**bph** genes of the thermophilic PCB degrader, *Bacillus* sp. JF8: characterization of the divergent ring-hydroxylating dioxygenase and hydrolase genes upstream of the Mn-dependent BphC

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**INTRODUCTION**

Catabolism of xenobiotic aromatic compounds by bacteria has been well studied, and micro-organisms capable of degrading these compounds appear to be ubiquitous. The biphenyl degradation pathway has been studied for its potential to co-metabolize polychlorinated biphenyls (PCBs), a family of anthropogenic, recalcitrant environmental pollutants, and the genes comprising the operon have been cloned and sequenced (Asturias & Timmis, 1993; Asturias et al., 1995; Furukawa et al., 1989; Hofer et al., 1993; Masai et al., 1995; Mukerjee-Dhar et al., 1998). The pathway is a four step process (Fig. 1) with the initial oxygenase, product of the **bphA** gene, crucially responsible for recognition and binding of the substrate. The biphenyl 1,2-dioxygenase (BphA) initiates the process by inserting two atoms of oxygen at carbon positions 2 and 3 of the aromatic ring, and the resulting dihydrodiol is dehydrogenated by the **bphB** gene product, dihydrodiol dehydrogenase. BphC (2,3-dihydroxybiphenyl 1,2-dioxygenase) cleaves 2,3-dihydroxybiphenyl at the *meta* position to give 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (6-phenyl HODA). This is hydrolysed by 6-phenyl HODA hydrolase, the **bphD** gene product, into 2-hydroxypenta-2,4-dienoate and benzoate.

*Bacillus* sp. JF8 is a thermophilic polychlorinated biphenyl (PCB) degrader, which utilizes biphenyl and naphthalene. A thermostable, Mn-dependent 2,3-dihydroxybiphenyl 1,2-dioxygenase, BphC_JF8, has been characterized previously. Upstream of **bphC** are five ORFs exhibiting low homology with, and a different gene order from, previously characterized **bph** genes. From the 5′ to 3′ direction the genes are: a putative regulatory gene (**bphR**), a hydrolase (**bphD**), the large and small subunits of a ring-hydroxylating dioxygenase (**bphA1A2**), and a *cis*-diol dehydrogenase (**bphB**). Hybridization studies indicate that the genes are located on a plasmid. Ring-hydroxylating activity of recombinant BphA1A2_JF8 towards biphenyl, PCB, naphthalene and benzene was observed in *Escherichia coli* cells, with complementation of non-specific ferredoxin and ferredoxin reductase by host cell proteins. PCB degradation by recombinant BphA1A2_JF8 showed that the congener specificity of the recombinant enzyme was similar to *Bacillus* sp. JF8. BphD_JF8, with an optimum temperature of 85°C, exhibited a narrow substrate preference for 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid. The Arrhenius plot of BphD_JF8 was biphasic, with two characteristic energies of activation and a break point at 47°C.

Furukawa et al., 1989; Hofer et al., 1993; Masai et al., 1995; Mukerjee-Dhar et al., 1998).

The initial enzymic steps in the aerobic degradation of many aromatic compounds are very similar, suggesting that they

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are AB113649 and AB181508.

A figure showing amino acid sequence comparisons is available as supplementary data with the online version of this paper.

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**Abbreviations:** HMSA, 2-hydroxymuconic semialdehyde; HOHDA, 2-hydroxy-6-oxohepta-2,4-dienoic acid; PCB, polychlorinated biphenyl; 6-phenyl HODA, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid.

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have a common, if distant, evolutionary origin. The enzymes of the naphthalene-degradation pathway can transform other polycyclic aromatic hydrocarbons, including indole and biphenyl (Barriault et al., 1994; Ensley et al., 1983; Resnick et al., 1996). However, 6-phenyl HODA hydrolase (in the biphenyl pathway), 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid hydrolase (HODA hydrolase; in the toluene pathway) and cis-2’-hydroxybenzalpyruvate aldolase (in the naphthalene pathway), are specific for their respective products.

Utilization of a thermophile for the degradation of xenobiotics in a biotechnological process offers several advantages, i.e. increased solubility of the pollutant, reduced risk of mesophile contamination and increased stability exhibited by thermophilic enzymes. Although thermophiles degrading aromatic compounds such as BTEX (benzene, toluene, ethylbenzene, xylene) and phenol/cresol have been isolated, there is limited molecular information about the genes/operons involved (Chen & Taylor, 1995; Dong et al., 1992; Duffner & Müller, 1998; Miyazawa et al., 2004; Mutzel et al., 1996; Natarajan et al., 1994). We have isolated and characterized a naphthalene- and biphenyl-utilizing Gram-positive thermophilic bacterium, Bacillus sp. JF8, which can degrade PCBs (Shimura et al., 1992). To study the structure, molecular organization, regulation and phylogeny of the genes/enzymes involved in degradation of biphenyl, we initially cloned and characterized the extradiol dioxygenase BphC_JF8 (Hatta et al., 1994). We have isolated and characterized a naphthalene- and biphenyl-utilizing Gram-positive thermophilic bacterium, Bacillus sp. JF8, which can degrade PCBs (Shimura et al., 1992). To study the structure, molecular organization, regulation and phylogeny of the genes/enzymes involved in degradation of biphenyl, we initially cloned and characterized the extradiol dioxygenase BphC_JF8 (Hatta et al., 2003). Unlike any other BphC reported so far, the thermostable BphC_JF8 exhibited manganese dependence. In most reports on extradiol dioxygenases that utilize metals other than Fe(II) (Boldt et al., 1995; Gibello et al., 1994; Miyazawa et al., 2004; Que et al., 1981), information on the flanking or adjoining genes, which might comprise an operon, is not available. Here we characterize the three genes that flank the gene encoding the Mn(II)-dependent BphC and that are responsible for the degradation of biphenyl in the thermophilic Bacillus sp. JF8.

**METHODS**

**Bacterial strains and culture conditions.** Bacillus sp. strain JF8 was grown on Castenholz D medium at 60 °C. Biphenyl was provided as vapour (Shimura et al., 1999), and 1-5 % agar was added for solid medium. Escherichia coli MV1184, DH5α and JM 109 (Takara Biomedicals) were used as hosts for plasmid construction and for gene expression at 37 °C. E. coli BL21(DE3) competent cells (Stratagene) and E. coli TOP10 chemically competent cells (Invitrogen) were also used. Lennox broth base (Invitrogen) at 20 g l⁻¹ was used as a complex medium. Ampicillin and chloramphenicol were added to the medium at concentrations of 100 µg ml⁻¹ and 30 µg ml⁻¹, respectively. IPTG and X-Gal were used at concentrations of 100 µg ml⁻¹ and 40 µg ml⁻¹, respectively.

**Molecular techniques.** Total DNA from strain JF8 was isolated by a modification of the procedure described by Marmur, while plasmid DNA from JF8 was isolated by the hot alkaline-pH method of Kado & Liu (Johnson, 1994). Standard procedures were used for plasmid DNA preparation and manipulation, and for agarose gel electrophoresis. E. coli MV1184, DH5α and JM109 were transformed by the CaCl₂ procedure (Sambrook et al., 1989). To clone the upstream region of bphC_JF8, initially a 4–6 kb SacI gene bank of Bacillus sp. JF8 was constructed in Charomid vector 9-42 (Nippon Gene) and was used to transfect E. coli DH5α using the Gigapack II packaging extract kit (Stratagene) following the manufacturer’s instructions. Colonies harbouring recombinant plasmids were transferred onto Hybond-N+ membranes (Amersham) and probed with a 1 kb HindIII–KpnI fragment encoding the C-terminal region of bphB and the N-terminal region of bphC. Southern blot hybridization was done with the non-radioactive DIG DNA hybridization kit (Roche Diagnostics) and probes were labelled according to the manufacturer’s instructions. SacI inserts from colonies that showed a positive hybridization signal were cloned into pBluescript II SK+ and KS+ vectors (Stratagene). The upstream region of the 4892 bp SacI fragment (pBSc5) was similarly isolated by constructing and screening a 6–7 kb HindIII gene bank. A Kilosequence deletion kit (Takara Biomedicals) was used to construct a series of deletion derivatives, whose nucleotide sequences were determined by the dideoxy termination method (Sanger et al., 1977). Sequences were analysed using the program OMEGA 2.0 (Oxford Molecular).

**Homology search and phylogenetic analysis.** A homology search was performed with BLAST 2.0 (gapped BLAST) (Altschul et al., 1997). Amino acid sequences retrieved from the protein database were aligned using CLUSTAL W version 1.9 (Thompson et al., 1994).

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**Fig. 1.** Biphenyl degradation pathway. I, biphenyl; II, 2,3-dihydroxy-4-phenylhexa-4,6-diene (dihydridiol compound); III, 2,3-dihydroxybiphenyl; IV, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (biphenyl meta-cleavage compound); V, 2-hydroxypenta-2,4-dienoic acid; VI, benzoic acid. BphA, biphenyl dioxygenase; BphB, dihydrodiol dehydrogenase; BphC, 2,3-dihydroxybiphenyl dioxygenase; BphD, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid hydrolase.
and a neighbour-joining tree constructed using the Blusum62 distance matrix, confidence levels being determined by bootstrap analysis. The results were depicted using Tree View version 1.5 (Page, 1996).

**Construction of expression plasmids.** To clone bpha1A2 of strain JF8 into the expression vector pET-21a (+) (Novagen), PCR was used to engineer a SacI restriction site and a Shine–Dalgarno sequence in front of the ATG start codon of bpha1A, and a unique restriction site immediately after the termination codon of bpha2. The HindIII, EcoRV and EcoRI restriction sites in the multicloning site of pBluescript II SK+ were removed using the DNA blunting kit (Takara Biomedicals) to give pSK+ and HE and the 3052 bp SacI–PstI fragment (encoding bpha1A2) from pBSc5 was ligated into pSK+ and HE to give pBScPA. A SacI site followed by an efficient Shine–Dalgarno sequence, AAGGAG (underlined in the primer), was introduced 8 bp upstream of the ATG start site of bpha1A (double underlined in the primer) using the primer 5′-GAGCTC-AAGGAGTGATAGGAGAAAGATGCGAAC-3′. The reverse primer used (giving a 800 bp product) was 5′-GTCGAC-CCGGATACCCCATGATAGGAGAAAGATGCGAAC-3′ (double underlined in the primer) using the primer 5′-GAGCTC-AAGGAGTGATAGGAGAAAGATGCGAAC-3′, which extended up to an internal HindIII site (underlined in the primer) in the bpha1A gene. The PCR product was cloned into the pCR 2.1-TOPO vector using the TOPO TA cloning kit to give pCRA2, and the DNA sequence was verified. After digestion with SacI and HindIII the 800 bp product was cloned into the pCRA1 and HindIII site of pBSCPA to give pBSPcA2. To introduce a Xhol site after the termination codon of bpha2A, the PCR primers used were 5′-GAGCTC-GATAGGAGAAAGATGCGAAC-3′ (forward primer) and 5′-GTCGAC-CCGGATACCCCATGATAGGAGAAAGATGCGAAC-3′ (reverse primer). This amplified a 392 bp product, from an internal EcoRV restriction site (underlined in the forward primer) in bpha2A to the TAG stop codon followed by the introduced Xhol site (underlined in the reverse primer). The PCR product was cloned into the pCR 2.1-TOPO vector using the TOPO TA cloning kit to give pCRA2, and the DNA sequence was verified. After digestion with EcoRV and Xhol, the 392 bp product was cloned into the similarly digested pBSPcA2 to give pBSPcPA3. Digesting pBSPcPA3 with SacI/Xhol gave a 1-9 kb fragment containing the bpha1A2 genes preceded by an efficient Shine–Dalgarno sequence, which was cloned into pET-21a (+) to give pET21A1A2. To test if the ORFs were being translated into proteins of the predicted size, the bpha1A2 genes were introduced into E. coli BL21(DE3) after initial transformation into a non-expression host. The culture was grown on Lennox broth at 37 °C to OD560 1.0, then the T7-mediated gene expression was induced by adding IPTG to a final concentration of 1 mM. The cultures were allowed to grow for 2–3 h and cells were harvested from 1 ml culture. The cell pellet obtained was resuspended in 500 μl 3× reducing SDS sample buffer (New England Biolabs) and heated. Some of the sample (3–10 μl) was analysed by SDS-PAGE (Laemmli, 1970) using broad range prestained protein markers (New England Biolabs).

The upstream region of the 4892 bp SacI fragment (pBSc5) was isolated as an overlapping 7 kb HindIII fragment and cloned into the vector pUC118 to give pBUH7. A 4-5 kb PstI–HindIII fragment from pBUH7, containing a functional hydrolyase gene, was subcloned into pUC118 to give pBUPH2.

PCR primers were used to amplify the bphB gene of strain JF8. The PCR product was cloned into the BamHI/EcoRI site of the vector pBluescript II SK+, and the DNA sequence of the PCR fragment was verified. The plasmid was then introduced into E. coli MV1184. The BamHI–EcoRI fragment encoding the bpha1 gene was cloned into the low copy number vector pSTV29 (Takara Biomedical) to give pSTV29 B, which is compatible with pET-21a (+), and therefore, the bpha1 gene could be introduced into E. coli BL21(DE3) along with the bpha1A2 genes.

The plasmids, pUAD1, with a 1-2 kb Stu–HindIII fragment encoding bpha1D, and pUAD2, with a 1-10 kb SacI–SpfI fragment encoding ethD1 of Rhodococcus sp. RHA1 (Yamada et al., 1998), were used in thermostability studies.

**Biotransformation experiments with recombinant proteins and identification of metabolites.** Liquid cultures of E. coli BL21(DE3) carrying the bpha1A2 and bphB genes were inoculated into Lennox broth containing 100 μg ampicillin ml–1 and 30 μg chloramphenicol ml–1. They were grown at 37 °C to an OD560 0-9 (about 4 h), and IPTG to a final concentration of 1 mM was added. The cultures were incubated for a further 2–3 h. The cells were collected by centrifugation, washed with ice-cold 50 mM phosphate buffer (pH 7.5) and resuspended in the same buffer after adjusting cell density to OD560 1-0 for resting cell experiments. Biphenyl, naphthalene and phenanthrene dissolved in ethyl acetate were supplied at a final concentration of 500 μM, while benzene was supplied as vapour. PCB congeners dissolved in DMSO were supplied at a concentration of 25 μM. The cells were incubated at 37 °C for 2–4 h, after which the culture was acidified to pH 2-0 with HCl, and extracted twice with an equivalent amount of ethyl acetate. The extract was evaporated to dryness, resuspended in a minimal amount of ethyl acetate, derivatized with BSTFA (N,O-bis(trimethylsilyl)tri fluoracetamide) + TMCS (trimethylchlorosilane) (Supelco) prior to GC-MS analysis. E. coli BL21(DE3) cells containing the vectors pET-21a (+) and pSTV29 were used as controls in all experiments. To obtain a mass fragmentation pattern of authentic standards, 2,3-dihydroxybiphenyl, 1,2-dihydroxynaphthalene and catechol were added to control samples, which were acidified, extracted with ethyl acetate and then derivatized with BSTFA+TMCS. In the case of PCB congeners, the ethyl acetate extract was directly used for analysis by GC-MS.

Extracted samples were analysed by GC (Hewlett Packard; model 6890) equipped with an HP-5ms capillary column (50 m, 0.25 mm, 0.33 μm; Hewlett Packard) and a mass selective detector (Hewlett Packard; model 5972A), integrated by Hewlett Packard MS Chemstation software. Some of the sample (1 μl) was injected by an auto-injector (Hewlett Packard; model 7673) in the splitless mode. Identification of PCB congeners has been described previously (Shimura et al., 1999). For the identification of PCB congeners, naphthalene, biphenyl, naphthalene and phenanthrene, the initial oven temperature of 60 °C was programmed to increase by 20 °C min–1 to 300 °C, where it was held for 5 min. The injector temperature was 250 °C and the transfer line temperature was 280 °C. The carrier gas was helium and its flow rate was kept constant at 1 ml min–1. Ions at m/z 188,190, 222,224; and 256,258 were chosen for monitoring PCB congeners, while the electron-ionization mass spectra for the hydroxylated derivatives were obtained using the scanning mode (50–650 m/z).

**Purification of native and recombinant 6-phenyl HODA hydrolase.** E. coli MV1184 harbouring pBUPH2 (BphD_JF8) was grown overnight in 1-5 litres of Lennox broth containing 100 μg ampicillin ml–1 and 1 mM IPTG. The cells were collected by centrifugation at 4000 g for 10 min. For native 6-phenyl HODA hydrolase, cells of strain JF8 were grown overnight in Lennox broth with biphenyl. The cells were washed twice with 25 mM phosphate buffer (pH 7-5) and resuspended in the same buffer. The cells were disrupted by a French pressure cell (Amino) and centrifuged at 12000 g for 30 min and then at 105 000 g for 60 min. The supernatant was recovered and used as crude extract.

For enzyme purification, all manipulations were carried out at 10 °C in 25 mM potassium phosphate buffer (pH 7-5) containing 1 mM β-mercaptoethanol (buffer A) unless otherwise stated. The enzyme activities of the eluted fractions were assayed against 6-phenyl HODA (as described below). For DEAE-Toyopearl chromatography, the crude extract was loaded onto a DEAE-Toyopearl column (2-6 × 15 cm)
previously equilibrated with buffer A. Proteins were eluted with a linear gradient of KCl from 0 to 0.5 M, in a total volume of 800 ml buffer A. Active fractions, eluted around 0-2 M KCl, were collected. For phenyl sepharose column chromatography, the collected fractions were dialysed against buffer A containing 100 M ammonium sulfate. The resulting protein solution was loaded onto a phenyl sepharose HP 26/10 column (Amersham Biosciences) equilibrated with buffer A containing 100 M ammonium sulfate. The enzyme was eluted with 600 ml of a gradient of 0--0.5 M ammonium sulfate. The enzyme was eluted at around 0.1 M ammonium sulfate. For MonoQ column chromatography, the active fractions eluted from the phenyl sepharose column were pooled and dialysed against buffer A. After the column was washed with 60 ml buffer A, the enzyme was eluted with 400 ml of a linear gradient of KCl from 0 to 0.25 M. The enzyme eluted at around 0.15 M KCl. Protein concentration was estimated by the method of Bradford (1976) using BSA as a standard. The purity and size of the enzyme proteins were estimated by SDS-PAGE according to the method of Laemmli (1970). Protein staining of the gel was performed with Coomassie brilliant blue R-250.

**BphD assay and kinetic measurements.** The hydrolyase activity was assayed by monitoring the decrease in A344 for 6-phenyl-HODA, ε 13 200 cm⁻¹ M⁻¹, A388 for HOHDA, ε 32 000 cm⁻¹ M⁻¹ and A375 for 2-hydroxyxymuconic semialdehyde (HMSA), ε 36 000 cm⁻¹ M⁻¹ (Asturias & Timmis, 1993). 6-Phenyl HODA, HOHDA and HMSA were prepared from 2,3-dihydroxybiphenyl, 3-methylcatechol and catechol by incubating with BphC_JF8. The assay was performed at 25 or 60 °C in 50 mM phosphate buffer (pH 7.5). The reaction was initiated by the addition of 5 μl enzyme solution to the reaction mixture. One unit of activity was defined as the amount of enzyme required to degrade 1 μmole of meta-cleavage compound min⁻¹.

The substrate concentration used was 0-4-2.5 μM for 6-phenyl HODA, and 1-300 μM for HOHDA and HMSA. The activation energy (Ea) was estimated using the Arrhenius equation for the temperature range of 20–75 °C. The value of Ea was determined from the slope of the straight line that resulted when the logarithm of the reaction constant, k, was plotted against 1/T. To determine thermal stability, a temperature range of 30–80 °C was utilized. In this case crude extract of recombinant BphD_JF8, BphD_RHA1 and EtbD_RHA1 were used.

**N-terminal sequence analysis.** Purified native BphD_JF8 was subjected to N-terminal amino acid sequencing by the Edman degradation process using a model 477A protein sequencer (Applied Biosystems) in accordance with the manufacturer’s instructions.

**Nucleotide sequence accession number.** The nucleotide sequence reported here has been submitted to the DDBJ and GenBank nucleotide sequence databases under accession nos AB113649 and AB181508.

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**RESULTS**

### Cloning the DNA upstream of bphC and localization of the bph gene cluster on a plasmid

From a 4 kb HindIII fragment of Bacillus sp. JF8, a gene encoding bphC was previously characterized (Hatta et al., 2003). By chromosome walking we cloned an overlapping 5 kb SacI fragment and then a 7 kb HindIII fragment from the upstream region of the meta-cleavage gene. Sequencing a 5 kb region upstream of the meta-cleavage gene showed five putative ORFs in the same orientation (Fig. 2; DDBJ and GenBank accession no. AB113649). Their deduced amino acid sequences were compared to other proteins in the SWISS-PROT, GenBank and EMBL databases and the functions of the individual ORFs deduced based on homology to previously described genes. Table 1 lists proteins that exhibit the highest sequence similarity to the deduced amino acid sequence of the genes cloned from Bacillus sp. JF8, and includes well known Bph proteins for comparison. Sequencing a 2 kb region further upstream (DDBJ and GenBank accession no. AB181508) revealed a single ORF that exhibited homology to transposases in insertion sequence elements of other bacteria.

To examine whether the bph gene cluster resided on the chromosome or on the approximately 40 kb plasmid, pBT40, an internal fragment of the bphC gene was used as a probe in hybridization experiments. Fig. 3 shows hybridization to pBT40. The probe did not hybridize to the total DNA of Bacillus sp. JF8N (Shimura et al., 1999), a mutant of strain JF8 that lacks the pBT40 and has lost the ability to utilize biphenyl (Fig. 3).

### Sequence analysis

The deduced amino acid sequence of the ORF 34 bp upstream of the bphC gene exhibited homology to the cis-diol dehydrogenases of naphthalene, toluene and biphenyl catabolic pathways, and was designated a biphenyl dihydrodiol dehydrogenase (BphB_JF8). In a phylogenetic tree of 18 short-chain dehydrogenases that convert the cis-diol products of dioxygenases to (methyl and chloro) catechols, and exhibit a 60–30 % identity to biphenyl dihydrodiol dehydrogenase of JF8 (results not shown), BphB_JF8 clusters with PhdE_KP7 (Saito et al., 2000) and NarB_12038 (Kulakov et al., 2000). BphB_JF8 could be expressed in E. coli cells giving a product of about 27 kDa, which is in

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**Fig. 2.** A partial restriction map and the gene order of the bph locus of Bacillus sp. JF8.
agreement with the predicted molecular mass of 29 kDa (results not shown).

The deduced amino acid sequence of the two ORFs upstream of the BphB_JF8-encoding gene showed similarity to the large (α) and small (β) subunits of terminal oxygenases from multicomponent primary dioxygenases, and the gene products were designated BphA1 and BphA2, respectively. The consensus sequence Cys-X1-His-X16-Cys-X2-His of a Rieske-type [2Fe-2S] cluster binding site can be identified in BphA1_JF8. The motif Glu-X3–4-Asp-X2-His-X3–5-His, separated from the Rieske type site by 90–100 aa, is the potential mononuclear non-haem iron coordination site (Kauppi et al., 1998). In BphA1_JF8, the initial Glu of the above-mentioned motif is replaced with Asp (also observed in some naphthalene- and phenanthrene-degrading proteins, and in TdnA1_UCC and AtdA3_YAA, Fig. 7), while other amino acids of the motif are conserved.

An unrooted phylogenetic tree of the α subunit of 35 well-studied ring-hydroxylating dioxygenases exhibiting 50–25 % identity to BphA1_JF8 (Fig. 4) shows BphA1_JF8 clustering not with the biphenyl dioxygenase, but with naphthalene dioxygenase NarAa_12038 (Larkin et al., 1999), NidA_124 (Treadway et al., 1999), NidA_PYR1 (Khan et al., 2001), aromatic ring hydroxylation dioxygenase E from Rhodococcus sp. RHA1 (ArhdE_RHA1) (Kitagawa et al., 1998). The function of BphR is not verified.

### Table 1. Genes of the bph locus of Bacillus sp. JF8 and their products

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*The DDBJ and GenBank accession number is AB113649.
†The function of BphR is not verified.

Fig. 3. Analysis of the localization of the biphenyl-degrading genes on the plasmid Southern blot hybridization using a bphC probe. (a) Electrophoresis of total DNA on an agarose gel. (b) Southern blot hybridization of the bphC probe to the total DNA. Lane M, Charomid vectors 9-42 (42 kb), 9-20 (20 kb); lane 1, total DNA of Bacillus sp. JF8; lane 2, total DNA of Bacillus sp. JF8N.
et al., 2001), phthalate dioxygenase PhtAa_12B (Eaton, 2001), and phenanthrene dioxygenase PhdA_KP7 (Saito et al., 2000). DitA1 from the diterpenoid-degrading Pseudomonas abietaniphila BKME-9 (Martin & Mohn, 1999) also appears to belong to the same cluster. In an unrooted phylogenetic tree of the $b$ subunit of 35 ring-hydroxylating dioxygenase, BphA1_JF8 clusters with the same enzymes (results not shown).

Upstream of BphA1_JF8, separated by 73 bp, is an ORF exhibiting homology to hydrolyses of ring fission products of the meta-cleavage pathway, which was designated BphD_JF8. The HODA hydrolases are classified into two major groups (Hernaez et al., 2000; Nandhagopal et al., 2001), one involved in degradation of substrates with large C-6 side chains (6-phenyl HODA hydrolase) (group I), while in the other (group II/III), enzymes are involved in the
degradation of monocyclic compounds with short alkyl side chains (HOHDA hydrolase and HMSA hydrolase). The unrooted phylogenetic tree of 24 well known group I and group II/III hydrolases (Fig. 5) shows the JF8 hydrolase clustering with neither group, but rather in a separate group along with CarC_CA10 (Sato et al., 1997) and DxnB_RW1 (Armengaud et al., 1998).

The deduced amino acid sequence of the ORF (224 aa) 33 bp upstream of the hydrolase gene exhibits 32% identity with the putative naphthalene-degrading regulatory protein (Kukalov et al., 2005), 29% identity with the CarR of the carbazole-degrading operon (Inoue et al., 2004) and low homology to several proteins involved in the regulation of the bph operon (Beltrametti et al., 2001; Mouz et al., 1999).

Fig. 5. Phylogenetic tree from the alignment of BphD_JF8 with related proteins. Protein sequences of 24 hydrolases, including BphD_JF8, were used. The numbers on some branches refer to percentage confidence estimated by bootstrap analysis with 1000 replicates. Bar, 0.1 nucleotide substitutions per site. The sequence abbreviations, enzyme substrate, species and DDBJ/EMBL/GenBank references are as follows: DxnB_RW1, dibenzop-dioxin, Spingomonas sp. RW1, X72850; CarC_CA10, carbazole, Pseudomonas resinivorans CA10, AB047548; EtbD1_RHA1, ethylbenzene, Rhodococcus sp. RHA1, AB004320; EtbD2_RHA1, ethylbenzene, Rhodococcus sp. RHA1, AB004321; CumD_IP01, cumene, Pseudomonas fluorescens IP01, D83955; McbF_JS705, chlorobenzene, Ralstonia sp. JS705, AJ006307; TodF_F1, toluene, Pseudomonas putida F1, M64080; AtlD_YAA, aniline, Acinetobacter sp. YAA, AB008831; DmpD_CF600, phenol, Pseudomonas sp. CF600, X52805; XylF_TOL, toluate, P. putida, M64747; NahN_AN10, salicylate, Pseudomonas stutzeri AN10, AF039534; BphD_KS702, biphenyl, Rhodococcus sp. RHA1, AB030672; ThnD_TFA, tetralin, Sphingomonas macroalcaligenes TFA, AF20496; CarC_CB3, carbazole, Sphingomonas sp. CB3, AF060489; BphD_KKS102, biphenyl, Pseudomonas sp. KKS102, M26433; BphD_TA421, biphenyl, Rhodococcus sp. TA421, AB014348; BphD_M5, biphenyl, Pseudomonas sp. M5, D85851; BphD_TA421, biphenyl, Rhodococcus erythropolis TA421, AB014348.
Ohtsubo et al., 2001; Watanabe et al., 2000), all of which belong to the GntR-family of transcriptional regulators (Fujita & Fujita, 1987; Haydon & Guest, 1991; Rigali et al., 2002). It may encode a regulatory protein involved in regulation of the JF8 bph operon and was designated BphR_JF8.

There is an ORF (420 aa, 53 mol% G+C) 260 bp upstream of the putative bphR exhibiting homology (43–44% identity) to the transposase for insertion sequence element IS256 in transposon Tn4001 of Staphylococcus aureus (Ito et al., 2003) and the transposase for insertion sequence ISRM5 of Sinorhizobium meliloti strain 1021 (Capela et al., 2001).

Expression and characterization of the bphA1A2 genes
A plasmid pET21_A1A2 was constructed and introduced into E. coli BL21(DE3) for the expression of the bphA1A2 genes. SDS-PAGE analysis indicated that the expressed BphA1A2 proteins were of the expected size (results not shown). The bphB gene, on a plasmid (pSTV29_B) compatible with pET-21a(+), was introduced into the E. coli containing the plasmid pET21_A1A2. Resting cells of E. coli BL21(DE3) expressing recombinant BphA1A2 and BphB proteins exhibited biphenyl-transforming activity indicating that non-specific ferredoxin and ferredoxin reductase supplied by the host cell complemented the recombinant proteins. GC-MS analysis of the extracted supernatant revealed the presence of a molecular ion with the molecular mass (m/z 330) and signature (fragment ion of m/z 315, 242, 227, 212 and 165) observed in authentic samples of trimethylsilanized 2,3-dihydroxybiphenyl (results not shown). Benzene, naphthalene and phenanthrene were also used as substrates for resting cell assays. Transformation products catechol and 1,2-dihydroxy-naphthalene were detected by GC-MS analysis in the case of benzene & naphthalene, respectively, and the molecular masses and signature patterns of the extracted compounds were consistent with authentic standards (results not shown). Bacillus sp. JF8 does not utilize benzene as a carbon source, nevertheless, benzene was a substrate for BphA1A2B. Although 1,2-dihydroxy-naphthalene is known to be unstable in aqueous solutions (Eaton & Chapman, 1992), we could identify the derivatized compound, albeit at very low concentrations. No transformation products were produced. In a mixture of 2,5,2'-, 2,4,4'-, 2,3,3'- and 2,4,3'-trichlorobiphenyl, 2,3,3'-trichlorobiphenyl was completely transformed, 2,5,2'-trichlorobiphenyl did not show any transformation while the other two congeners were partly transformed. Table 2 compares transformation of the above-mentioned congeners by the recombinant BphA1A2 with that of Bacillus sp. JF8.

Characterization of the hydrolase activity
6-Phenyl HODA hydrolase was purified to homogeneity from biphenyl-induced cells of Bacillus sp. JF8. The N-terminal amino acid sequence of the purified 6-phenyl HODA hydrolase was determined to be MHEQIQEKV DVDGI, which agrees with the DNA sequence data of cloned bphD.

Recombinant 6-phenyl HODA hydrolase was purified 42-fold with an overall yield of 54%. (Table 3). The molecular mass of the enzyme was determined to be 33 kDa by SDS-PAGE (Fig. 6), which is in agreement with the value calculated from the deduced amino acid sequence of BphD_JF8. Recombinant BphD_JF8 exhibited a high affinity for 6-phenyl-HODA as evidenced by a Kₘ of 0.71±0.07 µM at the physiological growth temperature of 60 °C, while at 25 °C, the Kₘ was 0.85±0.15 µM. At 60 °C, the specific activity of BphD_JF8 for 6-phenyl-HODA was 1.34 U mg⁻¹, Vₘₜₙₐₓ was 2.1 U mg⁻¹, while Kₘ was calculated to be 1.11 s⁻¹. BphD_JF8 exhibited faint activity towards HOHDA and HMSA. The optimum temperature of the enzyme was 85 °C. Fig. 7(a) compares thermal stability of BphD_JF8 with a mesophilic HOHDA hydrolase (EthD1_RHA1) and 6-phenyl HODA hydrolase (BphD_RHA1) after 30 min incubation at temperatures ranging from 30–80 °C. The Arrhenius plot was discontinuous with an inflection temperature of 47 °C (Fig. 7b). The activation

Table 2. Degradation of PCB congeners in a defined mixture

<table>
<thead>
<tr>
<th>Congener</th>
<th>Recombinant BphA1A2 (%) degradation*</th>
<th>Bacillus sp. JF8 (%) degradation†</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,2'-</td>
<td>8±5</td>
<td>14±5</td>
</tr>
<tr>
<td>3,3'</td>
<td>100±0</td>
<td>100±0</td>
</tr>
<tr>
<td>4,4'</td>
<td>100±0</td>
<td>100±0</td>
</tr>
<tr>
<td>2,3,3'</td>
<td>100±0</td>
<td>100±0</td>
</tr>
<tr>
<td>2,4,4'</td>
<td>20±2</td>
<td>35±6</td>
</tr>
<tr>
<td>2,4,3'</td>
<td>59±3</td>
<td>81±3</td>
</tr>
<tr>
<td>2,5,2'</td>
<td>0±0</td>
<td>0±0</td>
</tr>
</tbody>
</table>

*Congeners were supplied at a concentration of 25 µM, and the cells were incubated with PCB for 2 h at 37 °C. Experiments conducted in triplicate.
†Shimura et al. (1999).
energy for hydrolysis of 6-phenyl-HODA by BphD_JF8 was determined to be 7.5 kcal mol\(^{-1}\) (31.4 kJ mol\(^{-1}\)) for the temperature range of 20–46 °C, while it was 2.9 kcal mol\(^{-1}\) (12.1 kJ mol\(^{-1}\)) at the higher range (50–75 °C).

**DISCUSSION**

The plasmid-encoded upper-pathway bph genes from a thermophile have been cloned and characterized. The bph genes of *Bacillus* sp. JF8 are atypical, exhibiting low identity to the well studied and widely distributed mesophilic bph operons reported so far (Asturias & Timmis, 1993; Asturias et al., 1995; Furukawa et al., 1989; Hofer et al., 1993; Masai et al., 1995). Although the in vivo roles of the genes could not be confirmed as we were unable to transform *Bacillus* sp. JF8, loss of the plasmid encoding the cloned meta-cleavage genes resulted in the loss of ability of JF8 to utilize biphenyl, indicating the cloned genes are involved in biphenyl metabolism. In bacillus, reports of plasmids with host-benefiting traits like resistance to antibiotics (Hoshino et al., 1985; Muller et al., 1986) or the ability to degrade environmental pollutants (Niazi et al., 2001) are rare. *Bacillus* sp. JF8 possesses a plasmid of about 40 kb, pBt40, which confers on its host the ability to utilize biphenyl. These are believed to be the first plasmid-borne degradation genes for a xenobiotic compound identified in

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total activity* (units)</th>
<th>Specific activity (units mg(^{-1}))</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>420</td>
<td>13·4</td>
<td>0·032</td>
<td>100</td>
</tr>
<tr>
<td>DEAE-Toyopearl</td>
<td>42·3</td>
<td>11·0</td>
<td>0·26</td>
<td>82</td>
</tr>
<tr>
<td>Phenyl-sepharose</td>
<td>8·6</td>
<td>9·5</td>
<td>1·11</td>
<td>71</td>
</tr>
<tr>
<td>Mono Q</td>
<td>5·4</td>
<td>7·2</td>
<td>1·34</td>
<td>54</td>
</tr>
</tbody>
</table>

*One unit of activity was defined as the amount of protein that converts 1 µmol 6-phenyl HODA min\(^{-1}\) at 60 °C.

![Fig. 6](https://www.microbiologyresearch.org/)

**Fig. 6.** SDS-PAGE of purified 6-phenyl HODA hydrolase. Lane M, molecular mass standards; lane 1, 4 µg 6-phenyl HODA hydrolase purified from *E. coli*. The molecular mass of the protein standards are shown on the left.

**Fig. 7.** Effect of temperature on the activity of BphD_JF8. (a) Thermostability of BphD_JF8 (■) in comparison with mesophilic BphD_RHA1 (○) and EtbD1_RHA1 (▲) after 30 min incubation. (b) Discontinuous Arrhenius plot of BphD_JF8. The logarithm of the specific activity (V) (units ml\(^{-1}\)) was plotted against the reciprocal of absolute temperature (T). The values shown, 2.9 kcal mol\(^{-1}\) (12.1 kJ mol\(^{-1}\)) and 7.5 kcal mol\(^{-1}\) (31.4 kJ mol\(^{-1}\)), are the activation energies calculated from the linear part of the plot (20–46 °C and 50–70 °C). The arrow indicates the inflection temperature.
a thermophile. Also, the recombinant large and small sub-units of the ring-hydroxylating dioxygenase were found to exhibit a congener specificity resembling that of Bacillus sp. JF8 indicating that these proteins are responsible for the PCB-degrading ability of JF8. Although the multicomponent dioxygenase and dihydrodiol dehydrogenase genes cloned exhibit homology to recently isolated naphthalene and phenanthrene genes, the presence of a functional hydroxylase gene that was induced by biphenyl and was specific for 6-phenyl HODA indicates the pathway specificity of the cloned genes.

The genetic organization of the bph locus in JF8 is uncommon with the meta-cleavage product hydroxylase gene (bphD), which carries out the fourth step in the degradation of biphenyl, directly linked to the ring-hydroxylating dioxygenase, upstream of the bphA1A2 cluster, forming a single transcriptional unit, while bphB, which encodes the second enzyme (dihydrodiol dehydrogenase) in the pathway, is transcribed as a separate unit with bphC. The genes encoding the ferredoxin (bphA3) and ferredoxin reductase (bphA4) components of the ring-hydroxylating dioxygenases were not present in the bph locus. Sequencing regions 3 kb upstream and downstream of the bph locus failed to locate bphA3A4. It is possible that they are located elsewhere on the plasmid or they may be supplied by the cellular housekeeping genes. The interchangeability of electron-transfer components between evolutionary related primary dioxygenases has been reported previously (Furukawa et al., 1993; Romine et al., 1998), and a tolerance between redox and oxygenase partners has been proposed to function as an evolutionary process for multicomponent oxygenases (Harayama et al., 1992).

Indels (insertions or deletions) are signature sequences in proteins that can be used as phylogenetic markers to determine the course of evolutionary history (Gupta, 1998). We compared the amino acid sequences in the regions of the Rieske-type [2Fe-2S] cluster and the non-haem iron-coordination site in the 35 ring-hydroxylating dioxygenases shown in Fig. 4 to check for the presence of indels (see the Supplementary Figure available with the online journal). While the amino acids around the Rieske-type [2Fe-2S] cluster binding site are highly conserved, the environment of the Fe(II) ligands revealed differences, and indels flanked by regions conserved in all sequences could be used to categorize the dioxygenases into four groups (group I to IV), which appears to reflect the clustering on the phylogenetic tree (Fig. 4). Probably the geometry of the ligands of the active site where oxygen activation is thought to occur differs depending on the range of substrates that can be oxidized.

Consistent with the occurrence of BphD_JF8 in a pathway responsible for the catabolism of biphenyl, the enzyme exhibited a narrow substrate preference and hydrolysed 6-phenyl HODA. The narrow substrate specificity could be the key determining factor governing the selectivity of the pathway with respect to the aromatic compound degraded. BphD_JF8 bears the characteristic catalytic residues of nucleophile-acid-histidine of the x/y hydroxylase family (Nardini & Dijkstra, 1999). Of the polar residues (Asn-46, Asn-109, Gln-266) in the substrate-binding pocket of BphD_RHA1, determined to be sites for potential electrostatic interaction (Nandhagopal et al., 2001), the analogous Asn residues (Asn-43, Asn-104) are conserved in BphD_JF8, while Gln-266 is replaced by a non-polar methionine residue, and this is also true for DxnB_RW1 (Armengaud et al., 1998) and CarC_CA10 (Sato et al., 1997), which cluster with BphD_JF8 on the phylogenetic tree.

As BphD_JF8 is from a thermophile with an optimum growth temperature of 60 °C, it was expected that the optimum temperature for the enzyme would be higher than that of hydroxylases from mesophiles. The optimum temperature was 85 °C for BphD_JF8 compared to 48 °C for CumD_IP01 (Saku et al., 2002), and 65 °C for ThnD_TFA (Hernaez et al., 2000) and BphD_RHA1 (Hatta et al., 1998). Temperature stability of mesophilic HODA hydroxylases have been rarely reported. XylF_TOL, the HMASA hydroxylase retained 50% activity after 15 min at 42 °C (Diaz & Timmis, 1995), while CumD_IP01 retained 80% activity at 50 °C for 30 min (Saku et al., 2002). Compared to XylF_TOL, CumD_IP01, BphD_RHA1 and EtbD1_RHA1, the temperature stability of BphD_JF8 is higher. Surprisingly, the stability of BphD_JF8 was found to be lower than ThnD_TFA from the mesophilic tetralin degrader, Sphingomonas macrogoltabidas, which retained 78% residual activity at 70 °C after 2 h (Hernaez et al., 2000), while BphD_JF8 retained 41% residual activity at 70 °C after 30 min.

Molecular analysis of catabolic pathways in bacteria degrading xenobiotic compounds indicates that bacteria might have adapted to the appearance of these compounds by expressing new functions to counteract potential toxic effects, or by using the available compounds as alternative sources of carbon or nitrogen (Copley, 2000; Top & Sringael, 2003). Adaptation to the presence of xenobiotic compounds could involve horizontal gene transfer, mutations and gene rearrangements (van der Meer et al., 1992), and the construction of pathways by assembling pre-existing genes or gene modules (referred to as a patchwork assembly or pathway assembly) has been observed (Copley, 2000; Springael & Top, 2004; van der Meer, 1997). To account for the divergent phylogenetic clustering of the bph genes from Bacillus sp. JF8 and the variance in gene order, we hypothesize that the locus was created by the recruitment of genes from a variety of catabolic pathways. While BphC and BphD appear to be specific for the products of the biphenyl pathway (although their genes do not cluster with the bph genes on a phylogenetic tree), biotransformation experiments indicate that BphA and BphB have relaxed substrate specificity and they cluster with proteins that attack polyaromatic hydrocarbons. The recruitment and fusion of a meta-cleavage enzyme and hydroxylase specific for 2,3-dihydroxybiphenyl and 6-phenyl-HODA, respectively,
with a ring-hydroxylating dioxygenase and dehydrogenase with broad substrate specificity could have given rise to the bph operon of JF8. The presence of an ORF with homology to the transposase genes of insertion sequences upstream of the bph genes implies the likely involvement of transposition in the evolution of the operon.

REFERENCES


