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Temperature-dependent differences in community structure of bacteria involved in degradation of petroleum hydrocarbons under sulfate-reducing conditions

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Running headline:

Anaerobic *p*-xylene-degrading enrichment at low temperature

Abstract

Aim: To characterize the microbial community involved in anaerobic degradation of petroleum hydrocarbon under low and moderate temperature conditions.

Methods and Results: Sulfate-reducing enrichment cultures growing on crude oil and *p*-xylene were established at low and moderate temperatures. Bacterial community structures of the cultures were characterized by 16S rRNA gene-based analysis, and organisms responsible for degradation of *p*-xylene were investigated by analysis of the *bamA* gene, involved in anaerobic degradation of aromatic compounds. The PCR-denaturing gradient gel electrophoresis analysis indicated significant differences in microbial community structures among the cultures, depending on the temperatures of incubation. Difference depending on the temperatures was also observed in the cloning analysis of the *bamA* gene performed on the *p*-xylene-degrading enrichment cultures. Majority of clones detected in the culture of moderate temperature were related to *Desulfosarcina ovata*, whereas more diverse *bamA* gene sequences were obtained from the culture incubated at low temperature.

Conclusions: Temperature-dependent differences in microbial community were demonstrated by the analyses of two genes. It was suggested that sulfate-reducing bacteria of phylogenetically different groups might be involved in the degradation of petroleum hydrocarbons in different temperature environments.

Significance and Impact of the Study: The present study is the first report of *p*-xylene-degrading sulfate-reducing enrichment culture at low temperature. The results of the experiments at low temperature were distinctly different from those reported in previous studies performed at moderate temperatures.

Keywords: sulfate-reducing bacteria; low temperature; petroleum hydrocarbon; *p*-xylene; anaerobic

biodegradation; *bamA* gene

Introduction

As with other general organic compounds, biodegradation of petroleum hydrocarbon is more effective under aerobic conditions than under anaerobic conditions. In crude oil-contaminated sites, however, anaerobic conditions are readily formed because of oxygen consumption by aerobes. Therefore, elucidating the mechanism of anaerobic degradation is important to restore crude oil-contaminated environments and to predict the fate of hazardous hydrocarbons. In water and sediments, anaerobic conditions are formed more easily because of limited diffusion of oxygen (Cozzarelli *et al*. 1995; Lovley 2001). Since seawater contains an abundant supply of sulfate (25~30 mmol l^{-1}), it is expected that anaerobic degradation by sulfate-reducing bacteria is important in the cases of marine pollution (Kleikemper *et al*. 2002). Marine pollution by crude oil can occur by various events, such as oil tanker accidents, oil field exploitation at the bottom of the sea, and release of effluents from marine vessels (Head and Swannell 1999). In a large part of the sea, sediment is always at low temperature (Finster 2008). In addition, the risk of crude oil pollution in the cryosphere has recently increased because of the exploitation of the North Sea oil field and use of the Northern Sea Route as a transport line for oil. In spite of the importance suggested by these facts, there are only a few reports on petroleum hydrocarbon degradation by sulfate-reducing bacteria under low temperature conditions. Although there is growing knowledge about sulfate-reducing bacteria which utilize hydrocarbons at moderate temperatures, it is uncertain whether results of these studies are applicable to processes in the cold environments.

Among a variety of hydrocarbons in crude oil, monoaromatic compounds such as benzene, toluene, ethylbenzene, and xylene are notably toxic and have relatively high solubilities in water

(Chakraborty and Coates 2004). Consequently, these compounds have been noteworthy pollutants. With regard to sulfate-reducing bacteria, strains that degrade toluene (Rabus *et al*. 1993; Beller *et al*. 1996;), *o*-xylene, *m*-xylene (Harms *et al*. 1999; Morasch *et al*. 2004), and ethylbenzene (Kniemeyer *et al*. 2003) have already been isolated. However, *p*-xylene and benzene-degrading sulfate-reducing bacteria have not yet been isolated in pure culture. Some benzene-degrading enrichment cultures have been established under sulfate-reducing conditions, and their phylogenetic compositions were investigated in previous studies (Phelps *et al*. 1998; Kleinsteuber *et al*. 2008; Musat and Widdel 2008; Laban *et al*. 2009). Similarly, sulfate-reducing enrichment cultures which degrade *p*-xylene have been established from an aquifer (Morasch and Meckenstock 2005) and marine sediments (Nakagawa *et al*. 2008), but bacterium involved in *p*-xylene degradation was identified only in the latter study.

In the present study, sulfate-reducing enrichment cultures growing on crude oil and *p*-xylene were established at low and moderate temperatures. The enrichment cultures were characterized by bacterial community structure analysis. In addition to the 16S rRNA gene-based analysis, cloning analysis of the *bamA* gene was also performed to detect bacteria responsible for the degradation of *p*-xylene. The *bamA* gene encodes 6-oxocyclohex-1-ene-1-carbonyl-coenzyme A hydrolase, one of the key enzymes for anaerobic degradation of aromatic compounds (Kuntze *et al*. 2008).

Materials and methods

Anoxic cultivation under sulfate-reducing conditions

Throughout the present study, the defined bicarbonate-buffered, sulfide-reduced saltwater medium (Widdel and Bak 1992) was used for cultivation under sulfate-reducing conditions. All culture

bottles were sealed with butyl rubber stoppers, and N_2-flushed sterile syringes were used for aseptic and anoxic transfer of cultures. Increase in dissolved sulfide concentration in the aqueous phase was monitored with the methylene blue formation method (Cline 1969). In cases of cultivation with pure hydrocarbon species, each hydrocarbon was diluted to 2% vol vol^{-1} (*p*-xylene, *o*-xylene, *m*-xylene, benzene, ethylbenzene, and toluene) or 4% vol vol^{-1} (*n*-hexane) with 2,2,4,4,6,8,8-heptamethylnonane to mitigate the toxic effects (Rabus *et al*. 1993). The diluted hydrocarbon solutions were added to 25-fold volume of the basal medium.

Establishment of hydrocarbon-degrading enrichment cultures

As inocula for enrichments, marine sediments were collected from Sanban-ze, situated in Tokyo Bay, Japan (Tabuchi *et al*. in press). The sampling was performed on 20 June 2004. At the time of sampling, the temperature of the sediment was 25.8ºC, and pH of the overlying water was 8.18. The sediment sample was obtained from the surface to a depth of approximately 10 cm, by using a plastic core sampler. The sample was transported on ice to the laboratory and stored at 4ºC for 10 days until further processing.

In each 150-ml serum bottle, 5 ml of sediment was inoculated into 115 ml of the medium, and small amount of sodium dithionite was added as an additional reductant. As the sole source of carbon and energy, 2 ml of crude oil was added in each culture. The same crude oil was also used in previous studies of hydrocarbon-degrading enrichment cultures (Koizumi *et al*. 2002; Nakagawa *et al*. 2002; Nakagawa *et al*. 2008). The bottles were incubated in the dark at 8ºC and 28ºC and were manually shaken several times every week. After the increase in sulfide concentration and growth of cells were confirmed, portions of the crude oil-degrading enrichment cultures (1 ml) were inoculated

into 115 ml of medium supplemented with *p*-xylene. The enrichment cultures on *p*-xylene were incubated at the same temperatures as those of the original enrichments.

Denaturing gradient gel electrophoresis analysis of enrichment cultures

From an aliquot of each well-grown enrichment culture (10 to 30 ml), cells were harvested by centrifugation. Total DNA was extracted from the cells by the method previously described (Wilson 1990). The extracted DNA was suspended in nuclease-free water and then stored at –20ºC. Fragments of the 16S rRNA gene were amplified using the primer pair 341F and 907R (Muyzer *et al*. 1993). A 40-base 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 94ºC; 25 cycles of denaturation (2 min at 94ºC), annealing (1.5 min at 45ºC), and extension (2 min at 72ºC); and a final extension for 10 min at 72ºC.

DGGE analysis was performed as described previously (Muyzer *et al*. 1996). The PCR products were separated on a 1.5-mm-thick polyacrylamide gel (6% wt vol^{-1}) with a linear gradient of denaturants from 20% to 50% (100% was defined as 7 mol l^{-1} urea and 40% vol vol^{-1} formamide). The electrophoresis was run in 0.5× TAE buffer (20 mmol l^{-1} Tris, 10 mmol l^{-1} acetic acid, 0.5 mmol l^{-1} EDTA; pH 8.3) kept at 60ºC, at a constant voltage of 200 V for 4 h. Major DGGE bands were excised from the gel and DNA fragments in the gel pieces were reamplified with the same primer pair. The reamplified products were subjected DGGE again to verify their purity. The sequences of the bands were determined on both strands by using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA).

The sequences of the DGGE bands were compared to those in the public database by using the

basic local alignment search tool (BLAST) software located at the website of the National Center for Biotechnology Information. The sequences were then aligned with reference sequences from the public database using the ClustalX program. A phylogenetic tree was constructed by the neighbor-joining method using the software MEGA version 3.1 (Kumar *et al*. 2004). Bootstrap analysis was performed for 1000 replicates.

Cloning analysis of the *bamA* gene

The PCR-based analysis of aromatic compound-degrading gene was performed with the DNA extracted from the *p*-xylene-degrading enrichment cultures. The fragments of *bamA* gene were amplified using the primer pair BamA-SP9 and BamA-ASP1 (Kuntze *et al*. 2008). The PCR conditions were as follows: initial denaturation for 5 min at 94ºC; 35 cycles of denaturation (30 sec at 94ºC), annealing (45 sec at 60ºC), and extension (1 min at 72ºC); and a final extension for 10 min at 72ºC. The PCR products were purified using the Rapid PCR Purification System (Marligen Bioscience, MD, USA). The purified amplicons were cloned into plasmid vector pCR® 2.1-TOPO® using the TOPO TA Cloning® kit (Invitrogen, CA, USA). The recombinant vectors were transformed into TOP10 One Shot Chemically Competent cells (Invitrogen, CA, USA). The transformed cells were plated on Luria-Bertani agar plates containing 50 μg ml^{-1} kanamycin. For the blue/white assay, 40 mg ml^{-1} X-gal in dimethylformamide was spread onto the plates prior to plating cells. From each plate, white colonies were randomly selected and the inserts were directly amplified from cells with the vector primers M13F (-20) and M13R. The PCR conditions were as follows: initial denaturation for 10 min at 96ºC; 30 cycles of denaturation for 1 min at 94ºC, annealing for 1 min at 55ºC, and extension for 3 min at 72ºC; and the final extension for 7 min at 72ºC. The nucleotide sequences of

the PCR products were determined as described above. The amino acid sequences were deduced from the obtained nucleotide sequences by using the standard genetic code. On the basis of the deduced sequences, the clones were grouped into operational taxonomic units (OTUs); clones with identical amino acid sequence were regarded as members of same OTU. The deduced protein sequences were aligned with reference sequences from DDBJ database using ClustalX program. The phylogenetic trees were constructed as described above.

Isolation of sulfate-reducing bacteria and hydrocarbon utilization of strains

Two strains of sulfate-reducing bacteria were isolated from *p*-xylene-degrading enrichment cultures via cultivation using aqueous substrates. Portions of the *p*-xylene-degrading enrichment cultures (0.5 ml) were inoculated into 50 ml of media containing 2.5 mmol l^{-1} benzoate or 10 mmol l^{-1} acetate and incubated at the respective temperatures. One of the novel strains, 28bB2T was obtained from benzoate-utilizing culture of 28ºC. The benzoate-utilizing culture was subjected to agar shake dilution (Widdel and Bak 1992), and an isolated colony was picked. The resulting culture was further subjected to serial dilution with liquid medium supplemented with toluene. The other strain, 8aA2 was obtained from acetate-utilizing culture of 8ºC by agar shake dilution.

For phylogenetic analysis of the isolates, the 16S rRNA gene fragments were amplified with primer pair 27f and 1492r (Lane 1991), directly from cells harvested by centrifugation. In addition, PCR amplification of the *bamA* gene was also performed as described above. The sequencing of the PCR products and phylogenetic analysis were carried out as described above.

The utilization of hydrocarbons under sulfate-reducing conditions was tested for the strains 28bB2T, 8aA2, and *Desulfobacter psychrotolerans* DSM 17155 obtained from the Deutsche Sammlung von

Mikroorganismen und Zelkulturen (DSMZ). The hydrocarbons utilization tests for the novel strains were performed at 26ºC or 28ºC, corresponding to the optimum temperatures for growth of respective strains on aqueous substrates. The test for *Desulfobacter psychrotolerans* was also performed at 26ºC.

Nucleotide sequence accession numbers

The nucleotide sequences obtained in this study have been assigned the DDBJ/EMBL/GenBank accession numbers AB587695 to AB587723.

Results

Establishment of crude oil-degrading sulfate-reducing consortia

In the cultures, hydrocarbons (crude oil or *p*-xylene) and sulfate were added as the sole electron donor and electron accepter, respectively. Therefore, increase in sulfide indicates progression of hydrocarbon degradation coupled with sulfate reduction. The changes in the concentration of dissolved sulfide in the enrichment cultures on crude oil are shown in Fig. 1. The sulfide concentration of the enrichment incubated at 8ºC gradually increased to 6.1 mmol l^{-1} in 301 days, after which the production of sulfide seemed stopped. In contrast, the sulfide concentration in the culture on the same substrate incubated at 28ºC reached 23.2 mmol l^{-1} at day 248. From the crude oil-degrading enrichment cultures of two temperatures, the *p*-xylene-degrading enrichment cultures were established at respective temperatures. After 217-day incubation, sulfide in the enrichment cultures of 8ºC and 28ºC reached 7.5 mmol l^{-1} and 12.1 mmol l^{-1}, respectively.

The microbial communities in the cultures were characterized by PCR-DGGE of 16S rRNA genes. The DGGE band profiles differed clearly depending on the cultivation temperature and substrate (Fig. 2). The results of the phylogenetic analysis of DGGE bands are shown in Table 1. All sequences, except that of band 28oil-1, belonged to the class *Deltaproteobacteria*. The exceptional band, 28oil-1, belonged to the phylum *Bacteroidetes*. The band 8oil-1 from the crude oil-degrading culture of 8ºC was related to an uncultured *Desulfobacula* sp. detected from cold Antarctic sediments (Purdy *et al*. 2003). The band 8pX-1 from the *p*-xylene-degrading culture of 8ºC was quite closely related to *Desulfobacter psychrotolerans*, a psychrotolerant sulfate-reducing bacterium (Tarpgaard *et al*. 2006). The bacterium corresponding to the band 8oil-2 was related to a sulfate-reducing bacterium that degrades toluene (Küver *et al*. 2001). The sequence of the band 8oil-3 was identical to that of the *n*-alkane-degrading sulfate-reducing bacterium isolated from the *p*-xylene-degrading sulfate-reducing consortium (Higashioka *et al*. 2009) in the corresponding region. The band 28pX-1, which was dominant in the *p*-xylene-degrading consortium established at 28ºC, was related to bacteria belonging to the *Desulfosarcina-Desulfococcus* group.

The dominant band 8pX-1 was quite closely related to *Desulfobacter psychrotolerans* (Table 1), but it has not been reported whether this bacterium has the ability to oxidize monoaromatic hydrocarbons. As a result of hydrocarbon utilization test, *Desulfobacter psychrotolerans* could not grow on *p*-xylene, *o*-xylene, *m*-xylene, benzene, ethylbenzene, and *n*-hexane.

Cloning analysis of the *bamA* gene

The *p*-xylene-degrading cultures of two different temperatures were subjected to the cloning analysis targeting the *bamA* genes. In total, 30 clones each were sequenced for the cultures of two

temperatures. As a result of phylogenetic analysis, it was revealed that the clone library of 28ºC was dominated by OTUs closely related to *Desulfosarcina ovata* (23 of 30 clones, Fig. 3). The amino acid sequence of the most frequently detected OTU (SPX28-1) was identical to that of *Desulfosarcina ovata*. On the other hand, more diverse OTUs were detected from the culture of 8ºC and there was no obvious domination by specific lineage (Fig. 3). There was no OTU shared by the two libraries.

Isolation and characterization of sulfate-reducing bacteria

As an attempt to isolate organism responsible for *p*-xylene degradation, the enrichment cultures growing on *p*-xylene were inoculated into media containing benzoate. In a previous study, it was suggested that benzoate may be useful to obtain pure culture of *p*-xylene-degrading bacterium from enrichment cultures (Higashioka *et al*. 2009). Growth on benzoate was observed only in the culture of 28ºC, and the resulting benzoate-utilizing culture was subjected to the agar shake dilution. After 24-day incubation at 28ºC, an isolated colony was picked and inoculated into a liquid medium supplemented with benzoate. As a result of purity check, however, it turned out that the resulting culture contained several species of bacteria. The culture did not show capability to degrade *p*-xylene, but toluene-dependent sulfate reduction was observed in it. Thus this culture was further subjected to repeated serial dilution with toluene as a sole substrate, resulting in acquisition of a novel strain, 28bB2T. The strain 28bB2T was phylogenetically distinct from the dominant DGGE band in original *p*-xylene-degrading culture, and closely related to *Desulfosarcina ovata* with sequence similarities of 99% (1467/1475 bp) (Fig. 4). In addition, strain 28bB2T yielded amplicons of the *bamA* gene fragments. The nucleotide sequence obtained was very similar to that of *Desulfosarcina ovata*, and

deduced amino acid sequence of BamA was identical to that of *Desulfosarcina ovate* (Fig .3). The strain 28bB2T utilized toluene as an electron donor, whereas did not utilize *p*-xylene or *n*-hexane. *Desulfosarcina ovata* can utilize *o*-xylene as electron donor, but the strain 28bB2T could not grow on *o*-xylene.

The result of DGGE analysis suggested that *p*-xylene-degrading enrichment culture established at 8ºC was dominated by organism closely related to *Desulfobacter* species. To isolate organism corresponding to the DGGE band 8pX-1, the enrichment culture of low temperature was inoculated into medium containing acetate, known to be preferred by members of the genus *Desulfobacter*. In contrast to the case of benzoate, sufficient growth was observed on acetate at 8ºC. This acetate-utilizing culture was analyzed with DGGE, and it was confirmed that bacterium corresponding to 8pX-1 was further enriched in this culture (data not shown). This acetate-utilizing culture was subjected to the agar shake dilution, and the strain 8aA2 was obtained. Despite dominance of *Desulfobacter*-like organism in the acetate-utilizing culture, strain 8aA2 was closely related to *Desulfobacterium autotrophicum* HRM2, (Fig. 4) with sequence similarities of >99% (1407/1410 bp). In the PCR amplification of the *bamA* gene, no product was obtained from the strain 8aA2. The strain 8aA2 did not grow on *p*-xylene, toluene, or *n*-hexane.

Discussion

Among monoaromatic hydrocarbons, *p*-xylene is one of the most persistent chemicals (Rabus *et al.* 1996). In the present study, *p*-xylene-degrading enrichment cultures were established at 8ºC and 28ºC from crude oil-degrading enrichment cultures at the respective temperatures. This is the first report of *p*-xylene-degrading sulfate-reducing enrichment culture at low temperature.

The dominating effect of temperature on community structure has been repeatedly demonstrated in

various phylogenetic and functional groups of bacteria. On the other hand, availability of electron donor and acceptor is also known as major factor which determine distributions of bacteria. The combination of electron donor and acceptor tested in the present study, hydrocarbon and sulfate, is highly selective and only specific kinds of organisms are thought to grow under such conditions. If these restrictions are too strict and only a few kinds of bacteria can grow under that condition, structure of community comprising these limited members should remain unchanged irrespective of temperature. In the present study, however, community structures differed depending on temperature, and organisms closely related to cold-adapted bacteria were detected in the cultures of low temperature (Table 1). These results suggest that low temperature also acted as selective pressure, and thus imply presence of hydrocarbon degraders belonging to cold-adapted lineage, which cannot be dominant at moderate temperatures.

It should be taken into account, however, that detected organism did not necessarily utilize hydrocarbon and sulfate. One of the DGGE bands detected in the crude oil-degrading cultures belonged to the phylum *Bacteroidetes*, which includes decomposers of biopolymers (Kirchman 2002). At the present time, no sulfate reducer is known to belong to this phylum. A member of this lineage was also detected as a DGGE band in a sulfate-reducing enrichment culture which degraded ethylbenzene (Nakagawa *et al*. 2002). The bacteria corresponding to these DGGE bands might have utilized constituents produced by other bacteria.

In the *p*-xylene-degrading cultures of two temperatures, phylogenetically distinct bacteria were detected as the most intense DGGE bands (Fig. 2, Table 1). The DGGE band 28pX-1, dominant in the culture of 28ºC, belonged to the *Desulfosarcina-Desulfococcus* group. This group includes physiologically versatile sulfate-reducing bacteria capable of degrading a variety of complex organic compounds (Kube *et al*. 2005). As to mesophilic sulfate reducers isolated from marine environment,

strains that degrade monoaromatic hydrocarbons mostly belong to this group (Harms *et al*. 1999). The significant involvement of this lineage in degradation of monoaromatics was also suggested by the studies on enrichment cultures performed at around 28ºC (Nakagawa *et al*. 2002; Nakagawa *et al*. 2008). In contrast, dominant band in 8ºC-enriched cultures was related to *Desulfobacter* species (Table 1). In this lineage, capability of hydrocarbon degradation has not been found or suggested so far, and *Desulfobacter psychrotolerans* could utilize none of hydrocarbons tested in the present study. Although it is still unclear whether organism corresponding to the band 8pX-1 degrades *p*-xylene, there might be hydrocarbon-degrading sulfate reducers belonging to lineage missed in previous studies.

A clear difference in community structure was also observed in the results of cloning analysis of the *bamA* gene, involved in hydrocarbon degradation. A bacterium corresponding to the predominant OTU in the culture of 28ºC (SPX28-1) was successfully isolated, as the strain 28bB2T. However, the strain could not degrade *p*-xylene. Further, their amino acid sequence was also identical to that of *Desulfosarcina ovate*. The bacterium can grow on *o*-xylene, a substrate that cannot be used by the strain 28bB2T. These results indicate that OTUs defined in this study can include physiologically distinct bacteria, even though the threshold of sequence identity was set at 100% for the grouping. This finding may be very important to interpret results of *bamA*-based analysis in future studies. Anyhow, significance of the *Desulfosarcina-Desulfococcus* group in the culture of 28ºC was consistently suggested in the analysis of functional gene. In contrast, various types of *bamA* gene were detected in the culture of 8ºC dominated by *Desulfobacter*-like bacterium. Although all these OTUs belonged to the *Azoarcus/Syntrophus* cluster as with other sulfate reducer (Kuntze *et al*. 2008), microorganisms carrying these diverse *bamA* sequences could not be identified. In the culture of low temperature, *p*-xylene might be degraded by diverse organisms which were not detected in the

analysis of 16S rRNA gene because of their low respective abundances.

The strains isolated in the present study did not grow on *p*-xylene. These organisms might have survived in the enrichment cultures owing to their tolerance to toxic effects of hydrocarbons. The strain 28bB2T must have ability to resist toxicity of monoaromatic hydrocarbons, since it could grow on toluene. The strain 8aA2 was very closely related to *Desulfobacterium autotrophicum* HRM2, whose genome sequence has been determined (Strittmatter *et al*. 2009). In the genome of that strain, proteins involved in resistance to organic solvents are encoded.

Conclusion

The present study indicated temperature-dependent differences in microbial community associated with hydrocarbon degradation. The results from the culture of 28ºC were similar to those of the previous studies, but results observed at 8ºC differed significantly from them. This discrepancy may indicate that the knowledge based on experiments at moderate temperatures is not applicable to cold environments, which occupy large part of marine sediment. Further investigations under low-temperature conditions are desirable to address marine pollution by hydrocarbons.

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Table 1 Closest relatives of sequences obtained from 16S rRNA gene-targeting DGGE analysis of hydrocarbon-degrading enrichment cultures

Bands	Accession number	Closest database match		
		Taxonomic name (accession number)	Origin	Similarity
8oil-1	AB587695	Uncultured *Desulfobacula* sp. (AY177795)	Antarctic sediment	96% (496/512 bp)
8oil-2	AB587696	*Desulfobacula toluolica* (AJ441316)	Marine sediment	99% (550/551 bp)
8oil-3	AB587697	Delta proteobacterium PL12 (AB468588)	A *p*-xylene-degrading sulfate-reducing consortium	100% (515/515 bp)
28oil-1	AB587698	Bacterium Oil-K-3 (AB081536)	A crude oil-degrading sulfate-reducing consortium	94% (442/470 bp)
8pX-1	AB587699	*Desulfobacter psychrotolerans* (DQ057079)	Sediment from the northern part of The North Sea	99% (548/552 bp)
28pX-1	AB587700	Uncultured delta proteobacterium (AB121109)	Cold seep sediment	97% (536/551 bp)

Figure captions

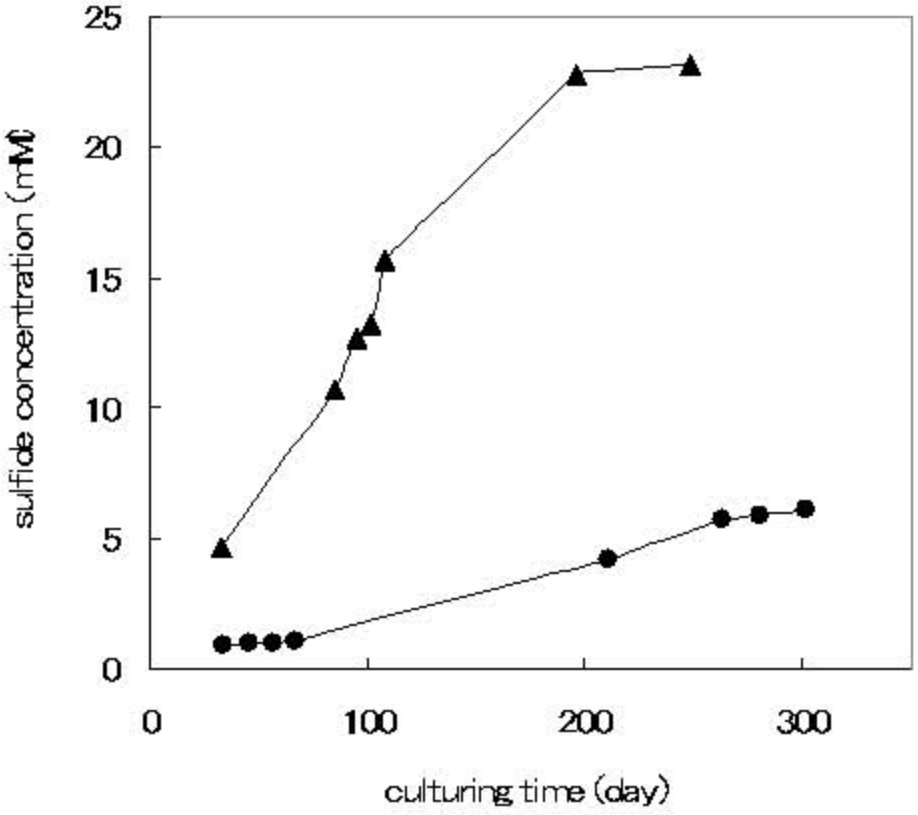
Fig. 1 Sulfide production in the crude oil-degrading enrichment cultures. Temperature: (●) 8ºC, (▲) 28ºC

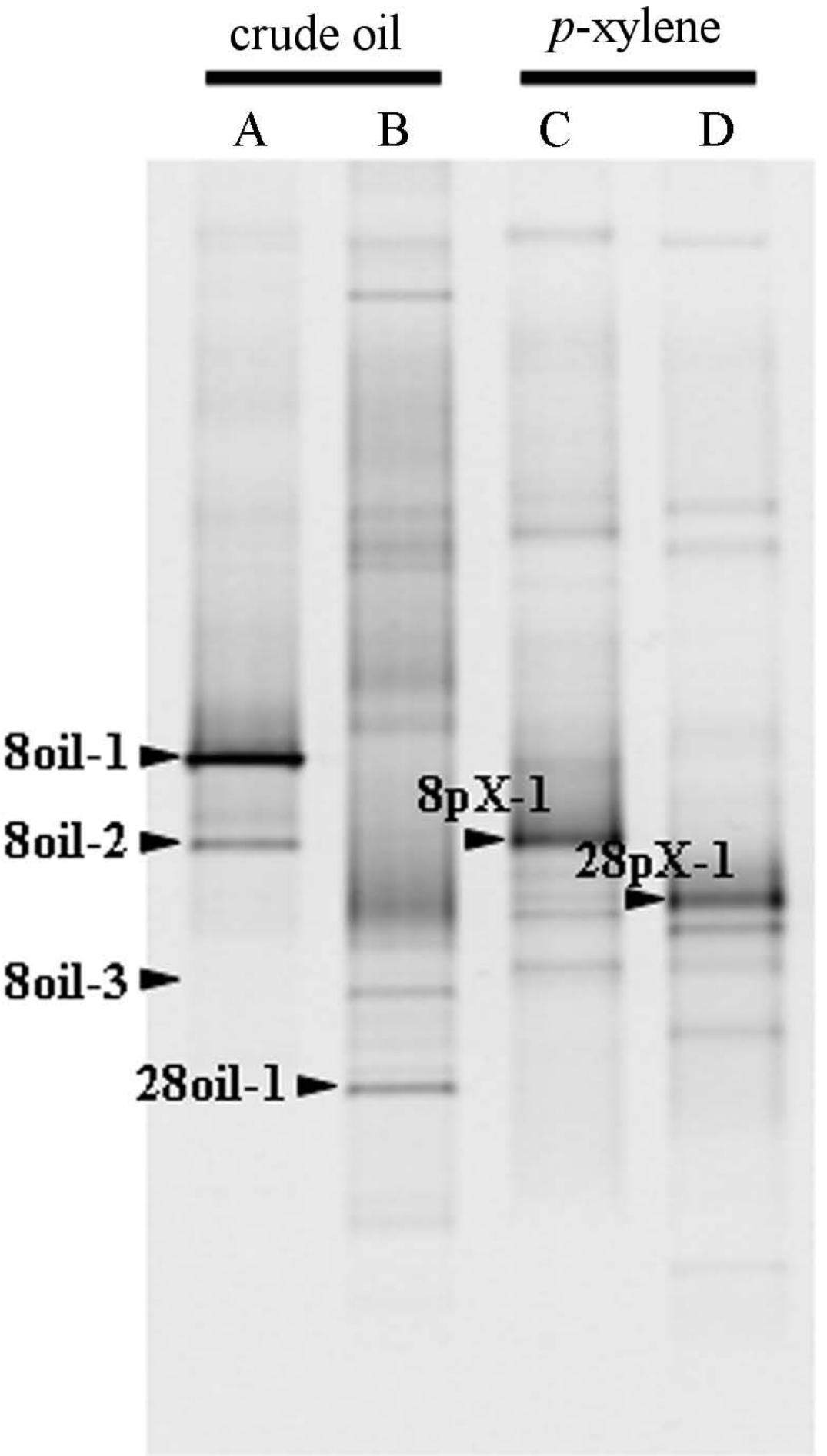
Fig. 2 DGGE profiles of 16S rRNA gene fragments of enrichment cultures. A, 8ºC-crude oil culture; B, 28ºC-crude oil culture; C, 8ºC-*p*-xylene culture; D, 28ºC-*p*-xylene culture

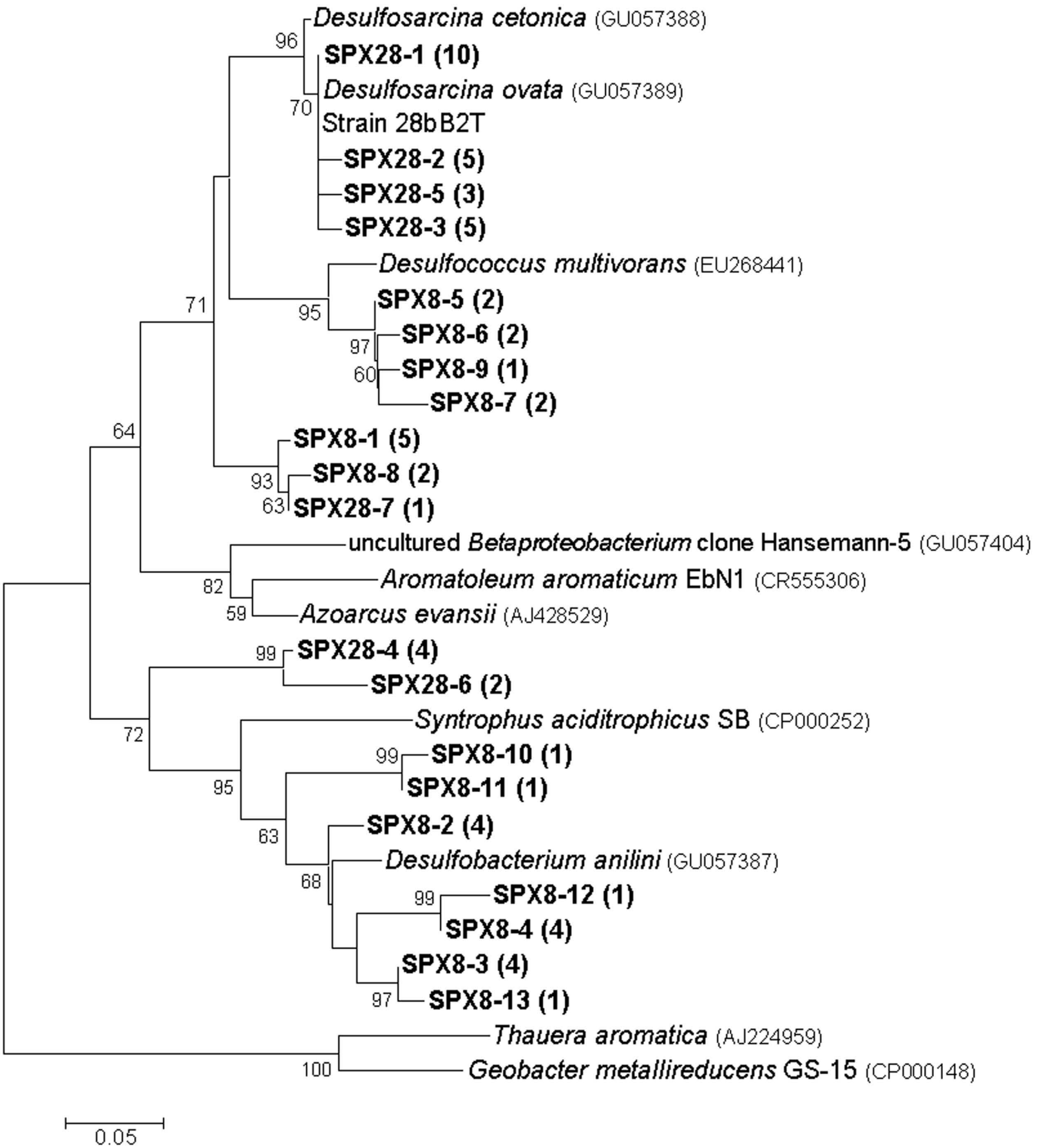
Fig. 3 Phylogenetic tree indicating the affiliations of the *bamA* clones obtained in this study. The tree was generated by the neighbour-joining methods. The scale bar represents 0.05 amino acid substitution per position. Bootstrap values greater that 50 are shown. Names of OTU prefixed “SPX8” and “SPX28” indicate OTU detected in the culture incubated at 8ºC and 28ºC respectively, and numbers in parentheses indicate number of clones belonging to each OTU.

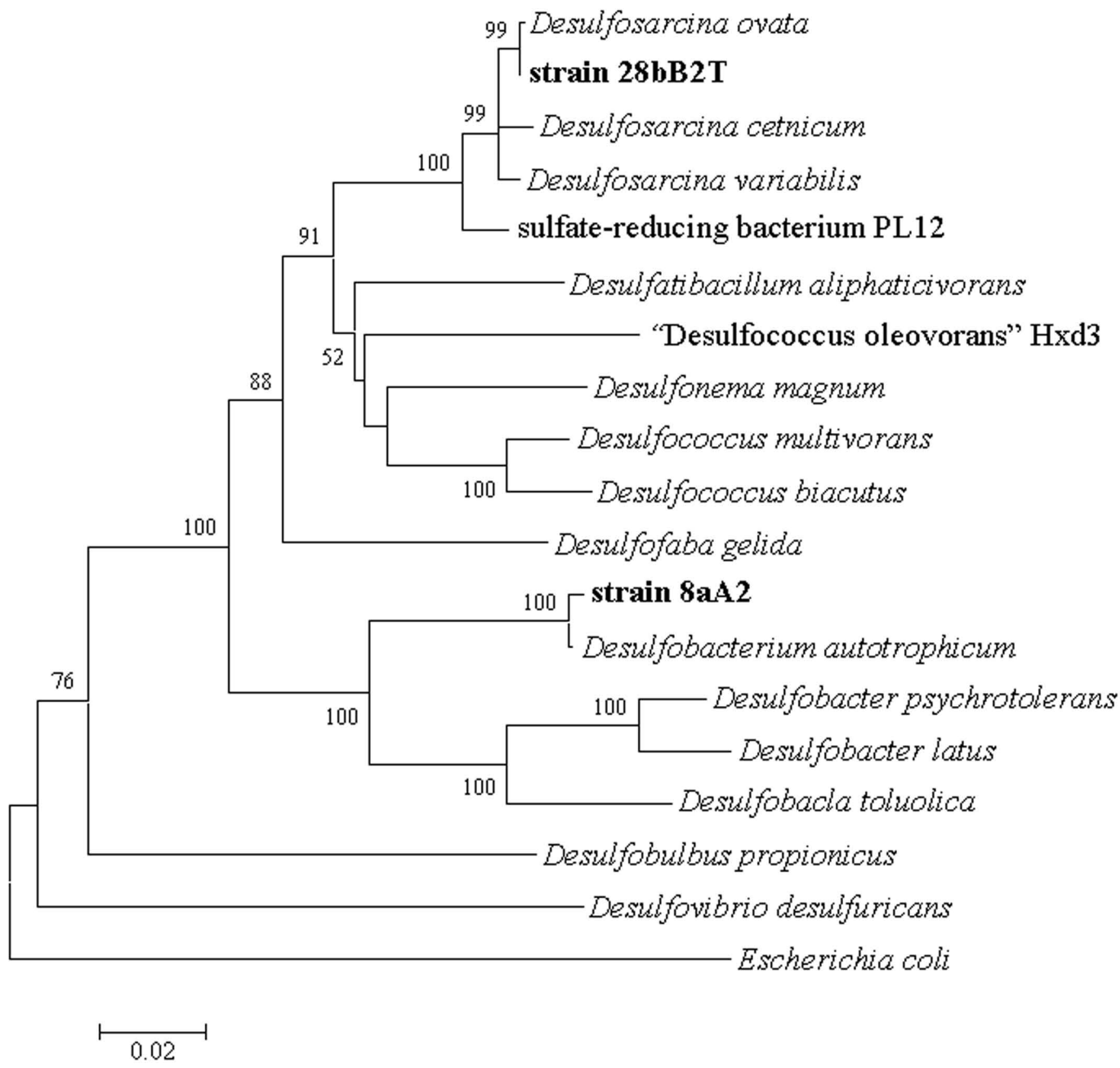
Fig. 4 Phylogenetic tree showing the affiliation of the 16S rRNA gene sequences of isolated strains within the class *Deltaproteobacteria*, with *Escherichia coli* as outgroup. The tree was generated by the neighbor-joining method. The scale bar represents 2% estimated divergence. Bootstrap values greater than 50 are shown.

Fig. 1 Higashioka *et al*.




crude oil
p-xylene
A
B
C
D
8oil-1
8oil-2
8oil-3
28oil-1
8pX-1
28pX-1


Desulfosarcina cetonica (GU057388)
96
SPX28-1 (10)
Desulfosarcina ovata (GU057389)
70
Strain 28bB2T
SPX28-2 (5)
SPX28-5 (3)
SPX28-3 (5)
Desulfococcus multivorans (EU268441)
71
SPX8-5 (2)
95
SPX8-6 (2)
97
SPX8-9 (1)
60
SPX8-7 (2)
64
SPX8-1 (5)
SPX8-8 (2)
93
63
SPX28-7 (1)
uncultured Betaproteobacterium clone Hansemann-5 (GU057404)
Aromatoleum aromaticum EbN1 (CR555306)
82
59
Azoarcus evansii (AJ428529)
99
SPX28-4 (4)
SPX28-6 (2)
Syntrophus aciditrophicus SB (CP000252)
72
99
SPX8-10 (1)
95
SPX8-11 (1)
SPX8-2 (4)
63
Desulfobacterium anilini (GU057387)
68
99
SPX8-12 (1)
SPX8-4 (4)
SPX8-3 (4)
97
SPX8-13 (1)
Thauera aromatica (AJ224959)
100
Geobacter metallireducens GS-15 (CP000148)
0.05


99
Desulfosarcina ovata
strain 28bB2T
99
Desulfosarcina cetnicum
100
Desulfosarcina variabilis
sulfate-reducing bacterium PL12
91
Desulfatibacillum aliphaticivorans
"Desulfococcus oleovorans" Hxd3
52
88
Desulfonema magnum
Desulfococcus multivorans
100
Desulfococcus biacutus
100
Desulfofaba gelida
100
strain 8aA2
Desulfobacterium autotrophicum
76
100
Desulfobacter psychrotolerans
Desulfobacter latus
100
Desulfobacla toluolica
Desulfobulbus propionicus
Desulfovibrio desulfuricans
Escherichia coli
0.02