BACTERIAL DEGRADATION AND BIOREMEDIATION OF CHLORINATED HERBICIDES AND BIPHENYLS

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ABSTRACT

Chlorinated herbicides (*e.g. s*-triazines) and polychlorobiphenyls (PCBs) are persistent organic pollutants (POPs) that are widely distributed in the environment. *s*-Triazine herbicides are used in agriculture and forestry in diverse regions of the world. PCBs were produced worldwide for industrial applications, and an important amount of these compounds have been released into the environment. PCBs and *s*-triazines are toxic compounds that could act as endocrine disrupters and cause cancer. Therefore, environmental pollution with *s*-triazines and PCBs is of increasing concern. Bioremediation is an attractive technology for the decontamination of polluted sites. Microorganisms play a main role in the removal of POPs from the environment. Diverse bacteria able to degrade *s*-triazines and PCBs have been characterized. Bacterial degradation of *s*-triazine herbicides involves hydrolytic reactions catalyzed by amidohydrolases encoded by the *atz* genes. Anaerobic and aerobic bacteria are capable of biotransforming PCBs. Higher chlorinated PCBs are subjected to reductive dehalogenation by anaerobic microorganisms. Lower chlorinated biphenyls are oxidized by aerobic bacteria. Genome analyses of PCB-degrading bacteria have increased the knowledge of their metabolic capabilities and their adaptation to stressful conditions. For the removal of *s*-triazines and PCBs from the environment, efficient bioremediation processes have to be established. In this report, bacterial degradation of *s*-triazines and PCBs is described and novel strategies to improve bioremediation of these POPs are discussed.

Keywords: bacterial degradation, *s*-triazines, PCBs, catabolic genes, genome, bioremediation.

INTRODUCTION

Environment preservation is one of the aims of the sustainable development. Environmental pollution has increased in many regions due to industrialization. Chlorinated herbicides (*e.g. s*-triazines) and polychlorobiphenyls are POPs that are widely distributed in the environment. In recent years, *s*-triazine herbicides and PCBs have been detected in aquatic systems in Central and Southern Chile (Cooman *et al.*, 2005; Palma-Fleming *et al.*, 2008).

Pesticides have been used for agriculture and forestry, increasing strongly the productivity. *s*-Triazines are herbicides used worldwide for the control

of weeds in agriculture, forestry and non crop soils. *s*-Triazines are endocrine disrupters and potential human carcinogens (Birnbaum and Fenton, 2003; Hayes *et al.,* 2006). PCBs have been used not only as dielectric fluids in capacitors and transformers, but also as flame retardants, plasticizers and ink solvents. Commercial mixtures typically consist of 40-70 congeners. PCBs have been sold under trade names such as Aroclor (Monsanto, USA, Canada and UK), Clophen (Bayer, Germany), Phenoclor (Prodelec, France and Spain), Sovol and Sovtol (Orgsteklo, Orgsintez, former Soviet Union) and Kanechlor (Kanegafuchi, Japan). More than 1.7 million tons of PCBs were produced worldwide, and an important amount of these compounds have been released into the environment (Seeger and Pieper, 2009). PCB congeners have been reported to cause cancer (Mayes *et al.,* 1998) and serious effects on endocrine, immune, nervous and reproductive systems (Faroon *et al.,* 2001).

Worldwide reduction and elimination of POPs discharge into the environment has been promoted by the Stockholm Convention in 2001. Bioremediation is an attractive technology for the decontamination of polluted sites. Microorganisms play a main role in the removal of POPs from the environment. This report describes bacterial degradation of *s*-triazine herbicides and PCBs and discusses the strategies to optimize degradation and bioremediation of these POPs.

BACTERIAL DEGRADATION OF *s*-TRIAZINE HERBICIDES

In diverse regions of the world, bacteria capable of degrading *s*-triazines have been isolated. *s*-Triazine-degrading bacterial strains belonging to *Pseudomonas*, *Arthrobacter*, *Chelatobacter*, *Agrobacterium, Rhodococcus*, *Stenotrophomonas, Pseudaminobacter* and *Nocardiodes* genera have been characterized (Topp *et al*., 2000; Rousseaux *et al*., 2001; Hernández *et al*., 2008a, 2008b, 2008c). In the 1980s, *Pseudomonas* sp. strains A, D and F and *Klebsiella pneumoniae* strains 90 and 99 able to use *s*-triazines as nitrogen source were isolated from a municipal sewage in Switzerland (Cook and Hütter, 1981; Cook *et al*., 1985). Atrazine-degrading strain *Pseudomonas* sp. strain YAYA6 was isolated by enrichment with atrazine from a mixture of garden soil, compost and coarse sand in Switzerland (Yanze-Kontchou and Gschwind, 1994). *Pseudomonas* sp. strain ADP was isolated from a soil exposed to herbicide spills and was able to mineralize atrazine (Mandelbaum *et al.,* 1995). Several Gram-negative and Gram-positive atrazine-degrading bacteria were isolated from atrazine polluted agricultural soils in USA (Radosevich *et al*., 1995; Struthers *et al*., 1998; Strong *et al*., 2002). From French agricultural soils treated with atrazine, diverse Gram-negative and Gram-positive bacteria able to degrade atrazine were isolated and characterized (Rousseaux *et al.,* 2001). Atrazine-degrading bacterium *Arthrobacter* sp. strain AD1 has been isolated from industrial wastewater in China (Cai *et al*., 2003). Gram-negative strain CDB21 that is able to degrade simazine has been isolated from an agricultural soil in Japan (Iwasaki *et al.,* 2007). Recently, bacterial strains able to use simazine as the sole nitrogen source for growth were isolated from agricultural soils in Central Chile (Hernández *et al*., 2008a, 2008b). These isolates belong to *Pseudomonas*, *Stenotrophomonas* and *Arthrobacter* genera. An efficient *s*-triazine-degrading strain, *Pseudomonas* sp. strain MHP41, was further characterized (Hernández *et*

al., 2008b) and successfully applied for bioremediation (Morgante *et al.,* 2010).

Pseudomonas sp. ADP has been a model bacterium for the study of *s*-triazine degradation. The catabolism of *s*-triazines in bacteria is illustrated in Figure 1. The upper *s*-triazine catabolic pathway converts simazine or atrazine into cyanuric acid. The enzymes of the upper catabolic pathway are encoded by the *atzA, atzB* and *atzC* genes (de Souza *et al*., 1998). Atrazine chlorohydrolase AtzA catalyzes hydrolytic dechlorination of simazine to yield hydroxysimazine. This product is further degraded through deamination by the AtzB hydrolase to *N*-etilammelide and *N*-etilamine. In the last reaction, *N*-etilammelide is deaminated by AtzC hydrolase producing cyanuric acid and a molecule of *N*-etilamine. The initial hydrolase TrzN, which has broader substrate specificity than AtzA, has been reported in Gram-positive strains *Arthrobacter aurescens* TC1, *Nocardioides* sp. C190 and *Nocardioides* sp. SP12 (Topp *et al*., 2000; Piutti *et al*., 2003; Smith *et al*., 2005), and also in Gram-negative bacteria such as *Sinorhizobium* and *Polaromonas* strains (Devers *et al*., 2007). The lower *s*-triazine catabolic pathway mineralizes cyanuric acid (Figure 1) (Martínez *et al*., 2001).

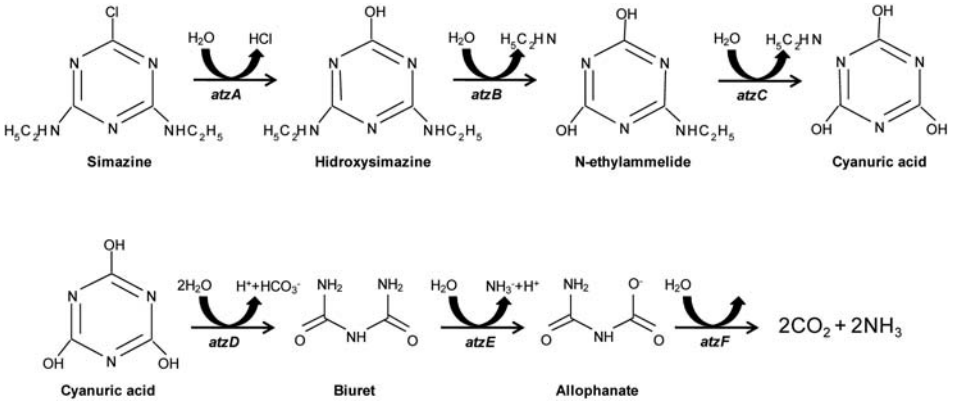

Figure 1. Degradation of *s*-triazines by bacteria. The upper *s*-triazine catabolic pathway converts simazine into cyanuric acid. The lower *s*-triazine catabolic pathway mineralizes cyanuric acid. The catabolic *atz* gene encoding the respective enzyme is indicated at each catabolic step.

The enzymes of the lower catabolic pathway are encoded by the *atzD, atzE* and *atzF* genes (Strong *et al*., 2002). Cyanuric acid is degraded by cyanuric acid amidohydrolase AtzD to biuret, which is further converted by biuret hydrolase AtzE into allophanate. Finally, allophanate is transformed by allophanate hydrolase AtzF into carbon dioxide and NH_3. Cyanuric acid amidohydrolase TrzD, which catalyzes the ring cleavage of cyanuric acid, has also been reported (Karns, 1999; Rousseaux *et al*., 2001; Devers *et al*., 2007). The *trzD* gene has

been detected in *Pseudomonas, Chelatobacter, Aminobacter, Acidovorax, Klebsiella, Alcaligenes* and *Ralstonia* strains (Karns, 1999; Rousseaux *et al*., 2001; Fruchey *et al.,* 2003; Devers *et al.,* 2007).

BACTERIAL DEGRADATION OF PCBs

Anaerobic and aerobic bacteria are able to biotransform PCBs. The reductive dehalogenation of highly and moderately chlorinated PCBs by anaerobic microorganisms generally involves selective dechlorination from *para* and *meta* positions (Figure 2). Nevertheless, *ortho* dechlorination of PCBs has also been described (Figure 2). Bacterial strains belonging to *Dehalococcoides* and *Dehalobium* genera have been associated to halogenation of PCBs (Cutter *et al*., 2001; Wiegel and Wu, 2000; Fennell *et al*., 2004).

Diverse aerobic bacteria capable of oxidizing PCBs have been reported (Pieper and Seeger, 2008). Bacterial strains of *Pseudomonas*, *Burkholderia*, *Comamonas*, *Cupriavidus, Sphingomonas*, *Acidovorax*, *Rhodococcus, Corneybacterium* and *Bacillus* genera have been characterized (Furukawa and Fujihara, 2008; Seeger and Pieper, 2009). *Burkolderia xenovorans* LB400 is able to degrade a broad range of PCBs (Haddock *et al*., 1995; Seeger *et al*., 1995a, 1995b, 1997, 1999, 2001) and is a model bacterium for PCB degradation. *Rhodococcus jostii* RHA1 is another potent PCB-degrading soil bacterium (Seto *et al*., 1995; Warren *et al*., 2004; McLeod *et al*., 2006).

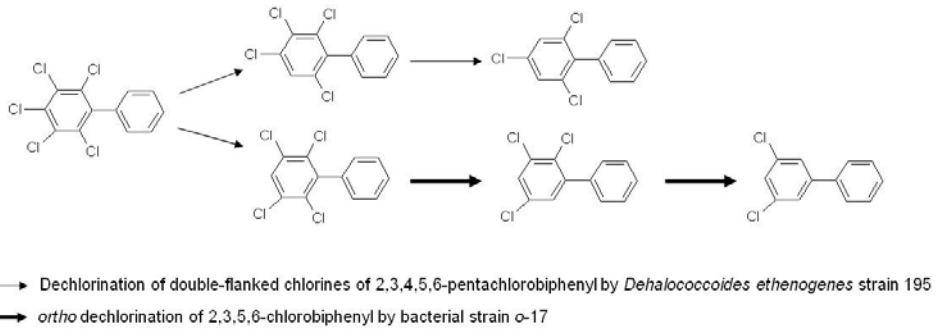

Figure 2. Anaerobic reductive dehalogenation of PCBs by bacteria. The dehalogenation of a pentachlorobiphenyl by anaerobic bacteria is illustrated (Pieper and Seeger, 2008).

The upper biphenyl pathway is involved in the degradation of PCBs into chlorobenzoates (CBAs) and 2-hydroxypenta-2,4-dienoates (Figure 3). Biphenyl degradation is initiated by a multicomponent Rieske non-heme iron oxygenase. Studies on several biphenyl 2,3-dioxygenases (BphAs) have revealed considerable differences in their PCB selectivity as well as their preference of the oxidized ring (McKay *et al*., 1997; Seeger *et al*., 1999, 2001). The dehydrogenation of (chlorinated) cis-2,3-dihydro-2,3-dihydroxybiphenyls (biphenyl 2,3-dihydrodiol) to give (chlorinated) 2,3-dihydroxybiphenyl, is catalyzed by cis-2,3-dihydro-2,3-dihydroxybiphenyl dehydrogenase (BphB).

2,3-Dihydroxybiphenyl 1,2-dioxygenase (BphC) cleaves the aromatic nucleus adjacent to the hydroxyl substituents (*meta*-cleavage) of 2,3-dihydroxy-biphenyl forming 2-hydroxy-6-phenyl-6-oxohexa-2,4-diene-oate (HOPDA). HOPDA hydrolase (BphD) hydrolyzes HOPDA to yield 2-hydroxypenta-2,4-dienoateand benzoate. The lower biphenyl catabolic pathway oxidizes 2-hydroxypenta-2,4-dienoate to pyruvate and acetyl-CoA (Figure 3) (Seeger *et al*., 1997). 2-Hydroxypenta-2,4-dienoate is transformed by 2-hydroxypenta-2,4-dienoate hydratase (BphH), an acylating acetaldehyde dehydrogenase (BphI) and 4-hydroxy-2-oxovalerate aldolase (BphJ) into acetyl-CoA, which enters the Krebs cycle.

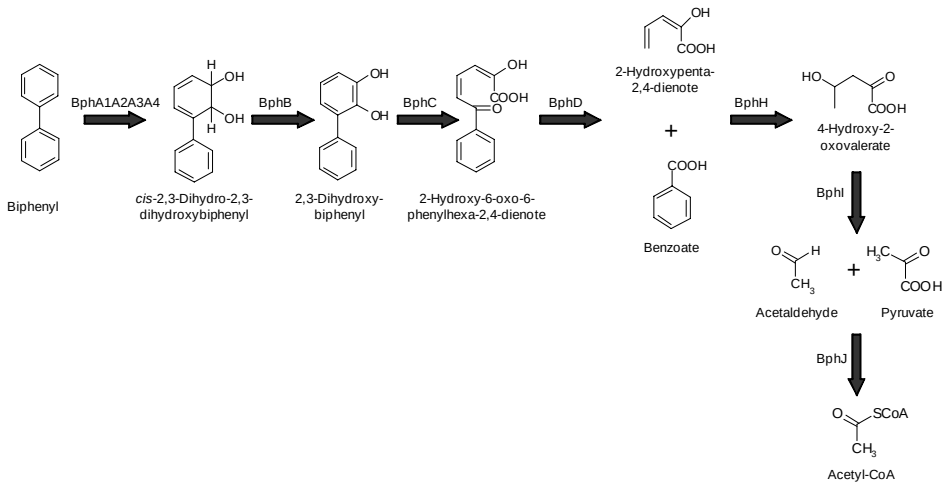

Figure 3. Aerobic bacterial degradation of biphenyl. BphA: Biphenyl 2,3-dioxygenase; BphB: *cis*-2,3-dihydro-2,3-dihydroxybiphenyl dehydrogenase; BphC: 2,3-dihydroxybiphenyl 1,2-dioxygenase; BphD: 2-hydroxy-6-phenyl-6-oxohexa-2,4-dieneoate (HOPDA) hydrolase; BphH: 2-hydroxypenta-2,4-dienoate hydratase; BphI: acylating acetaldehyde dehydrogenase; BphJ: 4-hydroxy-2-oxovalerate aldolase.

GENOME STUDIES

Genome analyses of bacteria have increased the knowledge of their metabolic capabilities and their adaptation to stressful conditions. The genomes of the PCB-degrading strains *B. xenovorans* LB400 (Chain *et al*., 2006) and *R. jostii* RHA1 (McLeod *et al*., 2006) have been sequenced. The general features of the genomes of strains LB400 and RHA1 are shown in Table 1. Strain LB400 has a genome of 9.73 Mbp distributed over two circular chromosomes and a circular megaplasmid. The genome of strain RHA1 has a size of 9.70 Mbp arranged on a linear chromosome and three linear plasmids. The genomes of strains LB400 and RHA1, which inhabit soil and plant rhizosphere niches, have evolved by

Table 1. Comparison of the genome features of the PCB-degrading bacteria *B. xenovorans* LB400 and *R. jostii* RHA1.

Genome feature	*Burkholderia xenovorans* LB400	*Rhodococcus jostii* RHA1
Genome size	9.73 Mbp	9.7 Mbp
Chromosomes	2 circular (4.9 and 3.36 Mbp)	1 linear (7.8 Mbp)
Plasmids	1 circular (1.47 Mbp)	3 linear (1.12, 0.44 and 0.33 Mbp)
Predicted coding sequences	8,958	9,145
Oxygenases	134	203
Central aromatic pathways	11	8
Peripheral aromatic pathways	20	26

different evolutionary mechanisms. More than 20% of the genome of strain LB400 was recently acquired via horizontal gene transfer (HGT). In contrast, strain RHA1 evolved mainly through ancient acquisition and gene duplication and HGT has been less important (McLeod *et al*., 2006). The genomes of *s*-triazine-degrading bacterial strains have not yet been sequenced.

The *bph* genes encoding enzymes of the biphenyl catabolic pathways of strains LB400 and RHA1 are located on mobile genetic elements. In strain LB400, *bph* genes are located in a genomic island on the megaplasmid, indicating that these genes were acquired via HGT (Chain *et al.,* 2006). In strain RHA1, the *bph* genes are encoded on two plasmids (McLeod *et al.,* 2006). *B. xenovorans* LB400 and *R. jostii* RHA1 have an unusually high metabolic versatility for degradation of aromatic compounds. Based on the study of *B. xenovorans* LB400 genome, it was predicted that it possesses at least 20 peripheral pathways and 11 central pathways for degradation of aromatic compounds (Chain *et al.,* 2006). The genes that encode enzymes of central aromatic pathways are usually clustered. In strain RHA1, 8 central and 26 peripheral aromatic pathways were identified (McLeod *et al.,* 2006). The genes encoding 11 of the 26 peripheral aromatic pathways are located on the plasmids. Predicted protein-encoding genes in strains LB400 (8,958) and RHA1 (9,145) are exceptionally rich in oxygenases (Table 1). Bacterial metabolism of aromatic compounds is usually initiated by oxygenases, which catalyze the incorporation of two oxygen atoms into the aromatic ring. BphAs commonly belong to the toluene/biphenyl branch of Rieske non-heme iron dioxygenases (Gibson and Parales, 2000; Pieper and Seeger, 2008). Dioxygenases are critical for the successful metabolism of PCBs and related aromatic compounds.

The knowledge of the bacterial physiology during degradation of POPs and related aromatic compounds is useful to design improved bioremediation processes. Analyses of the genome and proteome-wide defenses against PCBs in strain LB400 showed the induction of the molecular chaperones DnaK and GroEL during (chloro) biphenyl degradation (Agulló *et al.,* 2007) and of DnaK and HtpG by 4-CBA, a dead-end metabolite of the biphenyl upper pathway (Martínez *et al.,* 2007). Therefore, exposure to these compounds constitutes stressful conditions for this bacterium. Interestingly, strain LB400 has the potential to synthesize and degrade polyhydroxyalkanoates (Chain *et al.,* 2006). It has been reported that the degradation of these polymers increases the survival of bacteria under stressful conditions.

BIOREMEDIATION OF *s*-TRIAZINE HERBICIDES

Bioremediation is an attractive technology for herbicide removal in polluted environments. Selected *s*-triazine-degrading strains have been applied for bioremediation processes in the environment. An interesting method for the detection and enumeration of *s*-triazine-degrading microorganisms in soil has been described (Dinamarca *et al*., 2007). Bioaugmentation trials with bacterial strains increased the *s*-triazine degradation in soils (Mandelbaum *et al.,* 1993; Struthers *et al.,* 1998; Newcombe and Crowley, 1999). Inoculation with *Pseudomonas* sp. ADP, *Pseudaminobacter* sp. strains C147, C195 and C223, and *Nocardioides* sp. strain C190 increased atrazine mineralization in soil (Topp, 2001). Bioremediation of *s*-triazines by a bacterial consortium has also been described (Newcombe and Crowley, 1999). Recently, Morgante *et al.* (2010) reported that bioaugmentation with *Pseudomonas* sp. strain MHP41 increased simazine removal and the number of simazine-degrading microorganisms in agricultural soils. Additionally, fluorescent *in situ* hybridization analysis revealed that bioaugmentation increased the relative abundances of the phylogenetic groups *Acidobacteria* and *Planctomycetes* in these soils.

BIOREMEDIATION OF PCBs

Biostimulation of the native microflora and bioaugmentation with selected microorganisms have been applied for the removal of PCBs from contaminated environments. PCB bioremediation, specifically in soil or sediments, is limited by a number of factors including PCB availability, incomplete catabolic breakdown, low expression of catabolic genes, and toxicity of PCBs and their metabolic intermediates (Cámara *et al.*, 2004; Ohtsubo *et al.*, 2004; Agulló *et al.*, 2007; Vasilyeva and Strijakova, 2007; Pieper and Seeger, 2008).

The tendency of POPs to bind tightly to soil is a limiting factor for efficient bioremediation (Fava and Piccolo, 2002; Flores *et al.*, 2009). To increase PCB bioavailability, diverse surfactants have been used. The effect of both chemically synthesized non-ionic surfactants (*e.g.* Tween and Triton X-100) and biosurfactants (*e.g.* lipopeptides, maltose ethers and saponins) have been studied (Fava and Di Gioia, 1998; Golyshin *et al.,* 1999; Singer *et al.*, 2000). Due to their lower toxicity and higher biodegradability, biosurfactants are more suitable than synthetic surfactants for application in bioremediation processes (Makkar and Rockne, 2003). Application of biosurfactants such as cyclodextrins or humic substances (a natural occurring

biosurfactant) increased PCB degradation from 20 to 60% in polluted soils (Fava *et al.,* 1998; 2003; Fava and Piccolo, 2002).

The expression of the catabolic genes of PCB-degrading microorganisms is a key factor for PCB biodegradation in contaminated soils. Biphenyl has been used as inducer of *bph* genes of PCB-degrading strains (Singer *et al.*, 2003; Vasilyeva and Strijakova, 2007). To avoid the use of biphenyl, which could be toxic for bacteria (Cámara *et al.,* 2004), other natural substrates have been applied for the induction of the catabolic *bph* genes (Ohtsubo *et al.*, 2004; Pieper, 2005). Interestingly, plant terpenes increased PCB degradation in soils (Singer *et al.*, 2000). Rhizoremediation has also been used for the removal of PCBs (Vasilyeva and Strijakova, 2007; Macková *et al*., 2009). Some plants enhanced *in situ* PCB degradation (Villacieros *et al*., 2005). The increased activity of PCB-degrading strains in the plant rhizosphere is associated with the presence in the root exudates of inducers (*e.g.* flavonoids) of the genes encoding the PCB-degrading enzymes (Narasimhan *et al*., 2003; Leigh *et al*., 2006; Macková *et al*., 2007).

Native PCB-degrading bacteria are generally not able to degrade CBAs (Blasco *et al.,* 1995; Martínez *et al.*, 2007). Further degradation of CBAs by environmental microorganisms could indirectly result in accumulation of toxic metabolites, such as the antibiotic protoanemonin (Blasco *et al.,* 1995; 1997; Skiba *et al.,* 2002), decreasing the overall PCB degradation. The use of microbial consortium of PCB-degrading and CBA-mineralizing bacteria has increased bioremediation of PCBs (Ohtsubo *et al.*, 2004; Pieper, 2005). Additionally, the development of improved biocatalysts for the remediation of PCB-contaminated environments has been reported. Bacterial strains with enhanced PCB-degrading capabilities have been constructed by metabolic engineering (Rodriguez *et al.*, 2006; Wittich and Wolff, 2007; Saavedra *et al.*, 2010). The genetically modified bacterial strain *C. necator* JMS34, which has been constructed by the combination of the (chloro)biphenyl pathways, the CBA pathway and the chlorocatechol pathway, gained new catabolic abilities for mineralizing PCBs. Noteworthy, the recombinant strain JMS34 mineralized PCBs without accumulation of CBAs (Saavedra *et al.,* 2010). In bioremediation trials, strain JMS34 efficiently degraded PCBs in contaminated soils. The knowledge of catabolic processes and the analysis of enzyme activities involved in the degradation of PCBs are crucial to avoid accumulation of toxic metabolites and to optimize PCB-bioremediation processes.

CONCLUSIONS

Significant advances have been achieved in the last years in the elucidation of the genetic and biochemical basis of bacterial degradation of *s*-triazines and PCBs. The knowledge of the genome and the proteome-wide defenses against POP toxicity of the bacteria, permits to optimize microorganisms and conditions for improved POP degradation and bioremediation. The design of improved bioremediation strategies is needed for an efficient removal of chlorinated herbicides and biphenyls from the environment and a sustainable development.

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