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ORIGINAL PAPER

Anaerobic degradation of *p*-xylene in sediment-free sulfate-reducing enrichment culture

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Abstract Anaerobic degradation of *p*-xylene was studied with sulfate-reducing enrichment culture. The enrichment culture was established with sediment-free sulfate-reducing consortium on crude oil. The crude oil-degrading consortium prepared with marine sediment revealed that toluene, and xylenes among the fraction of alkylbenzene in the crude oil were consumed during the incubation. The PCR-denaturing gradient gel electrophoresis (DGGE) analysis of 16S rRNA gene for the *p*-xylene degrading sulfate-reducing enrichment culture showed the presence of the single dominant DGGE band pXy-K-13 coupled with *p*-xylene consumption and sulfide production. Sequence analysis of the DGGE band revealed a close relationship between DGGE band pXy-K-13 and the previously described marine sulfate-reducing strain oXyS1 (similarity value, 99%), which grow anaerobically with *o*-xylene. These results suggest that microorganism corresponding to pXy-K-13 is an important sulfate-reducing bacterium to degrade *p*-xylene in the enrichment culture.

Keywords *p*-Xylene · Sulfate-reducing bacteria (SRB) · Anaerobic · *Deltaproteobacteria*

Abbreviations

DGGE	Denaturing gradient gel electrophoresis
BTEX	Benzene, toluene, ethylbenzene and *o*-, *m*-, and *p*-xylenes
SRB	Sulfate-reducing bacteria

Introduction

Contamination of harbor sediments by various petroleum hydrocarbons is widespread due to the gasoline and diesel fuel spills as the result of human activity. Among these hydrocarbons, benzene, toluene, ethylbenzene and *o*-, *m*-, and *p*-xylenes (BTEX) are characterized by their toxicity to organisms and are widely distributed in oil-contaminated soils, groundwater, and sediments as a result of their relatively high solubility into water compared to other petroleum hydrocarbons (Tissot and Welte 1984). Therefore, BTEX have been the focus of biodegradation and bioremediation studies as one of the typical monoaromatic hydrocarbons contained in petroleum and gasoline (Colberg and Young 1995).

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In the last decade, it had been reported that anaerobic degradation of BTEX occurred under sulfate-reducing condition in sediments and in enrichment cultures (Phelps and Young 1999). Toluene- (Beller et al. 1996; Rabus et al. 1993), ethylbenzene- (Kniemeyer et al. 2003), and *o*-, *m*-xylene-degrading sulfate-reducing bacteria (SRB) (Harms et al. 1999) have been isolated, respectively. However, although the degradation of *p*-xylene under sulfate-reducing condition has been confirmed with an enrichment culture from the gasoline-contaminated sediments (Edwards et al. 1992), to our knowledge, no investigation has yet been reported about the characterization of microbial community present in a *p*-xylene-degrading sulfate-reducing enrichments.

The aims of this present study were to characterize the microbial community structure of the enrichment culture containing with *p*-xylene as the sole electron donor and carbon source under sulfate-reducing conditions, by using PCR-denaturing gradient gel electrophoresis (DGGE) analysis based on 16S rRNA gene.

Materials and methods

Source of bacteria

The mesophilic enrichment of SRB growing on Kuwait crude oil as the sole source of organic substrates were established from the petroleum contaminated sediments collected at Shuaiba, Kuwait, in 1995 (Sato et al. 1998). The mesophilic enrichments of SRB growing with *p*-xylene as the sole electron donor and carbon source were established from the water phase of the consortium acclimated with crude oil as a sole source of carbon and energy. The enrichment culture with *p*-xylene was sediment-free and stable resulting from five times repeated dilution for over 3 years.

Media and conditions of incubation

For enrichments, a defined bicarbonate-buffered, sulfide-reduced saltwater medium was prepared essentially with the same salt component as natural seawater (Widdel and Bak 1992). The serum bottles (150 ml) sealed with butyl rubber stoppers under a headspace of N_2-CO_2 (80:20 [vol:vol]) were used for cultivation. The enrichment of crude oil-degrading microbes was routinely incubated with 5 ml of crude oil. To avoid toxicity, *p*-xylene was diluted (0.1–20% [vol vol^{-1}]) with 2,2,4,4,6,8,8-heptamethylnonane, which served as the carrier phase (Rabus et al. 1993). The enrichment was routinely incubated with a carrier phase containing 2% *p*-xylene in 2,2,4,4,6,8,8-heptamethylnonane. Five milliliter of carrier phase was added into the serum bottle containing 115 ml of medium. The bottle was sealed with a butyl rubber stopper, and then inverted to avoid contact between the stopper and the carrier phase. From the grown enrichment culture, 3 ml of liquid culture medium was then transferred to the freshly prepared medium via N_2-flushed sterilized syringes. The culture was incubated horizontally at 25–28°C under the dark, and shaken by hand for a few second per week. Chemicals were of analytical grade. The samples were checked with phase-contrast microscopy (Axioplan 2, ZEISS).

Chemical analysis

Dissolved sulfide concentration in the aqueous phase of enrichment was measured colorimetrically using the methylene blue reaction (Cline 1969). Aromatic compounds in crude oil were measured by a column-switching technique to the identification of monomethylated polycyclic aromatic hydrocarbons (Matsuzawa et al. 1990) using a high performance liquid chromatograph (HPLC) systems consisting of a HPLC pump (PU-980, JASCO Co), a 4-way high-pressure switching valve, Silica-Gel column (Nucleosil 50-5, 4.6 mm^2 × 250 mm; pore size 5 nm, particle diameter 5 μm, specific area 450 m^2 g^{-1}), and UV and RI detector. *p*-Xylene in 2,2,4,4,6,8,8-heptamethylnonane was measured as described previously (Nakagawa et al. 2002).

Extraction of nucleic acids, PCR, and DGGE analysis

Nucleic acids of microorganisms were prepared by the following method (Wilson 1990). After incubation the cell with sodium dodesyl sulfate and proteinase K at 37°C for 1 h, nucleic acids were extracted by hexadecyltrimethyl ammonium bromide solution and phenol-chloroform. After the extraction of nucleic acids, the precipitated DNA was resuspended in double-distilled water (RNase, DNase free)

and then stored at –20°C. DNA fragments encoding 16S rRNA gene of the domain *Bacteria* were amplified by using primers 341F-GC and 907R and PCR conditions as described by Muyzer et al. (1993). The amplification products were analyzed by electrophoresis in 2% (wt vol^{-1}) Nusieve 3:1 agarose (FMC, Rockland, ME, USA) gels containing ethidium bromide (1 μg ml^{-1}). DGGE was performed as described as described previously (Nakagawa et al. 2002). DGGE bands were excised from the gels, and reamplified. The PCR products of second amplification were electrophoresed again in a DGGE gel to check the purity of the bands, and then purified using QIAquick™ PCR purification kit (Qiagen, Hiden, Germany). The sequence of the DNA extracted from the DGGE band was determined for both strands.

Phylogenetic analysis

Similarities of 16S rRNA gene sequences were investigated in the databases of the National Center for Biotechnology Information and the DNA Data Bank of Japan using BLAST (Altschul et al. 1997). The nucleotide sequences were multiply aligned by using the CLUSTAL W program in MEGA4 software (Tamura et al. 2007). Phylogenetic trees were calculated from confidently aligned regions of homologous nucleic acid sequences by use of the MEGA4 software.

Nucleotide sequence accession number

DDBJ/EMBL/GenBank accession number of pXy-K-13 is AB081553.

Results and discussion

Establishment of enrichment culture on *p*-xylene under sulfate-reducing condition

Crude oil-utilizing sulfate-reducing enrichment culture without any sediment particles was firstly established by repeated passages into the sulfide-reduced bicarbonate-buffered defined medium containing crude oil as a sole source of carbon and energy for more than 3 years. During 165 days of incubation, toluene, and xylenes decreased among the alkylbenzene fraction of crude oil in all oil-degrading sulfate-reducing consortia. With the crude oil-utilizing sulfate-reducing enrichment culture as inoculums, we then established a *p*-xylene-degrading sulfate-reducing enrichment culture that was sediment-free and stable after repeated dilution for over 3 years.

p-Xylene degradation by SRB

Rod shaped cells (0.6 μm with 2.2–2.9 μm length) were observed as the dominant population throughout incubation with 2% *p*-xylene under phase-contrast microscopy. *p*-Xylene in the carrier phase was consumed accompanying the accumulation of sulfide (Fig. 1). The molar ratio of dissimilation (sulfide/*p*-xylene) was 4.94 after 719 days of incubation. It is stoichiometrically assumed that 5.25 mmol of sulfide is formed per mmol of *p*-xylene, according to the following equation for complete oxidation of *p*-xylene: $C_6H_4(CH_3)_2 + 5.25SO_4^{2-} + 2.5H + 3H_2O \rightarrow 8HCO_3^- + 5.25H_2S$. Indeed, 4.94 mmol of sulfide is formed per mmol of *p*-xylene. This measured degradation balance is in agreement with the above stoichiometry. With 4% of *p*-xylene in the carrier phase, the enrichment culture showed the highest maximum sulfide production rate (Fig. 2). No production of sulfide and growth was occurred at 0% and 20% of *p*-xylene. These results demonstrated that *p*-xylene was utilized by SRB in the enrichment culture.

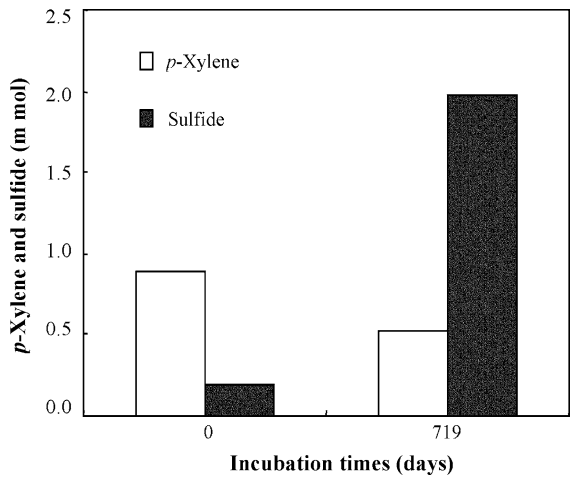

Fig. 1 Consumption of *p*-xylene in carrier phase and production of sulfide in water phase of *p*-xylene-degrading sulfate-reducing consortium. The concentration of *p*-xylene in carrier phase was 2% (vol vol^{-1}) in 2,2,4,4,6,8,8-heptamethylnonane

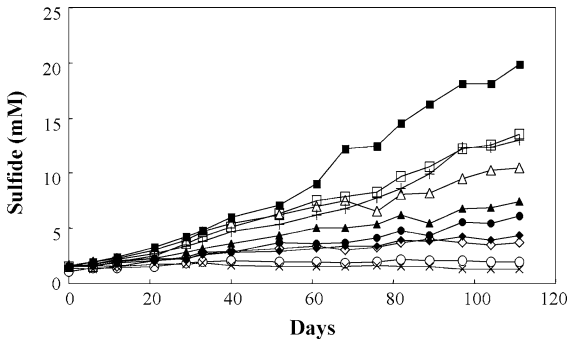

Fig. 2 The effect of different *p*-xylene concentrations on sulfide production by the enrichment culture. ○, 0% of *p*-xylene in the carrier phase; ◇, 0.1%; Δ, 0.5%; □, 1%; +, 2%; ■, 4%; ▲, 6%; ♦, 8%; ●, 10%; ×, 20%

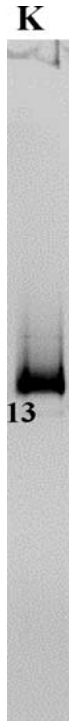

Fig. 3 DGGE profile of PCR amplified 16S rDNA fragment from the enrichment culture on *p*-xylene

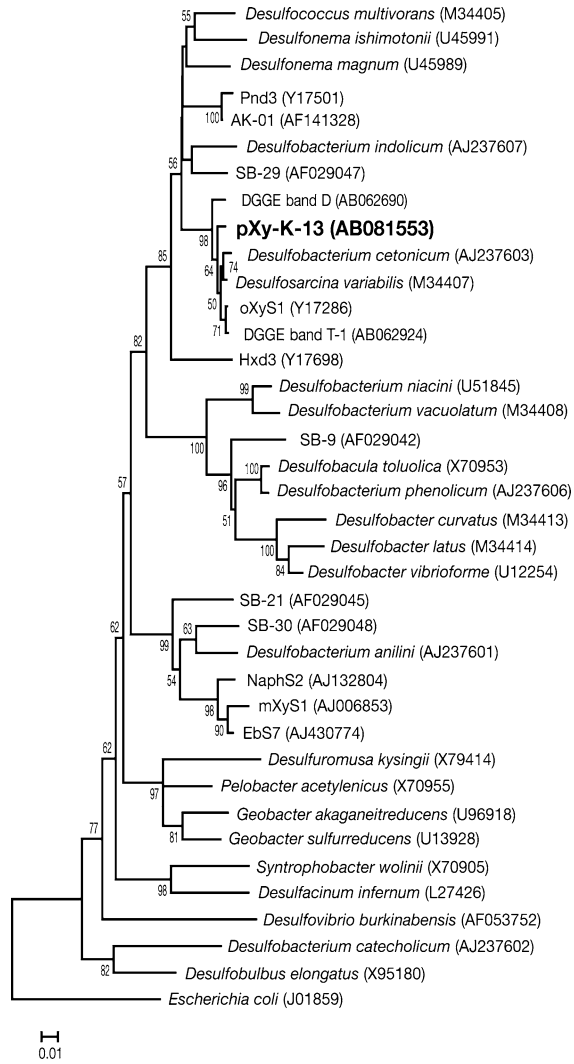

Fig. 4 Phylogenetic tree of the DGGE band (in bold) from *p*-xylene-degrading sulfate-reducing enrichment culture and of the various *Deltaproteobacteria* based on partial 16S rRNA gene sequences. The scale bar represents estimated 1% sequence divergence. Bootstrap values are shown for branches with more than 50 bootstrap support

DGGE and phylogenetic relationship based on 16S rRNA gene sequences

The DGGE profile obtained from the enrichment culture with *p*-xylene in the stationary phase consisted of the single dominant DGGE band designated pXy-K-13 (Fig. 3). The sequence of DNA from band pXy-K-13 was closely related to a strain of *o*-xylene-degrading sulfate reducer oXyS1 (Harms et al. 1999) within the *Deltaproteobacteria*, and clone T-1 derived from consortium enriched on toluene (Koizumi et al. 2002), with similarity of 99% (Fig. 4).

SRB corresponding to the band pXy-K-13 probably have a capability of oxidizing completely *p*-xylene to CO_2 as a terminal product, since the band pXy-K-13 was closely related to a strain of *o*-xylene-degrading SRB oXyS1 which has capable to oxidize completely *o*-xylene to CO_2 (Harms et al. 1999). Moreover, the long time needed to accumulate sulfide in aqueous phase of *p*-xylene-degrading sulfate-

reducing consortium is in good agreement with the long incubation times to degrade *p*-xylene for 119 days (Phelps and Young 1999), and 72 days (Edwards and Grbic-Galic 1992) previously indicated to occur with sulfate as terminal electron acceptor. Similarly, the highest maximum sulfide production rate ($\mu_{max} = 0.0020\ h^{-1}$) estimated from the *p*-xylene-degrading sulfate-reducing consortium was less than those of the toluene-degrading SRB (μ_{max} = ca. $0.0277\ h^{-1}$) (Rabus et al. 1993), and the *o*-xylene-degrading SRB (μ_{max} = ca. $0.0227\ h^{-1}$) (Harms et al. 1999).

Conclusions

We successfully established the culture enriched on *p*-xylene under sulfate-reducing condition. The presence of the single dominant DGGE band pXy-K-13 coupled with *p*-xylene consumption and sulfide production suggests that microorganism corresponding to pXy-K-13 is an important SRB to degrade *p*-xylene in the enrichment culture.

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