformation and the isolation of plasmids were carried out as described by Sambrook et al. (18). Figure 2 shows the structures of constructed plasmids discussed in this paper. The vector for pKH20 (14) was pUC18 (pMB9 replicon; carries a gene encoding ampicillin resistance [Ap^r]) (17), and for pKH21, -200, -201, -202, -203, -204, -303, and -304 the vector was pUC119 (pMB9 replicon; carries a gene encoding Ap^r) (26).

Overexpression plasmids (pKH400 and pKH401) were constructed from pAQN, which has the same structure as pAQI

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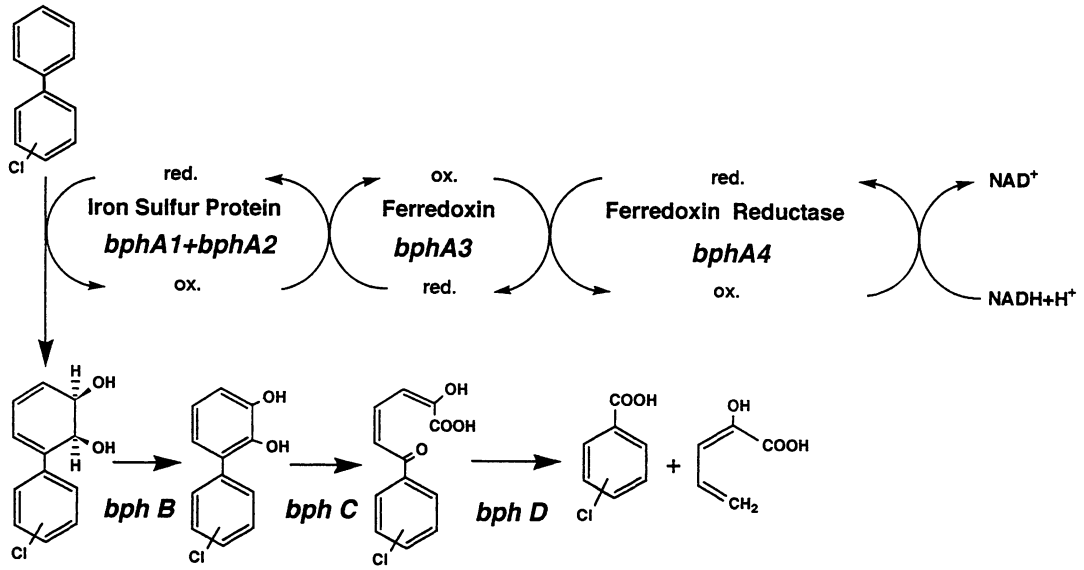

FIG. 1. Proposed catabolic pathway for degradation of biphenyls and PCBs. The following genes encode the indicated enzymes: *bphA1A2*, iron-sulfur protein; *bphA3*, ferredoxin; *bphA4*, ferredoxin reductase; *bphB*, dihydrodiol dehydrogenase; *bphC*, 2,3-dihydroxybiphenyl dioxygenase; and *bphD*, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid hydrolase.

(pMB9 replicon; *lacI*^{q}, aqualysin I gene of *Thermus aquaticus*; carries a gene encoding Ap^r) (23) except for differences in the aqualysin I-coding region. The plasmid pAQN was digested with *Eco*RI and *Hin*dIII to replace the 1.8-kb aqualysin I-coding fragment with the 2.2-kb *Eco*RI-*Hin*dIII fragment (equivalent to the 2.2-kb *Dra*I-*Bam*HI fragment containing open reading frame 1 [ORF1] and ORF2) from pKH203 and the 1.5-kb *Eco*RI-*Hin*dIII fragment (equivalent to the 1.5-kb *Hin*dIII-*Bam*HI fragment containing ORF2) from pKH204, respectively. The resulting plasmids, pKH400 and pKH401, contained the origin of replication from pUC18 and the *lacI*^{q} gene and expressed ORF1 and ORF2 together and ORF2 alone under the control of the *tac* promoter, respectively.

Nucleotide sequence determination. Unidirectional deletion

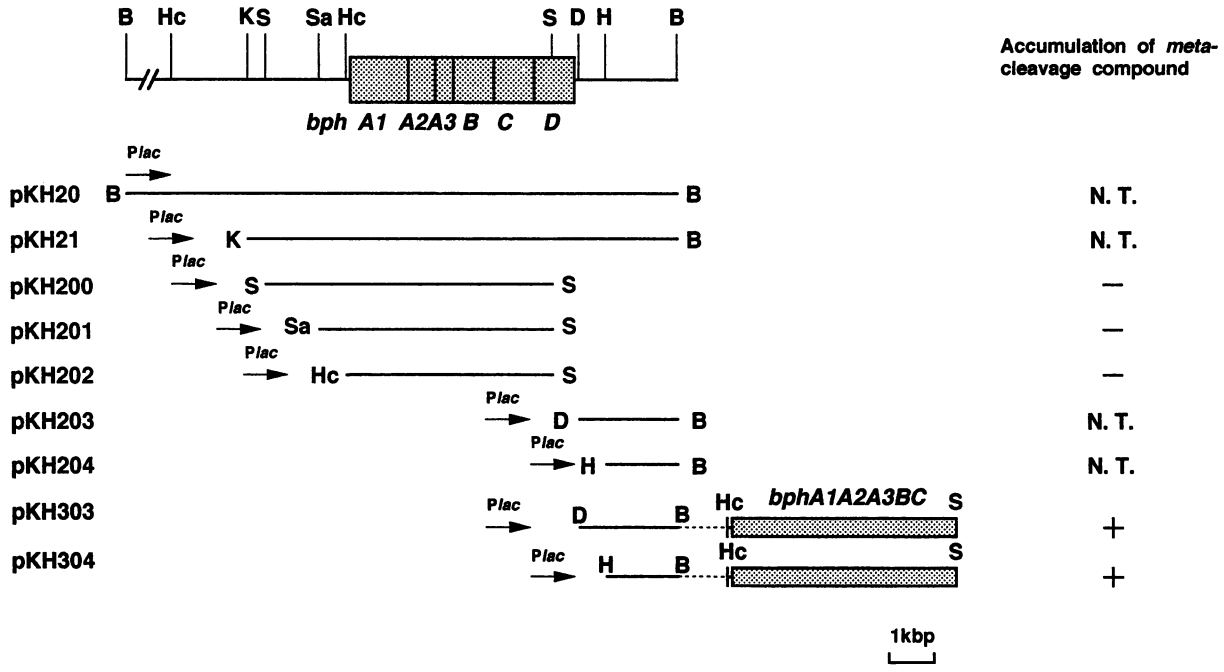

FIG. 2. Structures of the constructed plasmids and assay of biphenyl dioxygenase activity. Each DNA fragment from pKH20 was inserted downstream of the *lac* promoter (P*lac*) of pUC119. The directions of transcription by the *lac* promoter of pKH20, -21, -200, -201, -202, -203, -204, -303, and -304 are indicated by arrows. Biphenyl dioxygenase activities of *E. coli* harboring the pKH plasmids were measured as described in Materials and Methods. B, *Bam*HI; D, *Dra*I; H, *Hin*dIII; Hc, *Hin*cII; K, *Kpn*I; S, *Sma*I; Sa, *Sal*I; N. T., not tested.

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TGACGCTGGACTTCCTGGCCAACGGCTGAGCGCCGTTTCCACTTTTCCCGCAGAGCAAAC 60
* translational stop codon of the bphD
CAGTAAGGCCACCTCCCACAGGGGGAGGCGCGGCCCTGCTTTTCCATAAAACCGGCCTCT 120
TGGTCCATTTCTTTAAAAAAACGCGAGGAGACTCCCATGAAACCCTTTTCCCGCACCCTG 180
                         ORF1   M K P F S R T L

CTACCGCTGGCCTGCGTTGCGTTGTTTGGCAGTGCCCATGCCCAAACCACACCTAACGTC 240
L P L A C V A L F G S A H A Q T T P N V

CAAACTTCGCCCTGGTCGCTGGCTGTGGGCGCCGTGCATTTAGGCTTCAACACCAAGGCC 300
Q T S P W S L A V G A V H L G F N T K A

GACCTGTATGCCGGTGGGCCCGTCCCTGGTGCTGGTGTAGACGCCAGCAGCGACACCATA 360
D L Y A G G P V P G A G V D A S S D T I

GCGGGCCTGGAAATCGCCTATTCCTTTAACCCTAACTGGACGGCTCGGCTGGACCTCGGC 420
A G L E I A Y S F N P N W T A R L D L G

ACACCCGTCAAGACAAATCTTTCCGGCACGGGCAACCTCGGGGCTCTAGGGCGTCTCGGT 480
T P V K T N L S G T G N L G A L G R L G

GGCGTGAAGGGCGGGCCGGCAATTCTGACACTCACCTATTCGCCTGGCATGTGGGGCCAA 540
G V K G G P A I L T L T Y S P G M W G Q

TCGCCCATTCTTTGGCGGCGGCATGACCTACCTCAAGGTATTCAGCACCAGCAACGGTGC 600
S P I L W R R H D L P Q G I Q H Q Q R C

TCTGCAGAACCTCAAGGTGGACGACAACTTTGCGCGCCTTCATCGTCGGCGCCGACTGGC 660
S A E P Q G G R Q L C A P S S S A P T G

CTTTGGCTGACGCTACAGCCTCACCTTCACGCTGCAGAAACTTTACCTCAAGACCACTGC 720
L W L T L Q P H L H A A E T L P Q D H C

CAGCGGCACCATGGGCGGCCAACCGGTCACGGCCAACGTGCGACTGGACCCGCTGGTAAG 780
Q R H H G R P T G H G Q R A T G P A G K

CTTCCTCGGCCGTGCGCAAGCAATTCTGATTCTGGTCCGGAGAGCGATTTTTGTTGCGCC 840
L P R P C A S N S D S G P E S D F C C A

GCAACGCTTGCAGCTCGATGCTCCGCTGCAACGTTCGGCGCAAAGCGACAAACATGCAAA 900
A T L A A R C S A A T F G A K R Q T C K

GTGATGCGTTAGTGCTATTTGCTGCTGAATTGGGCAGCCCTTTTTTCCATTGAGTCGTCG 960
V M R *

AGTAACCTCACTATGTCACAAGAAGCATTGAAAGCCCCCGTTGTAGTTCTCGGTGCAGGC 1020
   ORF2     M S Q E A L K A P V V V L G A G

TTGGCCTCCGTCTCCTTTGTGGCCGAACTGCGCCAGGCGGGCTACCAGGGGCTGATCACC 1080
L A S V S F V A E L R Q A G Y Q G L I T

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V V G D E A E R P Y D R P P L S K D F M

GCCCATGGCCGACCGCCGAGAAGATCCGCCTTGACTGCAAGCGCGCGCCTGAGGCTGAAT 1200
A H G R P P R R S A L T A S A R L R L N

GGCTGCTGGGCGTCACGGCGGCAGTCGTTCGATCCGCAGGCCACACACGGTGGCGCTGTC 1260
G C W A S R R Q S F D P Q A T H G G A V

GTCGACGGGCGCACCCTTCCCTATGGGCACGCTGGTGCTGGCCACGGGTGCAGCGCCGCG 1320
V D G R T L P Y G H A G A G H G C S A A

CGCGTACCGACCTTGCAGGGCGCCACCATGCCGCGGGTACATACGCTGCGCACGCTGGAA 1380
R V P T L Q G A T M P R V H T L R T L E

GATGCGCGGCGCATCCAGGCCGGGCTGCGCCCGCAGTCGCGACTTTTGATCGTAGGCGGC 1440
D A R R I Q A G L R P Q S R L L I V G G

GGTGTGATCGGGCTCGAACTCGCCGCCACGGCGCGCACTGCTGGTGTCCACGTGAGCCTT 1500
G V I G L E L A A T A R T A G V H V S L

GTCGAAACTCAGCCGCGCCTCATGAGCCGCGCTGCACCGGCCACGCTGGGCACTTCGTCG 1560
V E T Q P R L M S R A A P A T L G T S S

CGCGCTACCACGCGGCCGCAAGGGGTCGATCTGCGCTTTGAGCGCAGCGTCACGGGCTCT 1620
R A T T R P Q G V D L R F E R S V T G S

GTCGATGGCGTGGTGCTGCTCGACGATGGCACGCGCATCGCGGCCGACATGGTGGTTGTG 1680
V D G V V L L D D G T R I A A D M V V V

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G H R R V G Q Q L A R A A G L A C D D G

ATCTTCGTCGATCGCTCACTCGGGCGTACCACCTGTCCCGTCGTCTATGCTCTTGGCGAC 1800
I F V D R S L G R T T C P V V V Y A L G D

GTAACGGCCAAGCGCAACCCTCTAAGCGGGCGTTTCGAGCGGATCGAAACCTGGTCGAAC 1860
V T A K R N P L S G R F E R I E T W S N

GCGCAGAACCAGGGCATCGCGGTGGCGCGCCACTTGGTCGATCCAACCGCGCCTGGATAT 1920
A Q N Q G I A V A R H L V D P T A P G Y

GCCGAGTTGCCGTGGTATTGGTCCGACCAGGGTCGCGTGCGCATCCAGGTGCGGGGCTTG 1980
A E L P W Y W S D Q G R V R I Q V R G L

CGAGCGCGACGAGGAAATCGTGCGTGGCGAAGTCTCACTCGACGCCCCCAAGTTCACGCT 2040
R A R R G N R A W R S L T R R P Q V H A

CATCGAGCTTGCAAAAGGCGCATTGTGGGCGCGACCTGCGTGAACAACGCGCGCGACTTC 2100
H R A C K R R I V G A T C V N N A R D F

GCACCGCTGCGGCGCCTGCTCGGTCGGCGCCAAGCCCGGACCGCGCCGCGCTCCGCCGAC 2160
A P L R R L L G R R Q A R T A P R S A D

CCGGCCACCGACTTGCGCAAGCTCGCTGCCGCCGTTGCCGCGTGAGTGCCTGCGTATGAC 2220
P A T D L R K L A A A V A A *

GCCGCATAGCTCAGAGAAAGCGTGCCCCTGCACGGCACGGCTCGGTGTGTCAAGAATGCG 2280
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TTCAGTGGGGATCC
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FIG. 3. Nucleotide sequence of the downstream region of *bphD* and the deduced amino acid sequences of ORF1 and ORF2. The deduced amino acid sequences of ORF1 and ORF2 are shown in one-letter code, and asterisks indicate stop codons. The putative Shine-Dalgarno sequences complementary to the 3′ ends of the 16S rRNAs of both *Pseudomonas aeruginosa* and *E. coli* (19) are double underlined.

mutants of pKH203 were constructed with exonuclease III and mung bean nuclease as described by Henikoff (10), with a slight modification. DNA sequencing from both strands was carried out with the *Bca*BEST dideoxy sequencing kit (Takara Shuzo Co., Kyoto, Japan). The nucleotide sequences were analyzed with GENETYX software (version 21.0; Software Development Co. Ltd., Tokyo, Japan).

Assay of biphenyl dioxygenase activity. Plasmids pKH200, -201, -202, -303, and -304 were introduced into *E. coli* MV1190, and the transformants were cultivated in 10 ml of L broth. The cell density was monitored by measuring the turbidity of the culture at 550 nm. When the turbidity reached a value of 0.6, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 5 mM, and cultivation was continued for another 3 h. Then 0.01% biphenyl was added, and the cells were cultivated for 30 min. After centrifugation, the A_{430} of the supernatant was measured.

Analysis of plasmid-encoded polypeptides. The overexpression plasmids pKH400 (containing ORF1 and ORF2) and pKH401 (containing ORF2) were introduced into *E. coli* MV1190. After cells were induced with IPTG, by the method described above, the cells from 1 ml of each culture were harvested by centrifugation at 1,500 × *g* for 10 min and washed with an equal volume of phosphate-buffered saline (NaCl [8.0 g/liter], KCl [0.2 g/liter], Na_2HPO_4 [1.44 g/liter], KH_2PO_4 [0.24 g/liter]) (pH 7.4). The cells were boiled at 100°C for 5 min in the loading buffer (0.125 M Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate [SDS], 5% 2-mercaptoethanol, 7.5% glycerol, 0.005% bromophenol blue), and then 5 μl of each lysate generated by this treatment was loaded directly onto an SDS–12.5% polyacrylamide gel. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was done according to the method of Laemmli (15). Electrophoresis was performed at 20 mA

until the tracking dye reached the bottom of the gel. Proteins were visualized by staining with Coomassie brilliant blue R250.

Preparation of crude extract. *E. coli* MV1190 cells harboring plasmids pUC119, pKH200, pKH304, and pKH21 were cultivated in 200 ml of L broth at 30°C. When the turbidity at 550 nm reached a value of 0.6, IPTG was added to a final concentration of 1 mM, and the cells were incubated for another 6 h. Then the cells were harvested, washed twice with cold phosphate-buffered saline (pH 7.4), and resuspended in 10 ml of 25 mM potassium phosphate buffer (pH 7.5) containing 10% ethanol and 10% glycerol. The cell suspensions were sonicated and centrifuged at 220,000 × *g* for 1 h, and then they were used as crude extracts. The protein concentrations of the crude extracts were estimated with a protein assay kit (Bio-Rad Laboratories, Richmond, Calif.).

Assay of cytochrome *c* reduction activity. Assays of the ferredoxin reductase activities of the crude extracts were performed by measuring cytochrome *c* reduction activity in the presence of a cofactor (NADH or NADPH) (21). Assay mixtures (1.5 ml) were composed of 25 mM phosphate buffer (pH 7.5), 77 μM cytochrome *c*, and 20 to 50 μl of the crude extracts. The reaction was initiated by the addition of a cofactor (NADH or NADPH) to a final concentration of 0.2 mM, and then the A_{550} of the mixture was monitored. One unit of activity was defined as the amount of enzyme required to reduce 1 μmol of cytochrome *c* per min. An extinction coefficient (amount of reduced cytochrome *c* minus amount of oxidized cytochrome *c*) of 21,000 M^{-1} cm^{-1} (24) was used for calculations. The cytochrome *c*, NADH, and NADPH were obtained from Wako Pure Chemical Industries Ltd., Osaka, Japan.

Nucleotide sequence accession number. The nucleotide sequence data in this paper have been submitted to the DDBJ, EMBL, and GenBank nucleotide sequence data bases under accession number D16831.

RESULTS

Nucleotide sequencing of the downstream region of *bphD*. The downstream region of *bphD* was sequenced for about 2.4 kb, and the sequence is shown in Fig. 3. Two open reading frames (ORF1 and ORF2) were found in this region by computer analysis, and the deduced amino acid sequences of ORF1 and ORF2 are also shown in Fig. 3.

Amino acid sequences which were similar to those of ORF1 and ORF2 were found in the SWISS-PROT amino acid sequence data bank (European Molecular Biology Laboratory release 23.0). In the case of ORF2, four amino acid sequences which encode ferredoxin reductases were found. These were TodA from *P. putida* F1 (28), a P4 gene product from *P. putida* BE-81 (11), BphA4 from *Pseudomonas pseudoalcaligenes* KF707 (22), and BphG from *Pseudomonas* strain LB400 (5) (Table 1). The deduced amino acid sequence of ORF1 did not show significant homology with the sequence of any protein in the data bank.

The alignment of the deduced amino acid sequences of ORF2 and TodA is shown in Fig. 4. The amino acid identity is 32.6%. The consensus sequences of the putative flavin adenine dinucleotide (FAD)- and NAD-binding sites of TodA are conserved in the deduced amino acid sequence of ORF2 (16) (see Discussion).

Assay of biphenyl dioxygenase activity. Figure 2 shows the structures of constructed plasmids which were used for determination of biphenyl dioxygenase activity. Accumulation of a *meta* cleavage compound was not detected in *E. coli* harboring pKH200, -201, or -202 (containing *bphA1A2A3BC*), while *E. coli* harboring pKH303 and -304 (containing ORF2 and *bphA1A2A3BC*) accumulated a *meta* cleavage compound under the same conditions. These results suggest that the mixture of the products of ORF2 and BphA1A2A3 exhibits biphenyl dioxygenase activity.

TABLE 1. Homology of the deduced amino acid sequence of ORF2 and the sequences of four ferredoxin reductases of dioxygenase systems

Ferredoxin reductase	% Identity with:				
	ORF2	TodA	P4 gene product	BphA4	BphG
ORF2	100	32.6	31.6	31.2	31.2
TodA[a]		100	96.8	52.7	52.5
P4 gene product[b]			100	50.2	50.0
BphA4[c]				100	99.8
BphG[d]					100

[a] From *P. putida* F1 (28).
[b] From *P. putida* BE-81 (11).
[c] From *P. pseudoalcaligenes* KF707 (22).
[d] From *Pseudomonas* strain LB400 (5).

SDS-PAGE analysis of products of ORF1 and ORF2. When the products of the 2.2-kb *Dra*I-*Bam*HI fragment (containing ORF1 and ORF2) and the 1.5-kb *Hin*dIII-*Bam*HI fragment (containing ORF2) were overexpressed under the control of the *tac* promoter, 24- and 44-kDa polypeptides were identified by SDS-PAGE (Fig. 5). Their molecular weights approximately match those determined on the basis of the deduced amino acid sequences of ORF1 and ORF2 (26,185 and 44,101, respectively).

Assay of ferredoxin reductase activity. To determine the ferredoxin reductase activity of the product of ORF2, cyto-

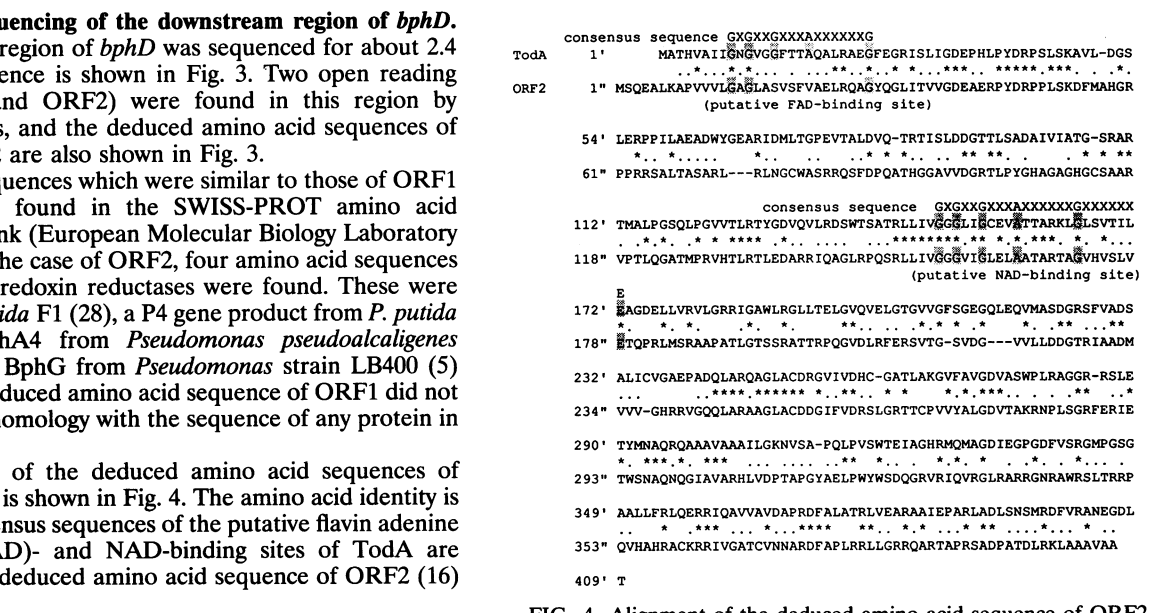

FIG. 4. Alignment of the deduced amino acid sequence of ORF2 with the sequence of TodA. Identical and similar amino acids are indicated by asterisks and dots, respectively. The consensus sequences of the putative FAD- and NAD-binding sites are shown above the amino acid sequences. The amino acid residues which match with the consensus sequences are in shaded boxes.

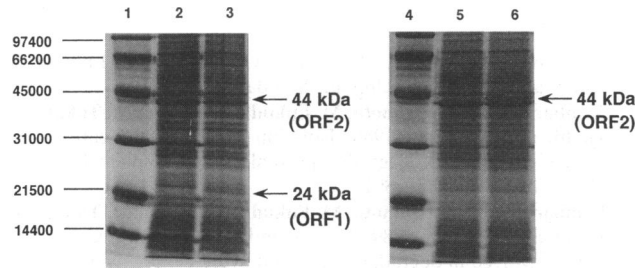

FIG. 5. SDS-PAGE analysis of products of ORF1 and ORF2 overexpressed in *E. coli* MV1190. The conditions for induction and electrophoresis are described in Materials and Methods. Total proteins of *E. coli* MV1190 harboring pKH400 (containing ORF1 and ORF2) (lanes 2 and 3) and pKH401 (containing ORF2) (lanes 5 and 6) are shown. Lanes 2 and 5, noninduced cells; lanes 3 and 6, cells induced with IPTG. The low-range molecular weight standards (lanes 1 and 4) (Bio-Rad Laboratories) were used to estimate the sizes of products of ORF1 and ORF2 (indicated by arrows).

chrome *c* reduction activity was measured by using the crude extracts of *E. coli* harboring pUC119 (vector), pKH200 (*bphA1A2A3BC*), pKH304 [(ORF2)*bphA1A2A3BC*], and pKH21 [*bphA1A2A3BCD*(ORF1,ORF2)] (Table 2). When NADH was used as a cofactor, cytochrome *c* reduction activity of the crude extract from pKH200 was at the same level as that from vector pUC119, while the crude extracts from pKH304 and pKH21 showed cytochrome *c* reduction activities increased by 11.4- and 7.1-fold over the level of reduction activity of the control, respectively. When NADPH was used as a cofactor, the cytochrome *c* reduction activity of the crude extract from pKH304 was at the same level as those of the crude extracts from pUC119 and pKH200.

DISCUSSION

In this study, the downstream region of the *bphD* was sequenced, and two open reading frames (ORF1 and ORF2) were found in this region by computer analysis. The deduced amino acid sequence of ORF2 showed homology with the sequences of four ferredoxin reductases (TodA [28], the P4 gene product [11], BphA4 [22], and BphG [5]) of dioxygenase systems. We also found that the consensus sequences of the putative FAD- and NAD-binding sites of TodA (16) were conserved in this amino acid sequence. When ORF2 was inserted just upstream of *bphA1A2A3BC*, the level of cytochrome *c* reduction activity was increased 11.4-fold relative to the level of the control. These data clearly suggest that ORF2 encodes ferredoxin reductase of biphenyl dioxygenase in KKS102; ORF2 is designated *bphA4*.

TABLE 2. Cytochrome *c* reduction activity of the product of ORF2

Cofactor	Plasmid	Genotype	Amt of cytochrome *c* reduction activity (mU/mg)
NADH	pUC119		5.8
	pKH200	*bphA1A2A3BC*	8.3
	pKH304	(ORF2)*bphA1A2A3BC*	94.6
	pKH21	*bphA1A2A3BCD*(ORF1 ORF2)	59.1
NADPH	pUC119		41.7
	pKH200	*bphA1A2A3BC*	37.7
	pKH304	(ORF2)*bphA1A2A3BC*	50.6
	pKH21	*bphA1A2A3BCD*(ORF1 ORF2)	NT[a]

[a] NT, not tested.

Cytochrome *c* reduction activity of the crude extract from pKH304 was at the same level as those of the crude extracts from pUC119 and pKH200 when NADPH was used as a cofactor (Table 2). On the other hand, when NADH was used as a cofactor, the crude extracts from pKH304 and pKH21 clearly showed higher cytochrome *c* reduction activity levels than those from pUC119 and pKH200. These data suggest that BphA4 is a NADH-dependent ferredoxin reductase.

The reductase components of toluene and benzene dioxygenase systems contain FAD as their prosthetic groups and are specific for NADH (2, 21). The putative ADP-binding sites of FAD (designated FAD-binding sites) and NAD (designated NAD-binding sites) in the ferredoxin reductases of toluene and benzene dioxygenases were inferred from amino acid sequence comparison with other oxidoreductases (16). The consensus sequence of the putative FAD-binding site is GlyX GlyXXGlyXXXAlaXXXXXXGly, where X represents any amino acid, and this sequence is located close to the N-terminal region of these ferredoxin reductases (16). Alignment of BphA4 and TodA showed that an amino acid sequence similar to the consensus sequence of the putative FAD-binding site was located close to the N-terminal region of BphA4 (Fig. 4). Solutions of partially purified BphA4 were yellow and showed absorption spectra typical of FAD, with maxima at around 375 and 450 nm (12). These observations suggest that BphA4 probably contains FAD as a prosthetic group and that the putative FAD-binding site is located close to the N-terminal region of BphA4. The consensus sequence of the putative NAD-binding site is GlyXGlyXXGlyXXXAlaXXXX XXGlyXXXXXXGlu, and this sequence is located in some 150 residues along the ferredoxin reductases of toluene and benzene dioxygenases (16). An amino acid sequence which completely matched the consensus sequence of the putative NAD-binding site was found in BphA4 (Fig. 4), and the cytochrome *c* reduction activity of BphA4 was dependent on NADH rather than NADPH as a cofactor. These observations suggest that the putative NAD-binding site of BphA4 is located in some 150 residues along BphA4 beginning at the N terminus.

As *bphA4* was identified in this study, the order of biphenyl- and PCB-degrading genes in KKS102 is *bphA1A2A3BCD* (ORF1)*A4*. The G+C content of the sequenced 2.4-kb region is 63.7%, and this value is similar to that of the region encoding *bphA1A2A3BCD* (62.0%). There is no apparent termination sequence (e.g., a stem-and-loop structure) between *bphD* and ORF1, and the crude extract of *E. coli* harboring pKH21, which contains *bphA1A2A3BCD*(ORF1)*A4*, shows cytochrome *c* reduction activity (Table 2). These results suggest that these eight genes are in one transcript.

Recently, Fetzner et al. (6) suggested that toluene dioxygenase, benzene dioxygenase, pyrazon dioxygenase, and naphthalene dioxygenase (20) have three-component systems which consist of terminal oxygenase, ferredoxin, and ferredoxin reductase components. The genes for these three components are all clustered (25), while *bphA4* and *bphA1A2A3* are separated by 3.5 kb in KKS102. As there is no report showing that the genes for these three components are not clustered in other strains, the gene organization of biphenyl and PCB degradation in KKS102 has a unique structure.

BphA4 showed a low level of homology with four other ferredoxin reductases (TodA [28], the P4 gene product [11], BphA4 [22], and BphG [5]) (Table 1). However, BphA4 had about 70% amino acid similarity to these ferredoxin reductases

when identical amino acids were included with similar amino acids. These data suggest that BphA4 probably is of the same type as these ferredoxin reductases.

The deduced amino acid sequence of ORF1 does not show significant homology with the sequences of any other proteins in the SWISS-PROT data bank, and its function is unknown. As the crude extract of *E. coli* harboring pKH304, which does not contain ORF1, showed cytochrome *c* reduction activity, the biphenyl dioxygenase system does not need the product of ORF1. More detailed studies are necessary to elucidate the function of ORF1.

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