

Short Communication

Microbial community structure of methanogenic benzene-degrading cultures enriched from five different sediments

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Due to its carcinogenicity and widespread use in industry, benzene is one of the most considerable hazardous contaminants in the environment (Salanitro, 1993). Benzene can be easily degraded by microorganisms in the subsurface environment under aerobic conditions, whereas it is much more stable under anaerobic conditions (Eweis et al., 1998). Bioremediation of benzene by aerobic bacteria is commonly applied to treat contaminated sites (Lovley, 1997). However, benzene-contaminated sites can become anaerobic because of oxygen consumption by heterotrophic bacteria. Moreover, anaerobic bioremediation should be much less costly and require less energy input than aerobic bioremediation (Vogt et al., 2011). Anaerobic benzene degradation must be a better option for soil and groundwater treatment if it can be realized.

Pure cultures of anaerobic benzene-degrading bacteria have been isolated under nitrate-reducing (Coates et al., 2001; Dou et al., 2010; Kasai et al., 2006) and iron-reducing (Holmes et al., 2011; Zhang et al., 2012) conditions. To date, no single isolates have been reported to degrade benzene under sulfate-reducing or methanogenic conditions, while benzene degradation by mixed microbial populations (consortia) has been observed under those conditions. As seen in the case of other organic contaminants, benzene is likely to be degraded by syntrophic interactions between different trophic groups of microorganisms under sulfate-reducing and methanogenic conditions (Vogt et al., 2011). Ulrich and Edwards (2003) and

Chang et al. (2005) reported dominance of members of the phylum *Firmicutes* in benzene-degrading consortia under methanogenic conditions. We have also found dominance of *Firmicutes* populations in a methanogenic benzene-degrading enrichment culture (Sakai et al., 2009). In these cultures, *Deltaproteobacteria* cells were also commonly found in abundance (Sakai et al., 2009; Ulrich and Edwards, 2003). These results suggested that methanogenic benzene-degrading microbial communities may have similar community structures, which might include indispensable organisms for benzene degradation under these conditions.

We previously reported that members of the order *Desulfobacterales* in *Deltaproteobacteria* assimilated benzene-derived carbon in a stable isotope probing (SIP) study for two different methanogenic benzene-degrading enrichment cultures (Noguchi et al., 2014; Sakai et al., 2009). In addition, the abundance of *Desulfobacterales*-related phylotypes increased in number only when benzene was added to the methanogenic benzene-degrading enrichment cultures (Masumoto et al., 2014). These studies strongly implied that the bacterial populations detected were very likely to be benzene-degrading microorganisms. However, they constituted only 2.8% of the communities, even though they had been fed only with benzene for more than three years (Noguchi et al., 2014). Other bacteria likely contribute to the benzene degradation either directly or indirectly. Additionally, the microbial community struc-

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ture remained complex and the roles of most members remain unknown. It is necessary to understand the microbial consortia that support benzene degradation in these cultures.

In this study, we established five benzene-degrading enrichment cultures from sediments sampled at four geographically different sites, and the microbial community structures of the enriched cultures and their seed sediments were analyzed using 16S rRNA gene-based amplicon pyrosequencing. The enrichment cultures were compared to find common constituents of the microbial communities. Microorganisms that showed increased abundance after enrichment were screened to identify microorganisms of importance for benzene degradation.

Muddy sediment samples were collected from four different sites in Japan as seeds for methanogenic benzene-degrading enrichment cultures between October 2007 and July 2008. Two samples were taken from paddy fields cultivating edible lotus root (lotus paddy field) at different sampling times in Tsuchiura, Ibaraki (for cultures TA and TB). Another sample was obtained from a lotus paddy field in Iwakuni, Yamaguchi (for culture IW). River sediments were collected from Shinshibakawa River (for culture SN) in Kawaguchi, Saitama, and lake sediments were collected from Lake Tega (for culture TE) in Abiko, Chiba. The sampling location for each enrichment culture is shown in Fig. S1.

Replicate sediment cultures for each sediment were prepared in serum bottles by suspending sediment in sterilized ultrapure water in the ratio of 0.5–1 g wet sediment/ml water. The headspace was filled with $\text{CO}_2:\text{N}_2 = 1:4$ mixed gas or $>99.9\%$ N_2 gas. Both Na_2S and L-cysteine were added as reducing agents at a final concentration of 0.3 g l^{-1} . The bottles were sealed with Teflon-coated butyl rubber stoppers (Maruemu, Osaka, Japan) and aluminum crimp caps (Maruemu). Benzene was added at $0.1\text{--}100 \text{ mg l}^{-1}$ to the cultures. Abiotic control samples were prepared for each incubation condition by autoclaving samples at 121°C for 60 min on 3 consecutive days. The cultures were incubated, in the dark, at 25°C without shaking. During the enrichment process, benzene was replenished to $20\text{--}40 \text{ mg l}^{-1}$, whenever the concentrations dropped below the detection limit ($0.3 \mu\text{mol l}^{-1}$), to continue the growth and enrichment of benzene-degrading microorganisms until sample collection (days 1217–1492) and community analysis (the details of the preparation are noted in Supplementary Materials).

Benzene degradation and methane production were monitored by a gas chromatography-flame ionization detector (GC-FID: GC2010; Shimadzu Corporation, Kanagawa, Japan). The analytical method used was the same as described in Masumoto et al. (2012). The concentration of benzene in the liquid phase was calculated on the basis of Henry's coefficient (0.18 for benzene) from the headspace concentration.

Microbial community structure was analyzed by amplicon pyrosequencing targeting the 16S rRNA gene, for both the sediments before enrichment and the slurries in the enrichment cultures. Cultures were sampled at day 1,458 for cultures TA and SN, day 1,217 for cultures TB and TE, and day 1,492 for culture IW. DNA was extracted

from 0.5 g of the original sediments and 0.5–1 ml of the enrichment culture slurries, using ISOIL for Beads Beating (Nippon Gene, Tokyo, Japan). The extracted DNA was amplified using the universal primers with tag sequences to distinguish different samples. The primer targeted the V4 region of the 16S rRNA genes (*Escherichia coli* positions 519–802) of both bacteria and archaea. Polymerase chain reaction (PCR) amplification and purification of the amplicons were conducted as previously described (Noguchi et al., 2014) with slight modification (Supplementary Materials).

All amplicons from the samples before and after enrichment were pooled into four tubes with three other sample amplicons not related to this research and sent to Macrogen Japan Co. (Tokyo, Japan) to be analyzed by a Roche 454 FLX Titanium platform. Denoising and quality control of the sequence reads generated, as well as their *de novo* clustering and taxonomy assignment, were performed using Quantitative Insights Into Microbial Ecology (QIIME) ver. 1.5.0 on VirtualBox (Caporaso et al., 2010, QIIME Team, 2011, <http://qiime.org/>), as previously described (Noguchi et al., 2014). The taxonomy file and reference sequence file of the 16S Greengenes rRNA gene released in May 2013 (Greengenes, <http://greengenes.lbl.gov/>) were used to assign taxonomy for representative sequences as operational taxonomic units (OTUs).

The OTU composition of each enrichment culture was subjected to non-metric multi-dimensional scaling analysis (nMDS) to visualize dissimilarities between samples. The relative abundance of each OTU in each sample was square-root transformed, and the similarity between each pair was determined using the Bray-Curtis index. The MDS plot was drawn with the Plymouth Routines In Multivariate Ecological Research (PRIMER) 6 software package (Clarke, 1993, <http://www.primer-e.com/>) along with the data transformation and similarity calculations.

The nucleotide sequences determined in this study have been deposited under DNA Data Bank of Japan (DDBJ). The accession number for this submission is DRA003728. The BioProject referred to this research is PRJDB3983, and the BioSample referred to the samples analyzed are SAMD00034846–SAMD00034855.

In all five cultures, feeding benzene as a sole source of carbon, benzene concentrations first dropped below the detection limit ($0.3 \mu\text{mol l}^{-1}$) on days 220–350 concurrent with CH_4 production. The abiotic control showed no benzene decrease and no methane production. Benzene degradation was repeatedly observed by replenishing benzene concentrations in the cultures to approximately $20\text{--}40 \text{ mg l}^{-1}$ every 15–60 days (Fig. S2). The benzene degradation rate was the fastest in culture TB, and the slowest in culture IW. At days 1217–1492, samples were collected from each culture and subjected to community analysis.

Amplicon pyrosequencing was applied to the total DNA extracted from the original sediments before enrichment and the slurries in the enrichment cultures. On average, 38,417 and 37,472 reads, and 3,769 and 2,421 OTUs were obtained after denoising for the original sediments and the enrichment cultures, respectively. The relative abundances of the sequence reads determined by pyrosequencing of partial 16S rRNA gene amplicons were summarized at the

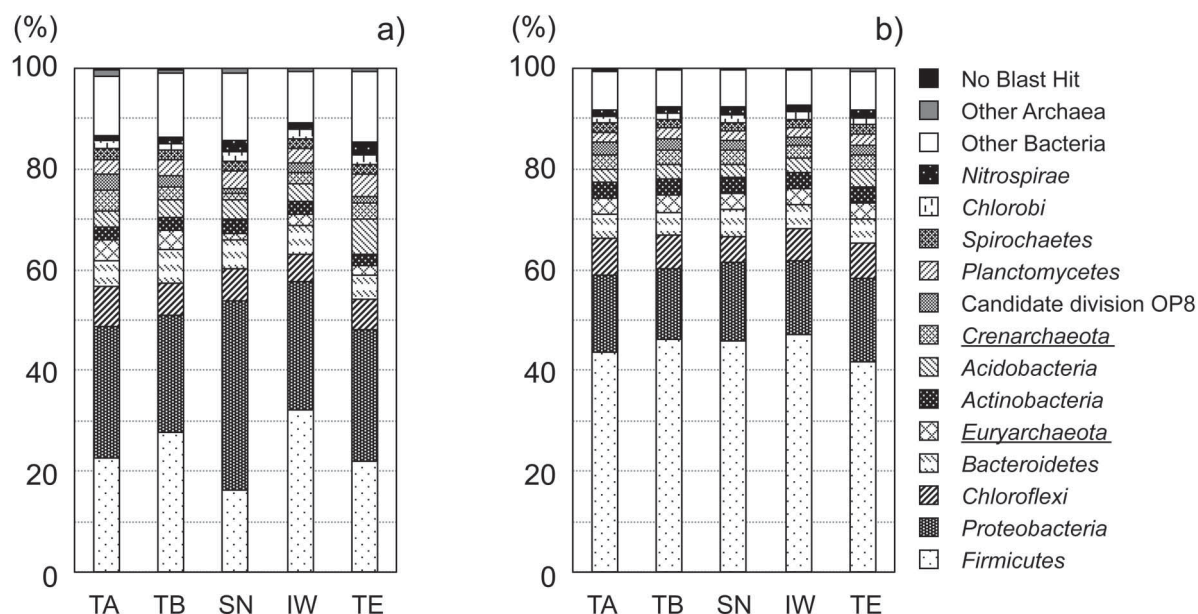


Fig. 1. Phylum level composition of five methanogenic benzene-degrading enrichment cultures and their original sediments.

Samples are (a) original sediments (before incubation), (b) benzene-degrading enrichment cultures. Phyla whose proportions were more than 1% are shown separately with symbols. Archaeal phyla are underlined in the graph legends. The abbreviations stand for culture name; TA: Tsuchiura-A, TB: Tsuchiura-B, SN: Shinshibakawa, IW: Iwakuni, TE: Teganuma.

phylum level (Fig. 1). In all of the five original sediments of the methanogenic benzene-degrading cultures (Fig. 1a), *Proteobacteria* ($27.6 \pm 5.7\%$) and *Firmicutes* ($24.2 \pm 6.1\%$) were the two most abundant phyla (average \pm standard deviation). In most samples, *Chloroflexi*, *Bacteroidetes*, and *Acidobacteria* were the third to fifth most abundant phyla, and their average relative abundances were 6.5 ± 0.9 , 5.6 ± 0.7 and $4.3 \pm 1.5\%$, respectively.

As shown by the OTU numbers above, the microbial community structures of the enriched cultures were still complex even after cultivation with benzene as a sole carbon source for more than 3 years (Fig. 1b). The average Chao1 value was calculated as 3555.9 ± 451.8 for the original sediments and 2263.5 ± 121.3 for the enriched cultures when 9580 sequences were sampled. The community structures after enrichment closely resembled each other and were less different from each other than from the original sediments. The populations of the four most abundant phyla, *Firmicutes*, *Proteobacteria*, *Chloroflexi*, and *Bacteroidetes*, appeared in the same order of relative abundance in all five cultures. Their average relative abundances were 44.9 ± 2.2 , 15.2 ± 0.9 , 6.4 ± 0.7 , and $4.8 \pm 0.3\%$, respectively. The sequence data suggested that the relative abundance of members of the *Firmicutes* increased during enrichment, while that of *Proteobacteria* decreased. Some *Firmicutes* should have important roles in the benzene-degrading enrichment cultures.

To evaluate the similarity of community structures of the five enrichment cultures and the five original sediments, nMDS plots were drawn based on the community composition at the OTU level (Fig. 2). While any chosen pair of the five enriched cultures showed a greater than 50% similarity, the original sediments showed a similarity slightly greater than 30%. The nMDS analysis clearly demonstrated that the microbial community structures became more similar to each other after the enrichment

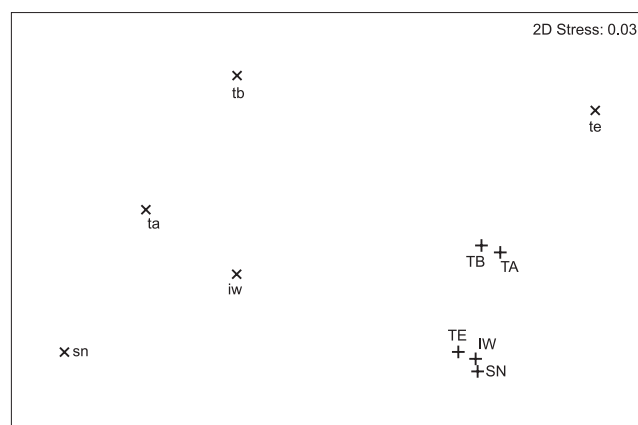


Fig. 2. Non-metric multi-dimensional scaling plot showing the similarity of methanogenic benzene-degrading enrichment cultures and their original sediments in two-dimensional space (stress = 0.03).

Lowercase letters indicate the original sediments (ta, tb, sn, iw, te), and capital letters indicate the enriched cultures (TA, TB, SN, IW, TE).

process. The similarity between the original TA and TB samples (ta and tb in Fig. 2, respectively), two cultures enriched from the lotus paddy field sediments located very near to each other but sampled on different occasions, was not much higher than the other pairs of unrelated samples. Thus, we may treat these samples as two independent samples.

The average benzene degradation rate for the last two benzene additions (for IW, until day 1378) was 0.2, 0.4, 0.2, 0.1, 0.3 mg/L/day, for TA, TB, SN, IW, TE, respectively (Fig. S2). While the degradation rate for TA, SN, and IW was about half of that for TB and TE, we could not find any clear difference in community structures.

Because the degradation of benzene became well estab-

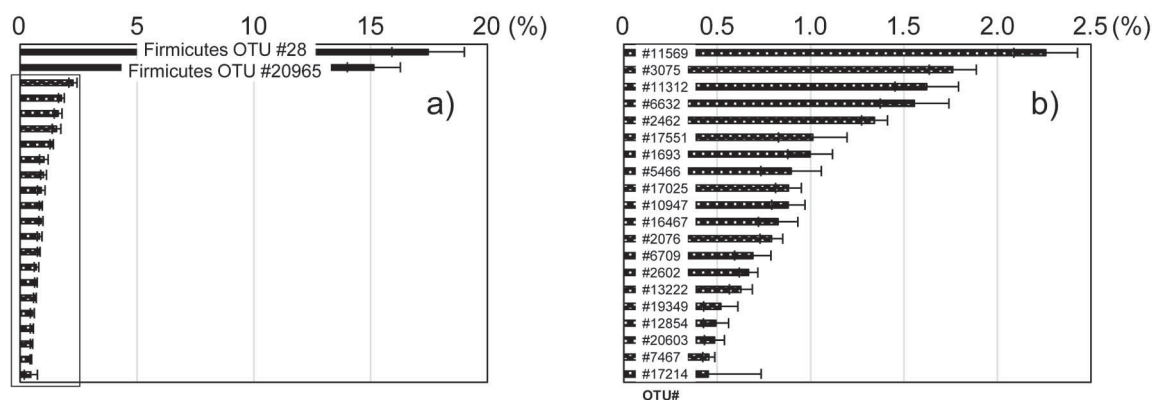


Fig. 3. The average relative abundance of major OTUs in methanogenic benzene-degrading enrichment cultures shown in the order of average relative abundance.

OTUs whose relative abundance was more than 0.5% on average in five cultures (22 OTUs) are included in the figures. (a) Two OTUs with high relative abundance (*Firmicutes* OTU, #28 and #20965) were eliminated from (b) to express the details of other minor OTUs. Error bars indicate the standard deviation of the five enrichment cultures used in this study.

lished only after the enrichment process, the microbial constituents common to the enriched cultures should represent important components of the methanogenic benzene-degrading consortia. This result indicated that microbes capable of degrading benzene, and those supporting the degradation under methanogenic conditions, were common in spite of the geographical differences in the origins of the cultures.

The relative abundance of 22 major OTUs, which were, on average, more than 0.5% in the five enriched cultures, is shown in Fig. 3a. The most and second most abundant OTU were the same for all five cultures. The most abundant OTU, OTU #28, belonged to the family *Planococcaceae* within the *Firmicutes/Bacilli* ($17.5 \pm 1.6\%$) (Fig. 3a). The most closely-related known species of the OTU were two *Sporosarcina*, *S. globispora* strain 785 (accession number: NR_029233) and *S. psychrophila* strain W16A (NR_036942), with 100% (296 bp) similarity to those in the benzene-degrading culture. *S. globispora* and *S. psychrophila*, are capable of organic acid production by fermentation (Larkin and Stokes, 1967; Nakamura, 1984). The second most abundant OTU, OTU #20965, was assigned to the family *Peptostreptococcaceae* within the *Firmicutes/Clostridia* ($15.1 \pm 1.1\%$). The most closely related known species of the *Peptostreptococcaceae*-related OTU was *Eubacterium acidaminophilum* strain DSM 3953 (NR_024922) with 99% (296 bp), which can ferment glycine and produces acetate, ammonia, and CO_2 . It can also be co-cultured with hydrogen-using sulfate-reducers, methanogens, or homo-acetogens (Zindel et al., 1988). We could not find any related species to both OTUs that were capable of using aromatic compounds and thus these strains are probably not using benzene directly.

The other OTUs accounted for less than 2.5% of the total population (Fig. 3b). The relative abundance of the other major OTUs was also very similar among the cultures. The relative standard deviation (RSD) of the relative abundances were below 0.3% for the OTUs shown in Fig. 3b. The major OTUs in the enriched cultures were almost completely common among the cultures.

The ratio of change in the relative abundances for the

major OTUs after enrichment is summarized in Table 1. The relative abundance of 21 of 22 OTUs, whose relative abundance was on average more than 0.5% in the five enriched cultures, increased through the enrichment process. These 21 OTUs accounted for 51% of the total reads, representing more than half of the total bacterial and archaeal population for the major phylotypes. The abundance of the two major OTUs in the enriched cultures, *Planococcaceae*-related OTU #28 and *Peptostreptococcaceae*-related OTU #20965, more than doubled from their abundances in the original cultures (9.1 ± 2.9 and $7.7 \pm 2.3\%$, respectively), where they were already the most abundant OTUs. In addition to the fact that the close relatives of both bacterial OTUs were organic compound fermenters, their abundance in both the original sediments and enriched cultures suggests that they are likely to be able to grow on general organic matter in sediment under anaerobic conditions. This result implies that the majority of the metabolic processes occurring in anaerobic methanogenic cultures might not be directly related to the substrate added to the cultures. Such organic matter might be produced as intermediates of the benzene degradation or as part of the original sediments or cell turnover in the enrichment culture.

The OTUs that had high rates of increase were rather minor ones. They were: OTU #17214 in the family *Clostridiaceae* of *Firmicutes*, OTU #6709 in the family *Hyphomicrobiaceae* of *Alphaproteobacteria*, and OTU #5499 in unclassified *Deltaproteobacteria*. OTU #17214, assigned to *Clostridiaceae* under *Clostridiales*, shared 100% identity with *Youngiibacter multivorans* (NR_104785, recently reclassified from *Acetivibrio multivorans*). *Y. multivorans* is a cinnamate-fermenting bacterium that can also ferment crotonate, pyruvate, acetoin, and carbohydrates (Tanaka et al., 1991). The bacteria identified as OTU #17214 were possibly involved in the fermentation of organic matter that increased after benzene addition, but it was not clear whether they were directly involved in benzene degradation. OTU #6709 was assigned to *Hyphomicrobiaceae* under *Rhizobiales*. Of the known isolated species of *Hyphomicrobiaceae*,

Table 1. The relative abundance ratio of OTUs before and after enrichment. OTUs whose relative abundance was more than 0.5% on average in five enrichment cultures are listed in the table.

#OTU	Phylum	Class	Order	Family	Ratio (Enriched/Original)		Abundance % (Enriched)	
					Average	SD	Average	SD
17214	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	5.1	5.0	0.5	0.3
6709	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	2.4	1.3	0.7	0.1
5466	Proteobacteria	Deltaproteobacteria	—	—	2.3	0.5	0.9	0.2
11312	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	2.2	0.7	1.6	0.2
19349	Bacteroidetes	Bacteroidia	Bacteroidales	—	2.2	0.9	0.5	0.1
20965	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	2.1	0.8	15.1	1.1
11569	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	2.1	0.6	2.3	0.2
28	Firmicutes	Bacilli	Bacillales	Planococcaceae	2.1	0.6	17.5	1.6
2602	Firmicutes	Clostridia	Clostridiales	anaerobic bacterium EtOH8	2.0	0.7	0.7	0.0
20603	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	1.9	0.5	0.5	0.1
16467	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	1.8	0.5	0.8	0.1
2462	Crenarchaeota	MCG	unidentified crenarchaeote clone pGrC26	—	1.8	0.9	1.3	0.1
1693	Proteobacteria	Deltaproteobacteria	uncultured hydrocarbon seep bacterium BPC076	—	1.7	0.8	1.0	0.1
13222	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	1.7	0.5	0.6	0.1
6632	Proteobacteria	Deltaproteobacteria	Desulfuromonadales	Geobacteraceae	1.6	0.3	1.6	0.2
3075	Bacteroidetes	Bacteroidia	Bacteroidales	—	1.6	0.3	1.8	0.1
17025	Euryarchaeota	Thermoplasmata	group E2	DHVEG-1	1.5	0.7	0.9	0.1
12854	Bacteroidetes	Bacteroidia	Bacteroidales	—	1.5	0.6	0.5	0.1
7467	Spirochaetes	Spirochaetes	Spirochaetales	Spirochaetaceae	1.3	0.2	0.5	0.0
10947	Acidobacteria	Acidobacteria-6	clone CCU21	—	1.2	0.2	0.9	0.1
17551	Chloroflexi	bacterium Ellin6529	—	—	1.1	0.1	1.0	0.2
2076	Candidate division OP8	OP8_1	clone SHA-124	—	0.9	0.5	0.8	0.1

Pseudolabrys taiwanensis, *Rhodoplanes elegans*, and *Rhodoplanes roseus* were the most closely-related strains to OTU #6709. *P. taiwanensis* and two members of *Rhodoplanes* are capable of the utilization of organic acids and denitrification (Hiraishi and Ueda, 1994; Kämpfer et al., 2006). The function of bacteria included in OTU #6709 could not be determined from the physiology of bacteria that were phylogenetically close to this OTU. The third OTU, which had a high similarity with the *Desulfobacterales*-related phylotypes, was the same as the previously determined putative benzene degrader (Noguchi et al., 2014; Sakai et al., 2009). This phylotype was identified as the most abundant strain that assimilated ^{13}C -labeled carbon when ^{13}C -benzene was dosed into the methanogenic benzene-degrading enrichment culture SN using SIP (Noguchi et al., 2014). Moreover, this OTU had a high similarity with a partial sequence of the 16S rRNA gene of clone OR-M2, which was consistently detected in over ten methanogenic benzene-degrading enrichment cultures derived from two petroleum contaminated sites in North America (Luo et al., 2015). We found the same phylotype in all of our enrichment cultures. The putative benzene-degrading bacteria are quite ubiquitous in the environment and are very important for methanogenic benzene degradation. The increase in relative abundance of this OTU by enrichment supported the findings from our previous studies (Noguchi et al., 2014; Sakai et al., 2009).

In this study, the microbial community structures of five anaerobic benzene-degrading enrichment cultures were compared with those of the original seed sediments. As a result, all of the community structures became very similar to each other after enrichment. In addition, abundance of almost all major OTUs, which were also common among the cultures, increased along with the development of benzene-degrading communities. In these OTUs, *Desulfobacterales*-related bacterial OTU, previously determined putative benzene degraders, were included. In future, the roles of commonly found bacteria in benzene-degrading enrichment culture should be further investigated. Some can also be used as members of co-cultures when the putative benzene degrader is further enriched and purified.

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Supplementary Materials

Supplementary figures are available in our J-STAGE site (<http://www.jstage.jst.go.jp/browse/jgam>).

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