Prospects for using combined engineered bacterial enzymes and plant systems to rhizoremediate PCBs

Michel Sylvestre

Institut National de la Recherche Scientifique, INRS-Institut Armand-Frappier, Laval, Quebec Canada H7V1B7

Correspondence to: Michel Sylvestre: Michel.Sylvestre@iaf.inrs.ca; Tel. 1-450-687-5010 (8829); Fax: 1-450-686-5309

Runing title: Engineered bacteria and plants to rhizoremediate PCBs

Summary

The fate of polychlorinated biphenyls (PCBs) in soil is driven by a combination of interacting biological processes. Several investigations have brought evidence that the rhizosphere provides a remarkable ecological niche to enhance the PCB degradation process by rhizobacteria. The bacterial oxidative enzymes involved in PCB degradation have been investigated extensively and novel engineered enzymes exhibiting enhanced catalytic activities toward more persistent PCBs have been described. Furthermore, recent studies suggest that approaches involving processes based on plant-microbe associations are very promising to remediate PCB-contaminated sites. In this review emphasis will be placed on the current state of knowledge regarding the strategies that are proposed to engineer the enzymes of the PCB-degrading bacterial oxidative pathway and to design PCB-degrading plant-microbe systems to remediate PCB-contaminated soil.

Key words: Polychlorinated biphenyls, PCBs, phytoremediation, rhizoremediation, genetic engineering, directed evolution, biphenyl dioxygenase, plant secondary metabolites, flavonoids, transgenic plants.

Introduction

Although polychlorinated biphenyls (PCBs) are now out of production worldwide, they still persist in the environment. Because of the high cost of landfill or incineration, biological degradation of PCBs has received a lot of attention over the years. Bacteria can co-metabolize PCBs oxidatively through the biphenyl catabolic pathway (Fig. 1). This pathway has been thoroughly investigated (Pieper and Seeger, 2008; Sylvestre and Toussaint, 2011). However, bioremediation strategies based solely on the use of naturally occurring PCB-degrading soil bacteria are not viable for three major reasons: 1- There are several bottlenecks along the biphenyl catabolic pathway that need to be overcome to expand their PCB substrate range; 2- full expression of the biphenyl pathway enzymes requires the presence of biphenyl or of some other fortuitous inducers; 3- PCBs are strongly hydrophobic, poorly bioavailable and both PCBs and bacteria are unevenly distributed in soil. Natural selection being a very slow process, several directed evolution approaches have been proposed and applied successfully to overcome many of the bottlenecks preventing efficient degradation of PCBs through the biphenyl catabolic (Furukawa, 2006; Pieper and Seeger, 2008; Sylvestre, 2004). On the other hand, the poor bioavailability and the requirement for an inducer may be overcome through processes based on the interactions between plants and their associated rhizobacteria (Mackova *et al.*, 2007; Singer *et al.*, 2003; Van Aken *et al.*, 2010; Villacieros *et al.*, 2005). Plant may help remove contaminants from the subsurface and transfer them to the rhizosphere zone where the bacterial density is higher. They also release exudates containing nutrients that may serve as growth substrates for their associated rhizospheric and endophytic bacteria. In return these bacteria produce plant growth stimulators or they suppress pathogens through competition and antibiotics production (Doty, 2008; Lugtenberg and Kamilova, 2009). These exudates also contain plant secondary metabolites (PSMs) that may act as signal chemicals to promote or induce the bacterial enzymes involved in PCB degradation (Singer, 2006).

In recent years most of the bottlenecks of the biphenyl catabolic pathway have been identified and we have acquired better insights about the plant signal chemicals acting as effectors of the biphenyl catabolic pathway of soil bacteria. This information will help design strategies to engineer bacterial enzymes and plants for achieving successful plant-microbe remediation processes. In this review we will summarize some of the recent advancements with regards to the bacterial enzymes involved in PCB degradation and how they can be engineered to broaden their PCB substrate range. We will also summarize recent progresses

about the role that plants and engineered plants may play to achieve successful plant-microbe PCB remediation processes. We will focus principally on the rhizoremediation process. Recent reviews covering the role that endophyte bacteria may play in PCB remediation are available (Doty, 2008; McGuinness and Dowling, 2009) and this issue will not be discussed here.

Engineering bacterial enzymes to degrade PCBs

In the rhizophere zone surrounding plants roots, PCBs are co-metabolized by the biphenyl catabolic pathway of aerobic soil bacteria. Four enzymatic steps are required to transform biphenyl/chlorobiphenyls into corresponding benzoate/chlorobenzoates (Fig. 1). Chlorobenzoates are then degraded through other pathways (Sylvestre and Toussaint, 2011). The first step of the upper biphenyl/chlorobiphenyls pathway is catalyzed by the biphenyl 2,3-dioxygenase (BPDO). The enzyme introduces one molecule of oxygen onto vicinal *ortho-meta* carbons of the aromatic ring. The metabolite, *cis*-2,3-dihydro-2,3-dihydroxybiphenyl is re-aromatized by the 2,3-dihydro-2,3-dihydroxybiphenyl-2,3-dehydrogenase (2,3-DDHBD). The catechol metabolite is then cleaved by the 2,3-dihydroxybiphenyl-1,2-dioxygenase (2,3-DHBD) to generate 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA), which is hydrolyzed by the HOPDA hydrolase to produce benzoic and pentanoic acids.

BPDO catalyzes the first step of the pathway and therefore, its PCB substrate range will determine the range of congeners the pathway will metabolize. For this reason it has been thoroughly investigated. BPDO is an interesting enzyme. Besides PCBs, this Rieske-type dioxygenase (RO) metabolizes many other biphenyl analogs including 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (DDT) (L'Abbée, 2011) and heterocyclic aromatics such as dibenzofuran (Mohammadi *et al.*, 2011), chlorodibenzoflurans (Kumar *et al.*, 2012) and phenylpropanoids (Chun *et al.*, 2003; Misawa *et al.*, 2002; Pham *et al.*, 2012; Seeger *et al.*, 2003; Seo *et al.*, 2011). Understanding how the BPDO catalytic pocket interacts with its substrates to bind them and orient their reactive carbons toward the protein reactive atoms will help design novel biocatalysts useful in biotechnological processes for the destruction of persistent pollutants or for green production of chemicals. BPDO comprises three components (Fig. 1). The catalytic component, which is a RO protein (BphAE), is a hetero hexamer made up of three α (BphA) and three β subunits (BphE). The other two components are the

ferredoxin (BphF) and the ferredoxin reductase (BphG) which are involved in electron transfer from NADH to BphAE. The catalytic center of the enzyme is located on the C-terminal portion of BphAE α subunit which also carries the major structural determinants for substrate specificity and regiospecificity. In the environment, three phylogenetically distinct clusters of BphAE have evolved (Standfuß-Gabisch *et al.*, 2012; Vézina *et al.*, 2008; Witzig *et al.*, 2006) and each of them seems to have acquired a distinct PCB degrading pattern (Standfuß-Gabisch *et al.*, 2012). The structure of a representative BphAE (also called BphA1A2) from each of these three clusters has now been elucidated. Thus the protein databank (PDB) coordinate file of *Burkholderia xenovorans* LB400 BphAE is available (Kumar *et al.*, 2011) as well as those of *Pandoraea pnomenusa* B356 BphAE (Gomez-Gil *et al.*, 2007; L'Abbée, 2011) and of *Rhodococcus jostii* RHA1 BphA1A2 (Furusawa *et al.*, 2004).

B. xenovorans LB400 BphAE ($BphAE_{LB400}$) has been thoroughly investigated because this organism is considered as one of the best PCB degrader of natural occurrence (Kumar *et al.*, 2011). Using directed-evolution approaches, we evolved $BphAE_{LB400}$ and obtained $BphAE_{p4}$ (Barriault and Sylvestre, 2004) and $BphAE_{RR41}$ (Mohammadi and Sylvestre, 2005), two variants that metabolize a much broader range of substrates than the parent enzyme (Barriault and Sylvestre, 2004; Viger *et al.*, 2012). Structural analyses showed that the Thr335Ala substitution, common to both variants relieves intramolecular constraints on the Val320Gly321Gln322 segment lining the catalytic pocket allowing for significant movement of this segment during substrate binding, thus increasing the space available to accommodate larger substrates (Kumar *et al.*, 2011). In addition, we found that the combined Asn338Gln and Leu409Phe substitutions of $BphAE_{RR41}$ alters a substrate-induced mechanism required to retune the alignment of protein atoms involved in the chemical steps of the reaction (Mohammadi *et al.*, 2011). This mechanism speeds up the electron transfer process during the catalytic reaction. As a result, $BphAE_{RR41}$ was able to catalyze the oxygenation of dibenzofuran (Mohammadi *et al.*, 2011) and chlorodibenzofurans (Kumar *et al.*, 2012) as well as of PCBs (Viger *et al.*, 2012) more efficiently than the parent enzyme. $BphAE_{RR41}$ was able to metabolize 17 of the 18 tested di- to penta-chlorinated PCBs, most of which, including the toxic 3,3',4,4'-tetrachlorobiphenyl are congeners that the parent $BphAE_{LB400}$ metabolized very poorly (Viger *et al.*, 2012). Therefore, these mutant enzymes are regarded as promising tools to be transferred into PCB-degrading rhizobacteria to broaden their PCB substrate range.

The second enzyme of the pathway, the 2,3-DDHBD can oxidize a very wide range of dihydrodiol substrates, including 3,4-dihydro-3,4-dihydroxybiphenyl and metabolites

produced from the dioxygenation of aromatic and heteroaromatic compounds such as naphthalene (Barriault *et al.*, 1999), dibenzofuran (Mohammadi and Sylvestre, 2005) and flavonoids (Misawa *et al.*, 2002; Pham *et al.*, 2012; Toussaint *et al.*, 2012). Recently, crystal structure analysis of the 2,3-DDHBD from *P. pnomenusa* B356 revealed that the substrate binding loop of the enzyme is highly mobile and conformational changes are induced during ligand-binding where the disorganized loop becomes organized, forming a well defined cavity to accommodate a wide range of substrates which explains its versatility (Dhindwal *et al.*, 2011).

The next enzyme, the 2,3-DHBD is less versatile than the 2,3-DDHBD as it is unable to cleave *meta-para* hydroxylated dihydroxybiphenyl metabolites (Eltis *et al.*, 1993). These metabolites are produced during catalytic oxygenation of some PCB congeners such as 2,2',5,5'-tetrachlorobiphenyl that do not offer any free vicinal *ortho-meta* carbons for oxygen attack. Therefore, this enzyme feature is a major drawback for efficient removal of complex PCB mixtures. Fortunately, the homologous enzyme 1,2-dihydroxynaphthalene dioxygenase (Dox G) of *Pseudomonas* sp. C18 was found to catalyze the ring cleavage of these *meta-para*-hydroxylated metabolites more efficiently than the 2,3-DHBD (Barriault *et al.*, 1998). Later, DoxG variants exhibiting higher ability to cleave 3,4-dihydroxybiphenyl metabolites were obtained by directed evolution (Fortin *et al.*, 2005), showing the feasibility of engineering extradiol dioxygenases to expand the range of PCB substrates metabolized by the biphenyl pathway. A second limitation of the 2,3-DHBD is its high sensitive to 3-chlorocatechol which was shown to interfere with PCB metabolism (Sondossi *et al.*, 1992). However the catecholic inhibition of 2,3-DHBD does not restrict PCB degradation in engineered bacteria that can degrade chlorobenzoates efficiently (Saavedra *et al.*, 2010). Altogether, the bottlenecks resulting from 2,3-DHBD limitations can be overcome through genetic engineering.

The last step of the *bph* pathway is catalyzed by a hydrolase. The enzyme has been crystallized from *R. jostii* RHA1 (Nandhagopal *et al.*, 2001) as well as from *B. xenovorans* LB400 (Horsman *et al.*, 2006) and the catalytic mechanism is quite well understood (Horsman *et al.*, 2007; Ruzzini *et al.*, 2012). With respect to PCB degradation, HOPDAs bearing chlorine atoms on the phenyl ring are in general good substrates for the HOPDA hydrolase isolated from strain LB400 (Seah *et al.*, 2000). However 3- and 4-chloro-HOPDA produced from chlorobiphenyls that bear chlorine on both rings, are poor substrates for this enzyme (Seah *et al.*, 2001). The steady state turnover toward these substrates is limited by steric features, resulting in non productive binding of the substituted HOPDA (Bhowmik *et al.*,

2007). However, HOPDA hydrolases obtained from various bacteria respond differently to chloroHOPDAs (Seah *et al.*, 2001). Remarkably, DxnB2, the homologous enzyme of the HOPDA hydrolase from the dibenzofuran catabolic pathway of *Sphingomonas wittichii* RW1 catalyzes the hydrolysis of 3-Cl HOPDA (Seah *et al.*, 2007). Therefore, in a manner similar to 2,3-DHBD, the limitations of the HOPDA hydrolase toward some of the chlorinated HOPDAs may be overcome by engineering bacterial strains expressing several homologs exhibiting various specificities. On the other hand studies conducted several years ago revealed that the aliphatic side chain of chloroHOPDAs may be hydrolyzed non specifically by enzymes that do not belong to the biphenyl catabolic pathway (Massé *et al.*, 1989; Yagi, 1980). Therefore, it is likely that once the phenyl ring is cleaved, other soil bacterial enzymes may also, unspecifically metabolize the resulting chloroHOPDAs.

Together, the recent achievements show promising prospects for the use of engineered microbial enzymes to metabolize many of the most persistent PCBs found in commercial mixtures. Expressing these engineered enzymes in rhizobacteria or in plants may help improve significantly the PCB rhizoremediation process. In the next section we will summarize recent progresses about how we may exploit plants to promote PCB degradation in the rhizosphere.

Exploiting plants to promote PCB degradation by rhizosphere bacteria

Over the last two decades, several phytoremediation technologies have been considered for alleviating contaminated soils from PCBs. Plants can metabolize xenobiotics, including PCBs, through a three-phase process in which soil contaminants are hydroxylated, then conjugated with a plant molecule (*e.g.* sugar) before being sequestrated into a vacuole or in the cell wall (Van Aken *et al.*, 2010). However, it has been demonstrated that the first phase for PCB degradation in plants may result in the formation and accumulation of hydroxylated PCBs, some of which are more toxic than the parent compounds (Rezek *et al.*, 2008). Furthermore, plants do not have the capacity to mineralize PCBs, mainly due to a lack of catabolic enzymes required to do so, which results in their slow and/or partial destruction. In addition, the efficiency of a plant to metabolize PCBs is highly dependent on both the plant species and PCB congeners (Glick, 2010; Sylvestre and Toussaint, 2011).

Alternatively, harnessing the potential of plants and their root-associated rhizobacteria in order to promote PCB degradation has retained much attention in the past decade (Mackova *et al.*, 2009; Mackova *et al.*, 2007). PSMs released in root exudates may act as signal

molecules in the rhizosphere (Shaw *et al.*, 2006; Singer, 2006). Among the PSMs, several flavonoids and terpenes that share chemical similarity with biphenyl were found to act as co-metabolites or pathway inducers to stimulate the bacterial biphenyl degradation pathway (Singer, 2006). Several, investigations made on microbial populations associated with the naturally established vegetation of contaminated sites have provided evidence that plants PMSs released from living roots or via lysis of dead roots may provide growth substrates or inducers for PCB-degrading bacteria, supporting the notion of biostimulation through rhizoremediation (Leigh *et al.*, 2002; Leigh *et al.*, 2006). However, it is clear that in spite of the important role played by PSMs in the rhizoremediation process, studies that directly link quantities and composition of root exudates to biodegradation activities are scarce and needed. More direct evidence that PSMs from root exudates can promote PCB degradation in soil microcosms were obtained by Narasimhan *et al.* (2003) who showed that PCB removal by *Pseudomonas putida* PML2 which is a phenylpropanoid-utilizing and PCB-degrading rhizobacteria was significantly lower in the rhizosphere of an *Arabidopsis thaliana* mutant exuding less flavonoids than in the rhizosphere of the wild-type strain. More recently, Toussaint *et al.* (2012) have used a hydroponic culture system to show *Arabidopsis* root exudates induce the biphenyl catabolic pathway of *Rhodococcus erythropolis* U23A, a PCB-degrading rhizobacterium. Flavanone a major component of these root exudates was identified as being an effector of the biphenyl catabolic pathway of strain U23A. Remarkably, the biphenyl catabolic pathway of strain U23A was strongly induced when this phenylpropanoid was used as co-substrate along with sodium acetate. The level of induction was in the same range as when biphenyl was the co-substrate (Toussaint *et al.*, 2012). Although strain U23A was unable to grow on flavonoids, its biphenyl catabolic enzymes were also shown to metabolize flavanone efficiently to generate 4-oxo-2-chromanecarboxylic acid as ultimate metabolite. The inability of strain U23A to further transform 4-oxo-2-chromanecarboxylic acid explains why this strain is unable to grow on flavanone. On the basis of these results, we have proposed a model for explaining how plants may promote PCB degradation in the rhizosphere (Toussaint *et al.*, 2012). Labile chemicals such as the sugar moiety of the conjugated PSMs might provide a substrate on which to grow, whereas the flavonoids or other phenylpropanoids would then induce the biphenyl pathway of the PCB-degrading rhizobacteria.

In another recent study, Pham *et al.* (2012) showed that in a manner similar to strain U23A, the biphenyl catabolic pathway of *P. pnomenusa* B356 metabolized flavone, flavanone and isoflavone rather efficiently, producing as ultimate metabolite the

corresponding oxo-chromane- or oxo-chromene-carboxylic acids. None of these flavonoids could serve as growth substrates for strain B356, but remarkably, isoflavone acted as a strong inducer for the biphenyl catabolic pathway when it was provided as co-substrate along with sodium acetate (Pham *et al.*, 2012). On the other hand, *B. xenovorans* LB400 did not respond as well to those flavonoids (Pham *et al.*, 2012). Although these three flavonoids were metabolized by the biphenyl catabolic pathway of *B. xenovorans* LB400, the rate of metabolism was much slower than for strain B356. Furthermore, none of these flavonoids were able to induce the biphenyl catabolic pathway of strain LB400. In addition, the kinetic parameters of $BphAE_{LB400}$ and $BphAE_{B356}$ toward these simple flavonoids differed considerably. $BphAE_{LB400}$ metabolized these flavonoids very poorly whereas, the kinetic parameters of $BphAE_{B356}$ toward these flavonoids were in the same range as for biphenyl (Pham *et al.*, 2012). Hence the biphenyl dioxygenase from strain B356 is significantly better fitted to metabolize flavone, isoflavone and flavanone than the biphenyl dioxygenase of the well characterized *B. xenovorans* LB400. Together, these data provide evidence supporting the hypothesis brought forward by Focht (1995) and others (Shaw *et al.*, 2006) whereby the biphenyl catabolic pathways may have evolved in bacteria to serve ecological functions, perhaps related to the metabolism of plant secondary metabolites in soil. More importantly, these observations show clearly that soil bacteria differ considerably from each other in the way they respond and/or metabolize plant flavonoids. This observation is significant as it implies that more rational rhizoremediation approaches will be required since the success of the process will depend on the choice of appropriate bacterial strains responding to the PSMs produced by the plants to which they are associated.

Although the perspective of using rhizoremediation as a tool to remove persistent contaminants from the soil is quite appealing, most of the well characterized PCB-degrading bacteria are not endowed with the genetic background to colonize plants roots and, in addition, they cannot fully degrade complex PCB mixtures. However, it is possible to engineer rhizobacterial strains to express PCB-degrading genes efficiently in the rhizosphere (Villacieros *et al.*, 2005). Therefore the use of transgenic plants and/or bacteria may offer yet another potential approach to remediate PCB-contaminated soils (Macek *et al.*, 2008; Sylvestre *et al.*, 2009). By introducing one or several genes from organisms that can degrade xenobiotic pollutants to candidate plants or bacteria, it may be possible to design an efficient plant-microbe system to metabolize PCBs efficiently in soil.

Since accumulation of toxic metabolites produced by the plant's P-450 systems and release of *trans*-diols may hamper efficient PCB removal by combined plant-rhizobacterial

systems, it has been suggested that transgenic plants producing bacterial PCB-degrading enzymes can overcome these difficulties and be advantageously used for PCB-rhizoremediation processes (Novakova *et al.*, 2009; Sylvestre *et al.*, 2009). Analyses of tobacco plants transiently expressing *B. xenovorans* LB400 genes encoding the BPDO components or transformed with them (Mohammadi *et al.*, 2007) have shown that each component can be produced individually as active protein in plants. Furthermore, active BphAE and BphG were co-purified from *Nicotiana benthamiana* leaves agroinfiltrated with pGreen-*bphA+bphE* + pGreen-*bphG* (Mohammadi *et al.*, 2007). However, the simultaneous expression of all four BPDO genes in transgenic tobacco is hampered by genetic or physiological reasons. More work is therefore required to obtained a transgenic plant expressing together, the four genes required for BPDO activity.

Unlike BPDO, 2,3-DHBD consists of a single homo-octamer component. Therefore, a single gene is required to produce active enzyme. Macek *et al.* (2008) have recently discussed an approach to overcome the inability of plants to cleave dihydroxybiphenyls by cloning 2,3-DHBD into plants. Gene *bphC* from *P. pnomenusa* B-356 was successfully cloned in *Nicotiana tabacum* (Novakova *et al.*, 2009). Interestingly, plants expressing 2,3-DHBD were more resistant to PCBs than non-transgenic ones (Macek *et al.*, 2005; Novakova *et al.*, 2009). This feature might be attributed to the fact that 2,3-DHBD can remove 2,3-dihydroxy-chlorobiphenyls derived from PCBs (Mackova *et al.*, 2007) which are potentially toxic to plants. Although the toxicity of 2,3-dihydroxy-chlorobiphenyls to plants has never been examined directly, Camara *et al.* (2004) have shown they are toxic to bacterial cells, Novakova *et al.* (2009) have shown they are toxic to tobacco plants and Lovecka *et al.* (2004) have shown that monohydroxylated PCB metabolites are toxic to plants depending on number and position of the chlorine atoms. Furthermore, the toxicity of catechol to plants has been clearly demonstrated by Liao *et al.* (2006). These are among the many observations supporting the use of transgenic plants producing 2,3-DHBD for rhizoremediation of PCB-contaminated sites since these plants are likely to be more resistant to the PCB metabolites produced by plants and their associated rhizobacteria than the non-transgenic parents. Therefore, these plants are likely to remain healthier for a more efficient rhizoremediation process.

Conclusion

Together, we may bring the following conclusions about the use of genetically modified enzymes and plants to promote the PCB rhizoremediation process.

1- Several bottlenecks preventing efficient PCB degradation through the biphenyl catabolic pathway have been identified. Among these bottlenecks, the range of substrate used by the biphenyl dioxygenase and by the third and fourth enzymes (2,3-DHBD and HOPDA hydrolase) needs to be expanded. To date, important advancements have been achieved with regard to the engineering of BPDOs and 2,3-DHBD that can overcome the limitations of the wild-type enzymes involved in the co-metabolism of PCBs. Also, recently acquired insights about the mechanisms by which chloroHOPDAs inhibit HOPDA hydrolase and about the substrate specificities of HOPDA hydrolase homologs will help design novel engineered enzymes or bacteria able to overcome the limitations caused by chloroHOPDA inhibitions. In the case of BPDOs, novel engineered enzymes exhibiting a significantly broader substrate range than the wild-type enzyme have been described which are likely to be efficient for remediating persistent commercial PCB mixtures such as Aroclor 1254.

2- There are plenty of evidence that plant secondary metabolites may act as signal chemical to promote PCB degradation by rhizobacteria. However, current literature indicates that we will need to rationalize the rhizoremediation processes by choosing plants that produce the signal chemicals that are appropriate for the bacterial strains involved in the process.

3- Transgenic plants for an efficient rhizoremediation process may serve in several ways: 1- Engineered transgenic lines producing increased amounts of appropriate signal chemicals to induce the bacterial biphenyl catabolic pathway may be an approach to improve PCB degradation. 2- Engineering transgenic plants to produce BPDOs is an interesting approach to initiate PCB degradation by plants which will be pursued by the plant-associated rhizobacteria. 3- Engineering plants to produce 2,3-DHBD is feasible and this seems to be a promising approach to improve the health of the plants involved in the rhizoremediation process.

Still many issues have to be considered for a successful rhizoremediation process, many of which have been discussed in recent reviews (Dzantor, 2007; McGuinness and Dowling, 2009). A major issue that influences significantly the success of the rhizoremediation process is the ability of the implanted bacteria to compete with the autochthonous microflora. More studies will be required to understand how the presence of

pollutants may affect the microbial community structure of the rhizosphere and how this community responds to the introduction of exogenous bacteria. Fortunately, many new molecular techniques have recently been introduced to investigate the soil microbial community structure. For example, stable isotope probing, metagenomic combined to functional genes arrays as well as the ribosomal proteins analysis by MALDI-TOF mass spectrometry have helped provide more insights about the microbial community structure that arises in PCB-contaminated soils (Kurzawova *et al.*, 2012; Leigh *et al.*, 2007; Uhlik *et al.*, 2012). These approaches will certainly be very useful to investigate the microbial community of the rhizosphere in presence and absence of the pollutant and of exogenous bacteria.

Together the recent investigations about the bacterial PCB-degrading enzymes and the possible exploitation of plant-microbe interactions to promote the PCB degradation in soil are very encouraging. These investigations allow us to draw the general conclusion that engineering rhizobacterial strains to enhance their PCB degrading abilities is very well possible. However, we will need to design more rational approaches for the rhizoremediation of PCBs which may include the engineering of plants producing appropriate effectors to induce the biphenyl catabolic pathway of their associated rhizobacteria.

Acknowledgements

I would like to dedicate this review in memory of Martina Mackova of the Prague Institute of Chemical Technology who sadly passed away prematurely this summer. In doing so I would sincerely like to acknowledge the fact that she contributed significantly to developing and promoting the technology based on engineered plant-rhizobacterial systems to remediate PCBs.

References

Barriault, D., Durand, J., Maaroufi, H., Eltis, L.D., and Sylvestre, M. (1998) Degradation of polychlorinated biphenyl metabolites by naphthalene-catabolizing enzymes. *Appl Environ Microbiol* **64:** 4637-4642.

Barriault, D., and Sylvestre, M. (2004) Evolution of the biphenyl dioxygenase BphA from *Burkholderia xenovorans* LB400 by random mutagenesis of multiple sites in region III. *J Biol Chem* **279:** 47480-47488.

Barriault, D., Vedadi, M., Powlowski, J., and Sylvestre, M. (1999) *cis*-2,3-dihydro-2,3-dihydroxybiphenyl dehydrogenase and *cis*-1, 2-dihydro-1,2-dihydroxynaphathalene dehydrogenase catalyze dehydrogenation of the same range of substrates. *Biochem Biophys Res Commun* **260:** 181-187.

Bhowmik, S., Horsman, G.P., Bolin, J.T., and Eltis, L.D. (2007) The molecular basis for inhibition of BphD, a C-C bond hydrolase involved in polychlorinated biphenyls degradation: large 3-substituents prevent tautomerization. *J Biol Chem* **282:** 36377-36385.

Camara, B., Herrera, C., Gonzalez, M., Couve, E., Hofer, B., and Seeger, M. (2004) From PCBs to highly toxic metabolites by the biphenyl pathway. *Environ Microbiol* **6:** 842-850.

Chun, H.K., Ohnishi, Y., Shindo, K., Misawa, N., Furukawa, K., and Horinouchi, S. (2003) Biotransformation of flavone and flavanone by *Streptomyces lividans* cells carrying shuffled biphenyl dioxygenase genes. *J Mol Catal B Enzymatic* **21:** 113-121.

Dhindwal, S., Patil, D.N., Mohammadi, M., Sylvestre, M., Tomar, S., and Kumar, P. (2011) Biochemical studies and ligand bound structures of biphenyl dehydrogenase from *Pandoraea pnomenusa* strain B-356 reveal a basis for broad specificity of the enzyme. *J Biol Chem* **286:** 37011-37022.

Doty, S.L. (2008) Enhancing phytoremediation through the use of transgenics and endophytes. *New Phytol* **179:** 318-333.

Dzantor, E.K. (2007) Phytoremediation: the state of rhizosphere 'engineering' for accelerated rhizodegradation of xenobiotic contaminants. *J Chem Technol Biot* **82:** 228-232.

Eltis, L.D., Hofmann, B., Hecht, H.J., Lunsdorf, H., and Timmis, K.N. (1993) Purification and crystallization of 2,3-dihydroxybiphenyl 1,2-dioxygenase. *J Biol Chem* **268:** 2727-2732.

Focht, D.D. (1995) Strategies for the improvement of aerobic metabolism of polychlorinated-biphenyls. *Curr Opin Biotech* **6:** 341-346.

Fortin, P.D., MacPherson, I., Neau, D.B., Bolin, J.T., and Eltis, L.D. (2005) Directed evolution of a ring-cleaving dioxygenase for polychlorinated biphenyl degradation. *J Biol Chem* **280:** 42307-42314.

Furukawa, K. (2006) Oxygenases and dehalogenases: Molecular approaches to efficient degradation of chlorinated environmental pollutants. *Biosci Biotechnol Biochem* **70:** 2335-2348.

Furusawa, Y., Nagarajan, V., Tanokura, M., Masai, E., Fukuda, M., and Senda, T. (2004) Crystal structure of the terminal oxygenase component of biphenyl dioxygenase derived from *Rhodococcus* sp. strain RHA1. *J Mol Biol* **342:** 1041-1052.

Glick, B.R. (2010) Using soil bacteria to facilitate phytoremediation. *Biotechnol Adv* **28:** 367-374.

Gomez-Gil, L., Kumar, P., Barriault, D., Bolin, J.T., Sylvestre, M., and Eltis, L.D. (2007) Characterization of biphenyl dioxygenase of *Pandoraea pnomenusa* B-356 as a potent polychlorinated biphenyl-degrading enzyme. *J Bacteriol* **189:** 5705–5715.

Horsman, G.P., Bhowmik, S., Seah, S.Y., Kumar, P., Bolin, J.T., and Eltis, L.D. (2007) The tautomeric half-reaction of BphD, a C-C bond hydrolase. Kinetic and structural evidence supporting a key role for histidine 265 of the catalytic triad. *J Biol Chem* **282:** 19894-19904.

Horsman, G.P., Ke, J., Dai, S., Seah, S.Y., Bolin, J.T., and Eltis, L.D. (2006) Kinetic and structural insight into the mechanism of BphD, a C-C bond hydrolase from the biphenyl degradation pathway. *Biochemistry* **45:** 11071-11086.

Kumar, P., Mohammadi, M., Dhindwal, S., Pham, T.T., Bolin, J.T., and Sylvestre, M. (2012) Structural insights into the metabolism of 2-chlorodibenzofuran by an evolved biphenyl dioxygenase. *Biochem Biophys Res Commun* **421:** 757-762.

Kumar, P., Mohammadi, M., Viger, J.F., Barriault, D., Gomez-Gil, L., Eltis, L.D., Bolin, J.T., and Sylvestre, M. (2011) Structural insight into the expanded PCB-degrading abilities of a biphenyl dioxygenase obtained by directed evolution. *J Mol Biol* **405:** 531-547.

Kurzawova, V., Stursa, P., Uhlik, O., Norkova, K., Strohalm, M., Lipov, J., Kochankova, L., and Mackova, M. (2012) Plant-microorganism interactions in bioremediation of polychlorinated biphenyl-contaminated soil. *New Biotechnol* **10.1016/j.nbt.2012.06.004**.

L'Abbée, J.B., Tu, Y. B., Barriault, D., Sylvestre, M. (2011) Insight into the metabolism of 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (DDT) by biphenyl dioxygenases *Arch Biochem Biophys* **516:** 35-44.

Leigh, M.B., Fletcher, J.S., Fu, X.O., and Schmitz, F.J. (2002) Root turnover: An important source of microbial substrates in rhizosphere remediation of recalcitrant contaminants. *Environ Sci Technol* **36:** 1579-1583.

Leigh, M.B., Pellizari, V.H., Uhlik, O., Sutka, R., Rodrigues, J., Ostrom, N.E., Zhou, J., and Tiedje, J.M. (2007) Biphenyl-utilizing bacteria and their functional genes in a pine root zone contaminated with polychlorinated biphenyls (PCBs). *ISME J* **1:** 134-148.

Leigh, M.B., Prouzova, P., Mackova, M., Macek, T., Nagle, D.P., and Fletcher, J.S. (2006) Polychlorinated biphenyl (PCB)-degrading bacteria associated with trees in a PCB-contaminated site. *Appl Environ Microbiol* **72:** 2331-2342.

Liao, Y., Zhou, X., Yu, J., Cao, Y.J., Li, X., and Kuai, B.K. (2006) The key role of chlorocatechol 1,2-dioxygenase in phytoremoval and degradation of catechol by transgenic *Arabidopsis*. *Plant Physiol* **142:** 620-628.

Lovecka, P., Mackova, M., and Demnerova, K. (2004) Metabolic products of PCBs in bacteria and plants - comparison of their toxicity and genotoxicity. In Proceedings ESEB 2004, Verstraete, W.,(ed) London: Taylor and Francis Group, pp. 299-302.

Lugtenberg, B., and Kamilova, F. (2009) Plant-growth-promoting rhizobacteria. *Annu Rev Microbiol* **63:** 541-556.

Macek, T., Kotrba, P., Svatos, A., Novakova, M., Demnerova, K., and Mackova, M. (2008) Novel roles for genetically modified plants in environmental protection. *Trends Biotechnol* **26:** 146-152.

Macek, T., Sura, M., Pavlikova, D., Francova, K., Scouten, W.H., Szekeres, M., Sylvestre, M., and Mackova, M. (2005) Can tobacco have a potentially beneficial effect to our health? *Z Naturforsch [C]* **60:** 292-299.

Mackova, M., Prouzova, P., Stursa, P., Ryslava, E., Uhlik, O., Beranova, K., Rezek, J., Kurzawova, V., Demnerova, K., *et al.* (2009) Phyto/rhizoremediation studies using long-term PCB-contaminated soil. *Environ Sci Pollut Res Int* **16:** 817-829.

Mackova, M., Vrchotova, B., Francova, K., Sylvestre, M., Tomaniova, M., Lovecka, P., Demnerova, K., and Macek, T. (2007) Biotransformation of PCBs by plants and bacteria - consequences of plant-microbe interactions. *Eur J Soil Biol* **43:** 233-241.

Massé, R., Messier, F., Ayotte, C., Lévesque, M.-F., and Sylvestre, M. (1989) A comprehensive gas chromatographic/Mass spectrometric analysis of 4-chlorobiphenyl bacterial degradation products. *Biomed Environ Mass* **18:** 27-47.

McGuinness, M., and Dowling, D. (2009) Plant-associated bacterial degradation of toxic organic compounds in soil. *Int J Env Res Pub He* **6:** 2226-2247.

Misawa, N., Shindo, K., Takahashi, H., Suenaga, H., Iguchi, K., Okazaki, H., Harayama, S., and Furukawa, K. (2002) Hydroxylation of various molecules including heterocyclic aromatics using recombinant *Escherichia coli* cells expressing modified biphenyl dioxygenase genes. *Tetrahedron* **58:** 9605-9612.

Mohammadi, M., Chalavi, V., Novakova-Sura, M., Laliberte, J.F., and Sylvestre, M. (2007) Expression of bacterial biphenyl-chlorobiphenyl dioxygenase genes in tobacco plants. *Biotechnol Bioeng* **97:** 496-505.

Mohammadi, M., and Sylvestre, M. (2005) Resolving the profile of metabolites generated during oxidation of dibenzofuran and chlorodibenzofurans by the biphenyl catabolic pathway enzymes. *Chem Biol* **12:** 835-846.

Mohammadi, M., Viger, J.F., Kumar, P., Barriault, D., Bolin, J.T., and Sylvestre, M. (2011) Retuning Rieske-type oxygenases to expand substrate range. *J Biol Chem* **286:** 27612-27621.

Nandhagopal, N., Yamada, A., Hatta, T., Masai, E., Fukuda, M., Mitsui, Y., and Senda, T. (2001) Crystal structure of 2-hydroxyl-6-oxo-6-phenylhexa-2,4-dienoic acid (HPDA) hydrolase (BphD enzyme) from the *Rhodococcus* sp strain RHA1 of the PCB degradation pathway. *J Mol Biol* **309:** 1139-1151.

Narasimhan, K., Basheer, C., Bajic, V.B., and Swarup, S. (2003) Enhancement of plant-microbe interactions using a rhizosphere metabolomics-driven approach and its application in the removal of polychlorinated biphenyls. *Plant Physiol* **132:** 146-153.

Novakova, M., Mackova, M., Chrastilova, Z., Viktorova, J., Szekeres, M., Demnerova, K., and Macek, T. (2009) Cloning the bacterial *bphC* gene into *Nicotiana tabacum* to improve the efficiency of PCB phytoremediation. *Biotechnol Bioeng* **102:** 29-37.

Pham, T.T.M., Tu, Y., and Sylvestre, M. (2012) Remarkable abilities of *Pandoraea pnomenusa* B356 biphenyl dioxygenase to metabolize simple flavonoids *Appl Environ Microbiol* **78:** 3560-3570.

Pieper, D.H., and Seeger, M. (2008) Bacterial metabolism of polychlorinated biphenyls. *J Mol Microbiol Biotechnol* **15:** 121-138.

Rezek, J., Macek, T., Mackova, M., Triska, J., and Ruzickova, K. (2008) Hydroxy-PCBs, methoxy-PCBs and hydroxy-methoxy-PCBs: Metabolites of polychlorinated biphenyls formed in vitro by tobacco cells. *Environ Sci Technol* **42:** 5746-5751.

Ruzzini, A.C., Ghosh, S., Horsman, G.P., Foster, L.J., Bolin, J.T., and Eltis, L.D. (2012) Identification of an acyl-enzyme intermediate in a meta-cleavage product hydrolase reveals the versatility of the catalytic triad. *J Am ChemSoc* **134:** 4615-4624.

Saavedra, J.M., Acevedo, F., Gonzalez, M., and Seeger, M. (2010) Mineralization of PCBs by the genetically modified strain *Cupriavidus necator* JMS34 and its application for bioremediation of PCBs in soil. *Appl Microbiol Biotechnol* **87:** 1543-1554.

Seah, S.Y., Ke, J., Denis, G., Horsman, G.P., Fortin, P.D., Whiting, C.J., and Eltis, L.D. (2007) Characterization of a C-C bond hydrolase from *Sphingomonas wittichii* RW1 with novel specificities towards polychlorinated biphenyl metabolites. *J Bacteriol* **189:** 4038-4045.

Seah, S.Y., Labbe, G., Nerdinger, S., Johnson, M.R., Snieckus, V., and Eltis, L.D. (2000) Identification of a serine hydrolase as a key determinant in the microbial degradation of polychlorinated biphenyls. *J Biol Chem* **275:** 15701-15708.

Seah, S.Y.K., Labbe, G., Kaschabek, S.R., Reifenrath, F., Reineke, W., and Eltis, L.D. (2001) Comparative specificities of two evolutionarily divergent hydrolases involved in microbial degradation of polychlorinated biphenyls. *J Bacteriol* **183:** 1511-1516.

Seeger, M., Gonzalez, M., Camara, B., Munoz, L., Ponce, E., Mejias, L., Mascayano, C., Vasquez, Y., and Sepulveda-Boza, S. (2003) Biotransformation of natural and synthetic isoflavonoids by two recombinant microbial enzymes. *Appl Environ Microbiol* **69:** 5045-5050.

Seo, J., Kang, S.I., Kim, M., Han, J., and Hur, H.G. (2011) Flavonoids biotransformation by bacterial non-heme dioxygenases, biphenyl and naphthalene dioxygenase. *Appl Microbiol Biotechnol* **91:** 219-228.

Shaw, L.J., Morris, P., and Hooker, J.E. (2006) Perception and modification of plant flavonoid signals by rhizosphere microorganisms. *Environ Microbiol* **8:** 1867-1880.

Singer, A. (2006) The chemical ecology of pollutant biodegradation. Bioremediation and phytoremediation from mechanistic and ecological perspectives. In Phytoremediation

and Rhizoremediation Theoritical Background, Mackova, M., Dowling, D.N., Macek, T,(ed) Dordrecht Springer, pp. 5-19.

Singer, A.C., Smith, D., W.A., J., Hathuc, K., and Crowley, D.E. (2003) Impact of the plant rhizosphere and augmentation on remediation of polychlorinated biphenyl contaminated soil. *Environ Toxicol Chem* **22:** 1998-2004.

Sondossi, M., Sylvestre, M., and Ahmad, D. (1992) Effects of chlorobenzoate transformation on the *Pseudomonas testosteroni* biphenyl and chlorobiphenyl degradation pathway. *Appl Environ Microbiol* **58:** 485-495.

Standfuß-Gabisch, C., Al-Halbouni, D., and Hofer, B. (2012) Characterization of biphenyl dioxygenase sequences and activities encoded by the metagenomes of highly polychlorobiphenyl-contaminated soils. *Appl Environ Microbiol* **78:** 2706-2715.

Sylvestre, M. (2004) Genetically modified organisms to remediate polychlorinated biphenyls. Where do we stand? *Int Biodeterior Biodegradation* **54:** 153-162.

Sylvestre, M., Macek, T., and Mackova, M. (2009) Transgenic plants to improve rhizoremediation of polychlorinated biphenyls (PCBs). *Curr Opin Biotechnol* **20:** 242-247.

Sylvestre, M., and Toussaint, J.P. (2011) Engineering microbial enzymes and plants to promote PCB degradation in soil: Current state of knowledge. In Microbial Bioremediation of Non-metals Current Research, Koukkou, A.I.,(ed) Norfolk, U.K.: Caister Academic Press, pp. 177-196.

Toussaint, J.P., Pham, T.T.M., Barriault, D., and Sylvestre, M. (2012) Plant exudates promote PCB degradation by a rhodococcal rhizobacteria. *Appl Microbiol Biotechnol* **95:** 1589-1603.

Uhlik, O., Wald, J., Strejcek, M., Musilova, L., Ridl, J., Hroudova, M., Vlcek, C., Cardenas, E., Mackova, M., *et al.* (2012) Identification of bacteria utilizing biphenyl, benzoate, and naphthalene in long-term contaminated soil. *PloS one* **7:** e40653.

Van Aken, B., Correa, P.A., and Schnoor, J.L. (2010) Phytoremediation of polychlorinated biphenyls: New trends and promises. *Environ Sci Technol* **44:** 2767-2776.

Vézina, J., Barriault, D., and Sylvestre, M. (2008) Diversity of the C-terminal portion of the biphenyl dioxygenase large subunit. *J Mol Microbiol Biotechnol* **15:** 139-151.

Viger, J.F., Mohammadi, M., Barriault, D., and Sylvestre, M. (2012) Metabolism of chlorobiphenyls by a variant biphenyl dioxygenase exhibiting enhanced activity toward dibenzofuran. *Biochem Biophys Res Commun* **419:** 362-367.

Villacieros, M., Whelan, C., Mackova, M., Molgaard, J., SanchezContreras, M., Lloret, J., deCarcer, D.A., Oruezabal, R.I., Bolanos, L., *et al.* (2005) Polychlorinated biphenyl rhizoremediation by *Pseudomonas fluorescens* F113 derivatives, using a *Sinorhizobium meliloti* nod system to drive *bph* gene expression. *Appl Environ Microbiol* **71:** 2687-2694.

Witzig, R., Junca, H., Hecht, H.J., and Pieper, D.H. (2006) Assessment of toluene/biphenyl dioxygenase gene diversity in benzene-polluted soils: links between benzene biodegradation and genes similar to those encoding isopropylbenzene dioxygenases. *Appl Environ Microbiol* **72:** 3504-3514.

Yagi, O., Sudo, R. (1980) Degradation of polychlorinated biphenyls by microorganisms. *Water Pollution Control* **52:** 1035-1043.

Figure Captions

Fig. 1. Bacterial biphenyl catabolic pathway of *B. xenovorans* LB400. BphAE, BphF and BphG are respectively the oxygenase component, the ferredoxin and the ferredoxin reductase components of the biphenyl-2,3-dioxygenase (BPDO); 2,3-DDHBD is the 2,3-dihydro-2,3-dihydroxybiphenyl dehydrogenase encoded by *bphB* in strain LB400; 2,3-DHBD is the 2,3- dihydroxybiphenyl 1,2-dioxygenase encoded by *bphC* in strain LB400; HOPDA hydrolase is the 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase encoded by *bphD* in strain LB400.