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Molecular genetics and evolutionary relationship of PCB-degrading bacteria

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Abstract

Biphenyl-utilizing soil bacteria are ubiquitously distributed in the natural environment. They cometabolize a variety of polychlorinated biphenyl (PCB) congeners to chlorobenzoic acids through a 2,3-dioxygenase pathway, or alternatively through a 3,4-dioxygenase system. The *bph* genes coding for the metabolism of biphenyl have been cloned from several pseudomonads. The biochemistry and molecular genetics of PCB degradation are reviewed and discussed from the viewpoint of an evolutionary relationship.

Abbreviations: BP, biphenyl; *bph*, BP/PCB-degradative gene; 23DHBP, 2,3-dihydroxybiphenyl; HPDA, 2-hydroxy-6-oxo-6-phenylhexa 2,4-dienoic acid; KF707, *P. pseudoalcaligenes* strain KF707; LB400, *Pseudomonas* sp. strain LB400; PCB, polychlorinated biphenyls; Q1, *P. paucimobilis* strain Q1*tod*; toluene catabolic gene.

Introduction

Microbial transformations of PCBs, which includes both aerobic degradation and anaerobic dechlorination, have been well documented and recognized to be the major route of environmental degradation for these widespread pollutants (Furukawa 1982; Bedard et al. 1986; Brown et al. 1987; Pettigrew et al. 1990; Abramovicz 1990; Higson 1992). Biphenyl (BP)-utilizing bacteria are widely distributed in the environment and cometabolize various PCB-congeners by a BP-metabolic enzyme system (Ahmed & Focht 1973; Furukawa 1982; Furukawa 1986; Bedard et al. 1987; Barton & Crawford 1988). The major pathway of PCB-degradation by soil bacteria proceeds via 2,3-dioxygenation, where molecular oxygen is introduced at a non-chlorinated ring, or at a ring offering one pair of adjacent non-chlorinated 2,3- (or 5,6-) sites (Furukawa et al. 1979). A number of PCB congeners examined were degraded to the corresponding chlorobenzoic acids through ring meta-cleavage and subsequent hydrolysis. Since most of the BP-utilizing strains are unable to degrade chlorobenzoic acids any further, these compounds accumulate. Alternatively, catabolic intermediates including dihydrodiols, dihydroxy compounds, and the ring-cleaved compounds often accumulate, depending on the substrate specificities of BP-catabolic enzymes in various strains (Furukawa 1982). Several naturally occurring strains grow on 4-chlorobiphenyl.

Recently, strains have been constructed which are able to totally mineralize mono- and dichlorobiphenyls. The combination of BP- and chlorobenzoate-degraders by mating or in a multiple chemostat system, yielded strains able to mineralize 2-chloro-, 3-chloro-, 4-chloro-, 2,4-dichloro-, or 3,5-dichlorobiphenyls (Mokross et al. 1990; Havel & Reineke 1991; Hickey et al. 1992; Adams et al. 1993). These strains utilized the entire carbon skeleton as the carbon and energy source, and released stoichiometric amounts of chloride. Thus, BP-utilizing strains show different ranges of PCB degradation.

The biochemistry and genetics of PCB-degradation through a 2,3-dioxygenase pathway have been well studied. A 3,4-dioxygenase system has recently been found in certain PCB-degraders which possess wide substrate spectra including highly chlorinated congeners (Bedard et al. 1987). In the present paper, the molecular genetics of bacterial PCB degradation is reviewed.

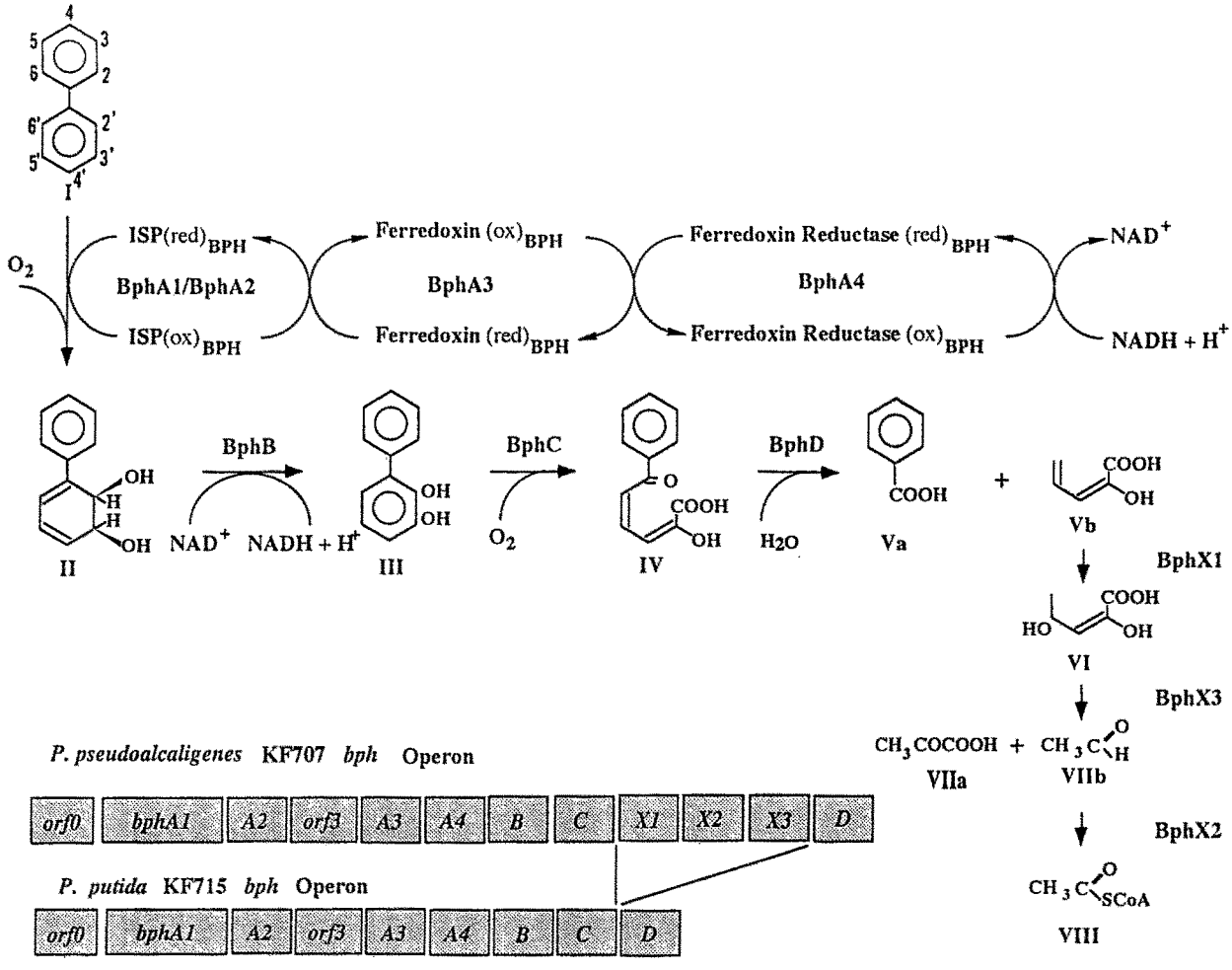

Fig. 1. Catabolic pathway of BP/PCB-degradation through 2,3-dioxygenase and the organization of *bph* operons in *P. pseudoalcaligenes* KF707 and *P. putida* KF715. (From Furukawa et al. 1991 ©Elsevier Science Publishers BV.)

PCB-degrading bacteria and modes of PCB-modification

PCBs found in an oxidative environment are more highly chlorinated biphenyls (mostly hepta- and hexachlorobiphenyls). This indicates, on one hand, that the less chlorinated biphenyls are more rapidly degraded than the more highly chlorinated congeners (Furukawa et al. 1978). On the other hand, however, anaerobic PCB dechlorination in aquatic sediments demonstrates that higher chlorinated PCBs are more susceptible to the reductive dechlorination (Brown et al. 1987). Thus, PCBs can be biodegraded by a combination of anaerobic dechlorination and aerobic breakdown of the biphenyl structure.

The principal route of aerobic degradation of PCBs in most soil bacteria appears to involve a 2,3-dioxygenase (Fig. 1) (Furukawa et al. 1982). In this reaction, molecular oxygen is introduced at the 2,3 position of the non-chlorinated or less chlorinated ring to produce a cis-dihydrodiol by the action of a BP dioxygenase (encoded by the *bphA* genes). The dihydrodiol is then dehydrogenated to a 2,3-dihydroxybiphenyl (23DHBP) by a dihydrodiol dehydrogenase (encoded by the *bphB* gene). The 23DHBP is then cleaved at the 1,2 position by a 23DHBP dioxygenase (encoded by the *bphC* gene). The meta-cleavage compound (a chlorinated derivative of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid) is hydrolyzed to the corresponding chlorobenzoic acid by a hydrolase (encoded by the *bphD* gene). Thus four enzymes are involved in the oxidative degradation of PCBs to chlorobenzoic acids through the 2,3-dioxygenase pathway.

Table 1. Biphenyl-utilizing strains and their growth characteristics on various biphenyl derivatives.

Strains	Growth on following substrate						
	BP	4ClBP	4MeBP	2BrBP	$2NO_2BP$	2OHBP	DM
Achromobacter xylosoxidans KF701	+++	-	+	-	-	++	-
Pseudomonas sp. strain KF702	+++	-	+	-	-	-	-
P. fluorescens KF703	+++	-	-	-	-	-	+
Moraxella sp. strain KF704	+++	-	-	-	-	-	+
P. paucimobilis KF706	+++	-	+	+	+	-	++
P. pseudoalcaligenes KF707	+++	-	+	-	-	-	-
Alcaligenes sp. strain KF708	+++	+	++	++	++	-	++
Unidentified strain KF709	+++	-	-	-	-	-	+
Pseudomonas sp. strain KF710	+++	-	-	-	-	-	-
Alcaligenes sp. strain KF711	+++	-	-	-	-	-	-
Pseudomonas sp. strain KF712	+++	-	-	-	-	-	-
P. stutzeri KF713	+++	-	-	-	-	-	-
Pseudomonas sp. strain KF714	+++	+	+	-	-	-	+
P. putida KF715	+++	+	+	-	+	++	+
P. paucimobilis Q1	+++	+	+	-	-	-	+
Arthrobacter sp. strain M5	+++	+	++	+	+	-	-

Symbols: +++, good growth; ++, moderate growth; +, poor growth: -, no growth or very poor growth.
Abbreviations: BP, biphenyl; 4ClBP, 4-chlorobiphenyl; 4MeBP, 4-methylbiphenyl; 2BrBP, 2-bromobiphenyl; $2NO_2BP$, 2-nitrobiphenyl; 2OHBP, 2-hydroxybiphenyl; DM, diphenylmethane.

The following results were obtained with respect to the correlation between PCB structures and biodegradability. They are based on the studies of many bacterial strains (Furukawa 1982). (i) Biodegradability decreases as chlorine substitution increases. (ii) Congeners with two ortho chlorines (i.e., 2,6- and 2,2'-) are extremely resistant to degradation. (iii) PCBs containing an unsubstituted ring are generally degraded more rapidly than isomers that are chlorinated on both rings. (iv) Tetra- and pentachlorobiphenyls containing a 2,3-chlorophenyl ring are more susceptible to biodegradation than other tetra- and pentachlorobiphenyls through an alternative pathway. (v) Ring fission generally occurs on the unchlorinated or lesser chlorinated ring of a congener.

On the other hand, two bacterial strains, isolated from PCB-contaminated sites, *Alcaligenes eutrophus* H850 and *Pseudomonas* sp. LB400, appear to degrade even highly chlorinated PCBs through the 3,4-dioxygenase pathway (Bedard et al. 1987), in which the mode of degradation of PCB congeners is very different from that of the above-mentioned 2,3-dioxygenase system as seen from the following results. (i) Tetra- and pentachlorobiphenyls containing a 2,5-dichlorophenyl ring are degraded more extensively than lower congeners chlorinated at both para positions, i.e., positions 4,4'. (ii) Many di-ortho-substituted congeners, including 2,2'-CB, 2,3,6-CB, 2,5,2'-CB, 2,5,2',5'-CB and 2,6,2',5'-CB, and several tri- and tetra-ortho-substituted congeners such as 2,4,6,2',5'-CB, 2,3,5,6,2',5'-CB and 2,3,6,2',3',6'-CB are degraded rather rapidly. (iii) Highly chlorinated biphenyls containing a 2,3-chlorophenyl group were less susceptible to degradation by these strains than those containing a 2,5-chlorophenyl ring. (iv) Both 2,4,4'-CB and 2,5,4'-CB were degraded via ring fission of the dichlorinated ring to yield 4-chlorobenzoic acid. (v) cis-3,4-dihydro-cis-3,4-dihydroxy-2,5,2',5'-tetra chlorobiphenyl was identified as a major metabolite of 2,5,2',5'-CB.

A 2,3-biphenyl dioxygenase pathway has also been demonstrated for *A. eutrophus* H850 and is responsible for the oxidation of some PCB congeners. Recently, it was shown that the biphenyl dioxygenase in *Pseudomonas* sp. LB400 possesses both 2,3- and 3,4-dioxygenase activities for PCB degradation (Erickson and Mondello 1993). The biodegradability of selected PCB congeners were compared with *Pseudomonas pseudoalcaligenes* KF707 which possesses only 2,3-dioxygenase and *Pseudomonas* sp. LB400 which possesses both activities of 2,3- and 3,4-dioxygenase (Gibson et al. 1993). LB400 degraded congeners contain-

ing chlorine substituents at the 2,5 positions on one ring and two or three chlorine substituents at the positions on the second ring, whereas KF707, showed little or no oxidation of these substrates. In addition to the above mentioned pathways, certain PCB congeners were catabolized in different fashions, depending upon the position and number of chlorine substituents (Furukawa et al. 1979). For instance 2,4,6-trichlorobiphenyl was quickly converted to dihydroxy compound and then to trihydroxy compound by *Acinetobacter* sp. P6. It is also true that PCB degradabilities are greatly different among various BP-utilizers. (Bedard et al. 1986).

Fungi, yeast and a cyanobacterium were found to metabolize BP and low chlorinated BP to monohydroxy and dihydroxy compounds as also demonstrated with a mammalian system (Furukawa 1982). The white rot fungus *Phanerochaete chrysosporium* has been found capable of degrading a number of xenobiotics, including PCBs (Aust 1990). Extensive anaerobic dechlorination has been demonstrated in Hudson River sediments, where the most highly chlorinated PCBs (Aroclor 1260) were dechlorinated to the less chlorinated ones (i.e., mono, di, and trichlorinated congeners) (Brown et al. 1987), although the dechlorinating anaerobes have not yet been isolated. It was demonstrated that dechlorination occurred primarily from the meta- and para-positions of the highly chlorinated congeners in Aroclor 1242, 1248, 1254 and 1260 (Quensen et al. 1988, 1990). This dechlorination resulted in significant benefits, including reduced toxicity and bioaccumulation as well as increased aerobic biodegradation of the dechlorination products.

Enzyme systems in the initial degradation of biphenyl and PCB

PCB-degradation of BP-utilizing bacteria is the result of cometabolism through the action of BP-metabolic enzymes (Table 1). BP dioxygenase is a multicomponent enzyme which catalyzes the initial oxidation of the BP molecule to the dihydrodiol by introducing two atoms of oxygen, as also shown for toluene dioxygenase (Zylstra & Gibson 1989; Subramanian et al. 1979) and naphthalene dioxygenase (Kurkela et al. 1988; Simon et al. 1993). The BP dioxygenase from *Pseudomonas* sp. LB400, which possesses *bphAEFG* genes (Erickson & Mondello 1992), was almost identical to the dioxygenase encoded by the *bphA1A2A3A4* region of strain KF707 (Taira et al. 1992) and has been partially purified (Haddock et al. 1993). Anion-exchange chromatography of cell extract yielded three proteins, which were similar to the terminal dioxygenase (composed of a large and a small subunit), the ferredoxin and the ferredoxin reductase of the multicomponent toluene dioxygenase from *P. putida* F1 (Zylstra & Gibson 1989). The terminal dioxygenase is involved in the direct introduction of molecular oxygen onto the BP ring and the ferredoxin and NADH-ferredoxin reductase mediate electron transport from NADH to the terminal dioxygenase (Fig. 1). The BP dihydrodiol compound produced is then dehydrogenated to the dihydroxy compound by a dehydrogenase that has not yet been purified. 23DHBP dioxygenase catalyzes the ring-meta-cleavage at the 1,2 position to produce 2-hydroxy-6-oxo-6-phenylhexa 2,4-dienoic acid (HPDA). The yellow meta-cleavage product is subsequently hydrolyzed to (chloro)benzoic acid by a hydrolase.

The 23DHBP dioxygenases were purified from *Pseudomonas pseudoalcaligenes* KF707 (Furukawa & Arimura 1987) and *Pseudomonas paucimobilis* Q1 (Taira et al. 1988). The enzyme from either KF707 or Q1 was colorless and contained the ferrous form of iron as the sole cofactor, which is the typical ion state for the extradiol cleaving enzymes. These enzymes possess many features in common such as molecular weight (ca. 260 kDa), subunit structure $[\alpha Fe(II)]_8$, and substrate specificity. Both 23DHBP dioxygenases were specific for only 23DHBP. They did not oxidize a positional isomer such as 3,4-dihydroxybiphenyl. Despite the above-mentioned close similarities between these two enzymes, there exists some discrepancy in terms of the substrate specificity. The 23DHBP dioxygenase of Q1 was able to oxidize catechol with a catalytic efficiency of almost one-quarter (1/4 V_{max}) of that with the natural substrate, 23DHBP, whereas catechol was virtually inert to the 23DHBP dioxygenase of KF707, (Taira et al. 1988). The 23DHBP dioxygenase from *Pseudomonas* sp. LB400 which showed a single amino acid difference from the KF707 enzyme (Ala^{185} to Val^{185}), has recently been crystalized (Eltis et al. 1993). Electron microscopy indicated that the enzyme is an octamer in which the subunits are arranged in two tetramer planes.

The HPDA hydrolase from *P. pseudoalcaligenes* KF707 was purified from BP-induced cells. The molecular mass of the native enzyme was 120 kDa and the size of the subunits 30 kDa, indicating that the HPDA hydrolase is a homotetramer (K. Furukawa, unpublished).

Molecular cloning of genes coding for PCB-degradation

Genes involved in the degradation of BP/PCB (designated as *bph*) were first cloned from *P. pseudoalcaligenes* KF707 (Furukawa & Miyazaki 1986) and then from several other soil bacteria such as *P. paucimobilis* Q1 (Taira et al. 1988), *Pseudomonas* sp. KKS102 (Kimbara et al. 1989), *Pseudomonas* sp. LB400 (Mondello 1989), *P. putida* OU83 (Khan & Walia 1989), *P. putida* KF715 (Hayase et al. 1990), *P. testosteroni* B356 (Ahmad et al. 1990) and *Arthrobacter* sp. M5 (Peloquin & Greer 1993). The 11.3 kb DNA fragment coding for the conversion of BP/PCB to (chloro)benzoic acids cloned from *P. pseudoalcaligenes* KF707 contained *bphA1A2A3A4* (BP dioxygenase genes), *bphB* (BP dihydrodiol dehydrogenase gene), *bphC* (23DBP dioxygenase gene) and *bphD* (HPDA hydrolase gene). In addition, there is a 3.5 kb-DNA segment (*bphX*) between *bphC* and *bphD* in the KF707 *bph* operon (Fig. 1) (Furukawa et al. 1992). A 9.4 kb DNA containing *bphABCD* was cloned from *P. putida* KF715. Interestingly, the 3.5 kb *pbhX* region, which exists in the KF707 *bph* operon, is missing between *bphC* and *bphD* in the KF715 *bph* operon despite the fact that the gene organization and nucleotide sequences of corresponding genes of the two operons are very similar (Fig. 1). Subcloning and deletion analysis revealed that the *bphA* region coding for BP dioxygenase is approximately 4 kb in size, followed by *bphB* (ca. 1 kb), and *bphC* (ca. 1 kb). The *bphD* is located just downstream of *bphC* gene in the KF715 *bph* operon. The *bph* gene clusters similar to KF707 *bphABCXD* operon were cloned from *Pseudomonas* LB400 (Mondello 1989). The organization of *bph* genes in *P. putida* OU83 (Khan & Walia 1989) and *P. testosteroni* B-356 (Ahmad et al. 1990) appears to be similar to the *P. putida* KF715 *bphABCD* in terms of gene organization.

Gene specific transposon mutagenesis of *bph* operon of *P. pseudoalcaligenes* KF707

The cloned *bphA, bphB* and *bphC* gene of *P. pseudoalcaligenes* KF707, coding for the conversion of BP/PCB into HPDA, were first randomly mutagenized by Tn*5*-B21 (Furukawa et al. 1991) (Fig. 2). Tn*5*-B21 is a Tn*5* derivative in which the neomycin resistant determinant (Nm^R) is replaced by a tetracycline determinant (Tc^R) originating from RP4 and the *lacZ* gene is lacking the transcriptional start signals (Simon et al. 1989). *Escherichia coli* S17-1 (chromosomally integrated RP4-2-Tc::*Mu*-Km::Tn7) (Simon et al. 1989) carrying Tn*5*-B21 mutagenized *bphABC* on a suicide pSUP vector was then filtermated with the parent strain KF707. Since the pSUP vector cannot replicate in the KF707, the Tc^R and $LacZ^+$ colonies were screened for those in which the chromosomal *bph* operon was recombined and replaced by the Tn*5*-B21 mutagenized *bph* DNA on the plasmid (Fig. 3). By this method, various double crossover mutants, in which Tn*5*-B21 was inserted gene-specifically, were obtained. Similarly, Tn*5*-B21 mutants were obtained from *P. fluorescens* KF703, *P. putrefaciens* KF710 and *Alcaligenes* sp. KF711 which possess very similar, if not identical, *bphABCXD* operons to that of *P. pseudoalcaligenes* KF707. The KF707 mutant strains KF730 (KF707 *bphA*::Tn*5*-B21), KF748 (KF707 *bphB*::Tn*5*-B21) and KF744 (KF707 *bphC*::Tn*5*-B21) were used to identify the catabolic intermediates formed from 4-chlorobiphenyl. As expected, KF730 did not attack 4C1BP since *bphA* was disrupted by transposon insertion. KF748 produced and accumulated the dihydrodiol compound and KF744 accumulated the dihydroxy compound from 4-chloroBP (Furukawa et al. 1991).

Using the transposon mutant KF744 (KF707*bphC*:: Tn*5*-B21), the substrate range of BP dioxygenase and the dehydrogenase of *P. pseudoalcaligenes* KF707 were examined for BP derivatives and BP-related compounds. KF744 converted the following compounds to the corresponding dihydroxy compounds: BP, 4-chloroBP, 4-bromoBP, 4-methylBP, 2-nitroBP, diphenylmethane, diphenylethane(dibenzyl), diphenylether and benzalacetophenone. The same cells, however, could not convert benzene, toluene and phenol. Thus, BP dioxygenase from *P. pseudoalcaligenes* KF707 possesses a wide substrate range for BP-derivatives and BP-related compounds, but not for benzene and its derivatives. Another transposon mutant, strain KF725 (a *bphC*::Tn*5*-B21 mutant of *Pseudomonas* sp. KF712), possesses a wider substrate specificity. This strain converted benzene and its derivatives to the corresponding dihydroxy compounds (Furukawa et al. unpublished).

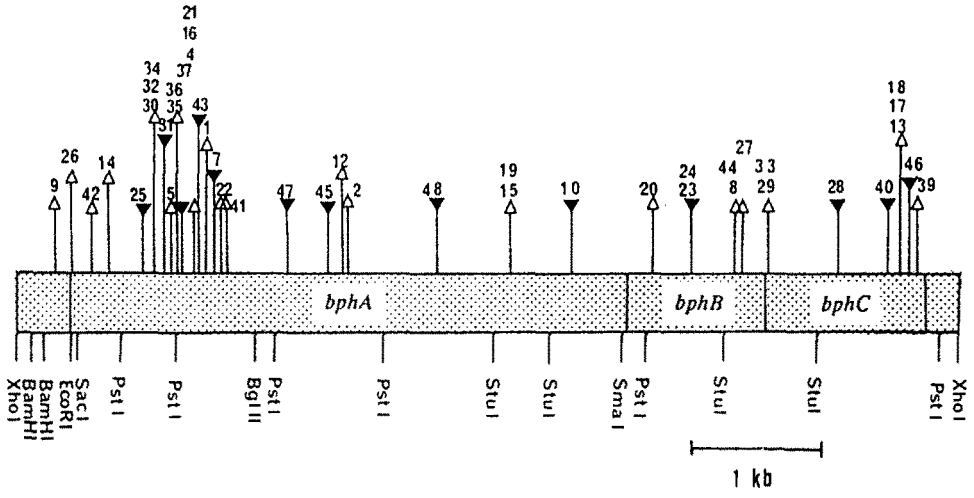

Fig. 2. Transposon Tn*5*-B21 insertion into the cloned *bphABC* cluster of *P. pseudoalcaligenes* KF707. The promoterless *lacZ* gene inserted with Tn*5*-B21 has the same orientation (△) to the direction of transcription of the *bphABC* sequences, or has an opposite orientation (▼). (From Furukawa et al. 1993, with permission. © 1993 American Society for Microbiology.)

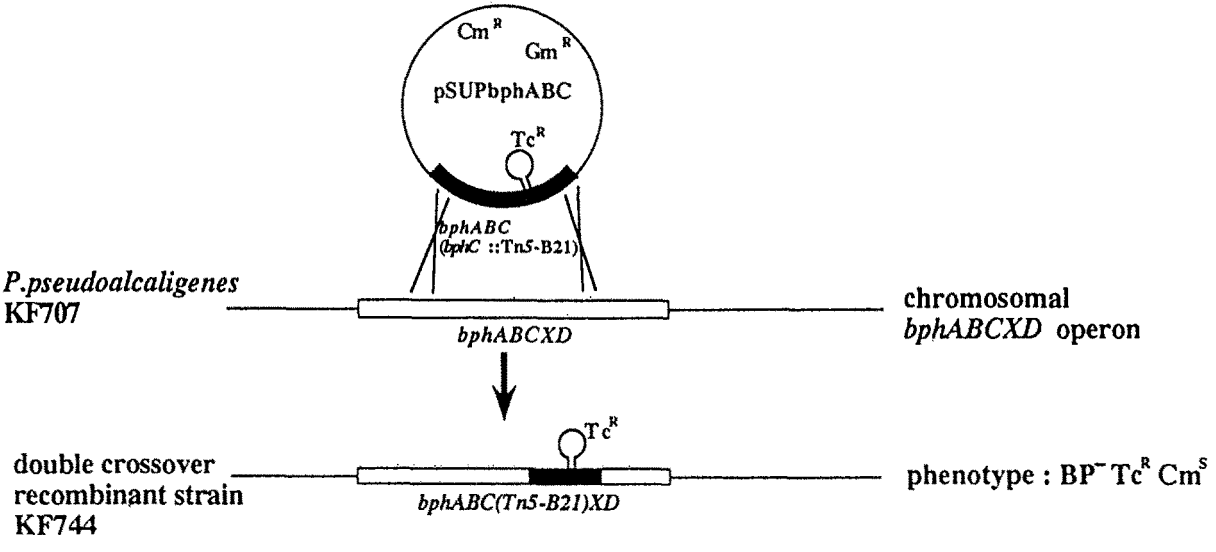

Fig. 3. Schematic double crossover recombination between vector-borne *bphABC* (*bphC*::Tn*5*-B21) and chromosomal *bph* operon of *P. pseudoalcaligenes* KF707. Recombination both upstream and downstream of the transposon insertion resulted in insertion of only Tn*5*-B21 into the *bph* operon. The recombinant shows a BP^-, Tc^R, $LacZ^+$ phenotype and accumulates dihydroxy compounds from various BP derivatives. (From Furukawa et al. 1991, with permission. © 1991 Elsevier Science Publishers BV.)

Sequence analysis of *bph* operon of *P. pseudoalcaligenes* KF707

The complete nucleotide sequence of the 11.3 kb DNA fragment containing the *bphABCXD* genes was determined (Taira et al. 1992, K. Furukawa et al. unpublished). The identified transcriptional initiation site was 104 bases upstream from the start codon of the *bphA1* gene (Taira et al. 1992). The *bphA* region encoded a cluster of five open reading frames (ORFs). The five proteins corresponding to these ORFs were detected by in vitro protein synthesis. Four ORFs were very similar to the *todC1C2BA* genes coding for the corresponding enzymes catalyzing the initial dioxygenation reaction of toluene. The third open reading frame (ORF3) of the *bphA* region, missing its counterpart in the toluene

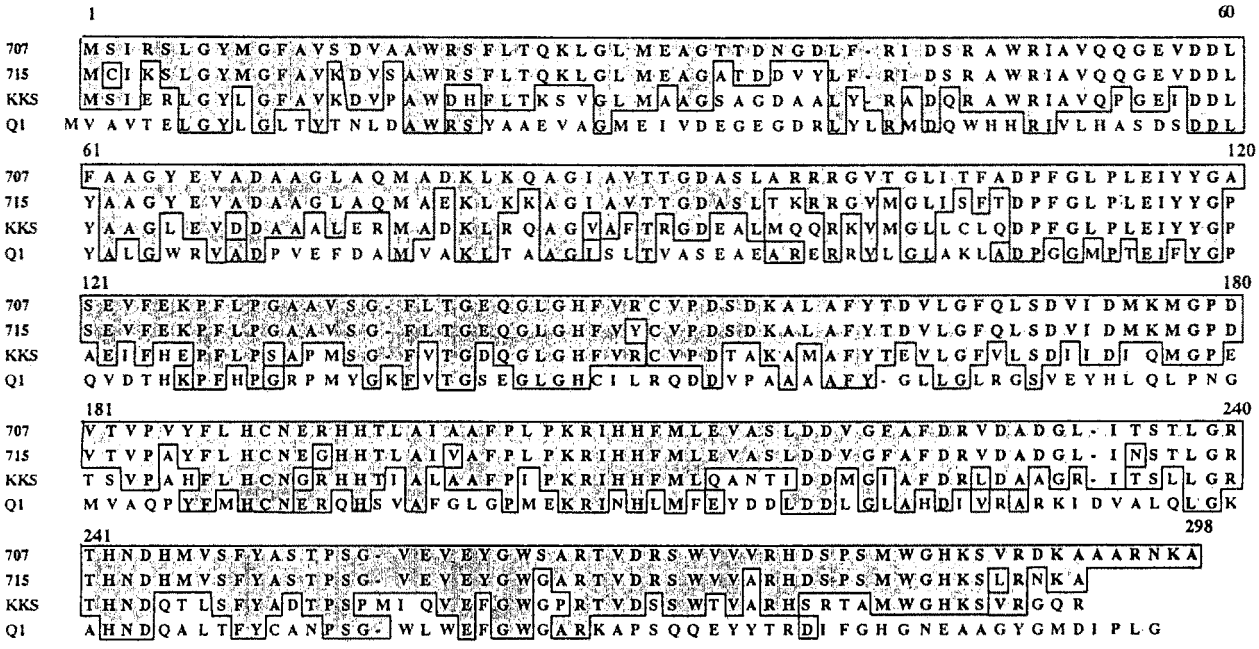

Fig. 4. Amino acid sequence homology of 23DHBP dioxygenases in various PCB degrading strains. 707, *P. pseudoalcaligenes* KF707; 715, *P. putida* KF715; KKS, *Pseudomonas* KKS102; Q1, *P. paucimobilis* Q1. Dashes represent missing amino acids when the sequences are maximally aligned. Identical amino acids within the four enzymes are boxed. (From Furukawa et al. 1989. © 1989, American Society for Microbiology).

dioxygenase gene cluster, was site specifically deleted. The resulting enzymatically active mutant revealed that ORF3 was not mandatory for the metabolism of BP and 4-chloroBP.

The structure of the *P. pseudoalcaligenes* KF707 *bphA1A2A3A4BCXD* operon is presented in Fig. 1. The products of *bphA1, A2, A3, A4, B, C,* and *D* are, respectively, terminal dioxygenase (large subunit), terminal dioxygenase (small subunit), ferredoxin, ferredoxin reductase, dehydrogenase, 2,3-dioxygenase, and hydrolase. The reduced form of the iron-sulfur protein (terminal dioxygenase) designated as ISP(red)$_{BPH}$ (the products of *bphA1* and *bphA2*), catalyzes the initial addition of both atoms of molecular oxygen to the BP ring. The resulting oxidized form of ISP(ox)$_{BPH}$ accepts electrons from ferredoxin(red)$_{BPH}$ (the product of *bphA3*) which is a one-electron carrier. In turn, ferredoxin(ox)$_{BPH}$ accepts electrons from a flavoprotein, ferredoxin reductase(red)$_{BPH}$ (the product of *bphA4*). The original source of electrons is NADH which initially transfers hydride equivalently to the flavoprotein ferredoxin reductase(ox)$_{BPH}$ (Taira et al. 1992). Many of the multicomponent dioxygenases involved in aromatic degradation contain a Rieske-type [2Fe-2S] cluster either associated with the oxygenase itself or as part of a small electron transport protein (Harayama & Kok 1992). The Rieske-type proteins contain a highly conserved sequence including two cysteine-histidine pairs separated by 16 or 17 amino acids. These cysteine-histidine pairs separated by 17 amino acids are also found in the putative translation products of the *bphA1* and *bphA3* genes of the *bph* operon.

The amino acid sequence of the *bphC*(KF707) gene deduced from the nucleotide sequence is presented in Fig. 4, together with, for comparison, the amino acid sequences encoded by *bphC*s from *P. paucimobilis* Q1 (Taira et al. 1988), *P. putida* KF715 (Kayase et al. 1990) and *Pseudomonas* sp. KKS102 (Kimbara et al. 1989). The overall identity between BphC(KF707) and BphC(KF715) is as high as 92%, while that between BphC(KF707) and BphC(KKS102) was 65%, and that between BphC(KF707) and BphC(Q1) as low as 38%.

The sequence of *bphD*(KF707) was also determined. The overall amino acid sequence identity between BphD(KF707) and BphD from *P. putida* KF715 was as high as 96% and between BphD(KF707) and BphD from *Pseudomonas* sp. KKS102, 79%.

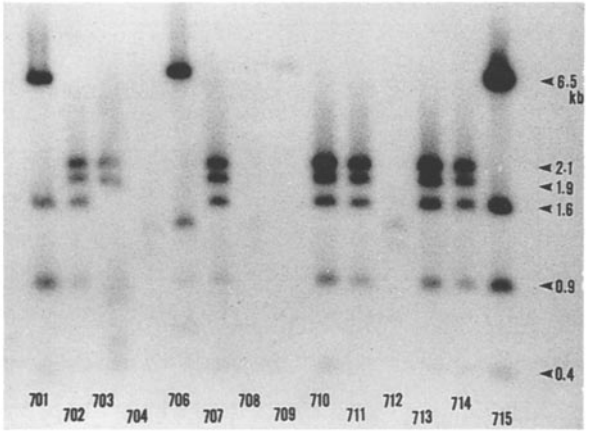

Fig. 5. Hybridization of *P. pseudoalcaligenes* KF707 *bphABC* genes with cellular DNA of various PCB-degrading strains. (Table 1). Total cellular DNAs were doubly digested with *Xho*I and *Pst*I and hybridized with the ^{32}P-labeled 6.8-kb *Xho*I fragment that includes the *bphABC* genes of KF707. 701, *Achromobacter xylosoxidans* KF701; 702, *Pseudomonas* sp. KF702; 703, *P. fluorescens* KF703; 704, *Moraxella* sp. KF704; 706, *P. paucimobilis* KF706; 707, *P. pseudoalcaligenes* KF707; 708, *Alcaligenes* sp. KF708; 709, Unidentified strain KF709; 710, *Pseudomonas* sp. KF710; 711, *Alcaligenes* sp. KF711; 712, *Pseudomonas* sp. KF712; 713, *P. stutzeri* KF713; 714, *Pseudomonas* sp. KF714; 715, *P. putida* KF715. (from Furukawa et al. 1989, with permission.)

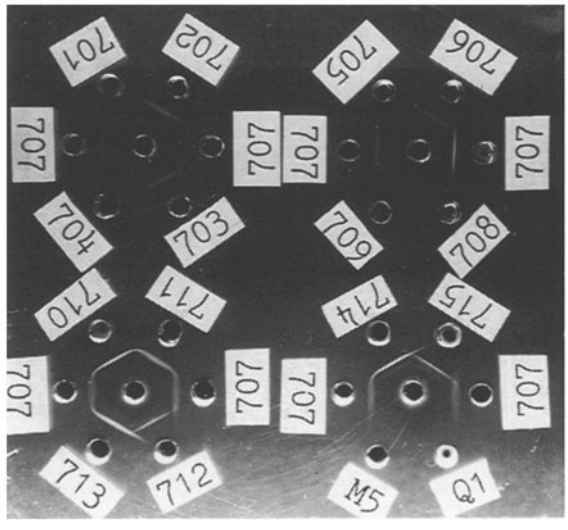

Fig. 6. Immunoprecipitation profile of 23DHBP dioxygenases from various PCB-degraders with antiserum prepared against the purified 23DHBP dioxygenase of *P. pseudoalcaligenes* KF707. The KF707 23DHBP dioxygenase antiserum was placed in the central wells. The cell extracts obtained from BP-grown cells were placed in the wells surrounding the antiserum. Numbers in the figure correspond to the strains in the legend of Fig. 5. KF704 and KF705 are the same strain. (From Furukawa et al. 1989, with permission.)

As mentioned previously, there is a 3.5 kb *bphX* region between *bphC* and *bphD* in the KF707 *bph* operon, which is missing in *P. putida* KF715 *bph* operon (K. Furukawa et al. 1992). The nucleotide sequences determined revealed at least three ORFs, designated as BphX1 (260 amino acids), BphX2 (303 amino acids) and BphX3 (337 amino acids). A protein data base search revealed that BphX1 is ca. 70% identical to DmpE and XylJ, which are the 2-hydroxypenta-2,4-dienoate hydratase involved in phenol catabolism of *Pseudomonas* sp. CF600 (Shingler et al. 1992) and the meta-cleavage pathway encoded by the *xyl* operon of pWW0 (Harayama & Rekik 1993), respectively. Similarly, BphX2 shares ca. 55% sequence identity with DmpF and XylQ [acetaldehyde dehydrogenase (acylating)] and BphX3 shares ca. 57% identity with DmpG and XylK (4-hydroxy-2-oxovalerate aldolase) (K. Furukawa et al. unpublished). In view of the observed similarity, the putative protein products of these ORFs may be involved in the metabolism of 2-hydroxypenta-2,4-dienoate to acetyl coenzyme A via 4-hydroxy-2-oxovalerate and acetaldehyde (Fig. 1).

Molecular relationship of chromosomal *bph* genes among soil bacteria

Using the *bphABC* and *bphD* genes of *P. pseudoalcaligenes* KF707 as probes, Southern blot analyses were carried out to test whether the *bph* genes in various BP-PCB strains (Table 1) are related. The results shown in Fig. 5 reveal that out of 15 strains tested almost identical *bph* genes are present in 7 strains, including KF707 itself (Furukawa et al. 1989). The *Xho*I-*Pst*I double digested genomic DNA from these strains showed identical fragment profiles. *bphABCD* genes similar to those of *P. putida* KF715 were observed in *Achromobacter* sp. KF701. Some other BP/PCB strains possessed weakly hybridized *bph* genes. Three strains did not show any homology. When the genomic DNAs were digested with various endonucleases, the DNAs flanking the *bph* genes hybridized differently in the strains which showed nearly identical *bphABCXD* operon to that of KF707. This indicates that the regions outside of the *bph* genes are unrelated in these strains.

The antibody raised against purified 23DHBP dioxygenase from *P. pseudoalcaligenes* KF707 was

used to examine the immunological cross reactivity of 23DHBP dioxygenases from various BP/PCB degraders listed in Table 1. The immunological cross reactivity corresponded well to the DNA homology. Thus the enzymes prepared from six strains (KF702, KF703, KF710, KF711, KF713 and KF714) with DNA sequences homologous to the *bph* DNA, showed clear fused precipitin bands against the KF707 antibody, without forming a spur with the KF707 enzyme. The 23DHBP dioxygenases from KF701, KF706 and KF715, which possess a homologous *bph* DNA segment, but with different restriction enzyme profiles, showed a precipitin band with the same antibody. However, they formed a spur with the KF707 enzyme (Fig. 6). Enzymes from KF704 and KF715 showed a weak precipit in band and formed a spur with the KF707 enzyme (Furukawa et al. 1989). The immunological cross-reactivities of hydrolases from various PCB-degraders with the antibody raised against hydrolase from KF707, coincided well with the results obtained with the 23DHBP dioxygenase described above (K. Furukawa et al. unpublished). These results imply that almost identical *bph* genes are present in different soil bacteria, but less homologous *bph* genes do also occur.

Comparison of BP-metabolic *bph* operons among BP/PCB strains

Bph gene clusters from several bacterial strains have recently been cloned and sequenced. This allowed us to compare the gene organization of *bph* operons and DNA and protein sequence homologies at nucleotide as well as the amino acid level of each component. The sequence data showed that *P. aeruginosa* JI104 possessed *bphA* genes (coding for BP dioxygenase) nearly identical with *bphA1A2A3A4* genes of KF707 (A. Kitayama pers. comm.). Another *bph* gene cluster from *Pseudomonas* sp. KKS102 appeared to have shuffled the ferredoxin reductase gene (corresponding *bphA4* in KF707) and *bphX* region, although other *bph* genes were organized similarly with those of KF707 (Y. Nagata pers. comm.). To our surprise, the sequence of the *bph* gene cluster of BP dioxygenase from *Pseudomonas* sp. LB400 (Erickson & Mondello 1992) was very similar to those of *bphA1A2A3A4* (Taira et al. 1992) despite the fact that the BP dioxygenase (LB400) showed oxidative activity for highly chlorinated PCBs (up to and including hexachlorobiphenyls) through 3,4-dioxygenation, while the KF707 enzyme showed narrow oxidative ability for PCBs through 2,3-dioxygenation as described previously.

The similarity among BP dioxygenase components is presented in Table 2. Nineteen two amino acids (one amino acid is missing in the KF707 enzyme) were different between the large subunits of the terminal dioxygenases (458 amino acids in KF707 and 459 amino acids in LB400), and only one amino acid was different between the small subunits. Ferredoxins of the two strains were identical as well as ferredoxin reductases (Erickson and Mondello 1993). An unknown orf (*orf1* in LB400 corresponding to *orf3* in KF707) was present in both *bph* operons. Only one amino acid difference was observed between these two (Taira et al. 1992; Erickson & Mondello 1992). Recently, the *bphB, bphC* and *bphD* genes of LB400 were sequenced (Hofer et al. 1993). One amino acid differed between the BphBs and BphCs. The *bphX* region (ca. 3.5 kb) in KF707 was also present in the LB400 *bph* operon.

Comparison of BP metabolic *bph* operon and toluene metabolic *tod* operon

P. putida F1 degrades toluene through cis-toluene dihydrodiol to 3-methylcatechol. The chemistry and biochemistry of the dihydrodiol pathway has been studied extensively by Gibson and his associates (1990). It is interesting to note that the similarity of the genetic organizations and nucleotide sequences of the individual genes between the BP-metabolic *bph* operon and the toluene metabolic *tod* operon is high. The organization of the KF707 *bph* operon and the F1 *tod* operon is presented in Fig. 7 (Furukawa et al. 1993). Comparison of the KF707 *bph* genes and their products with the corresponding *tod* genes and their products is summarized in Table 3. In spite of similarities between the *bph* and *tod* operons, some discrepancy was noticed. The third open reading frame (*orf3*) of the *bphA* region was missing its counterpart in the toluene dioxygenase gene cluster. *bphD* (a hydrolase gene) was located downstream of the *bphX* region in KF707, but *todF* (a hydrolase gene) was located upstream of *todC1*. The amino acid sequences of the large and small subunits of the terminal dioxygenase, ferredoxin, ferredoxin reductase, dehydrogenase and ring-meta-cleavage-2,3-dioxygenase showed around 60% identities between the two metabolic systems, but the similarity of the hydrolases (BphD and TodF) is as low as 35%. Fur-

Table 2. Comparison of amino acid sequences of biphenyl dioxygenase components of *P. pseudoalcaligenes* KF707 and *Pseudomonas* sp. LB400.

Component	KF707		LB400		Similarity of aa sequences	
	Product	aa size	Product	aa size	number of aa difference	%
Large subunit	BphA1	458	BphA	459	19(1)	95.6
Small subunit	BphA2	213	BphE	213[a]	1	99.5
Unknown	ORF3	139	ORF1	139	1	99.3
Ferredoxin	BphA3	109	BphF	109	0	100
Reductase	BphA4	408	BphG	408	0	100

[a] In the original paper (Erickson & Mondello 1992) the size of BphE was described to be 188 amino acids, however, our analytical data indicated that the size of BphE was the same as that of BphA2 (25 kDa). Thus, for comparison, we have used position 2861 as the starting point of the LB400 *bphE* gene. aa, amino acid(s).

Table 3. Comparison of the gene components and their products of biphenyl degradation in *Pseudomonas pseudoalcaligenes* KF707 and toluene degradation in *Pseudomonas putida* F1.

Enzymes	*Pseudomonas pseudoalcaligenes* KF707			*Pseudomonas putida* F1			Homology (%)	
	genes	nucleotides (G+C%)	amino acid residues	genes	nucleotides (G+C%)	amino acid residues	nucleo-tide	amino acid
Terminal dioxygenase (ISP large subunit)	*bphA1*	62.2	458	*todC1*	60.4	450	68	65
Terminal dioxygenase (ISP small subunit)	*bphA2*	58.2	188	*todC2*	58.7	187	64	60
Ferredoxin	*bphA3*	56.3	109	*todB*	57.0	107	62	60
Ferredoxin reductase	*bphA4*	69.0	408	*todA*	63.7	410	59	53
Dehydrogenase	*bphB*	61.1	277	*todD*	60.4	275	62	60
2,3-Dioxygenase	*bphC*	60.4	298	*todE*	58.3	291	61	55

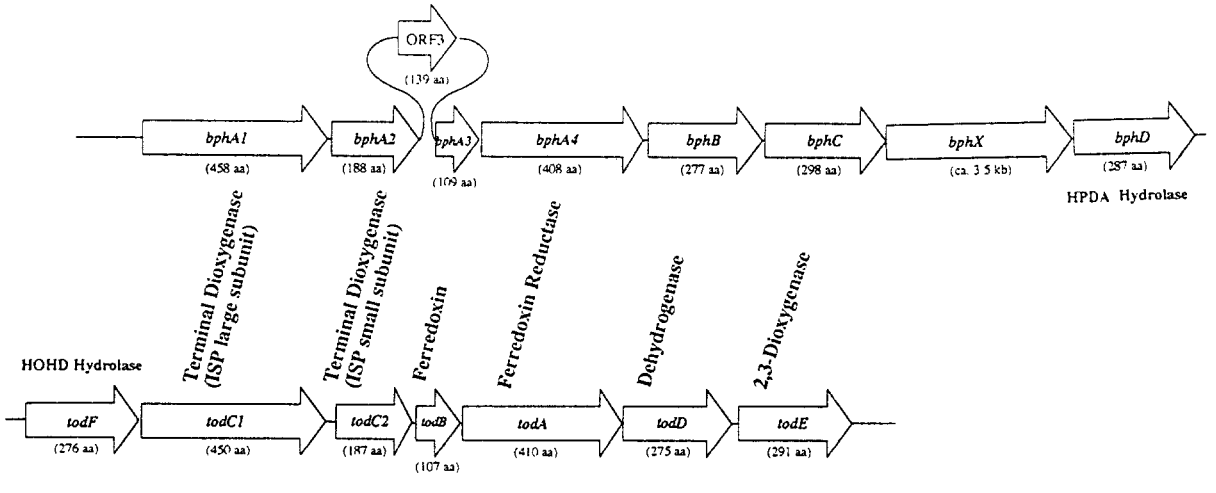

Fig. 7. Comparison of *bph* operon in *P. pseudoalcaligenes* KF707 and *tod* operon in *P. putida* F1. (from Furukawa et al. 1993, with permission.)

thermore, BphD is a homotetramer, whereas TodF is a homodimer.

We constructed a variety of hybrid gene clusters between *bph* and *tod* genes to analyze the function of the hybrid aromatic ring dioxygenase (Furukawa et al. 1993; Hirose et al. 1994). The *E. coli* cells expressed the hybrid gene clusters (*todC1::bphA2A3A4, todC1C2::bphA3A4*) and (*bphA1::todC2::bphA3A4*) and showed novel dioxygenation activities for benzene/toluene, biphenyl and their derivatives. The results show that: (i) the two subunits of terminal dioxygenase are critically involved in the subunit specificity for BP, benzene and their derivatives, and; (ii) the electron transport proteins, ferredoxin and ferredoxin reductase are exchangable with one another between BP dioxygenase and toluene dioxygenase complexes (Furukawa et al. 1993; Hirose et al. 1994).

Conclusions

BP utilizing bacteria seem to be distributed widely in the environment. They are mostly Gram negative soil bacteria, and include many *Pseudomonas* strains, but some Gram positive strains have also been isolated. Such BP-utilizing strains cometabolize many PCB congeners through the 2,3-dioxygenase pathway. Alternatively some bacterial strains degrade PCBs through the 3,4-dioxygenase pathway. Aromatic ring dioxygenases are considered to have evolved from a common ancestor, as evidenced with toluene dioxygenase, benzene dioxygenase, naphthalene dioxygenase and BP dioxygenase (Harayama & Kok 1992). Bph enzymes show relaxed substrate specificities for various BP derivatives and BP-related compounds and some Bph enzymes also attack benzene and its derivatives. Some BP/PCB degraders possess similar, if not identical, *bph* operons, whereas other *bph* genes show different degrees of homology. The presence of nearly identical *bph* operons flanked by unrelated DNA segments in different strains suggests that there is a certain transposition mechanism of *bph* genes among soil bacteria. In fact, a catabolic transposon, Tn*4371*, carrying BP degradation genes has recently been identified in *Alcaligenes eutrophus* A4 (Springael et al. 1993). The variation of homology of *bph* genes suggests that *bph* genes have accumulated mutations through a long historical period. It could be postulated that many degraders of aromatics might be involved in the final degradation of plant lignin, which is massively distributed in the environment and which consists of many polymeryzed aromatic moieties. BP/PCB degraders could be one such group of soil microorganisms as well as other organisms that degrade other aromatic compounds.

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