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Formation of hydroxylated and methoxylated polychlorinated biphenyls by *Bacillus subtilis*: New insights into microbial metabolism

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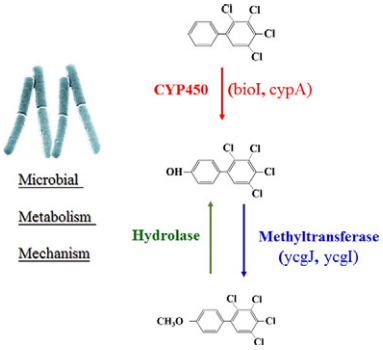
HIGHLIGHTS

- Interconversion between MeO-PCB and OH-PCB was observed in *Bacillus subtilis*.
- PCB can be transformed to OH-PCB by genes bioI and cypA encoding CYP450.
- Genes ycgJ and ycgI encoding methyltransferase induced the formation of MeO-PCB.

GRAPHICAL ABSTRACT



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ABSTRACT

The detoxification and degradation of polychlorinated biphenyls (PCBs) have been studied. However, little information is available about the biological mechanisms involved in the metabolism of hydroxylated polychlorinated biphenyls (OH-PCBs) and methoxylated polychlorinated biphenyls (MeO-PCBs) by specific microorganism. In this study, the simultaneous formation of OH-PCB (major metabolite) and MeO-PCB (minor metabolite) was found in *Bacillus subtilis* after exposure to PCB. Interconversion between MeO-PCB and OH-PCB was also observed and the demethylation ratio of MeO-PCB was higher than the methylation ratio of OH-PCB. The high-throughput RNA-sequencing (RNA-Seq) was conducted to analyze the genes involved in the metabolism processes. The potential metabolism pathways of PCB by *Bacillus subtilis* were proposed. PCB can be transformed to OH-PCB by Cytochrome P450 encoded by the genes bioI and cypA. The genes ycgJ and ycgI that are related with methyltransferase are potentially involved in the subsequent biotransformation from OH-PCB to MeO-PCB. MeO-PCB was prone to be transformed to OH-PCB by a group of hydrolases. This is the first study considering the mechanism involved in the interconversion between OH-PCBs and MeO-PCBs by microorganism. These findings broaden our insights into the biotransformation mechanism of PCBs in the environment.

1. Introduction

The ubiquity of polychlorinated biphenyls (PCBs) in the environment and their various toxicities to biota and human beings has

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attracted significant attention during past decades (Verner et al., 2014; Sun et al., 2016c). In recent years, hydroxylated analogues of PCBs (OH-PCBs) have been found prevalent in various species and habitats, occasionally with similar concentration level to PCBs (Hovander et al., 2002; Park et al., 2009; Routti et al., 2014; Nomiyama et al., 2014). Furthermore, for some endpoints, the toxicity of OH-PCBs was considered to be higher than that of PCBs (Meerts et al., 2002).

An overlooked metabolic pathway of PCBs has been proposed in our previous study (Sun et al., 2016b). After exposure to intact rice plants, OH-PCBs were detected as major metabolites of PCBs, while methoxylated analogues of PCBs (MeO-PCBs) were identified as minor metabolites. Moreover, the interconversion between OH-PCBs and MeO-PCBs in plant was observed. Rice played a key role in these transformation processes, while endophytes were jointly responsible for the hydroxylation of PCBs and demethylation of MeO-PCBs. Microorganisms are crucial to the Earth's biogeochemical cycles and the fate of various pollutants. Recently, we reported the occurrence of MeO-PCBs and OH-PCBs in sewage sludge from wastewater treatment plants in China (Sun et al., 2016d). It was assumed that the abundant and diverse microorganisms in sludge dominated the formation of these metabolites. However, up to now, there were no evidences of the formation of hydroxylated and methoxylated PCBs *in vivo* by specific living microorganism. Therefore, it was necessary to use a model microorganism as a probe to further study the biotransformation mechanism of PCBs.

Several mechanisms for the detoxification and degradation of xenobiotics, including PCBs, have been proposed (Cvancarova et al., 2012; Rezek et al., 2012). Cytochrome P450 (CYP450) and glutathione S-transferases (GSTs) mediated catabolic processes have been implicated in the metabolism mechanism for toxicants (Anzenbacher and Anzenbacherova, 2001; Huang et al., 2013). The studies of molecular biological mechanisms involved in the metabolism of PCBs showed that the transformation from PCBs to hydroxylated PCBs was evident in reactions catalyzed by CYP isoforms such as CYP2B and CYP1A (Matsusue et al., 1996; Zhai et al., 2013). Previous studies have demonstrated that the bph gene is responsible for the biodegradation of PCBs in *Rhodococcus* (Taguchi et al., 2007; Yang et al., 2007). Unfortunately, very little is currently known about the biological mechanisms of the interconversion between OH-PCBs and MeO-PCBs. The genes responsible for the hydroxylation and methoxylation of PCBs in living organisms were not identified. The genome-wide profiling of transcriptome has resulted in identification of many functional genes associated with metabolism in organisms (Zhang et al., 2016). The study on gene expression and regulatory mechanisms for transformation would facilitate our understanding of the fate of PCBs in the environment.

Bacillus subtilis (*B. subtilis*) is a group of multifunctional bacteria which has been extensively applied as the protection against crop root pathogens, and as biosorbents for wastewater treatment (Wang et al., 2010; Cao et al., 2011). As a result, *B. subtilis* is widespread in various environmental media, such as soil, water, and sewage sludge. The *B. subtilis* has been reported to be the potential degrader of various organic pollutants, such as polycyclic aromatic hydrocarbons (PAHs) and phthalate esters (PAEs) (Das and Mukherjee, 2007; Lily et al., 2009; Navacharoen and Vangnai, 2011). In this study, *B. subtilis* was selected as typical microorganism to study the metabolism of PCBs, OH-PCBs and MeO-PCBs. To gain insights into the molecular mechanism, we used the high-throughput RNA-sequencing (RNA-Seq) technology to identify the genes that are differently expressed in *B. subtilis* exposed to target contaminants. We further explored the relationship between PCB, OH-PCBs, and MeO-PCBs, and the metabolism pathway of PCBs was proposed. The findings of this study will help to understand the entire PCBs-responsive molecular events and figure out the mechanisms for regulating microorganism response to PCBs, OH-PCBs, and MeO-PCBs.

2. Experimental section

2.1. Chemicals and culture medium

The commercially available pairs of homologous MeO-PCBs and OH-PCBs are limited. In this study, three stock standards including 2,3,4,5-tetrachlorobiphenyl (CB-61), 4′-hydroxy-2,3,4,5-tetrachlorobiphenyl (4′-OH-CB-61), and 4′-methoxy-2,3,4,5-tetrachlorobiphenyl (4′-MeO-CB-61) were selected as target compounds according to the previous studies (Sun et al., 2016a, 2016b), and prepared in acetone at 100 μg/mL before use. The selection of these chemicals also based on the consideration for the purposes of comparison with their metabolism in plants (Sun et al., 2016a, 2016b). Surrogate standards were CB-101 and 4′-OH-CB-101 for neutral and phenolic chemicals, respectively. All these chemical standards were purchased from AccuStandard (New Haven, CT, USA). Luria Bertani (LB) was made using 10.0 g/L bacteriological tryptone, 5.0 g/L bacteriological yeast, and sodium chloride 10.0 g/L (pH 7.0). Solid media was prepared by adding agar at 15 g/L (1.5%). Prior to use, the medium was sterilized by autoclaving at 121 °C for 15 min. Hexane, dichloromethane (DCM), acetonitrile, methyl *tert*-butyl ether, and acetone used in this study were of HPLC grade or pesticide grade, and were purchased from Fisher Scientific (Pittsburgh, PA, USA). Acid silica gel was prepared by mixing 70 g of activated silica with 30 g of concentrated H_2SO_4. Anhydrous sodium sulfate was activated in advance. Deionized water (18.2 MΩ) from a Milli-Q system was used in all experiments. All the other experimental materials and reagents were of analytical reagent grade or higher purity.

2.2. Microorganism and exposure conditions

The *B. subtilis* NCIB-3610 were used as the model strain. The biotransformation experiments were carried out in batch mode in sterile Erlenmeyer flasks containing 50 mL of LB media at 37 °C under shaking condition (120 rpm). Cell concentrations in samples were analyzed (OD_{600} 1.53–1.62) prior to being added to 1.0 μg individual exposure compound in each reactor. Reactors were placed in the dark and fully wrapped with aluminum foil to prevent photolysis of the chemicals. According the studies using plant and sludge (Sun et al., 2016b, 2016d), as well as our preliminary experiment, the exposure time was set 24 h. Longer exposure time will not significantly promote the metabolism of PCBs, but may cause strong response of strains to stress. Additionally, blanks controls (strains in the absence of exposure compounds) and amicrobic controls (exposure compounds in the absence of strains) were conducted (Fig. SI-1). All the exposure and control treatments were prepared in triplicate.

2.3. Sample preparation and analysis

Chemicals in samples was spiked and repeatedly extracted with a hexane/methyl *tert*-butyl ether mixture (1:1; v/v). The extracts were combined and evaporated to dryness and redissolved in 20 mL of DCM. Acid silica gel was added and violently shaken for 5 min. An anhydrous Na_2SO_4 column was applied to remove the acid silica and an additional DCM (30 mL) was used to elute all the targeted compounds. The elution was collected and concentrated to dryness under a gentle of nitrogen. A total of 200 μL hexane was added to redissolve the target compounds.

Half the extract (100 μL in hexane) was used for the analysis of CB-61 and 4′-MeO-CB-61 by gas chromatograph/mass spectrometer (GC/MS) (7890B/5977B, Agilent, Santa Clara, CA, USA) with an electron ionization (EI) ion source. A DB-5 MS (J&W Scientific, Folsom, CA) capillary column (30 m, 0.25 mm i.d., 0.25 μm film thickness) was applied to separate the target compounds. Helium was used as the carrier gas at a constant flow of 1 mL/min. The oven was initially 90 °C and then increased to 210 °C at a rate of 20 °C/min, then increased to 300 °C at a rate of 6 °C/min. The selected ion monitoring (SIM) mode was applied for quantitative