ELSEVIER

Soil Biology & Biochemistry 37 (2005) 1401–1405

Soil Biology & Biochemistry

www.elsevier.com/locate/soilbio

Short communication

Effects of nutrients and surfactants on pyrene mineralization and *Mycobacterium* spp. populations in contaminated soil

Pui-Yi Cheung*, Brian K. Kinkle

Department of Biological Sciences, University of Cincinnati, Cincinnati, OH 45221-0006, USA

Received 3 July 2004; received in revised form 15 December 2004; accepted 16 December 2004

Abstract

The impact of nutrient (KNO_3 and KH_2PO_4) or surfactant (Triton X-100 and Tergitol NP-10) amendments on the mineralization of ^{14}C-pyrene and the indigenous *Mycobacterium* spp. community structure in a petroleum-contaminated soil were determined. All soil amendments enhanced pyrene mineralization with nutrients being slightly more effective than surfactants. 16S rRNA genes were PCR-amplified using *Mycobacterium* spp.-specific primers, separated by temperature gradient gel electrophoresis (TGGE), and prominent bands sequenced to compare the mycobacteria communities. The soil sample with the highest level of mineralization had no detectable changes in the community structure. Disappearance of a specific phylotype occurred in soils with lower mineralization rates. Phylogenetic analysis of sequenced TGGE bands indicated existence of novel strains.

Keywords: *Mycobacterium* spp.; Microbial communities; PA; Biodegradation; 16S rRNA gene; PCR-TGGE; Phylogenetic analysis

Polycyclic aromatic hydrocarbons (PAHs) are produced and released in large quantities into the environment during fossil fuel combustion, waste incineration, or as by-products of coal gasification and petroleum refining processes (Cerniglia, 1992). Several high-molecular-weight PAHs, for example, pyrene and benzo[a]pyrene, are known genotoxins (Cerniglia, 1992). A large number of described PAH-degrading bacteria are fast-growing species of *Mycobacterium* (Grosser et al., 1991; Jimenez and Bartha, 1996; Kelley et al., 1993). However, little is known about the diversity and population structure of indigenous fast-growing mycobacteria, present in PAH-contaminated and pristine soils. Biodegradation in natural environments could be limited by a number of ecological factors. The effect of inorganic nutrients on PAH degradation is unclear, with studies showing both increases and decreases in degradation, as well as no significant effects (Bauer and Capone, 1985; Carmichael and Pfaender, 1997; MacNaughton et al., 1999; Manilal and Alexander, 1991). Many PAHs and other hydrophobic pollutants of soil are often not bioavailable due to sorption by soil organic matter (Alexander, 1994). Previous studies have shown that surfactants, e.g. Triton X-100 and Tergitol NP-10, could increase the dissolution/availability of PAHs and, hence, the mineralization rates (Jimenez and Bartha, 1996; Grimberg et al., 1996; Tsomides et al., 1995; Volkering et al., 1995). However, inhibitory effects by surfactants on degradation have also been reported (Jimenez and Bartha, 1996; Volkering et al., 1998).

There have been only a few attempts to evaluate the impact of nutrient or surfactant additions on microbial community structure during bioremediation efforts (Colores et al., 2000; MacNaughton et al., 1999; Röling et al., 2002, 2004). In the current study, the effect of nutrient- and surfactant-amendments on the mineralization of pyrene by indigenous microorganisms in a historically petroleum-contaminated soil was measured. Concurrently, PCR amplification of 16S rRNA genes and temperature gradient gel electrophoresis (TGGE) were used to examine the population structure of the potential pyrene-degrading

* Corresponding author. Present address: Room 704, Public Health Laboratory, Public Health Laboratory Centre, 382 Nam Cheong Street, Shek Kip Mei, Kowloon, Hong Kong. Tel.: +852 2319 8318; fax: +852 2776 1446.

E-mail address: cheungpi@yahoo.com (P.-Y. Cheung).

0038-0717/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.soilbio.2004.12.008

strains of *Mycobacterium* spp. in the same soil, in an attempt to relate population shifts and the pyrene-degrading ability of the indigenous microorganisms to nutrient- or surfactant-amendments.

A historically contaminated silty clay soil (pH 7.6, 5.65% organic carbon, CEC of 20.3 meq 100 g^{-1}, and a PAH content of 86 mg g^{-1}) was sampled from a petroleum refinery site, screened to less than 10 mm, homogenized, and stored at 4 °C until use (Cheung and Kinkle, 2001). Soil pH was determined in a 1:1 ratio of soil and water. Organic carbon content was determined by the Walkley–Black method using $FeSO_4$ for titration (Nelson and Sommers, 1982). CEC was measured as according to Rhoades (1982). PAH content was determined by the Arthur D. Little Laboratory (Cambridge, Mass) using a gas chromatograph/mass spectrometer (GC/MS) operated in the selective ion monitoring (SIM) mode, after extraction from the soil by sonication with methylene chloride (Environmental Protection Agency (EPA) Method 8270B). To measure the effect of nutrient and surfactant additions on PAH mineralization, a series of 50 ml serum bottles each containing 5 g soil sample were divided into nine groups and received the following treatments. Sterile double-distilled water (ddH_2O) was added to Group 1 (control) soil to 80% of its water holding capacity (WHC). Sterile nitrate or phosphate solutions were added to Groups 2, 3, 4, or 5 soils to bring them up to 80% of WHC. Groups 2 and 3 were amended with 0.15 and 1.5 mg g^{-1} soil of KNO_3, respectively. Groups 4 and 5 were amended with 0.15 and 1.5 mg g^{-1} soil of KH_2PO_4, respectively. Two different non-ionic surfactants, Triton X-100 and Tergitol NP-10, which have been previously shown to increase PAH mineralization were selected for this study (Jimenez and Bartha, 1996; Grimberg et al., 1996; Tsomides et al., 1995; Volkering et al., 1995). Solutions of the surfactants were prepared in sterilized ddH_2O at concentrations both above and below their aqueous critical micelle concentration (CMC): Triton X-100 (Sigma, St Louis, MO), CMC = 1.3×10^{-2}% (v/v) (Sigma data); Tergitol NP-10 (Sigma, St Louis, MO), CMC = 4×10^{-3}% (v/v) (Sigma data). Aqueous solutions of surfactants were added to soils to bring them up to 80% WHC. Groups 6 and 7 were amended with solutions of Triton X-100 at 8×10^{-3}% and 3×10^{-2}% (volume of surfactant/volume of ddH_2O) (equivalent to 0.013 and 0.048 mg g^{-1} soil), respectively. Groups 8 and 9 were amended with solutions of Tergitol NP-10 at 3×10^{-3}% and 1.5×10^{-2}% (volume of surfactant/volume of ddH_2O) (equivalent to 0.0048 and 0.024 mg g^{-1} soil), respectively. All soils were subsequently amended with 0.01 μCi ^{14}C-pyrene (34 pmol g^{-1} soil; specific activity 58.7 mCi $mmol^{-1}$; pyrene-4, 5, 9, 10-^{14}C, Sigma, St Louis, MO) dissolved in 100 μl of acetone (carrier). Soils were then vortexed briefly and incubated in the dark at 24 °C. Pyrene degradation during incubation were measured by serum bottle radiorespirometry (Knaebel and Vestal, 1988) using a Tri-Carb Liquid Scintillation Analyzer (Packard Instrument Company, Downers Grove, IL).

To evaluate the effects of nutrients and surfactants on *Mycobacterium* spp. community structure, polypropylene jars (250 ml) containing 50 g soil sample were treated with nutrient and surfactant solutions as described above and then amended with unlabelled pyrene (Sigma, St Louis, MO) (34 pmol g^{-1} soil dissolved in 0.35 ml of acetone). Soils were then well mixed and the jars were screw-capped, sealed with parafilm, and incubated in the dark at 24 °C. At different time points during incubation, 10 g representative samples of each soil were taken after thorough mixing. Samples were extracted for total bacterial DNA (Zhou et al., 1996). Crude DNA extracts obtained were purified using Sepharose 4B spin columns (Jackson et al., 1997). Purified DNA was used as a template for PCR amplification of 16S rRNA gene with the forward primer MycF and reverse primer MycR. Amplified fragments (approximately 760 bp) were used for TGGE analysis with the DCode™ System (Bio-Rad, Hercules, CA) (Cheung and Kinkle, 2001). Prominent TGGE bands were excised and PCR-amplified with MycF and eubacterial reverse primer 519R (5′GA/TATTACCGCGGCG/TGCTG), which corresponds to *E. coli* positions 536–519. This amplified region enclosed the mycobacteria 16S rRNA gene hypervariable V2 region (*E. coli* positions 175–238) and the signature sequence for the fast-growing group of mycobacteria (*E. coli* positions 451–482). PCR products (approximately 400 bp) were purified before automated sequencing with Applied Biosystems Model 377 or 373 (Perkin–Elmer, Norwalk, CT). For phylogenetic analysis of the TGGE band sequences, maximum-likelihood and maximum-parsimony analyses as implemented by BioNumerics 4.0 (Applied Maths BVBA, Austin, Texas, US) were conducted using *Nocardia farcinica* (GenBank accession number X91041) as

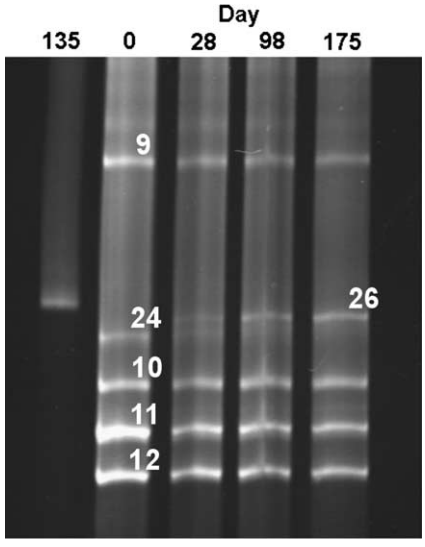

Fig. 1. TGGE separation of the 16S rRNA genes of fast-growing mycobacteria in unamended (control) soil sample. Lane 135, *Mycobacterium* sp. strain 135; lanes Day 0, 28, 98, and 175, soil extracted at days 0, 28, 98, and 175, respectively. Bands 9, 10, 11, 12, 24, and 26 correspond to bands CT-9, CT-10, CT-11, CT-12, CT-24, and CT-26, respectively.