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ORIGINAL PAPER

A novel *n*-alkane-degrading bacterium as a minor member of *p*-xylene-degrading sulfate-reducing consortium

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Abstract A *p*-xylene-degrading, sulfate-reducing enrichment culture was characterized by analyzing the response of its members to changes in the available substrate. The culture was inoculated into media containing other substrates, resulting in the establishment of benzoate-, acetate-, and lactate-utilizing enrichment cultures. PCR-denaturing gradient gel electrophoresis (DGGE) analysis of the enriched cultures targeting 16S rRNA genes showed quite simple band patterns. The predominant band from the benzoate-utilizing enrichment culture was identical to that from the original enrichment culture utilizing *p*-xylene. A single, dominant DGGE band was observed in common from the acetate- and lactate-utilizing enrichment cultures. A novel sulfate-reducing bacterium, strain PL12, was isolated from the lactate-utilizing enrichment culture. The 16S rRNA gene sequence of strain PL12 was identical to that of the dominant DGGE band in the acetate- and lactate-utilizing enrichment cultures and distinct from the dominant sequences in the original *p*-xylene-degrading and benzoate-utilizing enrichment cultures. Phylogenetic analysis of the 16S rRNA gene sequences showed that the isolate belonged to the family *Desulfobacteraceae* in the class *Deltaproteobacteria*. The isolated strain PL12 could utilize *n*-hexane and *n*-decane as substrates, but could not utilize benzoate, *p*-xylene and other aromatic hydrocarbons. These results suggest that the *p*-xylene degradation observed in the original enrichment culture was performed by the dominant bacterium corresponding to DGGE band pXy-K-13 (Nakagawa et al. 2008). The novel strain PL12 might have been utilizing metabolites of *p*-xylene.

Keywords Sulfate-reducing bacteria · *p*-Xylene · *n*-Alkane

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Introduction

Among monoaromatic hydrocarbons, the compounds benzene, toluene, ethylbenzene, and xylene (BTEX) are particularly toxic and have relatively high solubility in water. Because of these characteristics, BTEX are regarded as important pollutants and the

biodegradation of these compounds have been investigated intensively (Chakraborty and Coates 2004). The last decade has witnessed significant developments in our understanding of the anaerobic degradation of BTEX, including the identification of bacteria responsible for this degradation (Widdel and Rabus 2001). As for sulfate-reducing bacteria, strains with the ability to degrade toluene (Beller et al. 1996; Rabus et al. 1993), *o*-xylene, *m*-xylene (Harms et al. 1999; Morasch et al. 2004), and ethylbenzene (Kniemeyer et al. 2003) have been isolated in the past. The metabolic pathways of these bacteria have been elucidated, and the fumarate addition was identified as the common mechanism for the initial activation of monoaromatic hydrocarbons (Chakraborty and Coates 2004). However, bacteria capable of degrading benzene or *p*-xylene under sulfate reducing condition have not been isolated in pure culture yet. One of the available approaches is to analyze sufficiently established sulfate-reducing enrichment cultures, as has been demonstrated for benzene degradation (Musat and Widdel 2008; Phelps et al. 1998; Ulrich and Edwards 2003). The establishment of a sulfate-reducing enrichment culture for *p*-xylene degradation was reported recently (Nakagawa et al. 2008). By analyzing the established enrichment culture, single dominant DGGE band pXy-K-13 was identified, and partial 16S rRNA gene sequence of corresponding bacterium was obtained. However, the physiological characteristics of the dominant bacterium have not yet been investigated. In addition, the previous study provided no information on organisms other than the dominant organism in the enrichment culture.

In the present study, we analyzed the *p*-xylene-degrading, sulfate-reducing enrichment culture to investigate the substrate specificities of the dominant bacterium and other community members. As a result of the analysis, a novel sulfate-reducing bacterium distinct from the dominant bacterium was isolated. The isolated strain could not degrade *p*-xylene, but utilized *n*-alkane. Sulfate-reducing organisms capable of degrading *n*-alkanes have also attracted some interest (Rueter et al. 1994; So and Young 1999; Cravo-Laureau et al. 2004), and several novel strains have been isolated recently (Kniemeyer et al. 2007). The strain isolated in the present study was phylogenetically distinct from any of these bacteria.

Materials and methods

Enrichment, media, and conditions of incubation

The *p*-xylene-degrading, sulfate-reducing enrichment culture was established using a sediment from Shuaiba, Kuwait, used in a previous study (Nakagawa et al. 2008). The enrichment and cultivation methods were the same as those described by Nakagawa et al. (2008). A defined, bicarbonate-buffered, sulfide-reduced saltwater medium containing 28 mM sulfate (Widdel and Bak 1992) was used as the basal medium throughout this study. The serum bottles sealed with butyl rubber stoppers. The headspace of the bottles was filled with N_2–CO_2 (80:20 [vol vol^{-1}]) and sodium dithionite was added as additional reductant. To avoid the toxic effects, *p*-xylene was diluted to 2% (vol vol^{-1}) with 2,2,4,4,6,8,8-heptamethylnonane (Rabus et al. 1993). The diluted *p*-xylene was added to basal medium (4% [vol vol^{-1}]). From the grown enrichment culture, liquid culture medium was transferred to the freshly prepared medium (1% [vol vol^{-1}]) via the stoppers by means of N_2-flushed sterilized syringes. The culture bottles were incubated in horizontal position at 28°C under the dark, and were manually shaken several times every week. The dissolved sulfide concentration in the aqueous phase of the enrichment culture was monitored by the methylene blue formation reaction, as described previously (Cline 1969).

Response to change of substrate

Portions of the *p*-xylene-degrading enrichment culture (1.5 ml) were inoculated into 50 ml of media containing different substrates using N_2-flushed and sterilized syringes. The substrates tested were 2 mM benzoate, 10 mM acetate, and 20 mM lactate. The tests were carried out in sealed serum bottles with a headspace of N_2–CO_2 (80:20 [vol vol^{-1}]). The enrichment cultures were incubated at 28°C. Growth was confirmed by the increase in dissolved sulfide concentration in the aqueous phase determined as described above.

From a 10–30 ml aliquot of each well-grown enrichment culture, cells were harvested by centrifugation. Total DNA was extracted using the method described by Wilson (1990). The extracted DNA was resuspended in nuclease-free water (Sigma, Steinheim, Germany) and then stored at −20°C.

DNA fragments of the 16S rRNA gene of the domain *Bacteria* were amplified using the eubacterial primer 341F and the universal primer 907R (Muyzer et al. 1993). A 40-base pair GC clamp was attached to the 5′-end of the 341F primer for denaturing gradient gel electrophoresis (DGGE) analysis. The polymerase chain reaction (PCR) conditions were as follows: initial denaturation for 1 min at 94°C; 25 cycles of denaturation (2 min at 94°C), annealing (1.5 min at 45°C), and extension (2 min at 72°C); and the final extension for 10 min at 72°C. The amplification products were analyzed by electrophoresis in 1.5% (wt vol^{-1}) agarose S (Nippon Gene, Tokyo, Japan) gels containing ethidium bromide (1 μg ml^{-1}).

DGGE analysis of the PCR products was performed on 6% (wt vol^{-1}) polyacrylamide gels, as described by Muyzer et al. (1996), using D-code systems (Bio-Rad Laboratories, CA, USA). The denaturant gradient ranged from 20 to 50% (100% was defined as 7 M urea and 40% [vol vol^{-1}] deionized formamide) and the thickness of the gels was 1.5 mm. Electrophoresis was run in 0.5× TAE buffer (20 mM Tris, 10 mM acetic acid, 0.5 mM EDTA; pH 8.3) at a constant voltage of 200 V and temperature of 60°C, for 4 h. Subsequently, the gels were incubated for 10 min in an ethidium bromide solution (0.5 μg ml^{-1}), rinsed for 10 min in Milli-Q water, and then photographed under UV transillumination (wavelength, 312 nm) with a charge-coupled device camera (Image Server; ATTO, Tokyo, Japan).

The major DGGE bands were excised from the gel and reamplified. DGGE analysis of the reamplified products was performed to verify their purity. The PCR products obtained from each band were purified using the Rapid PCR purification System (Marligen Bioscience, MD, USA). The sequences of the bands were determined by cycle sequencing with a dye terminator (BigDye® Terminator v3.1 Cycle Sequencing Kit; Applied Biosystems, CA, USA).

Isolation and characterization of a novel sulfate-reducing bacterium

The lactate-utilizing enrichment culture was subjected to the agar shake dilution using 20 mM sodium lactate as the substrate (Widdel and Bak 1992). An isolated colony was picked and inoculated into a liquid medium supplemented with 20 mM sodium lactate. Agar shake dilution was performed again from this culture to obtain a pure culture of the novel strain.

The cell morphology of the isolate was observed using a phase-contrast microscope (Axioplan 2; ZEISS, Jena, Germany). Growth at various temperatures was examined using the basal medium supplemented with 20 mM lactate, and the growth of the culture was evaluated by monitoring the optical density of the culture (610 nm). Temperature limits for growth were determined by incubating the culture at 14 different temperatures ranging from 4 to 45°C.

The utilization of electron acceptors was determined by monitoring the growth in the basal medium without sulfate, and 20 mM lactate was used as an electron donor. The tested substances were 10 mM thiosulfate, 5 mM sulfite, 20 mM sulfate, 0.5 g l^{-1} elemental sulfur, 10 mM nitrate, 10 mM fumarate, or 10 mM poorly crystalline Fe (III) oxide. We evaluated the utilization of electron donors by monitoring the growth in the basal medium containing 10 mM lactate, 5 mM acetate, 5 mM formate, 5 mM propionate, 5 mM butyrate, 5 mM isobutyrate, 10 mM pyruvate, 10 mM fumarate, 2.5 mM benzoate, 5 mM citrate, 0.5 mM phenol. In the test of H_2 utilization, headspace of the bottle was filled with H_2:N_2:CO_2 (50:40:10 [vol vol^{-1}]), total pressure was 200 kPa. All tests were performed in tubes sealed with butyl rubber stoppers at 28°C.

The utilization of hydrocarbon was tested in flat bottles sealed with butyl stoppers. Toluene, benzene, *o*-xylene, *m*-xylene, and *p*-xylene were diluted to 2% (vol vol^{-1}) with 2,2,4,4,6,8,8-heptamethylnonane, which served as the carrier phase (Rabus et al. 1993). Ethylbenzene, cyclohexane, *n*-hexane, and *n*-decane were diluted to 4%. The diluted hydrocarbon solutions were added to basal medium (4% [vol vol^{-1}]). In the case of naphthalene, 0.6 g l^{-1} was added to the basal medium. The culture bottles were incubated in the dark in a horizontal position at 28°C and were manually shaken several times every week.

Total DNA of the strain was extracted from 25 ml of the culture medium using the technique described previously. The 16S rRNA gene fragments of the isolate were amplified with primers 27f and 1492r (Lane 1991). The fragments of the gene for alpha and beta subunits of dissimilatory sulfite reductase (*dsrAB*) were amplified with primers DSR1Fdeg and DSR4R-deg (Klein et al. 2001). Partial sequence of *dsrA* gene

was determined with primers DSR1Fdeg and newly designed reverse primer (5′-TTGATGCAATGCA TGCA-3′). The purification and sequencing of the PCR products were performed as described above.

Similarity analysis of the sequences obtained was carried out using the basic local alignment search tool (BLAST) software of the National Center for Biotechnology Information. The nucleotide sequences of the 16S rRNA gene and the amino acid sequence deduced from the nucleotide sequence of the *dsrA* gene were aligned with reference sequences from the DNA Data Bank of Japan (DDBJ) database using the ClustalX program. The phylogenetic trees were constructed by the neighbor-joining method with the software MEGA version 3.1 (Kumar et al. 2004). Bootstrap analysis was performed for 1000 replicates.

Stoichiometry of *n*-decane degradation with strain PL12

For quantitative analysis of *n*-alkane degradation, strain PL12 was grown on *n*-decane in flat bottle (100 ml) containing 65 ml basal medium under N_2–CO_2 (80:20 [vol vol^{-1}]). As a sole electron donor, 2.8 ml of the 4% *n*-decane solution was added. The bottle was inoculated with grown culture of strain PL12 (1% [vol vol^{-1}]) and sealed with butyl rubber stopper, and then incubated at 28°C in the dark. Concentration of dissolved sulfide in the aqueous phase was determined described above. Concentration of *n*-decane in carrier phase was measured by gas chromatography (GC-14A; Shimadzu, Kyoto, Japan) equipped with a flame ionization detector. The column used in the analysis was DB-5 (J & W Scientific, Folsom, USA; length 25 m, internal diameter 0.53 mm). The oven temperature program began at 50°C for 10 min, proceeded at a rate of 10°C min^{-1} to 250°C, and was held for 10 min. The injector and detector temperature were set at 250 and 350°C, respectively.

Nucleotide sequence accession numbers

The nucleotide sequences of strain PL12 isolated in this study have been assigned the DDBJ/EMBL/GenBank accession numbers (AB468588 and AB468589).

Results and discussion

Shift in community structure induced by change of substrate

The *p*-xylene-degrading enrichment culture was inoculated into a medium containing other aqueous substrates. We observed growth in all cases, i.e., benzoate-, acetate-, and lactate-utilizing enrichment cultures were established. The results of 16S rRNA gene-targeted PCR-DGGE analysis of these enrichment cultures are shown in Fig. 1. All three samples showed quite simple band patterns. In addition, the band patterns of the acetate- and lactate-utilizing enrichment cultures were quite similar to each other. These results suggest that the original *p*-xylene-degrading enrichment culture contained limited types of organisms. In fact, the sequence of band B1 from the benzoate-utilizing enrichment was identical to that of pXy-K-13, which was dominant in the original *p*-xylene-degrading enrichment culture (Nakagawa et al. 2008). The sequences of the other bands (B2, A1, and L1) were identical to each other, and distinct from that of pXy-K-13 (528/545 bp). These results suggest that the bacterium corresponding to pXy-K-13 could utilize benzoate, and that it was outcompeted by other specific bacteria in the presence of other less selective substrates. It is suggested that an isolation method using benzoate or *p*-xylene as the substrate may be useful in obtaining a pure culture of *p*-xylene-degrading bacteria from the sulfate-reducing consortium.

Isolation and characterization of the novel strain of sulfate-reducing bacteria

A novel strain of sulfate-reducing bacterium, i.e., PL12, was isolated from the lactate-utilizing enrichment culture by the agar shake dilution method. The partial 16S rRNA gene sequence (551 bp) was determined for this strain, and it was identical to the sequences obtained from DGGE bands B2, A1, and L1. This suggests that the isolated bacterium was dominant in the enrichment cultures established with acetate or lactate as the substrate. The cells of strain PL12 were motile, short (0.5 × 1–1.5 μm) with a rod-shaped morphology. They were distinct from the dominant bacterium in the *p*-xylene-degrading enrichment culture. As reported in the previous study

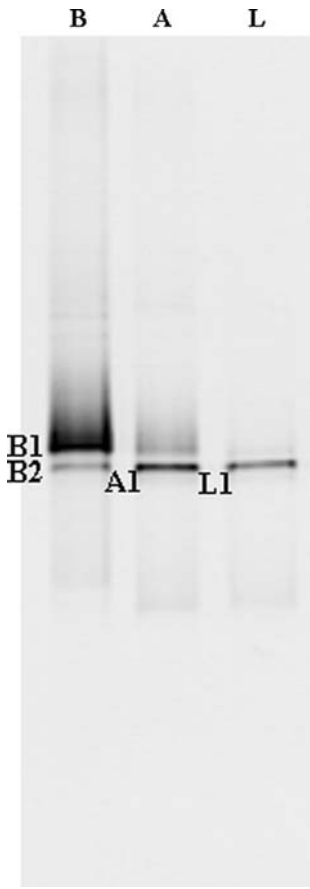

Fig. 1 DGGE profiles of PCR amplified 16S rRNA gene fragments from the enrichment cultures. *Lane B*, culture on benzoate; *A*, culture on acetate; *L*, culture on lactate

(Nakagawa et al. 2008), rod-shaped cells (0.6 × 2.2–2.9 μm) formed the dominant population in the enrichment culture on *p*-xylene. The temperature range for the growth of strain PL12 was 18–37°C, with optimum growth at 30–34°C.

Strain PL12 was able to use sulfate (20, 28 mM), thiosulfate (10 mM), sulfite (5 mM), and poorly crystalline Fe (III) oxide (10 mM) as an electron acceptor, but not nitrate (10 mM), fumarate (10 mM), or elemental sulfur (0.5 g l^{-1}).

Strain PL12 used the following substrates as electron donors and carbon sources (concentrations in mM, except where stated): lactate (10, 20), acetate (5), formate (5), propionate (5), butyrate (5), isobutyrate (5), pyruvate (10), fumarate (10). The isolate could grow autotrophically with H_2. The strain was not able to grow on the following substrates (concentrations in mM): benzoate (2.5), citrate (5), and phenol (0.5). Strain PL12 was able to oxidize the hydrocarbons *n*-hexane and *n*-decane. When either *n*-hexane or *n*-decane was added to the medium as the sole energy and carbon source, growth of cells and an increase in sulfide concentration were observed. Over the course of a 120-day incubation period, no sulfide production was observed with following substrates; toluene, benzene, *o*-xylene, *m*-xylene, *p*-xylene, ethylbenzene, cyclohexane, and naphthalene.

The stoichiometry of *n*-decane degradation by strain PL12 was tested by quantifying generated sulfide and consumed *n*-decane. After 384 days, 0.1 mmol *n*-decane was consumed, while 0.74 mmol sulfide was produced (Fig. 2). The complete oxidation of *n*-decane corresponds to the following theoretical reaction:

$$C_{10}H_{22} + 7.75SO_4^{2-} + 5.5H^+ \rightarrow 10HCO_3^- + 7.75H_2S + H_2O.$$

The observed molar ratio of consumed *n*-decane to produced sulfide is close to this reaction, suggesting that strain PL12 completely oxidized *n*-decane to CO_2.

In order to carry out detailed phylogenetic analysis, a nearly full length 16S rRNA gene sequence (1,526 bp) was obtained from strain PL12. It was revealed that this novel isolate belongs to the family *Desulfobacteraceae* in the class *Deltaproteobacteria* (Fig. 3). The closest phylogenetic relatives of strain PL12 were *Desulfosarcina variabilis*, *Desulfosarcina*

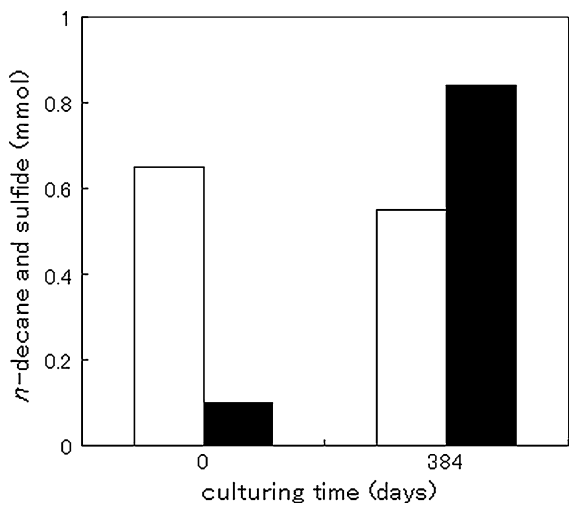

Fig. 2 Consumption of *n*-decane and concomitant sulfide production by strain PL12

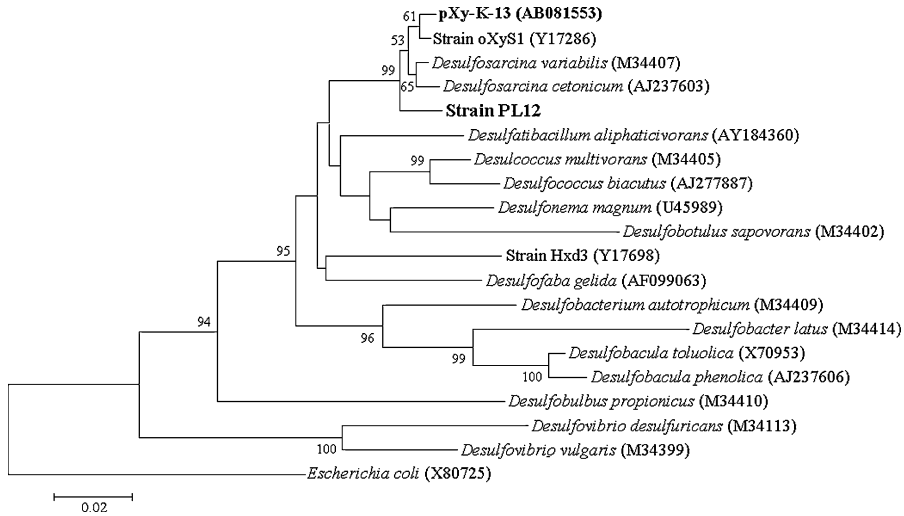

Fig. 3 Phylogenetic tree showing the affiliation of the 16S rRNA gene sequences of strain PL12 within the *Deltaproteobacteria*, with *Escherichia coli* as outgroup. GeneBank accession numbers are shown in parentheses. The tree was based on approximately 550 base pairs and was generated by the neighbour-joining methods. The *scale bar* represents 2% estimated divergence. Bootstrap values greater than 50 are shown

cetonicum, and the sulfate-reducing strain oXyS1, with sequence similarities of 97%. The most closely related *n*-alkane-degrading bacterium was *Desulfatibacillum aliphaticivorans* (92%). The phylogenetic position of the novel isolate within the family *Desulfobacteraceae* was supported by deduced DsrA sequence (221 amino acids) analysis (Fig. 4), indicating a close relationship to *Desulfosarcina cetonicum*. The phylogenetic and physiological characteristics of strain PL12 suggest that the isolate belongs to a new genus within *Desulfobacteraceae*. A more detailed characterization will be needed to reveal the role of this *n*-alkane-degrading bacterium in the *p*-xylene-degrading consortium.

Although strain PL12 could not utilize benzoate, a DGGE band corresponding to PL12 (band B2) was detected from the benzoate-utilizing enrichment culture. It is possible that intermediates suitable for PL12 metabolism might have been generated by organism/s corresponding to band B1 (and to pXy-K-13). This might explain the retention of the strain PL12 in the *p*-xylene-degrading enrichment culture.

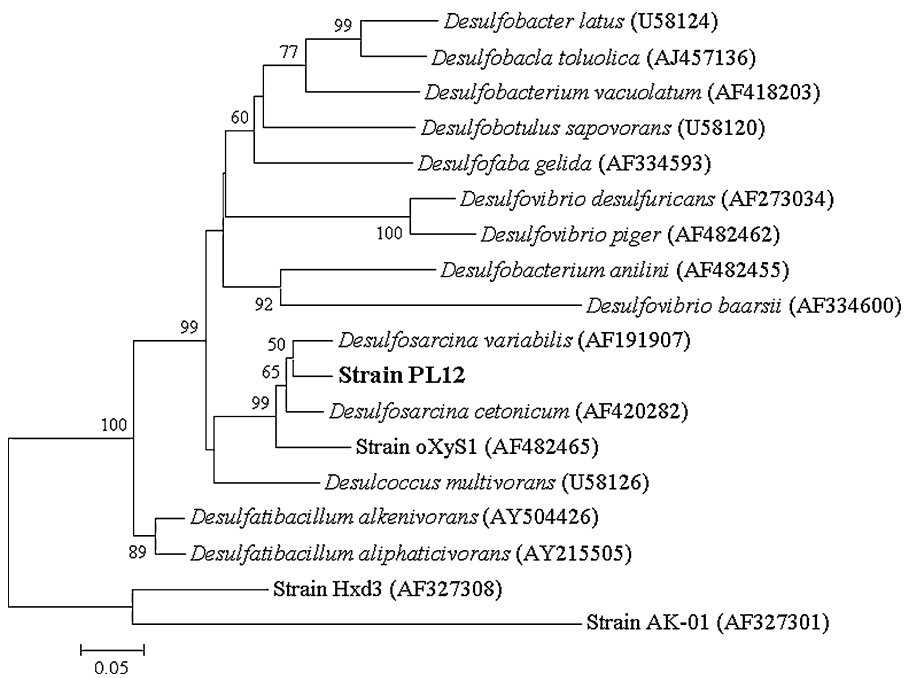

Fig. 4 Phylogenetic tree showing the affiliation of the deduced DsrA amino acid sequences of strain PL12. GeneBank accession numbers are shown in parentheses. The tree was based on approximately 220 amino acid positions and was generated by the neighbour-joining methods. The scale bar represents 5% estimated divergence. Bootstrap values greater than 50 are shown

In the present study, it was demonstrated that the original *p*-xylene-degrading enrichment culture did contain other bacteria besides the dominant pXy-K-13. However, only one other kind of sulfate-reducing bacterium was detected from the enrichment cultures of all the tested substrate; including lactate, which is known to be utilized by a number of sulfate-reducing bacteria. The detected organism was identified as the strain PL12, which could not utilize *p*-xylene. These results suggest that *p*-xylene degradation coupled with the sulfate reduction observed in the original enrichment culture was performed by the unisolated bacterium corresponding to pXy-K-13.

In the present study, the purity of the previously established *p*-xylene degrading enrichment culture was further emphasized. In addition, the results strongly suggest that the dominant bacterium is responsible for *p*-xylene degradation. Further investigation of the enrichment culture may provide more information on BTEX degradation under sulfate-reducing conditions. In addition, the results obtained in the present study may help in the isolation of *p*-xylene-degrading, sulfate-reducing bacteria. In addition to contributing to the understanding of BTEX degradation, the novel strain isolated in this study may prove useful in elucidating the anaerobic degradation of petroleum hydrocarbons.

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