Evolution of PCB(polychlorinated biphenyl)-degradative pathways and pathway engineering for effective biodegradation and bioremediation of PCB

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Polychlorinated biphenyls (PCBs) have been used widely for various industrial purposes since they were first introduced in 1929. Due to their low chemical reactivity, heat stability, nonflammability, and high electrical resistance, they have been used for more than half century as dielectric fluids in capacitors and transformers, hydraulic fluids, solvent extenders, and flame retardants[5]. It has been known that several hundred million pounds were released into environment, where they have become persistent by adsorbing to organics in soil and sediments because of its hydrophobicity, resulting in accumulating in a variety of biota including human being. PCBs are strictly regulated because of its potential toxicity (e.g., mutagenicity, endocrine disruption, etc.). Factors affecting microbial degradation of PCBs in the environment are: the physical and chemical nature of PCB components (degree of chlorination, position of substituted chlorines, and water solubility), occurrence of degrading organisms and their degrading ability, and environmental factors (photolysis, pH, temperature, oxygen, etc.). PCBs are generally recalcitrant but decontamination of PCBs in the environment may be possible through combustion, photolysis and microbial degradation. While PCBs, primarily accumulated in sediments, can be shielded from photolysis and incomplete combustion can generate polychlorinated dibenzofurans (PCDFs) that are more toxic than PCBs, the microbial degradation holds the greatest promise for inexpensive decontamination of polluted sites with PCBs[20].

By the way, bioremediation of PCBs in the environment through the microbial degradation can be accomplished in both anaerobic and aerobic conditions. Reductive dechlorination of PCBs in anaerobic conditions can significantly reduce the degree of chlorination and hence their toxicity. Moreover, this process makes the compounds more vulnerable to aerobic biodegradation in which a ring cleavage reaction occurs and subsequently generates chlorobenzoate and chlorinated aliphatics that are also biodegradable under an aerobic condition. For the effective bioremediation of PCBs, two aspects of biodegradability of the microorganisms should be taken into account: the organisms possessing degradative enzymes of broad substrate specificity because of the presence of congeners in PCBs and their increased degradation rate. The development of these aspects can be met by genetic modification to improve enzyme properties (or entire metabolic pathways) and pathway induction by natural substrates available in the environment.

In this article the potential utilities of genetic modification of and pathway induction by the aerobic PCB degraders will be reviewed that will facilitate biodegradation and bioremediation of PCBs in the environment.

Evolution of PCB-degradative pathway

Ecology of biphenyl degraders and the degradative genes

An aerobic mineralization of PCBs is performed through the participation of two groups of bacteria, that is, those that utilize biphenyl and cometabolize PCB congeners to chlorobenzoates and those that utilize chlorobenzoates. This may indicate the existence of catabolic gene pool for the PCB degradation in a consortium but not in a single organism[17]. In addition Adriaens[3] argued that there was a third uncharacterized group of microorganisms involved in the mineralization of PCBs, namely those that dehalogenate the 5-carbon aliphatic acids.

Enrichment of PCB degraders were successfully employed to isolate a variety of degraders. Here biphenyl was used as a sole carbon source because it is the backbone of PCBs

(consisting of congeners) and the PCBs themselves may not be used as a carbon source for the degrader's growth due to their strong recalcitrancy. A rapid screening procedure utilizing biphenyl as a carbon source allowed Bedard group[6] to isolate natural aerobic bacteria able to degrade PCBs in almost every contaminated soil they tested. The use of defined PCB mixture combined with the screening technique permitted the study of substrate specificity of degradative enzyme of each isolate[1, 6]. The results indicate that those natural isolates can degrade PCBs, are quite common in the environment, and that the organisms include many different types: most of the organisms (two thirds of the total isolates) were identified as *Pseudomonas* sp. However, more sophisticated analyses of the genus have elucidated that most of these organisms are members of the β subclass of the class Proteobacteria and few are true *Pseudomonas* strains[26]. The PCB degrading enzymes were also found in Gram-positive organisms such as *Rhodococcus* sp. which are belong to soil actinomycetes. The habitats of the actinomycetes were the PCB polluted-soil (*Rhodococcus globerulus* P6; [4]), wood-feeding termite gut (*Rhodococcus erythropolis* TA421; [23]) in which biphenyl moieties can be generated through breakdown of lignin, and a chlorinated heaxane -contaminated soil (*Rhodococcus* sp. RHA1; [24]). The strain RHA1 could also degrade the intermediate chlorobenzoates.

A microcosm enrichment approach was recently employed to isolate organisms which are representative of long-term biphenyl-adapted microbial communities[31], Here it was hypothesized that different biphenyl-degrading microbial communities would develop within each microcosm of physico-chemical, biological conditions and pollution history. However, the microorganisms isolated from the microorganism after 6 months of enrichment showed little taxonomic diversity, reflected by the presence of Rhodococcus opacus as a dominant organism. Here it was assumed that other biphenyl-degraders must have been outcompeted by the Gram-positive which would not have been isolated through the standard enrichment procedure.

Evolution of *bph* genes

The biochemical steps involved in the aerobic degradation of PCBs have been well characterized. The degradation usually involves initial addition of O_2 at 2,3-position by biphenyl dioxygenase enzyme, with subsequent dehydrogenation to the caltech followed by ring cleavage through the action of 2,3-dihydroxybiphenyl dioxygenase. The Fig. 1[13] illustrates how

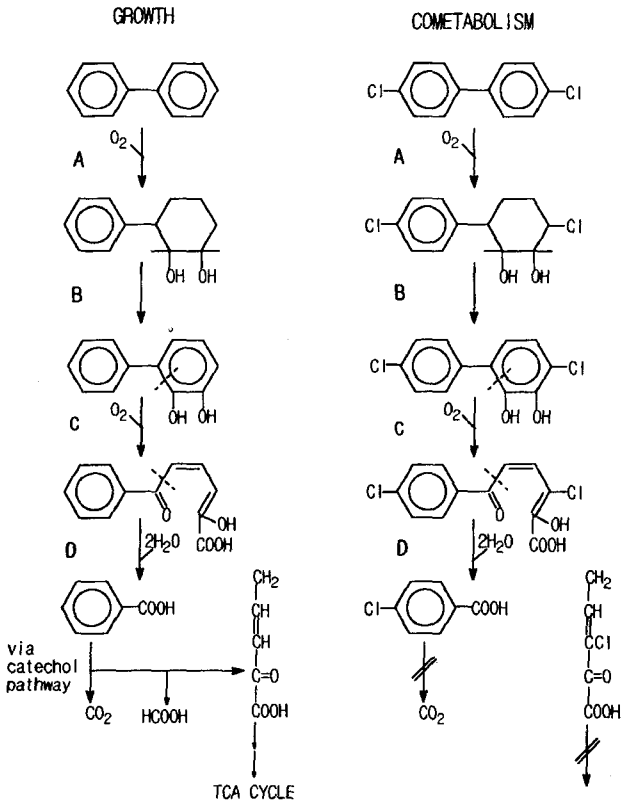

Fig. 1. Bacterial metabolism of biphenyl and cometabolism of 4, 4'-dichlorobiphenyl (Focht, 1993). The bph operon consists of *bphA, bphB, bphC,* and *bphD,* each of which encodes the enzyme for the degradation steps A, B, C, and D respectively. The enzymes in sequence are: biphenyl 2,3-dioxygenase (A); dihydrodiol dehydrogenase (B); 2,3-hydroxybiphenyl 1,2-dioxygenase (C); 2-hydroxy 6-oxo-6-phenylhexa-2,4-dienoic acid hydrase (D).

cometabolism of 4,4'-dichlorobiphenyl occurs concomitantly when cells are grown on biphenyl as an inducer the degradation pathway. Although PCBs typically attacks their 2,3-position, 3, 4-dioxygenase attack is also confirmed in both *Alcaligenes eutrophus* H850 and *Pseudomonas* sp. LB400[27]. In addition, the congener specificity clearly indicates two distinct classes of dioxygenases. A dioxygenase present in *Rhodococcus globerulus* P6 (previously designated *Acinetobacter* sp. strain P6, *Arthrobacter* sp. strain M5, and *Corynebacterium* sp. strain MB1) preferentially attacks the congeners of *para*-substitution while *Alcaligenes eutrophus* H850 and *Pseudomonas* sp. LB400 prefers 2,5-substitution patterns[30]. The 16S rRNA sequence analysis of PCB-degraders has also shown[32] that there are two groups of bacteria: one group able to degrade a broad range of PCBs but not the di-*para*-chlorine-substituted congeners including strain H850 and the other strongly able to degrade the di-para-chlorine-substituted PCBs including *Rho-*

Table 1. Grouping of PCB and mono-benzene ring degraders based on comparison of amino acid sequences of different *meta*-cleavage dioxygenases (modified from refs. 4, 21, 29). Combined lines indicate more close relationships in view of dendrogram based on the amino acid sequences

Group	*meta*-Cleavage dioxygenases coded by bphC family	Strain
I	XYLETOL; Catechol 2,3-dioxygenase	*Pseudomonas putida* mt-2
	BPHE; Catechol 2,3-dioxygenase	*Pseudomonas* sp. IC
	NAHH; Catechol 2,3-dioxygenase	*Pseudomonas putida* PpG1064
	DMPB; Catechol 2,3-dioxygenase	*Pseudomonas putida* CF600
	CDOKF711; Catechol 2,3-dioxygenase	*Acaligenes* sp. KF711
	TBUE; Catechol 2,3-dioxygenase	*Burkholderia pickettii*
	C23OII; Catechol 2,3-dioxygenase	*Pseudomonas putida* MT15
II	BPHC; 2,3-hydroxybiphenyl 1,2-dioxygenase	*Pseudomonas cepacia* LB400
	BPHC; 2,3-hydroxybiphenyl 1,2-dioxygenase	*P. pseudoalcaligenes* KF707
	BPHC; 2,3-hydroxybiphenyl 1,2-dioxygenase	*Pseudomonas putida* OU83
	BPHC; 2,3-hydroxybiphenyl 1,2-dioxygenase	*P. pseudoalcaligenes* KF715
	BPHC; 2,3-hydroxybiphenyl 1,2-dioxygenase	*Pseudomonas* sp. KKS102
	BPHC; 2,3-hydroxybiphenyl 1,2-dioxygenase	*Rhodococcus* sp. RHA1
	BPHC; 2,3-hydroxybiphenyl 1,2-dioxygenase	*Rhodococcus globerulus* P6
	BPHC; 2,3-hydroxybiphenyl 1,2-dioxygenase	*Rhodococcus erythropolis* TA421
	TODE; 3-methylcatechol 2,3-dioxygenase	*Pseudomonas putida* F1
	BPHC; 2,3-hydroxybiphenyl 1,2-dioxygenase	*Sphingomonas yanoikuyae* B1
	BPHC; 2,3-hydroxybiphenyl 1,2-dioxygenase	*Pseudomonas paucimobilis* Q1
	NAHC;1,2-dihydroxynaphthalene dioxygenase	*Pseudomonas putida* PpG7
III	CDO; catechol 2,3-dioxygenase	*Rhodococcus rhodochrous* CTM
IV	PHEB; catechol 2,3-dioxygenase	*Bacillus stearothermophilus*

dococcus globerulus P6 and other species of *Rhodococcus*. Various bacteria isolated from tropical soils were possessing multiple dioxygenases and most of them belonged to the beta subgroup of *Proteobacteria*[28].

The substrate specificity of PCB-degraders can determine kinds of the congeners they metabolize. This is probably due to differences in the genes encoding their PCB-degradative enzymes. Khan *et al.*[21] recently reported the phylogenetic tree of the various *meta*-cleavage dioxygenases which are essentially divided into two major groups based on the bphC gene family encoding the enzymes (Table 1).

The group I consists of bicyclic-ring substrate *meta*-cleavage dioxygenase genes while group II consists of monocyclic-ring substrate *meta*-cleavage dioxygenase genes. The two Gram-positive strains *Rhodococcus rhodochrous* CTM and *Bacillus stearothermophilus* are remotely related to the above two groups. As shown in the Table 1, it is interesting to see that biphenyl degradation pathway and the responsible enzymes/genes of *P. pseudoalcaligenes* KF707 are quite similar to those of the toluene degrader *Pseudomonas putida* F1[29]. The gene products of *bphABC* genes of *Rhodococcus* sp. RHA1 have also a amino sequence homology with *tod* enzyme of the strain F1[24]. The presence of multiple *bph* operons in *Rhodococcus* sp. RHA1(*bphA1A2A3A4*), *Rhodococcus erythropolis* TA421(*bphC1C2C3C4C5C6C7*), and *Rhodococcus globerulus* P6(*bphC1C2C3*) may indicate the genetic diversity of these Gram-positive organisms. Most of these operons are carried on plasmids which can mobilize the degradative gene horizontally and play an important role in the evolution of the catabolic pathway[4, 22, 24].

Genetic engineering and recombination of PCB-degradative pathways

The protein and pathway engineering will be important tools applied to enhance degradation and destruction of xenobiotics in the environment. The microbial degradation system for the xenobiotics are not readily available in the environment. so that modification for the increased activity of degradative enzymes and a generation of novel or hybrid degradation pathway will be necessary here.

Through the protein and pathway engineering, we can alter key enzymes or enzyme systems in a cell to increase degradation rates, broaden substrate specificity, sustain metabolism at

low concentrations, and improve biocatalyst stability during the biodegradation[9].

Improvement of enzyme properties

Improvement for the desirable properties of degradative enzyme may be obtained by broadening their substrate specificity and increasing their degradation rates.

The effective degradation of PCB congeners requires an enzyme system of broader substrate range because the narrow specificity of the enzyme system usually prevent a metabolism of certain congeners. By complementing the genes encoding the various components of the two dioxygenases, Furukawa *et al.*[15] constructed hybrid enzyme systems with broader substrate specificity (Fig. 2). Here introduction into *P. pseudoalcaligenes* KF707 of the genes *todC1* and *todC2*, encoding the terminal toluene dioxygenase components (Fig. 2C), resulted in a recombinant organism that could utilize it and cometabolize trichloroethylene. Similarly, when the large subunit of the terminal iron sulfur protein in the biphenyl dioxygenase was replaced with the similar gene from toluene dioxygenase (Fig. 2D), the new hybrid enzyme can oxidize both toluene and biphenyl. This indicates that it is possible to change the substrate specificity of the degradative enzymes by substituting components from related enzymes. As another similar example utilizing site-directed mutagenesis techniques, the GE researchers[10] have demonstrated that the introduction of amino acids present in the terminal component of biphenyl dioxygenase from KF707 into LB400 counter part could increase the activity of the latter enzyme against para-substituted substrates.

Improvement of the entire pathways

The degradation of PCBs inherently generates chlorobenzoates which cannot be usually degraded by biphenyl utilizers. To circumvent these problem, Focht group has developed new strains which are able to degrade mono- or dichlorobiphenyl and metabolize the resulting chlorobenzoate intermediates and grow on the PCBs[2, 25]. These novel strains were generated through the genetic exchange between two distinct parental strains under a amalgamated culture mating.

Without resorting to the designed mating, it was also possible to generate recombinant PCB-degrader by inoculation of a benzoate degrader into soil contaminated with PCBs[14]. Here it was hypothesized that the bph genes were transferred to the chlorobenzoate-degrading inoculant from indigenous biphenyl degraders. A genetic manipulation of rhizosphere-adapted

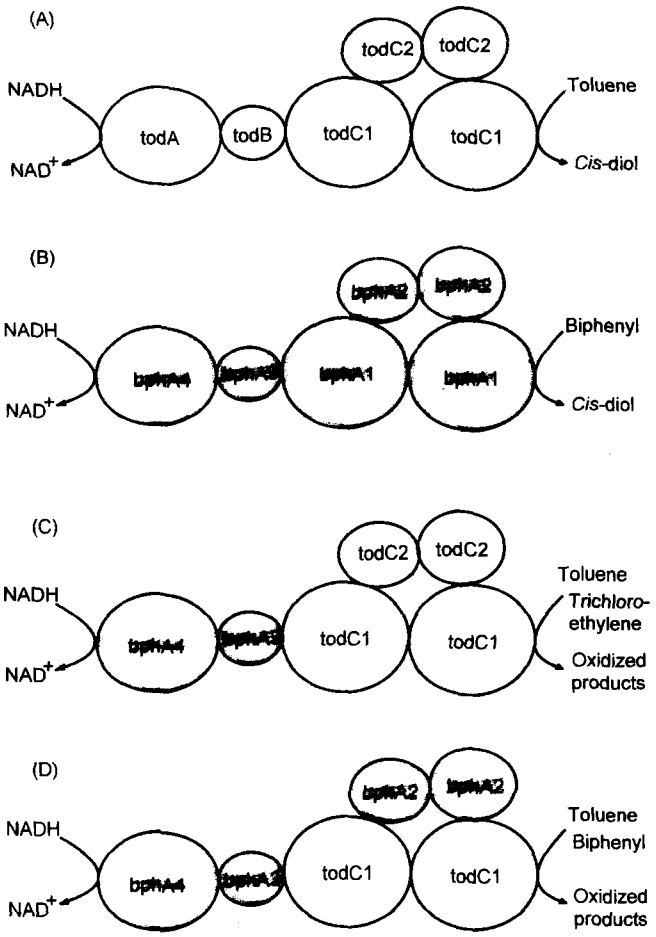

Fig. 2. The components of hybrid toluene/biphenyl dioxygenase (from ref. 9). (A) Wild type toluene dioxygenase; (B) Wild type biphenyl dioxygenase; (C) Hybrid enzyme system containing toluene dioxygenase terminal component (encoded by *todC1* and *todC2*) with the remaining components part of biphenyl dioxygenase; (D) Hybrid enzyme constructed by integrating the large subunit (encoded by *todC1*) from toluene dioxygenase and small subunit (encoded by *bphA2*) from biphenyl dioxygenase. Note the broadened substrate specificity of the hybrid enzymes in (C) and (D).

bacterium was attempted so that it could carry *bph* genes and expanding metabolic functions for the bioremediation of the pollutant (e.g., PCBs) [7].

Pathway induction by natural substrates for biodegradation and bioremediation

Biphenyl as a substrate: its utility and limitation

Biphenyl has usually been used to isolate and grow bacteria that partially degrade PCBs and to enhance biodegradation in soil[12] and sediments. Biphenyl, however, is not a normal constituent of soil and the hypothesis is plausible that biphenyl

is a good inducer for PCB-degradative pathway. By the way, biphenyl may not be a good candidate substrate for the pathway induction because of its health effects, cost and lower solubility. The easy isolation of biphenyl-degraders from natural environments allowed the hypothesis that the biphenyl/PCB pathway are clearly designed to attack natural substrate having structural similarity to biphenyl.

Plant terpenes as natural substrates in PCB biodegradation

Recently it was assumed that certain plant-origin compounds or root exudates could serve as natural substrates for induction of bph genes. These include flavonoids[11], and lignin[19], Donnelly *et al.*[8] showed that some plant flavonoids were as effective as biphenyl in inducing PCB-degrading enzyme, ience pathway. Although carvone, a chemical component of spearmint, was not utilized as a sole carbon source, it was quite effective in inducing PCB-degradative pathway by *Arthrobacter* sp. strain B1B[16]. Another report[17] has shown that various plant residues could significantly enhance the PCB biodegradation by indigenous PCB degraders in soil. Here several Gram-positive organisms were isolated and turned out to grow on terpenes including limonene and abietic acid and cometabolize PCBs (Aroclor1242).

The use of plant terpenes as an inducer for PCB degradative pathway allows clear advantages over biphenyl: good bioavailability, compatibility in natural environments, and their ubiquity in the environment.

Closing remarks

The future tasks ahead for the effective biodegradation and bioremediation of PCBs in the environment will be summarized as follows:

- To increase substrate specificity range and degradation rate of the PCB-degradative enzymes through genetic modification (e.g. site-directed mutagenesis).
- To recruit efficient degradative pathways through designed or natural genetic exchange mechanism to improve the rate and stability of biocatalyst during the biodegradation.
- To select best natural substrates (replacing biphenyl) for the efficient induction of PCB-degradative pathway and utilize them for in situ or ex situ remediation of PCBs.

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