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Biodegradation of 3,3′,4,4′-tetrachlorobiphenyl by *Sinorhizobium meliloti* NM

Xiaomi Wang [a,b], Ying Teng [a,*], Yongming Luo [a], Richard P. Dick [c]

[a] Key Laboratory of Soil Environment and Pollution Remediation, Institute of Soil Science, Chinese Academy of Sciences, Nanjing 210008, China
[b] University of Chinese Academy of Sciences, Beijing 100049, China
[c] School of Environment and Natural Resources, The Ohio State University, Columbus, OH 43210, USA

HIGHLIGHTS

- This report is the first on the degradation of PCB 77 by *Sinorhizobium meliloti*.
- *S. meliloti* NM could effectively utilize PCB 77 (0.25–5 mg L^{-1}) as a sole substrate.
- *S. meliloti* NM had maximum PCB 77 degradation in the presence of caffeic acid.
- Additional substrates might promote PCB 77-degradation via biofilm formation.
- The expression of benzoate metabolism-related genes was detected.

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ABSTRACT

A rhizobial strain, *Sinorhizobium meliloti* NM, could use 3,3′,4,4′-tetrachloro-biphenyl (PCB 77) as the sole carbon and energy source for growth in mineral salt medium. The degradation efficiency of PCB 77 by strain NM and the bacterial growth increased with a decrease in PCB 77 concentration (5–0.25 mg L^{-1}). The addition of secondary carbon sources, phenolic acids and one surfactant influenced PCB 77 degradation, rhizobial growth and biofilm formation. The highest degradation efficiency was observed in the presence of caffeic acid. Benzoate and chloride ions were detected as the PCB 77 metabolites. The up-regulation of benzoate metabolism-related gene expression was also observed using quantitative reverse transcription-polymerase chain reaction. This report is the first to demonstrate *Sinorhizobium* using coplanar tetrachlorobiphenyl as a sole carbon and energy source, indicating the potential wide benefit to the field of rhizobia-assisted bioremediation.

1. Introduction

Polychlorinated biphenyls (PCBs) are a family of 209 possible congeners differing in the number of chlorine atoms (1–10) attached to their biphenyl rings (Passatore et al., 2014). Their persistence and bioaccumulation in nature are a significant risk for the ecosystem and humans due to their carcinogenicity, mutagenicity and teratogenicity (Wang et al., 2014). The type of toxicity exerted by PCBs relies on their ability to form coplanar structures (Venkatachalam et al., 2008). The dioxin-like 3,3′,4,4′-tetrachlorobi phenyl (PCB 77) is one of the most toxic coplanar congeners and abundant within the food chain due to its wide presence in commercial mixtures (e.g., Aroclor) (Venkatachalam et al., 2008).

A broad range of physical, chemical and biological methods has been applied to detoxify PCBs-contaminated soils (Aken et al., 2010). Bioremediation is a promising technology due to its cost-effectiveness and ecological sustainability (Passatore et al., 2014). Some species of plants (e.g., poplar and *Solanum nigrum*) and microorganisms (e.g., *Burkholderia*, *Dehalococcoides* and white rot fungi) have been shown to biotransform or degrade PCBs (Passatore et al., 2014). As for bioremediation of PCB 77, *Populus deltoids* has been reported to metabolize PCB 77 to hydroxylated PCB 77 (OH-CB77) (Zhai et al., 2010). However, many microorganisms (e.g., *Rhodococcus* sp. RHA1 and *Phanerochaete chrysosporium*) that have strong abilities to degrade a broad range of PCBs exhibit no or low degradation of di-para-chlorine-substituted congeners (e.g., PCB 77) (Sakai et al., 2005; Vyas et al., 1994). Liang et al. (2014) found that bioaugmentation with *Burkholderia xenovorans*

* Corresponding author at: Key Laboratory of Soil Environment and Pollution Remediation, Institute of Soil Science, Chinese Academy of Sciences, East Beijing Road No 71, Nanjing, Jiangsu Province 210008, China. Tel.: +86 25 86881531; fax: +86 25 86881126.
E-mail address: yteng@issas.ac.cn (Y. Teng).

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LB400 was associated with improved degradation of 2,2,5,5-tetrachlorobiphenyl, but not PCB 77, in switchgrass rhizosphere. This finding indicates that the dioxin-like structure of PCB 77 renders it more toxic and resistant to microbial degradation than other tetrachlorinated biphenyls (Liang et al., 2014).

Nitrogen-fixing rhizobia as free-living or symbiotic cells are recently shown to remediate soils contaminated with aromatic hydrocarbons and phenolic compounds (Teng et al., 2015b). Damaj and Ahmad (1996) reported the potential of rhizobia to catabolize mono- and di-chlorobiphenyls. Transgenetic rhizobia that express biphenyl degradation genes have been constructed by Chen et al. (2005). The association between legumes and degradative rhizobia offers benefits for ecosystems by enhancing the fertility of the soil while simultaneously enhancing bioremediation of contaminated soils (Wei et al., 2008). PCBs-degrading *Mesorhizobium* sp. ZY1 and *Rhizobium* sp. have been shown to stimulate the phytoextraction of PCBs by forming synergistic association with legumes (Teng et al., 2015a; Xu et al., 2010). To date, research regarding aerobic microbial degradation of PCBs has mainly focused on the degradation of lower chlorinated congeners of PCB (chloride $\leqslant 3$) (Passatore et al., 2014; Teng et al., 2015b) with very little information about tetrachlorobiphenyl-degrading rhizobia, especially dioxin-like PCB 77.

Microbial remediation is most effective when environmental conditions are optimal to perform degradation reactions. To promote the effectiveness of microbial remediation, co-substrates and inducers (e.g., analogs) have been proposed. For example, the addition of secondary carbon sources (glucose and biphenyl) can enhance PCB degradation by promoting co-metabolism and biomass of *Pseudomonas* spp. (Murínová et al., 2014). Biofilm formation has been proposed to function in facilitating the utilization of dimethylformamide by *Paracoccus* sp. when in the presence of co-substrates (Nisha et al., 2015). Biofilm-mediated biodegradation is considered to be efficient, cost-effective and environmentally friendly due to their high microbial biomass and ability to immobilize pollutants (Pandey et al., 2002). Natural compounds (such as plant terpenes and flavanones) can also increase the degradation rates of PCBs, probably due to the increase of bacterial biomass and biphenyl dioxygenase expression (Dudášová et al., 2012; Toussaint et al., 2012). Phenolic acids (e.g., caffeic acid, vanillic acid, coumalic acid, ferulic acid, and hydroxybenzoic acid) are water-soluble plant secondary metabolites, generally existing in plant roots and rhizosphere (e.g., alfalfa) (Cheynier et al., 2013). However, the influence of phenolic acids on rhizospheric microbe-mediated transformation of PCBs remains largely unknown. Surfactants have also been proven to be effective for increasing bioavailability and uptake of PCBs by plants (Xia et al., 2009). As for rhizobia-mediated remediation, literature related to the effect of supplements on PCB degradation remains sparse.

Sinorhizobium meliloti NM has been previously proven to utilize 2,4,4′-trichlorobiphenyl (PCB 28) as a sole source of carbon and energy (Tu et al., 2011). Therefore, the objectives of the study were to determine: (1) the degradation capability of coplanar tetrachlorobiphenyl (PCB 77) by *S. meliloti* NM, (2) the effect of supplemental substrates (secondary carbon sources, phenolic acids and surfactant) on the removal efficiency of PCB 77 and biofilm formation by *S. meliloti* NM, (3) the metabolic intermediates of PCB 77 biodegradation, and (4) the transcription of degradation genes of rhizobial cells.

2. Methods

2.1. Chemicals

3,3′,4,4′-tetrachlorobiphenyl (PCB 77, 99% purity) was purchased from Accustandard (New Haven, CT. USA). A PCB 77 stock solution I (250 mg L^{-1}) was prepared by dissolving PCB 77 in acetone, and sterilized on 0.22 μm hydrophobic polyvinylidene fluoride (PVDF) membrane filters.

A mixture of bis (trimethylsilyl) trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) (99:1, v/v), obtained from Sigma–Aldrich (St Louis, MO, USA), was used as the derivatization reagent. HPLC grade *n*-hexane and ethyl acetate used for extraction were procured from Tedia (Ohio, USA). Secondary carbon sources (glucose, sucrose, acetate and succinate), surfactant (Tween 80) and other substrates (biphenyl and phenolic acids (caffeic acid, vanillic acid, coumalic acid, ferulic acid and 4-hydroxybenzoic acid)) were purchased from Sigma–Aldrich. The remaining chemical reagents were of analytical grade (Sinopharm Chemical Reagent Co., Ltd, Beijing, China) unless stated otherwise. All enzymes used in DNA manipulations were obtained from TaKaRa Biotechnology Co. Ltd. (Dalian, China).

2.2. Media

The yeast mannitol (YM) medium contained (L^{-1}) 10.0 g mannitol, 0.4 g yeast extract, 0.5 g K_2HPO_4, 0.2 g $MgSO_4{\cdot}7H_2O$, and 0.1 g NaCl, pH 7.2 (Tu et al., 2011). The mineral salts medium (MSM) contained (L^{-1}) 0.5 g NH_4NO_3, 0.5 g KH_2PO_4, 0.2 g $MgSO_4{\cdot}7H_2O$, 5 mg $FeSO_4{\cdot}7H_2O$, and 10 mL of trace elements solution at pH 7.0. The trace elements solution contained the following (L^{-1}): 500 mg EDTA·2Na, 10 mg $ZnSO_4{\cdot}7H_2O$, 5 mg $MnSO_4{\cdot}H_2O$, 30 mg H_3BO_3, 24 mg $CoSO_4{\cdot}7H_2O$, 5 mg $CuSO_4{\cdot}5H_2O$, 5 mg $Na_2MoO_4{\cdot}2H_2O$, and 50 mg $Ca(OH)_2$, pH 7.2. The solid media were prepared by adding 15 g L^{-1} agar into the liquid media. The media were sterilized by autoclaving at 121 °C for 20 min.

2.3. Bacterial strains and culture conditions

The rhizobial strain *S. meliloti* NM (strain ACCC17519) was obtained from the Agricultural Culture Collection of China (Tu et al., 2011). Rhizobial strains were routinely cultivated on YM agar and stored at 4 °C. For long-term storage, the cultures were maintained at −70 °C in 15% (v/v) glycerol with YM broth. *S. meliloti* TC1021 was used as a negative control.

2.4. Growth kinetics and biodegradation of PCB 77

The growth kinetics of *S. meliloti* NM were determined in screw-cap serum bottles over PCB 77 (as the sole substrate), according to Tu et al. (2011) with modifications. Uninoculated control bottles were kept to account for abiotic loss of PCB 77 (Blank control). Cells inactivated by autoclaving at 121 °C for 20 min were used as a negative control (Sterilized Sm NM, Fig. 1). In brief, *S. meliloti* NM was inoculated onto YM agar plates at 28 °C for 1 d, and then individual colonies were sub-cultured into YM broth medium at 28 °C on a rotary shaker at 180 rpm min^{-1} for 48 h (in the exponential phase). The cells were harvested by centrifugation at 5000 × g for 15 min, washed twice with sterile 0.2 mol L^{-1} phosphate buffered saline (PBS), pH 7.0, and re-suspended in the MSM medium to an OD of 0.50 at 600 nm (a density of 1.1×10^9 cell mL^{-1}; colony-forming units, CFU). For different treatments (final PCB 77 concentration at 0.25–15 mg L^{-1}), PCB 77 stock solution I (250 mg L^{-1}) was diluted to varied concentrations (stock solution II) in acetone and 150 μL of stock solution II was transferred into each sterile 20 mL serum bottle over the PCB 77 concentration range. 3 mL of the bacterial suspension were added to each bottle after acetone evaporated and incubated at 28 °C on a rotary shaker at 180 rpm in the dark. To avoid volatilization and photo-degradation of PCB 77, the vials were closed with aluminum foil lined caps. The samples