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Molecular Breeding of 2,3-Dihydroxybiphenyl 1,2-Dioxygenase for Enhanced Resistance to 3-Chlorocatechol

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3-Chlorobiphenyl is known to be mineralized by biphenyl-utilizing bacteria to 3-chlorobenzoate, which is further metabolized to 3-chlorocatechol. An extradiol dioxygenase, 2,3-dihydroxybiphenyl 1,2-dioxygenase (DHB12O; EC 1.13.11.39), which is encoded by the *bphC* gene, catalyzes the third step of the upper pathway of 3-chlorobiphenyl degradation. In this study, two full-length *bphC*s and nine partial fragments of *bphC*s fused to the 3′ end of *bphC* in *Pseudomonas pseudoalcaligenes* KF707 were cloned from different biphenyl-utilizing soil bacteria and expressed in *Escherichia coli*. The enzyme activities of the expressed DHB12Os were inhibited to varying degrees by 3-chlorocatechol, and the *E. coli* cells overexpressing DHB12O could not grow or grew very slowly in the presence of 3-chlorocatechol. These sensitivities of enzyme activity and cell growth to 3-chlorocatechol were well correlated, and this phenomenon was employed in screening chimeric BphCs formed by family shuffling of *bphC* genes isolated from *Comamonas testosteroni* KF704 and *C. testosteroni* KF712. The resultant DHB12Os were more resistant by a factor of two to 3-chlorocatechol than one of the best parents, KF707 DHB12O.

Key words: cassette PCR, 3-chlorocatechol, family shuffling, inhibition, *in vitro* protein evolution.

Abbreviations: DHB12O, 2,3-dihydroxybiphenyl 1,2-dioxygenase; CTAB, cetyltrimethylammonium bromide; TE, Tris-EDTA buffer, pH8.0; IPTG, isopropyl β-L-thiogalactopyranoside; KP buffer, potassium phosphate buffer, pH7.4; PCB, polychlorinated biphenyl; C23O, catechol 2,3-dioxygenase.

Protein engineering technologies such as site-directed mutagenesis and computer-assisted protein modeling have been used not only to modify protein function but also to identify the major structural features of a protein family (*1*). Site-directed mutagenesis of *Burkholderia* sp. LB400 *bphA* gene, which encodes a large subunit of biphenyl dioxygenase, results in an enzyme with broad substrate specificity and high specific activity (*2*). Recently, the new method for protein engineering, DNA shuffling, which mimics the evolution of proteins *in vitro*, has been developed (*3*). Family shuffling is one of the most effective methods (*4*), and the efficiency of making numerous chimeric shuffled genes has been improved by using either restriction enzymes (*5*) or single-stranded DNA (*6*) for preparation of gene fragments.

Both gram-negative and gram-positive biphenyl-utilizing bacteria, which are widely distributed in the natural environment, have been isolated and characterized (*7*, *8*). The degradation pathway of biphenyl (Fig. 1) is well characterized in *Burkholderia* sp. LB400 (*9*, *10*) and *Pseudomonas pseudoalcaligenes* KF707 (*11*). It has also been characterized in other biphenyl-utilizing strains. Mono- and polychlorinated biphenyls (PCBs) can be degraded by cometabolism with biphenyl (*12*). The major pathway for PCB degradation is initiated by the insertion of two atoms of oxygen at carbon positions 2 and 3 by biphenyl dioxygenase (Fig. 1, *13*). This enzyme exhibits a high degree of selectivity for the aromatic ring to be attacked, with the order of ring preference being non- > *ortho*- > *meta*- > *para*-substituted form mono- and dichlorinated congeners (*14*). In the case of monochlorinated biphenyls, only one of the two aromatic rings, the nonchlorinated ring, is preferentially attacked, and this produces chlorobenzoates and 2-hydroxypenta-2,4-dienoate. Monochlorobiphenyl-utilizing strains derive carbon and energy for growth from 2-hydroxypenta-2,4-dienoate. The fate of chlorobenzoates has not been well studied.

Chlorobenzoates have generally been regarded as dead-end metabolites in the utilization of monochlorinated biphenyls (*12*). Complete mineralization of mono-

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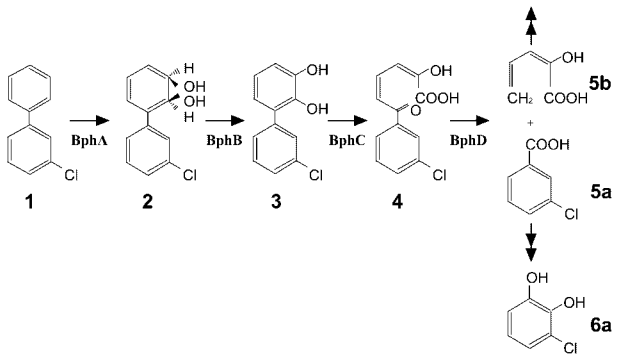

Fig. 1. **Upper pathway for metabolism of 3-chlorobiphenyl as encoded by the *bph* locus of biphenyl-utilizing bacteria.** Metabolites: 1, 3-chlorobiphenyl; 2, *cis*-2,3-dihydro-2,3-dihydroxy-3′-chlorobiphenyl; 3, 2,3-dihydroxy-3′-chlorobiphenyl; 4, 2-hydroxy-6-oxo-6-(3′-chlorophenyl)-hexa-2,4-dienoate; 5a, 3-chlorobenzoate; 5b, *cis*-2-hydroxypenta-2,4-dienoate; 6a, 3-chlorocatechol. Enzymes: BphA, biphenyl dioxygenase; BphB, 2,3-dihydro-2,3-dihydroxybiphenyl dehydrogenase; BphC, 2,3-dihydroxybiphenyl 1,2-dioxygenase; BphD, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase.

chlorobiphenyls requires two pathways. The upper pathway transforms chlorobiphenyls into chlorobenzoates, and the lower pathway degrades the chlorobenzoates. The biphenyl-utilizing bacteria normally do not possess the oxidative lower pathway for monocyclic ring products (*15*). When chlorobenzoates produced in the upper pathway of chlorobiphenyl degradation are removed from the cell culture, the efficiency of chlorobiphenyl degradation increases (*16*, *17*). *Pseudomonas cepacia* P166 strain degrades all three chlorobiphenyls into the respective chlorobenzoates, which are further metabolized to 3- or 4-chlorocatechols (*18*). Biphenyl-grown *Comamonas testosteroni* B-356 strain transforms chlorobenzoates into several products, including chlorocatechols and their cleavage products (*17*). Chlorobenzoates themselves or some of their metabolites are reported to interfere with the degradation of chlorobiphenyls. Among the three chlorobenzoates, 3-chlorobenzoate is the most effective inhibitor of biphenyl degradation (*17*). These metabolites are proposed to inhibit the 2,3-dihydroxybiphenyl 1,2-dioxygenase (DHB12O) activity in *C. testosteroni* B-356. DHB12O is encoded by the *bphC* gene and catalyzes the third step of biphenyl degradation, a *meta*-cleavage of 2,3-dihydroxy biphenyl. Although the inhibition is observed, the mechanism is still unresolved.

Family shuffling of two very homologous *bphA1* genes from *Burkholderia* sp. LB400 and *P. pseudoalcaligenes* KF707 created evolved biphenyl dioxygenases exhibiting enhanced degradation of PCBs and even monocyclic compounds (*19*, *20*). Three less similar *bphA1* genes from *Burkholderia* sp. LB400, *C. testosteroni* B-356, and *Rhodococcus globerulus* P6 were family-shuffled to create evolved biphenyl dioxygenase with extended substrate specificity (*21*). In this study, we have cloned several *bphC* genes from biphenyl-utilizing bacteria and demonstrated that 3-chlorocatecol in fact inhibited DHB12O activity. Five *bphC* genes were family-shuffled to generate chimeric DHB12Os more resistant to 3-chlorocatechol.

MATERIALS AND METHODS

Bacterial Strains and Plasmids—Biphenyl-utilizing gram-negative bacteria (*22*) and *Escherichia coli* strains used in this study are listed in Table 1. A biphenyl-utilizing strain was grown in the medium as described before (*23*) with slight modification. A basal minimal medium (PAS medium, pH 7.0) was solidified with 1.5% (w/v) Bacto-agar (Difco). One of the biphenyl-utilizing strains was streaked out on the PAS agar and incubated with a piece of solid biphenyl on the lid at 30°C. Five ml of PAS medium overlayed with 1 ml of biphenyl solution [0.1% (w/v) in mineral oil (Sigma)] was inoculated with a single colony grown on biphenyl as a sole carbon source, and then incubated at 30°C for 2 to 3 d. *E. coli* strains were grown in LB medium at 37°C. The plasmids used in this study are listed in Table 1. The concentration of ampicillin was 100 μg/ml.

Chemicals—3-Chlorocatechol and 2,3-dihydroxybiphenyl were purchased from Wako Pure Chemical Industries and Tokyo Kasei Kogyo, respectively.

Cloning of bphC Genes from Biphenyl-Utilizing Strains—Biphenyl-utilizing cells grown to confluently in the medium with biphenyl were harvested, and chromosomal DNA was isolated by use of cetyltrimethylammonium bromide (CTAB) (*24*). Chromosomal DNA was diluted with Tris-EDTA buffer, pH 8.0 (TE) to a concentration of 100 μg/ml and sonicated for 3 min. The *bphC* gene was PCR amplified from 1 μl of chromosomal DNA (100 ng) as a template by using a pair of degenerate primers, bphBA1 and bphDB2, or bphBA1 and bphC-R. The primer sequences were: bphBA1, 5′-*TGTAAAACGACGGCCAGT*GGATCCGARTAYACNGGNGCNTAYGT-3′; bphDB2, 5′-*CAGGAAACAGCTATGACC*AAGCTTCCNCCRTGNARCATDATNAC-3′; and bphC-R, 5′-**GAGGCATAGAACGACACCAT**RTGRTCRTTNGTRTG-3′. The underlined sequences in bphBA1 and bphDB2 correspond to *Bam*HI and *Hin*dIII restriction sites, respectively. The sequences shown in italics in bphBA1 and bphDB2 were identical to the sequence primers M13–21, 5′-TGTAAAACGACGGCCAGT-3′, and M13R, 5′-CAGGAAACAGCTATGACC-3′, respectively. The sequence in bold was complementary to the B7R primer used for the cassette PCR as mentioned below. PCR reaction was performed with LA Taq (Takara Bio) with 30 cycles of denaturation at 95°C for 45 s, annealing at 53°C for 1 min, and extension at 72°C for 4 min. The amplified products were separated on agarose gels, excised from gels, and purified with an E.Z.N.A.Gel Extraction kit (Omega Bio-tek). In the case of the combination of bphBA1 and bphC-R, 5′ partial *bphC* gene fragments were obtained. In order to isolate the full-length *bphC* genes, cassette PCR was performed (*25*). The 3′ end of *bphC* of KF707 ($bphC_{KF707}$), about 200 bp in size, was amplified with primers B7R and 707R from KF707 chromosomal DNA. The primer sequences were: B7R, 5′-ATGGTGTCGTTCTATGCCTC-3′; and 707R, 5′-CGGAAGCTTTAAAGTTTCATGCAGGGATT-3′. The underlined sequence corresponds to a *Hin*dIII restriction site. PCR was performed with 30 cycles of denaturation at 95°C for 45 s, annealing at 56°C for 1 min, and extension at 72°C for 1 min. The amplified 200-bp fragment was separated on agarose gel, excised from the gel, and purified with an E.Z.N.A.Gel Extraction kit.

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Table 1. **Bacterial strains and plasmids used in this study.**

Strain/plasmid	Genotype/description[a]	Source
Strains		
biphenyl-utilizing strain		
KF701	Bph$^+$, *Pseudomonas graminis*	*22*
KF702	Bph$^+$, *P. aeruginosa*	*22*
KF703	Bph$^+$, *P. putida*	*22*
KF704	Bph$^+$, *Comamonas testosteroni*	*22*
KF706	Bph$^+$, *Sphingomonas yanoikuyae*	*22*
KF707	Bph$^+$, *P. pseudoalcaligenes*	*22*
KF708	Bph$^+$, *Ralstonia basilensis*	*22*
KF709	Bph$^+$, *R. paucula*	*22*
KF710	Bph$^+$, *P. pseudoalcaligenes*	*22*
KF711	Bph$^+$, *P. pseudoalcaligenes*	*22*
KF712	Bph$^+$, *C. testosteroni*	*22*
KF713	Bph$^+$, *P. pseudoalcaligenes*	*22*
KF714	Bph$^+$, *Acinetobacter baumannii*	*22*
KF715	Bph$^+$, *P. putida*	*22*
KF751	Bph$^+$, *P. putida*	*22*
E. coli		
Top10F′	F′{*lacI*^{q}, Tn10(*tet*^{R})} *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ* Δ*M15* Δ*lacX74 deoR recA1 araD139* Δ(*ara-leu*)*7967 galU galK rpsL endA1 nupG*	Invitrogen
HMS174	F$^-$ *recA hsdR* Rif^R	Novagen
Plasmids		
pUC118	Cloning vector, Ap^R	*26*
pTrc99A	Expression vector, Ap^R	*32*
pUC701	1.1-kb *Bam*HI–*Hin*dIII PCR fragment containing *bphC* of KF701 in pUC118	This study
pUC702	1.1-kb *Bam*HI–*Hin*dIII PCR fragment containing *bphC* of KF702 in pUC118	This study
pUC704	1.2-kb *Bam*HI–*Hin*dIII PCR fragment containing *bphC* of KF704 in pUC118	This study
pUC706	1.1-kb *Bam*HI–*Hin*dIII PCR fragment containing *bphC* of KF706 in pUC118	This study
pUC712	1.2-kb *Bam*HI–*Hin*dIII PCR fragment containing *bphC* of KF712 in pUC118	This study
pUC751	1.1-kb *Bam*HI–*Hin*dIII PCR fragment containing *bphC* of KF751 in pUC118	This study
p99A701	1.1-kb *Bam*HI–*Hin*dIII fragment of pUC701 in pTrc99A	This study
p99A702	1.1-kb *Bam*HI–*Hin*dIII fragment of pUC702 in pTrc99A	This study
p99A704	1.2-kb *Bam*HI–*Hin*dIII fragment of pUC704 in pTrc99A	This study
p99A706	1.1-kb *Bam*HI–*Hin*dIII fragment of pUC706 in pTrc99A	This study
p99A712	1.2-kb *Bam*HI–*Hin*dIII fragment of pUC712 in pTrc99A	This study
p99A751	1.1-kb *Bam*HI–*Hin*dIII fragment of pUC751 in pTrc99A	This study
p99A715	1.3-kb *Bam*HI–*Hin*dIII PCR fragment containing *bphC* of KF715 in pTrc99A	This study

[a]Bph$^+$, phenotype able to grow on biphenyl as the sole carbon source; Ap^R, ampicillin resistance; *tet*^{R}, tetracycline resistance; Rif^R, rifampicin resistance

Two PCR products, a 900-bp fragment amplified by bphBA1 and bphC-R, and the 200-bp fragment, were mixed and subjected to the second PCR. The second PCR was performed with a pair of primers, M13–21 and 707R, with 30 cycles of denaturation at 95°C for 45 s, annealing at 54°C for 1 min, and extension at 72°C for 1 min. The final PCR products were digested with *Bam*HI and *Hin*dIII and cloned into pUC118 (*26*).

Phylogenetic Analysis—The PCR-amplified or cassette-PCR-amplified *bphC* gene fragment was directly nucleotide-sequenced by use of M13-21, M13R, or 707R as primers. The sequencing reaction was carried out with a DyeDeoxy Terminator cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions and analyzed with a 373A DNA sequencer (Applied Biosystems). The translated 240 amino acid sequences of BphC were aligned by using CLUSTAL W (*27*). A phylogenetic tree was constructed with the PHYLIP program package (*28*), using the neighbor-joining method (*29*).

The partial 16S rDNA fragments were PCR-amplified from the chromosomal DNA of KF strains with a pair of primers, pr0R, 5′-AGAGTTTGATCCTGGCTCAG-3′, and 9Rev, 5′-AAGGAGGTGATCCAGCC-3′. PCR was performed with 35 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 2 min. Amplified fragments of about 1.5 kb were separated on agarose gels, excised from the gels, and purified with an E.Z.N.A.Gel Extraction kit. The nucleotide sequences of purified fragments were directly determined by use of pr0R, 9Rev, and 16SA2 (5′-CGGTAATACAGAGGGTGC-AAG-3′) as primers and compared to the known 16S rDNA sequences in the European Ribosomal RNA database (*30*).

Expression of DHB12O in E. coli—The *Bam*HI–*Hin*dIII fragments containing *bphC* genes on pUC118 were recloned into the same restriction sites of pTrc99A (*32*). The resultant plasmids were transferred into *E. coli* HMS174. Overnight cultures of the transformants were diluted 1/50 in fresh LB medium supplemented with

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ampicillin and incubated at 37°C for 1.5 h. Isopropyl β-D-thiogalactopyranoside (IPTG) was then added at the final concentration of 1 mM, and the incubation was continued for 2 h. Cells were harvested, washed twice with 50 mM potassium phosphate buffer, pH 7.4 (KP buffer), re-suspended in 1/5 volume of KP buffer, and sonicated. Cell lysates were prepared by removing cell debris by centrifugation for 10 m at 10,000 ×*g*. Proteins in the cell lysates were analyzed by SDS-PAGE. DHB12O activity was measured as mentioned below.

Measurement of Enzyme Activity—DHB12O activity was assayed under the standard conditions in KP buffer at 25°C with 100 mM 2,3-dihydroxybiphenyl as the substrate, and the amount of the ring-cleavage product, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate, was determined spectrophotometrically at 434 nm (*32*). The molar extinction coefficient of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate is 21,700 M^{-1} cm^{-1}. The reaction was initiated by addition of an appropriate amount of cell lysate to the reaction mixture (1 ml). Specific activity is expressed as micromoles of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate formed per min per milligram of protein at 25°C. Protein concentration was determined by BCA protein assay reagent (Pierce).

Toxicity Test for Chlorobenzoate and Chlorocatechol—*Growth inhibition in the liquid culture:* One-milliliter aliquots of preincubated transformant cells of HMS174, HMS174/p99A701, HMS174/p99A702, HMS174/p99A704, HMS174/p99A706, HMS174/p99A712, HMS174/p99A715, HMS174/p99A751, and HMS174/pTrc99A were inoculated into 20 ml of M9-glucose with ampicillin and incubated for 2 h at 37°C. The expression of *bphC* genes was induced by incubation with IPTG for 2 h. Five-ml aliquots of IPTG-induced cells were transferred to three new tubes containing 50 μl of distilled water, 50 μl of 500 mM 3-chlorobenzoate, or 50 μl of 100 mM 3-chlorocatechol and incubated for 6 h. Cell growth was monitored by periodically measuring absorbance at 600 nm. The final concentrations of 3-chlorobenzoate and 3-chlorocatechol were 5 mM and 1 mM, respectively.

Growth inhibition on agar plates: Confluent transformant cells of HMS174 were diluted to 10^{-7}. Diluted cells of HMS174/p99A701, HMS174/p99A702, HMS174/p99A704, HMS174/p99A706, HMS174/p99A712, HMS174/p99A715, and HMS174/p99A751 or together with diluted cells of HMS174/pTrc99A were spread on LB agar plates supplemented with ampicillin, 0.1 mM IPTG, and 0.1 mM 3-chlorocatechol and incubated overnight at 37°C. Catechol was sprayed on the agar plates to identify active *bphC* clones. At the same time, the colony sizes were scored.

Inhibition of DHB12O activity: One-milliliter aliquots of preincubated transformant cells of HMS174, HMS174/p99A701, HMS174/p99A702, HMS174/p99A704, HMS174/p99A706, HMS174/p99A712, HMS174/p99A715, HMS174/p99A751, and HMS174/pTrc99A were inoculated into 20 ml of M9-glucose with ampicillin and incubated for 2 h at 37°C. The expression of *bphC* genes was induced by incubation with IPTG for 2 h. Five-ml aliquots of IPTG-induced cells were transferred to three new tubes containing 50 μl of distilled water, 50 μl of 500 mM 3-chlorobenzoate, or 50 μl of 100 mM 3-chlorocatechol and incubated for 3 h. The final concentrations of 3-chlorobenzoate and 3-chlorocatechol were 5 mM and 1 mM, respectively. Cells were harvested, washed with KP buffer twice, re-suspended in 500 μl of the same buffer, and sonicated for 30 s twice. Cell debris was then removed, and DHB12O activity in the cell lysate was measured.

Family Shuffling—Family shuffling by five *bphC* genes was carried out by the reported method (*5*) with modification. Five *bphC* genes on pTrc99A vector, $bphC_{KF702}$, $bphC_{KF704}$, $bphC_{KF706}$, $bphC_{KF712}$, and $bphC_{KF715}$, were PCR-amplified *with* a pair of primers, RV-M, 5′-GAGCGGATAACAATTTCACACAGG-3′, and Trc-B, 5′-TCTGTTTTATCAGACCGCTTC-3′. PCR was performed with 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min. Amplified fragments were separated on agarose gels and excised from the gels. Purified *bphC* gene fragments (200 ng) were digested with two different combinations of restriction enzyme(s): $bphC_{KF702}$ was digested with *Hae*III–*Hap*II (702A) and *Taq*I–*Tsp*509I (702B), $bphC_{KF704}$ with *Hha*I (704A) and *Taq*I (704B), $bphC_{KF706}$ with *Hae*III–*Hap*II (706A) and *Taq*I–*Tsp*509I (706B), $bphC_{KF712}$ with *Hha*I (712A) and *Taq*I–*Tsp*509I (712B), and $bphC_{KF715}$ with *Hae*III–*Hap*II (715A) and *Taq*I–*Tsp*509I (715B). Enzyme digestions with *Hae*III, *Hap*II, and *Hha*I and with *Taq*I and *Tsp*509I were performed overnight at 37°C and 65°C, respectively, and gene fragments generated by enzyme digestions were purified by a MinElute Reaction Cleanup kit (QIAGEN). Aliquots of each digestion were analyzed on agarose gels, and the gel pattern was compared with *in silico* gel pattern. After complete digestion was confirmed, equal volumes of fragments A and fragments B, *e.g.*, 702A and 704B, were added to reassembly reaction mixture. Reassembly without primers was carried out in 20 μl of reaction mixture by using *PfuTurbo* DNA polymerase (1 unit, Stratagene) for 15 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 1 min with 5 s increment after each cycle. Partially reassembled gene fragments of all 20 combinations (702A and 704B, 702A and 706B, 702A and 712B, 702A and 715B, 704A and 702B, 704A and 706B, 704A and 712B, 704A and 715B, 706A and 702B, 706A and 704B, 706A and 712B, 706A and 715B, 712A and 702B, 712A and 704B, 712A and 706B, 712A and 715B, 715A and 702B, 715A and 704B, 715A and 706B, and 715A and 712B) were mixed together and subjected to 25 more reassembly reactions. Appropriate amount of reassembly product was used as a template for PCR with primers RV-M and Trc-B to amplify the full-length *bphC* genes. The PCR products were separated on agarose gel, and the DNA fragments corresponding to the full-length *bphC* genes were isolated from the gel, digested with *Bam*HI and *Hin*dIII, and cloned into pTTQ18 (*33*).

Screening of 3-Chlorocatechol-Resistant Chimeric Clones—Family-shuffling products cloned onto pTrc99A were put into HMS174 and spread onto LB agar plates supplemented with ampicillin, 0.5 mM IPTG, and 0.2 mM 3-chlorocatechol. After spraying catechol on the colonies, the largest yellowish colonies were selected and plasmids were purified. Purified plasmids and one of the parents, p99A702, were used to re-transform HMS174. Confluent transformant cells (0.1 ml) were mixed with 3 ml of fresh LB with ampicillin and incubated for 2 h at 37°C. IPTG

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was added to the culture medium at the final concentration of 1 mM, and an incubation was continued for 2 h. One ml of cell suspension was transferred to a new tube containing 10 μl of distilled water, and another 1 ml was transferred to a new tube containing 10 μl of 100 mM 3-chlorocatechol (at final concentration of 1 mM), and both were incubated for 3 h. Cells were then harvested, washed twice with KP buffer, resuspended in 500 μl of KP buffer, and sonicated for 30 s twice. DHB12O specific activities in cell lysates prepared from cells incubated (a) without and (b) with 3-chlorocatechol were measured, and ratio (b)/ (a) was calculated. If the ratios of selected clones were statically higher than that of $bphC_{KF702}$, those clones were considered to be 3-chlorocatechol-resistant *bphC*s. The chimeric *bphC* genes on the resistant clones were PCR-amplified with M13-21 and M13R primers as mentioned before, and nucleotide sequences of chimeras were determined by use of the same primers.

Purification of DHB12O—Portions of 2.5 ml of confluent cells of HMS174 with different *bphC* genes were inoculated into 250 ml of fresh LB with ampicillin and incubated for 2 h at 37°C. DHB12O was overexpressed by cultivation of the cells with 1 mM IPTG for 3 h. Cells were harvested, washed with buffer and frozen at –80°C until use. The frozen cells were thawed on ice, resuspended in 25 ml of 10 mM ethylenediamine-HCl, pH 7.5 containing 10% isopropanol (EDP buffer), and sonicated for 30 s four times. Cell debris and membrane fractions were removed by centrifugation for 1 h at 100,000 ×*g*, and the supernatant was loaded on Mono Q HR 10/10 (8 ml, Amersham Biosciences) equilibrated with EDP buffer. Bound proteins were eluted with 160 ml of 0–0.5 M Na_2SO_4 gradient in EDP buffer at a flow rate of 3 ml m^{-1} and fractionated. Fractions containing DHB12O were mixed and proteins in these fractions were precipitated with 75% saturated ammonium sulfate. Precipitated proteins were dissolved in 5 ml of 10 mM ethylenediamine-HCl, pH 7.5 (ED buffer) containing 1 M $(NH4)_2SO_4$ and loaded on RESOURCE PHE (1 ml, Amersham Biosciences) equilibrated with the same buffer. Bound proteins were eluted with 20 ml of 1–0 M $(NH4)_2SO_4$ gradient in ED buffer at a flow rate of 2 ml m^{-1} and fractionated. DHB12O was eluted at the end of the gradient.

Kinetic Measurements—The k_{cat} and K_m values of DHB12O for 2,3-dihydroxybiphenyl were determined under the standard conditions, except that the concentration of each substrate was varied in the range of 0.25–100 μM. The competitive inhibition constant, K_i, was calculated from a Dixon plot. The concentrations of 2,3-dihydroxybiphenyl were 2 μM and 5 μM, and the concentration of 3-chlorocatechol was varied from 0.1 to 0.5 μM. An equation for competitive inhibition was used for fitting the data.

RESULTS

PCR Amplification of bphC Genes—Gene clusters responsible for biphenyl degradation have been characterized in several bacteria. By comparison of these gene clusters in gram-negative bacteria, biphenyl-utilizing gram-negative bacteria were classified into two types in terms of the order of the *bphB*, *bphC*, and *bphD* genes. In the first type, which is represented by KF707 and LB400, the *bphC* and *bphD* genes are separated by other genes; while in the other type, as in KF715 and KKS102, they are contiguous (Fig. 2A). In either case, the *bphB* gene is located just before the *bphC* gene. From the amino acid sequence alignments of BphB, BphC, and BphD, we found conserved amino acid residues and were able to design the degenerate primers, bphBA1, bphC-R, and bphDB2, respectively (Fig. 2B). By use of a combination of primers, bphBA1 and bphDB2, the entire *bphC* gene was amplified from KF715 (Fig. 2C, lane 4). The same set of primers was unable to amplify the *bphC* gene from KF707, in which the *bphD* gene is located far from the *bphB* gene (Fig. 2C, lane 2). The 3′ end-truncated *bphC* genes were amplified from both KF707 and KF715 with a pair of primers, bphBA1 and bphC-R (Fig. 2C, lanes 3 and 5). Based on these results, we applied each pair of primers to amplify the entire or truncated form of *bphC* genes from chromosomal DNA prepared from biphenyl-utilizing gram-negative bacteria.

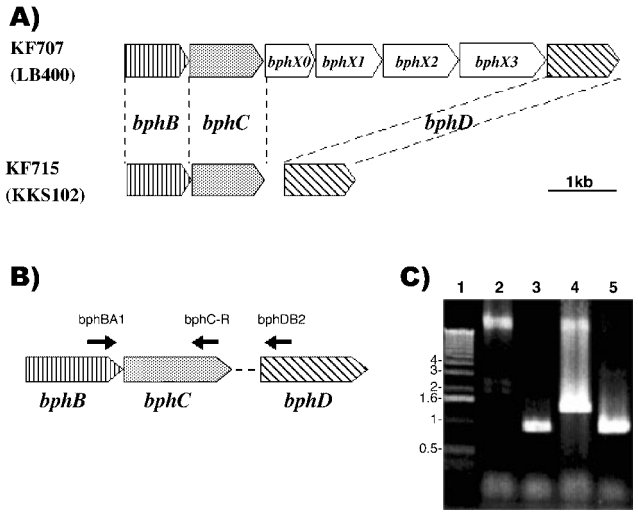

Fig. 2. **PCR amplification of $bphC_{KF707}$ and $bphC_{KF715}$ genes.** (A) Gene organizations of *bph* gene clusters around *bphC* genes. *bph* genes encoding: *bphB*, 2,3-dihydro-2,3-dihydroxybiphenyl dehydrogenase; *bphC*, 2,3-dihydroxybiphenyl 1,2-dioxygenase; *bphD*, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase; *bphX0*, glutathione S-transferase; *bphX1*, 2-hydroxypenta-2,4-dienoate hydratase; *bphX2*, acetaldehyde dehydrogenase; *bphX3*, 4-hydroxy-2-oxovalerate aldolase.(B) Primers for amplification of *bphC* genes. (C) PCR-amplified products were separated on 0.8% agarose gel. Lane 1, 1 kb DNA ladder (Bioneer, size in kb on the left). Chromosomal DNA isolated from KF707 (lanes 2 and 4) and KF715 (lanes 3 and 5) and different pairs of primers, bphBA1 and bphDB2 (lanes 2 and 3), and bphBA1 and bphC-R (lanes 4 and 5), were used.

Full-length *bphC* genes were amplified only from KF704 and KF712 with the pair of primers bphBA1 and bphDB2 (Fig. 3A). No amplified fragments were obtained from other KF strains, probably because they have KF707-type gene clusters for biphenyl degradation. When the other set of primers, bphBA1 and bphC-R, was used, the 900-bp truncated *bphC* genes were amplified from other KF strains, KF701, KF702, KF703, KF706, KF710, KF711, KF713, KF714, and KF751 (Fig. 3B). Since these amplified fragments contained the truncated forms of *bphC* genes, we applied cassette PCR with the

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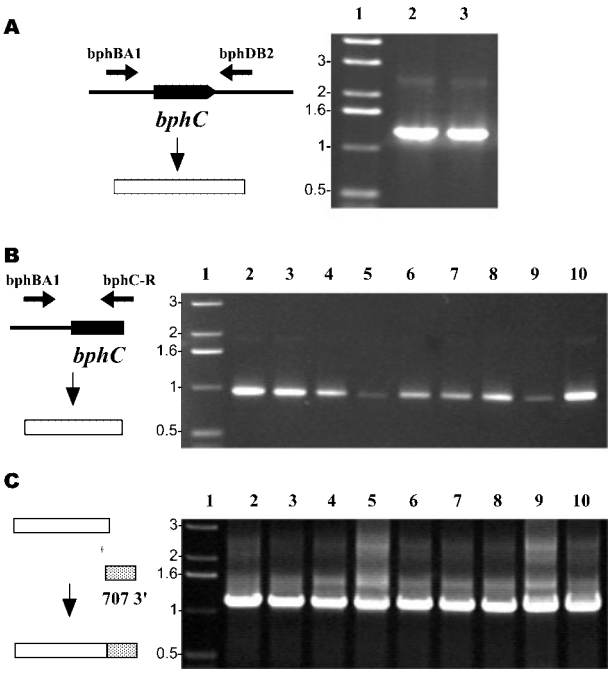

Fig. 3. **Cloning of *bphC* genes from different KF strains.** (A) Full-length *bphC* genes were amplified with a pair of primers, bphBA1 and bphDB2, from chromosomal DNA isolated from KF704 (lane 2) and KF712 (lane 3) as templates. Lane 1, 1 kb DNA ladder (Bioneer, size in kb on the left). (B) The 3′-end truncated *bphC* gene fragments were amplified with a pair of primers, bphBA1 and bphC-R, from chromosomal DNA isolated from KF701 (lane 2), KF702 (lane 3), KF703 (lane 4), KF706 (lane 5), KF710 (lane 6), KF711 (lane 7), KF713 (lane 8), KF714 (lane 9), and KF751 (lane 10). Lane 1, 1 kb DNA ladder (Bioneer, size in kb on the left). (C) Cassette PCR. The 3′ end of *bphC* fragment was PCR amplified from KF707 with primers B7R and 707R. This 200-bp fragment was fused to the 5′end of 900-bp *bphC* gene fragments amplified as shown in (B) by cassette PCR with a pair of primers, M13–21 and 707R. Lane 1, 1 kb DNA ladder (Bioneer, size in kb on the left).

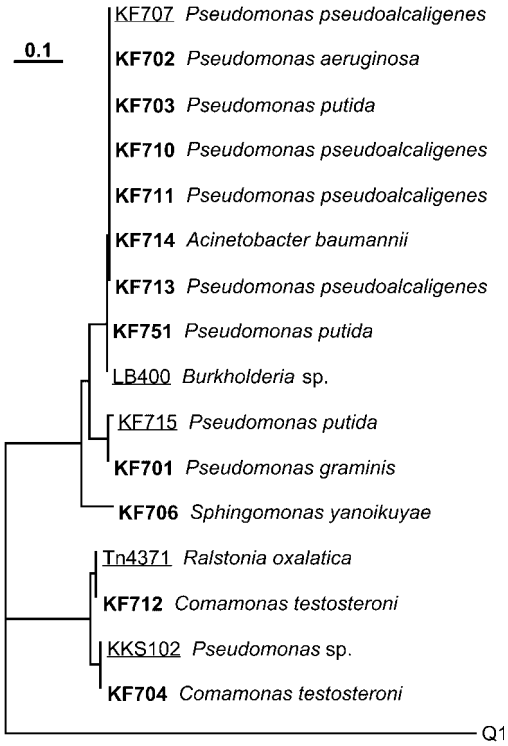

Fig. 4. **Phylogenetic tree based on BphC amino acid sequences.** Translated 240 amino acid sequences of BphCs were aligned by using ClustalW. The phylogram was drawn by TreeView (*51*). The scale bar means 0.1 amino acid substitutions per site. Species or genus identified by 16S rDNA sequences is shown on the right of the strain name. BphCs with underlined names were previously cloned and sequenced: KF707 (accession number: AAA25749), LB400 (CAA46910), KF715 (AAA25756), Tn4371 (CAD61144), KKS102 (AAA25750), Q1 (P11122). Accession numbers for 16S rDNA of KF707, KF702, KF703, KF710, KF714, KF715, KF701, KF706, KF712, and KF704, are AB109887, AB109752, AB109777, AB109888, AB109775, AB109776, AB109886, AB109749, AB109751, and AB109750, respectively.

known *bphC* gene to assemble the full-length *bphC* genes (*25*). The 200-bp gene fragment of $bphC_{KF707}$ was successfully added to the 3′ end of each *bphC* gene in frame (Fig. 3C). No PCR amplified fragments were obtained from KF708 and KF709 even by use of the pair of primers bphBA1 and bphC-R.

Phylogenetic Analysis of bphC Genes—The nucleotide sequences of the PCR amplified fragments were determined directly. The sequences of 3′ ends of the amplified fragments, except those from KF704 and KF712, exactly matched the $bphC_{KF707}$ sequence, indicating that the cassette PCR was successful. Based on the N-terminal 240 amino acid sequences deduced from the nucleotide sequences, the phylogenetic tree was constructed (Fig. 4). BphC of *Sphingomonas paucimobilis* Q1 strain was used as an outgroup. The N-terminal amino acid sequences of BphC in KF702, KF703, KF710, KF711, KF713, and KF714 were 100% identical to the sequence of $BphC_{KF707}$. Even the nucleotide sequences of *bphC* genes in these strains were identical to that of $bphC_{KF707}$ gene. The N-terminal $BphC_{KF751}$ was identical to $BphC_{LB400}$. The nucleotide sequence was also identical. As a result, BphCs in KF702, KF703, KF710, KF711, KF713, KF714, and KF751 belonged to the KF707/LB400 group. BphC in KF701 was very similar to $BphC_{KF715}$, differing by two amino acid residues. $BphC_{KF715}$ and $BphC_{KF701}$ formed another group. $BphC_{KF706}$ was different from the other BphCs. BphCs in KF704 and KF712, whose full-length gene fragments were PCR-amplified, belonged to another clade. $BphC_{KF704}$ and $BphC_{KF712}$ were 100% identical to $BphC_{KKS102}$ and $BphC_{Tn4371}$, respectively.

At the same time, biphenyl-utilizing KF strains containing these *bphC* genes were identified based on 16S rRNA sequences. Although most of the KF strains were identified as members of the genus *Pseudomonas*, KF714 was classified into the genus *Acinetobacter*. KF704 and KF712 were classified into the genus *Comamonas,* and KF706 was identified as a member of the genus *Sphingomonas*. These results demonstrated that *bphC* genes were distributed in wide variety of gram-negative bacteria, alphaproteobacteria, betaproteobacteria and gammaproteobacteria, and their phylogenetic distances based on BphC proteins and 16S rRNA were not correlated.

Cloning and Expression of bphC—The amplified fragments were digested with restriction enzymes, *Bam*HI and *Hin*dIII, and cloned into the same restriction sites of the cloning vector, pUC118. Since several *bphC* genes

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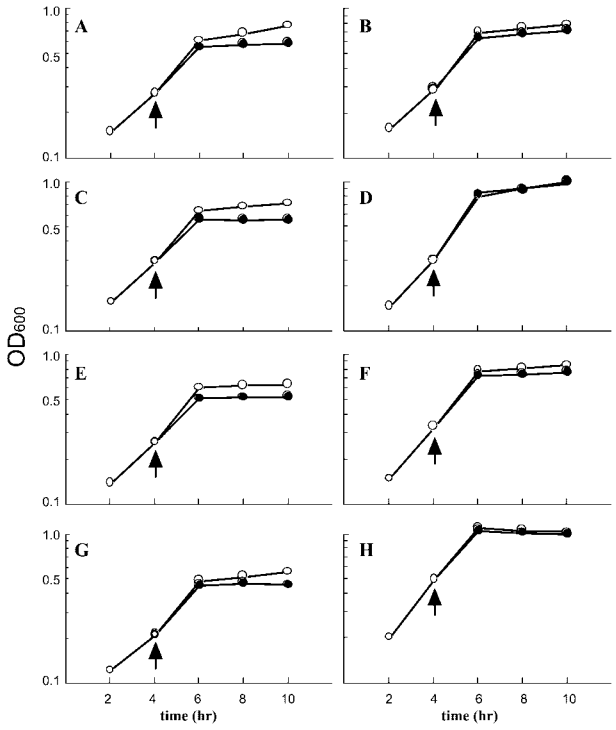

Fig. 5. **Growth inhibition of DHB12O-expressing *E. coli* by 3-chlorocatechol.** *E. coli* HMS174 cells containing p99A701 (A), p99A702 (B), p99A706 (C), p99A751 (D), p99A704 (E), p99A712 (F), p99A715 (G), and pTrc99A (H) were incubated in M9 minimal medium with glucose and ampicillin (0 time). IPTG was added at 2 h. At the time shown by the arrow, cells were divided into two portions, and 1 mM 3-chlorocatechol was added to one portion. Cell growth was monitored by measuring absorbance at 600 nm of cells incubated without 3-chlorocatechol (open circles) and with 3-chlorocatechol (closed circles).

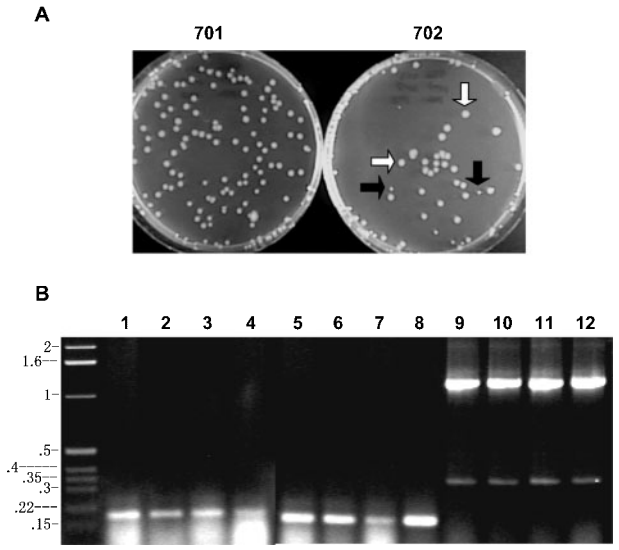

Fig. 6. **Colony morphology of DHB12O-expressing *E. coli* cells on the agar plate with 3-chlorocatechol.** (A) About 10^2 cells of HMS174/p99A701 and HMS174/pTrc99A (701) and HMS174/p99A702 and HMS174/pTrc99A (702) were spread on LB agar plates with ampicillin, 0.1 mM IPTG, and 0.1 mM 3-chlorocatechol and incubated overnight at 37°C. When catechol was sprayed, only small colonies (black arrows) on 702 plate turned yellow. Large colonies (white arrows) did not change color. No colonies on 701 plate turned yellow. (B) Colony PCR was carried out with a pair of primers, RV-M and Trc-B, for four large colonies on 701 (lanes 1–4), four large colonies on 702 (lanes 5–8) and four small colonies on 702 (lanes 9–12). A 1-kb DNA ladder (Invitrogen) is shown on the left with size in kb.

were identical, only *bphC* gene fragments from KF701, KF702, KF704, KF706, KF712, and KF751 were cloned, to make pUC701, pUC702, pUC704, pUC706, pUC712, and pUC751, respectively. The cloned *bphC* genes on these plasmids were confirmed to have no mutations in their nucleotide sequences. All *E. coli* HMS174 transformants with these plasmids showed the DHB12O activity. In order to increase the *bphC* expression level, *bphC* genes were re-cloned into the expression vector pTrc99A. The resultant plasmids, p99A701, p99A702, p99A704, p99A706, p99A712, and p99A751, and p99A715, in which $bphC_{KF715}$ was cloned onto pTrc99A, were transferred into *E. coli* HMS174. The specific activities in the IPTG-induced cells of HMS174 with p99A701, p99A702, p99A706, p99A751, p99A704, p99A712, and p99A715, were 3.41 ± 1.89, 15.79 ± 2.35, 2.16 ± 0.65, 16.93 ± 1.51, 13.36 ± 1.23, 17.24 ± 1.06, and 21.88 ± 5.79 unit/mg, respectively. No enzyme activities were observed in the IPTG-induced cells of HMS174 with pTrc99A. When the cell lysates of HMS174 cells with different *bphC* plasmids were analyzed on SDS-PAGE, clear BphC bands were observed with the expected molecular masses on the gel (data not shown). No corresponding protein band was detected in the cell lysate of the pTrc99A-containing HMS174 cells.

Growth Inhibition of BphC-Expressing Cells by 3-Chlorocatechol—Monochlorobiphenyls, 2-, 3-, and 4-chlorobiphenyls, are metabolized by biphenyl-utilizing bacteria to the respective chlorobenzoates. Chlorobenzoates or their degradation products, chlorocatechols, are known to be toxic to the cells (*18*), and 3-chlorobiphenyl is especially toxic. We tested to see whether cell growth of *E. coli* expressing only DHB12O would be diminished by 3-chlorobenzoate or 3-chlorocatechol. HMS174 cells with different *bphC* genes on pTrc99A or pTrc99A itself were incubated in the M9 minimal medium with glucose and ampicillin, and *bphC* gene product, DHB12O, was overexpressed by addition of IPTG. When cells were growing with IPTG in the mid log-phase, 3-chlorobenzoate or 3-chlorocatechol was added to the culture medium, and the cell growth in this medium was compared with that in the medium without either of compounds. All types of DHB12O-expressing cells grew as well with up to 5 mM 3-chlorobenzoate as without it (data not shown), indicating that 3-chlorobenzoate had no effects on the growth of DHB12O-expressing cells. On the contrary, 3-chlorocatechol had a range of effects on the cell growth. HMS174 cells with pTrc99A grew well even with 1 mM 3-chlorocatechol (Fig. 5, panel H), indicating that 3-chlorocatechol itself did not influence the cell growth or metabolism when DHB12O was not expressed. HMS174 cells with p99A701, p99A706, and p99A715 stopped growing almost completely in 2 h after addition of 3-chlorocatechol (Fig. 5, panels A, C, and G). When $bphC_{KF702}$ and $bphC_{KF751}$ were overexpressed in HMS174 cells, growth of

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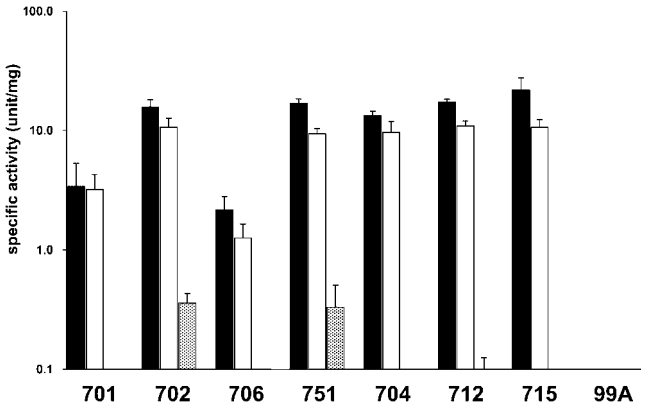

Fig. 7. **Specific activity of DHB12O.** HMS174 cells containing p99A701 (701), p99A702 (702), p99A706 (706), p99A751 (751), p99A704 (704), p99A712 (712), p99A715 (715), or pTrc99A (99A) were incubated with IPTG for 2 h. Cells were divided into three portions, each of which was incubated for 3 h with no compounds (black bars), 5 mM 3-chlorobenzoate (white bars), or 1 mM 3-chlorocatechol (gray bars). Cells were then harvested, washed, and sonicated, and DHB12O activities in cell lysates were measured. Specific activity is expressed as micromoles of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate formed per min per milligram of protein at 25°C. The values are averages of at least three measurements with SD.

these cells was not significantly influenced by 3-chlorocatecol (Fig. 5, panels B and D). In the case of HMS174 cells with p99A704 and p99A712, the growth inhibition by 3-chlorocatechol was intermediate (Fig. 5. panels E and F). When IPTG was not added into the minimal medium, no growth inhibition was observed in HMS174 cells expressing any DHB12O. Under these conditions, DHB12O was proved to be expressed at a lower level by measuring enzyme activity and SDS-PAGE analysis (data not shown).

Colony formation of DHB12O-expressing cells on the agar plate with 3-chlorocatechol was also tested. HMS174/pTrc99A and HMS174/p99A702, HMS174/p99A704, HMS174/p99A712, or HMS174/p99A751 were each grown in LB medium supplemented with ampicillin. Equal numbers of the two types of cells were then mixed and spread on an agar plate supplemented with ampicillin, 0.1 mM IPTG, and 0.1 mM 3-chlorocatechol. After incubation, two different sizes of colonies were found on the plate, small and large (see Fig. 6A for HMS174/pTrc99A and HMS174/p99A702). When catechol, one of the DHB12O substrates, was sprayed on colonies, all of the small colonies turned yellow, indicating DHB12O activity. None of the large colonies showed enzyme activity (data not shown). In the other combination of cells, HMS174/pTrc99A and HMS174/p99A704, HMS174/pTrc99A and HMS174/p99A712, and HMS174/pTrc99A and HMS174/p99A751, both large white colonies and small yellowish colonies were observed (data not shown). We picked four large white and four small yellowish colonies from the agar plate spread with cells of HMS174/pTrc99A and HMS174/p99A702 and checked the plasmid species in cells by colony PCR using a pair of primers designed on the cloning site of pTrc99A. Fragments of about 170 bp, which were in good agreement with the predicted size of amplified fragment from pTrc99A, were PCR-amplified from all four large colonies; and 1.2-kb fragments, which corresponded to the $bphC_{KF702}$ gene on pTrc99A, were amplified from all small colonies (Fig. 6B, lanes 5–12). Cells of HMS174/pTrc99A and HMS174/p99A701 were mixed and spread on the agar plate supplemented with ampicillin, 0.1 mM IPTG, and 0.1 mM 3-chlorocatechol. All colonies were large (Fig. 6A), and none of them showed DHB12O activity when catechol was sprayed (data not shown). We observed the same results with other combinations of cell cultures, HMS174/pTrc99A and HMS174/p99A706, and HMS174/pTrc99A and HMS174/p99A715. By colony PCR, fragments of about 170 bp were amplified from all four colonies, indicating that cells in these large colonies contained pTrc99A (Fig. 6B, lanes 1–4). These results were consistent with the results obtained in the liquid culture.

Inhibition of DHB12O Activity by 3-Chlorocatechol—One or both of 3-chlorobiphenyl metabolites 3-chlorobenzoate and 3-chlorocatechol are believed to inhibit DHB12O activity. So we tested the effect of both 3-chlorobenzoate and 3-chlorcatechol on DHB12O activity. DHB12O-overexpressing HMS174 cells were incubated with 5 mM 3-chlorobenzoate or 1 mM 3-chloroatechol for 3 h, and the residual DHB12O activities in cell lysates were measured. When HMS174 cells with overexpressed DHB12O were incubated with 3-chlorobenzoate, HMS174 cells containing any of the *bphC* genes showed almost same DHB12O activities as cells without 3-chlorobenzoate treatment, indicating that 3-chlorobenzate did not inhibit DHB12O activity at all (Fig. 7). On the contrary, 3-chlorocatechol at 1 mM severely inhibited the DHB12O activities. In particular DHB12O activities in cell lysates of HMS174/p99A701, HMS174/p99A706, and HMS174/p99A715, whose growth was completely inhibited by 3-chlorocatechol (Fig. 5 and 6), were below detectable level (Fig. 7). Substantial residual enzyme activities were detected in $DHB12O_{KF702}$ and $DHB12O_{KF751}$ (Fig. 7). DHB12O protein levels expressed in cells without inhibitors, with 3-chlorobenzoate, and with 3-chlorocatechol did not differ among HMS174 transformants (data not shown), indicating that *bphC* gene expression was not repressed by 3-chlorocatechol.

Screening of 3-Chlorocatechol-Resistant DHB12Os—We found some DHB12Os showed greater resistance to 3-chlorocatechol inhibition than others, and the degree of resistance was inversely correlated with the degree of growth inhibition. *E. coli* cells expressing the DHB12O that was more resistant to 3-chlorocatechol grew better in the culture medium with 3-chlorocatechol than these expressing DHB12O that was less resistant. We took advantage of this phenomenon to screen chimeric DHB12Os for those that were more resistant to 3-chlorocatechol than parental DHB12Os. We used $bphC_{KF715}$ and four newly cloned *bphC* genes in this study, $bphC_{KF702}$, $bphC_{KF704}$, $bphC_{KF706}$, and $bphC_{KF712}$, as parents for DNA shuffling to generate a large number of chimeric genes (*5*). Five *bphC* genes were digested with different restriction enzymes to generate two sets of gene fragments. These sets of *bphC* gene fragments were mixed together and reassembled into full-length *bphC* genes as described in MATERIALS AND METHODS, which were finally cloned into pTTQ18. DNA-shuffled *bphC* genes were transferred into HMS174, and transformants were

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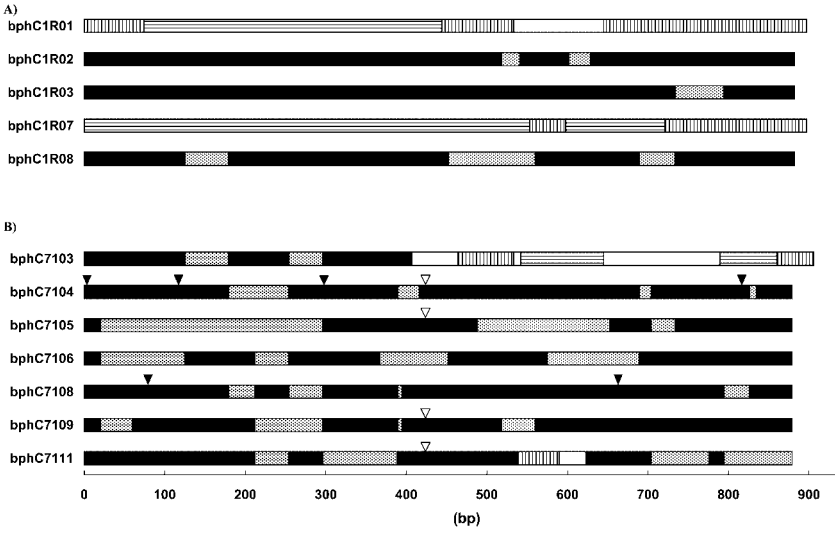

Fig. 8. **Family-shuffled *bphC* genes.** Five *bphC* genes, $bphC_{KF702}$, $bphC_{KF704}$, $bphC_{KF706}$, $bphC_{KF712}$, and $bphC_{KF715}$, were digested into small fragments with restriction enzymes and reassembled into full-length *bphC* genes by family shuffling. Shuffled *bphC* products were cloned into pTrc99A and transformed HMS174. Plasmids were purified from (A) five *bphC* active clones randomly selected from LB agar plates with ampicillin and (B) largest *bphC* active clones selected from LB agar plates with ampicillin, 0.5 mM IPTG, and 0.2 mM 3-chlorocatechol. Nucleotide sequences of *bphC* genes on these plasmids were determined. Sequences derived from $bphC_{KF702}$, $bphC_{KF704}$, $bphC_{KF706}$, $bphC_{KF712}$, and $bphC_{KF715}$ are indicated by boxes with vertical lines, gray boxes, open boxes, black boxes, and boxes with horizontal lines, respectively. Synonymous nucleotide substitution and nonsynonymous substitutions with amino acid changes (S2G, V27M, A39T, K99R, D220E, and S272C) are indicated by open and closed triangles, respectively.

grown on agar plates with ampicillin. When catechol was sprayed on transformants, about half of the colonies turned yellow. We purified plasmids from five randomly-selected yellowish colonies and determined the nucleotide sequences of cloned *bphC* genes (Fig. 8A). All of five clones were chimeras of parental *bphC* genes. Three clones, $bphC_{1R02}$, $bphC_{1R03}$, and $bphC_{1R08}$, were chimeras of two parents, $bphC_{KF704}$ and $bphC_{712}$, which were very similar (Fig. 8A). One clone, $bphC_{1R07}$, was a chimera of two very homologous parents, $bphC_{KF702}$ and $bphC_{KF715}$, and another clone, $bphC_{1R01}$, was a chimera of three parents, $bphC_{KF702}$, $bphC_{KF715}$, and $bphC_{KF706}$ (Fig. 8A).

When transformants were grown on agar plates with ampicillin, 0.5 mM IPTG, and 0.2 mM 3-chlorocatechol, colonies with different sizes were formed. By spraying catechol, as expected, almost all of the large colonies showed no enzyme activity. Cells in these colonies were likely to contain pTTQ18 or non-functional *bphC* genes.

Table 2. **DHB12O activity of shuffled *bphC* genes.**

bphC gene	DHB12O activity (unit/mg)[a]		ratio[b] (+/–)
	–3-chlorocatechol (–)	+3-chlorocatechol (+)	
$bphC_{KF702}$	7.61 ± 3.54	2.03 ± 1.65	0.27
$bphC_{7103}$	1.41 ± 0.62	0.41 ± 0.14	0.29
$bphC_{7104}$	2.08 ± 0.36	1.06 ± 0.16	0.51
$bphC_{7105}$	2.63 ± 0.38	1.35 ± 0.59	0.51
$bphC_{7106}$	2.15 ± 0.23	1.27 ± 0.07	0.59
$bphC_{7108}$	1.92 ± 0.21	0.97 ± 0.40	0.51
$bphC_{7109}$	1.98 ± 0.48	1.42 ± 0.74	0.71
$bphC_{7111}$	0.51 ± 0.22	0.18 ± 0.06	0.36

[a]DHB12O activity in HMS174 cells containing *bphC* genes incubated without or with 3-chlorocatehol was measured in KP buffer at 25°C with 100 mM 2,3-dihydroxybiphenyl as the substrate, and the amount of the ring-cleavage product, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate, was determined spectrophotometrically at 434 nm. The molar extinction coefficient of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate is 21,700 M^{-1} cm^{-1}. Specific activity expressed as micromoles of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate formed per min per milligram of protein at 25°C is the average of at least three measurements with SD. [b]DHB12O activity with 3-chlorocatechol (+) was divided by DHB12O activity without 3-chlorocatechol (–).

We selected the 12 largest yellowish colonies, which were thought to contain 3-chlorocatechol-resistant DHB12Os, and purified plasmids. The purified plasmids were used to re-transform HMS174. Transformants were grown in the liquid medium with 3-chlorocatechol, and the residual DHB12O activities were measured. The degree of inhibition by 3-chlorocatechol was calculated from the ratio of the residual activity to the DHB12O activity without 3-chlorocatechol (Table 2). Two transformants containing $bphC_{7103}$ and $bphC_{7111}$ showed almost same ratios as the parental clone containing $bphC_{KF702}$ (Table 2). The nucleotide sequences revealed that these chimeric *bphC* genes consisted of five and four parental genes, respectively (Fig. 8B). Other clones containing chimeric *bphC* genes, $bphC_{7104}$, $bphC_{7105}$, $bphC_{7106}$, $bphC_{7108}$, and $bphC_{7109}$, were generated from two parental genes, $bphC_{KF704}$ and $bphC_{KF712}$ (Fig. 8B), and showed higher ratios by a factor of 2 (Table 2).

One of the most resistant chimeras, $BphC_{7109}$, and the parent, $BphC_{KF702}$, were purified to homogeneity and kinetic parameters were determined. Both enzymes showed the similar affinities to 2,3-dihydroxybiphenyl (Table 3). Inhibition of enzyme activity by 3-chlorocatechol was monitored at different concentrations (Fig. 9). Based on the Dixon plot, the K_i values for 3-chlorocatechol of $BphC_{KF702}$ and $BphC_{7109}$ were 0.11 μM and 0.30 μM, respectively (Table 3). These data clearly demonstrated that the most resistant chimeric DHB12O isolated from shuffled clones was three times more resistant to 3-chlorocatechol than the parental DHB12O.

Table 3. **Steady-state kinetic parameters for 2,3-dihydroxybiphenyl and inactivation parameter for 3-chlorocatechol of DHB12Os.**

Enzyme	K_m (μM)[a]	k_{cat} (s^{-1})[a]	K_i (μM)[b]
$BphC_{KF702}$	5.4	360	0.11
$BphC_{7109}$	3.6	210	0.30

[a]Values are the average of at least three independent experiments. [b]Values were obtained from the Dixon plots in Fig. 9.

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DISCUSSION

Family shuffling is a very effective method to generate a large variety of chimeric genes from parental genes. As parents, we used five *bphC* genes whose gene products, DHB12Os, showed different sensitivities to 3-chlorocatechol. One group of parents, comprising $bphC_{KF702}$, $bphC_{KF715}$, and $bphC_{KF706}$, is homologous, with similarities of 85% to 91% between members. The other group comprises $bphC_{KF704}$ and $bphC_{KF712}$, which show 92% similarity. Similarity between the two groups is about 65%. Randomly selected shuffling products were chimeras of parents among the same groups (Fig. 8A), which makes sense because the sequence similarities between parents in the same group are much higher than the similarity between groups. Chimeric clones showing lower inhibition by 3-chlorocatechol were selected from an enormous number of shuffled chimeras. All were chimeras of $bphC_{KF704}$ and $bphC_{KF712}$ (Fig. 8B and Table 2). One of the chimeras, $BphC_{7109}$, was three times less inhibited by 3-chlorocatechol than the parent, $BphC_{KF702}$. When the second round of family shuffling was performed with six selected clones from the first round, $bphC_{7103}$, $bphC_{7104}$, $bphC_{7105}$, $bphC_{7106}$, $bphC_{7108}$, and $bphC_{7109}$, all resistant clones were also the chimeras of $bphC_{KF704}$ and $bphC_{KF712}$ (data not shown). The most resistant clone among parents was $BphC_{KF702}$, whose sequences were not included in the selected resistant clones. We do not yet know why only the chimeras of $BphC_{KF704}$ and $BphC_{KF712}$ were least inhibited by 3-chlorocatechol. While the activity of all other BphCs tested decreased rapidly during incubation with the substrate, 2,3-dihydroxybiphenyl , indication of the mechanism-based inactivation by oxidation of the active site Fe^{2+} to Fe^{3+}, both $BphC_{KF704}$ and $BphC_{KF712}$ did not show such rapid mechanism-based inactivation (data not shown). This might be a clue.

Chlorobenzoates produced by biphenyl degradation can be broken down in several ways. A bacterial consortium, designated LPS10, completely mineralizes 4-chlorobiphenyl without accumulation of 4-chlorobenzoate (*34*). In this consortium, *C. testosteroni* strain LPS10A mediates the breakdown of 4-chlorobiphenyl to 4-chlorobenzoate, and *Arthrobacter* sp. strain LPS10B mediates 4-chlorobenzoiated degradation. *R. globerulus* strain P6 cometabolizes 4,4′-dichlorobiphenyl primarily to 4-chlorobenzoate, which is, in a coculture with *Acinetobacter* sp. strain 4CB1, converted to inorganic chloride (*35*). Hybrid strains have been also constructed (*36*). A 3-chlorobiphenyl-mineralizing recombinant is produced by intergenic mating of *R. globerulus* strain P6, a biphenyl utilizer, and *Pseudomonas* sp. strain HF1, a chlorobenzoate degrader (*16*). The same kind of construct can mineralize all three monochlorobiphenyls (*37*). Different types of recombinant hybrid strains contain the upper pathway of biphenyl degradation and chlorobenzoate dehalogenase genes (*38*). Either a bacterial consortium or a hybrid strain can completely oxidize both aromatic rings. However, the fission of a nonchlorinated ring of chlorobiphenyls is enough for biphenyl-utilizing bacteria to grow. In this case, DHB12Os that are not severely inhibited by chlorocatechols are required for efficient degradation of chlorobiphenyls. So either finding bacteria possessing such DHB12Os or molecular breeding of resistant DHB12Os is very important.

3-Chlorobenzoate arises as intermediates during the degradation of 3-chlorobiphenyl (*12*). 3-Chlorobenzoate produced in biphenyl-utilizing strains is enzymatically or non-enzymatically converted to corresponding 3-chlorocatechol. 3-Chlorobenzoate or its metabolite, 3-chlorocatechol, affects chlorobiphenyl degradation. The inactivation of catechol 2,3-dioxygenase (C23O) by 3-chlorocatechol has been suggested to occur through irreversible covalent modification by an acyl chloride species generated by the ring cleavage reaction (*39*). The *meta*-cleavage product of 3-chlorocatechol has been reported to produce a reactive acyl halide (5-chloroformyl-2-hydroxypenta-2,4-dienoate) (*39*, *40*), which can quickly condense to macromolecules. This condensation is thought to inactivate proteins in cells, release chloride, and eliminate free metabolites, which results in growth inhibition (*18*). Some of biphenyl-utilizing bacteria possess the genes for the lower pathway of biphenyl degradation. *S. yanoikuyae* B1, *C. testosteroni* B-356 and *Pseudomonas* sp. strain IC contain two *meta*-cleavage dioxygenases, DHB12O and catechol 2,3-dioxygenase (C23O) (*41–44*). Since 3-chlorocatechol can act as a substrate for both *meta*-cleavage dioxygenases, we used *E. coli* strains expressing only *bphC* genes to make sure that the effect of 3-chlorocatechol is indeed on DHB12O. We cloned several *bphC* genes from biphenyl-utilizing gram-negative bacteria and showed that *bphC* gene products, DHB12Os, had different sensitivities to 3-chlorocatechol inhibition. Inhibition of DHB12O activity by 3-chlorocatechol has been demonstrated in several biphenyl-utilizing bacteria (*16–18*, *45*). DHB12O activity of *Burkholderia* sp. LB400 heterologously expressed in *E. coli* is completely inhibited by 400 μM 3-chlorocatechol (*46*). Activity of $BphC_{KF751}$, which is identical to $DHB12O_{LB400}$, was almost completely inhibited by 3-chlorocatechol at 1 mM. The inhibition of BphC activity by 3-chlorocatechol is thought to be caused by enzyme inactivation. The inactivation mechanism of DHB12O by 3-chlorocatechol has been suggested to occur through irreversible covalent modification by an acyl halide produced by the ring cleavage reaction (*18*, *39*). However, a recent result demonstrates that the mechanism-based inactivation of DHBD does not involve covalent modification (*46*). The inactivation appears to involve oxidation of the active site $Fe2^{+}$. One DHB12O from *Sphingomonas* sp. strain BN6, BphC1-BN6, can oxidize 3-chlorocatechol by a distal cleavage mechanism (*47*). It is very unlikely that the DHB12Os showing relatively high resistance to 3-chlorocatechol inhibition in this study are BphC1-BN6-type DHB12Os, because they were quite different from BphC1-BN6 in amino acid sequence and size. $DHB12O_{LB400}$ inactivated by 3-chlorocatechol can be readily reactivated *in vivo* and *in vitro*, once 3-chlorocatechol is removed from the reaction mixture. When the resting cells of DHB12O-expressed *E. coli* HMS174 were treated with 3-chlorocatechol, DHB12O enzyme activity was inhibited (Fig. 7). However, after removal of 3-chlorocatechol, DHB12O activity in washed cells was recovered within 30 min (data not shown). This is consistent with $DHB12_{LB400}$ reactivation data. *E. coli* cells do not contain a DHB12O-specific regeneration system, such as chloroplast-type

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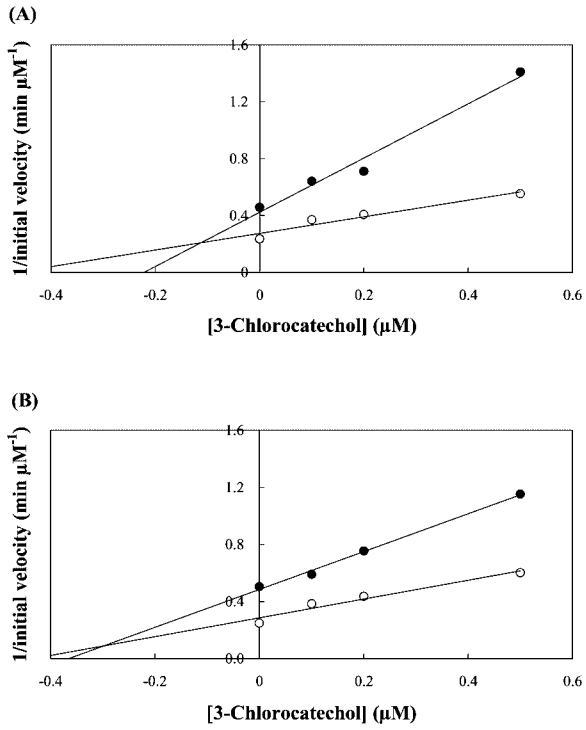

Fig. 9. **Dixon plots of 3-chlorocatechol inhibitory effect on DHB12O activity.** The rate of 2,3-dihydroxybiphenyl cleavage was determined using 2 μM (closed circles) and 10 μM (open circles) 2,3-dihydroxybiphenyl. Best-fit lines were obtained using an equation for competitive inhibition for (A) $BphC_{KF702}$ and (B) $BphC_{7109}$.

ferredoxin XylT in C23O (*48*), so the *in vivo* reactivation of 3-chlorocatechol-inactivated DHBDs in *E. coli* might use a nonspecific ferrous ion regeneration system.

Growth of *E. coli* cells expressing high level of DHB12O was inhibited in the presence of 3-chlorocatechol (Fig. 5). The fact that 3-chlorobenzoate had no effect on growth indicated that it is not converted to 3-chlorocatecol in *E. coli* cells, and only 3-chlorocatechol is an inhibitor of cell growth. When the expression level of the *bphC* gene was much lower, no growth inhibition was observed in the presence of 3-chlorocatechol (Fig. 5). These data indicate that it is not 3-chlorocatecol itself but a cleavage product of 3-chlorocatechol by DHB12O that affects the cell growth. Although the optical density of cultures increased after 3-chlorocatechol addition to the medium, cells expressing DHB12O were not viable, as judged from the results on the agar plate (Fig. 6). The same phenomenon has been observed in the growth of biphenyl-utilizing cells on monochlorinated biphenyls (*18*). Since recent data demonstrates that DHB12O is not covalently modified by acyl chloride produced by DHB12O-catalyzed cleavage of 3-chlorocatechol (*49*), the mechanism of growth inhibition by a reactive acyl halide is ruled out. The lack or reduction of viability of *E. coli* cells expressing a high level of DHB12 in the presence of 3-chlorocatechol can be explained by superoxide production (*49*). 3-Chlorocatechol is highly reactive with superoxide, and inactivation of DHB12O during catalytic turnover involves the dissociation and release of superoxide from the DHB12O-3-chlorocatechol-O_2 ternary complex. A burst of superoxide induces the cell death. The more DHB12O molecules there are, the more is the turnover and the bigger is the burst of superoxide. The superoxide production is also correlated to the 3-chlorocatechol concentration.

We cloned two full-length *bphC*s and nine partial *bphC* fragments fused to $bphC_{KF707}$ from biphenyl-utilizing bacteria. All these strains are gram-negative soil bacteria isolated from various locations in Japan. Southern hybridization using DNA fragment containing $bphABC_{KF707}$ genes as a probe indicates that KF702, KF703, KF710, KF711, KF713, and KF714 have the same or almost the same genome organizations as KF707 (*22*). The fact that the nucleotide sequences of 5′ partial *bphC* fragments of all these KF strains were 100% identical to that of $bphC_{KF707}$ (Fig. 4) supports the hybridization data. $bphC_{KF701}$ was two nucleotides different from $bphC_{KF715}$, which is in good agreement with the hybridization data showing that these two strains have only slightly different profiles. Although the probe hybridizes with chromosomal DNA of KF706, the profile is different from other KF strains. The phylogenetic distance of $BphC_{KF706}$ from BphCs of other KF strains clearly confirms this data (Fig. 4). While the DNA probe does not hybridize with DNAs of KF704, KF708, KF709, and KF712, the antiserum raised against $BphC_{KF707}$ weakly reacts with $BphC_{KF704}$ and $BphC_{KF712}$ (*22*). Phylogenetic analysis and PCR amplification of *bphC* gene fragments (Fig. 2) demonstrated that genome organizations around *bphC* genes in KF704 and KF712 are the same as in KKS102, and that $bphC_{KF704}$ and $bphC_{KF712}$ belong to a different clade from *bphC* genes in the KF707/LB400 group. These results of this study do not conflict with previous data.

We could not elucidate why the chimeric DHB12Os were more resistant to 3-chlorocatechol. The chimeric DHB12Os might be more easily reactivated in *E. coli* and probably in biphenyl-utilizing bacteria. Crystal structures of $BphC_{KKS102}$, which is identical to $BphC_{KF704}$, have been determined for three forms, the substrate-free form, the DHB12O-sustrate complex, and the DHB12O-substrate-nitric oxide complex (*50*). Comparison of the three structures reveals that His194 is the key residue during the *meta*-cleavage reaction. His194, along with other important residues for Fe^{2+} binding, His145, His209, and Glu260, were all conserved in two parents, $BphC_{KF704}$ and $BphC_{KF712}$, and all selected resistant chimeras. Although other amino acid residues must be involved in cleavage of 3-chlorocatechol, amino acid alignment of resistant DHB12Os isolated in this study did not reveal the important residues. Many aspects of the inhibition of extradiol-type dioxygenases and the relationship of this inhibition to productive catalysis remain to be elucidated.

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