Chemosphere 113 (2014) 141–145

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Technical Note

Bacterial acquisition of hexachlorobenzene-derived carbon in contaminated soil

Ondrej Uhlik [a,*], Michal Strejcek [a], Jan Vondracek [a], Lucie Musilova [a], Jakub Ridl [b], Petra Lovecka [a], Tomas Macek [a,*]

[a] Institute of Chemical Technology Prague, Department of Biochemistry and Microbiology, Faculty of Food and Biochemical Technology, Technicka 3, 166 28 Prague 6, Czech Republic
[b] Institute of Molecular Genetics, Czech Academy of Sciences, Department of Genomics and Bioinformatics, Videnska 1083, 142 20 Prague 4, Czech Republic

HIGHLIGHTS

- Bacteria in contaminated soil can gain carbon from hexachlorobenzene.
- *Methylobacterium* and *Pseudomonas* acquire HCB-derived carbon.
- Pentachlorophenol is a likely intermediate of HCB biodegradation.

ARTICLE INFO

Article history:
Received 20 February 2014
Received in revised form 22 April 2014
Accepted 23 April 2014
Available online 29 May 2014

Handling Editor: O. Hao

Keywords:
16S rRNA genes
Amplicon pyrosequencing
Stable isotope probing
Bioremediation
Pesticides
Pentachlorophenol 4-monooxygenase

ABSTRACT

Pesticides are a class of xenobiotics intentionally released into the environment. Hexachlorobenzene (HCB) was used as a fungicide from 1945, leaving behind many contaminated sites. Very few studies have examined the biodegradation of HCB or the fate of HCB-derived carbon. Here we report that certain bacterial populations are capable of deriving carbon from HCB in contaminated soil under aerobic conditions. These populations are primarily Proteobacteria, including *Methylobacterium* and *Pseudomonas*, which predominated as detected by stable isotope probing (SIP) and 16S rRNA gene amplicon pyrosequencing. Due to the nature of SIP, which can be used as a functional method solely for assimilatory processes, it is not possible to elucidate whether these populations metabolized directly HCB or intermediates of its metabolism produced by different populations. The possibility exists that HCB is degraded via the formation of pentachlorophenol (PCP), which is further mineralized. With this in mind, we designed primers to amplify PCP 4-monooxygenase-coding sequences based on the available *pcpB* gene sequence from *Methylobacterium radiotolerans* JCM 2831. Based on 16S rRNA gene analysis, organisms closely related to this strain were detected in ^{13}C-labeled DNA. Using the designed primers, we were able to amplify *pcpB* genes in both total community DNA and ^{13}C-DNA. This indicates that HCB might be transformed into PCP before it gets assimilated. In summary, this study is the first report on which bacterial populations benefit from carbon originating in the pesticide HCB in a contaminated soil.

1. Introduction

Hexachlorobenzene (HCB) was used as a fungicide starting in 1945 for crop, onion, and sorghum seeds protection. Although its intentional production was stopped, it can still be released unintentionally during some chemical syntheses (Barber et al., 2005). HCB is classified among persistent organic pollutants (POPs) by the Stockholm Convention on POPs, which aims to eliminate and restrict the production and use of these compounds (http://www.pops.int). In spite of its persistence, some studies have been published reporting bacterial transformation of HCB, e.g. by Prytula and Pavlostathis (1996), Yuan et al. (1999) or Brahushi et al. (2004). Very few studies, however, have investigated the identity of microorganisms associated with HCB metabolism. The HCB-transforming microorganisms identified so far include *Dehalococcoides* sp. CBDB1 (Jayachandran et al., 2003) and other *Dehalococcoides* strains (Taş et al., 2010), which are capable of HCB dehalorespiration, and genetically engineered *Sphingobium chlorophenolicum* ATCC 39723, which is capable of partial HCB degradation (Yan et al., 2006). The first bacterium reported to mineralize HCB is *Nocardioides* sp. PD653 with the proposed

* Corresponding authors. Tel.: +420 220 44 5136 (O. Uhlik). Tel.: +420 220 44 5139 (T. Macek).
E-mail addresses: ondrej.uhlik@vscht.cz (O. Uhlik), tomas.macek@vscht.cz (T. Macek).

http://dx.doi.org/10.1016/j.chemosphere.2014.04.110
0045-6535/

biodegradation pathway (Fig. 1) being initiated by the oxidative dehalogenation of HCB into pentachlorophenol (PCP). Further metabolites include tetrachlorohydroquinone and dichlorohydroquinone (Takagi et al., 2009). Although the strain PD653 was able to mineralize HCB, it was not clear whether this strain used HCB as a source of carbon and/or energy. Aerobic growth on HCB as the sole carbon source was detected in members of the genera *Azospirillum* and *Alcaligenes*, which dominated the HCB-utilizing community isolated from a contaminated site (Liu et al., 2009). Yet information is still missing which will help elucidate if HCB can be metabolized directly in soil under aerobic conditions. The aim of this study was to demonstrate the assimilation of HCB-derived carbon under aerobic conditions and identify the bacteria that acquired carbon from HCB. In order to achieve this aim, stable isotope probing (SIP) was employed, which benefits from the provision of a stable isotope (mostly ^{13}C)-labeled substrate to a community and subsequent tracking of the label in cellular biomarkers, such as nucleic acids (for reviews see Chen and Murrell, 2010; Madsen, 2010; Uhlík et al., 2013). In this study, the microbial community of an aged contaminated soil was provided with ^{13}C-HCB, and populations incorporating ^{13}C were identified by pyrosequencing analysis of 16S rRNA genes amplified from ^{13}C-labeled DNA.

2. Materials and methods

2.1. Soil samples

The contaminated soil was collected from a chemical factory in Neratovice, Czech Republic. Due to industrial production, the site has experienced variable levels of multiple contaminants (exact data are not available). The actual amount of HCB at the time of sampling was 38.1 mg kg^{-1} dry matter. Other contaminants included DDT (9.5 mg kg^{-1}), lindane (261.5 mg kg^{-1}), and heavy metals. The soil samples were used for the isolation of metagenomic DNA, amplification of 16S rRNA genes and their pyrosequencing as well as SIP experiments. DNA isolation was performed with PowerMax Soil DNA Isolation Kit (MoBio Laboratories, USA) according to the manufacturer's instructions.

2.2. SIP and ^{13}C-DNA isolation

SIP microcosms were established in triplicates in sterile 100 mL serum bottles (Sigma–Aldrich, USA). First, 200 μL of either fully ^{13}C-labeled or unlabeled HCB (Alsachim, France and Sigma–Aldrich, USA, respectively) solution in ether (corresponding to the amount 400 μg of HCB) was pipetted onto the bottom of each bottle allowing ether to completely evaporate in the fume-hood. Second, 2 g of homogenized, sieved soil was placed into each of the bottles and moistened with 400 μL of mineral salt solution [in g L^{-1}: 1 $(NH_4)_2SO_4$, 2.7 KH_2PO_4, 10.955 $Na_2HPO_4 \cdot 12H_2O$, 0.03 $Ca(NO_3)_2$, 0.01 $FeSO_4$, 0.2 $MgSO_4$]. The bottles were sealed to maintain stable atmosphere with continuous sublimation of HCB, which was allowed to be gradually trapped by indigenous soil microbes during the incubation at 25 °C in the dark. Harvesting of the microcosms was accomplished after 2 and 4 weeks.

Isolated metagenomic DNA (^{13}C-enriched as well as control unlabeled DNA) was subjected to isopycnic centrifugation performed on Discovery 90 Ultracentrifuge with TFT 80.2 rotor (Sorvall, USA) at conditions of 145 000×*g* for 72 h in 1.5 mL cuvettes. Using Beckman Fraction Recovery System (Beckman Coulter, USA) and Harvard Pump 11 Plus Single Syringe (Harvard Apparatus, USA), each gradient was fractionated into 50 μL fractions (with flow rate 200 μL min^{-1}). Buoyant densities (BDs) of fractions were determined using Digital Handheld Refractometer (Reichert Analytical Instruments, NY, USA) based on refractive indices of fractionated gradient constructed with no DNA. After DNA from each fraction was retrieved by isopropanol precipitation with glycogen, 16S rRNA genes were quantified by real-time qPCR in relation to a standard curve constructed with *Pseudomonas stutzeri* JM300 genomic DNA (Ginard et al., 1997). Real-time PCR conditions were as follows: each 12 μL reaction contained iQ SYBR Green Supermix (Bio-Rad, USA), 0.4 μM primers 786f: 5′-GATTAGATACCCTGGTAG-3′ and 939r: 5′-CTTGTGCGGGCCCCCGTCAATTC-3′ (Baker et al., 2003), and 2 μL of template DNA. Cycling program was set to 95 °C for 5 min, 30 cycles of 95 °C for 40 s, 55 °C for 40 s, 72 °C for 60 s, and a final extension at 72 °C for 10 min. Fractions with ^{13}C-enriched DNA were compiled and further analyzed as ^{13}C-DNA (or 'heavy' DNA).

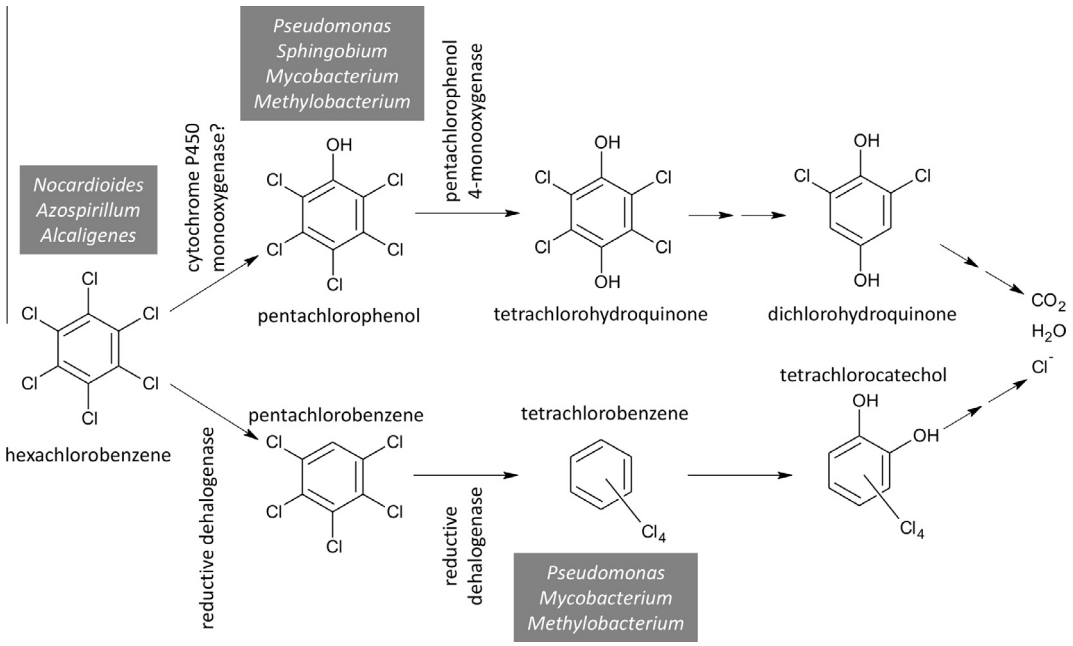

Fig. 1. Summary of possible degradation pathways for HCB together with bacterial genera which acquire carbon from certain intermediates based on sequenced genomes and data available in the literature. Enzymes for the key reaction steps are displayed.