Journal of Bioscience and Bioengineering
VOL. 127 No. 1, 45–51, 2019

www.elsevier.com/locate/jbiosc

Microbial community structure in deep natural gas-bearing aquifers subjected to sulfate-containing fluid injection

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Received 28 December 2017; accepted 18 June 2018
Available online 3 August 2018

In the natural gas field located in central Japan, high concentrations of natural gases and iodide ions are dissolved in formation water and commercially produced in deep aquifers. In the iodine recovery process, the produced formation water is amended with sulfate, and this fluid is injected into gas-bearing aquifers, which may lead to infrastructure corrosion by hydrogen sulfide. In this study, we examined the microbial community in aquifers subjected to sulfate-containing fluid injection. Formation water samples were collected from production wells located at different distances from the injection wells. The chemical analysis showed that the injection fluid contained oxygen, nitrate, nitrite and sulfate, in contrast to the formation water, which had previously been shown to be depleted in these components. Sulfur isotopic analysis indicated that sulfate derived from the injection fluid was present in the sample collected from near the injection wells. Quantitative and sequencing analysis of dissimilatory sulfite reductase and 16S rRNA genes revealed that sulfate-reducing bacteria (SRB), sulfur-oxidizing bacteria, and anaerobic methanotrophic archaea (ANME) in the wells located near injection wells were more abundant than those in wells located far from the injection wells, suggesting that fluid injection stimulated these microorganisms through the addition of oxygen, nitrate, nitrite and sulfate to the methane-rich aquifers. The predominant taxa were assigned to the ANME-2 group, its sulfate-reducing partner SEEP-SRB1 cluster and sulfur-oxidizing Epsilonproteobacteria. These results provide important insights for future studies to support the development of natural gas and iodine resources in Japan.

[**Key words:** Amplicon pyrosequencing; Deep biosphere; Microbial corrosion; Sedimentary saline aquifer; Sulfate reduction; Sulfur oxidation]

The largest natural gas deposit in Japan, where gas components are dissolved in formation water, is located in and around the center of the Boso Peninsula. The reservoir rocks were deposited in deep marine environments during the Plio-Pleistocene period (1,2) and are separated from surface inputs unless they are subject to the infiltration of meteoric water or targeted for borehole drilling. Natural gas is composed mostly of biogenic methane (99% of the total gases) and is not associated with oil (3,4). Gas-associated formation water originated as paleo-seawater and is characterized by higher concentrations of bicarbonate, bromide, iodide, ammonium ions and total organic carbon, a comparable concentration of chloride, and an almost complete depletion of nitrate and sulfate in comparison with present-day seawater (4). Our previous study revealed the presence of diverse viable methanogens and suggest that the aquifers in this gas field are regarded primarily as methanogenic environments (4). In this gas field, iodide ions are also recovered as molecular iodine for industrial use, accounting for approximately 80% and 30% of the total production in Japan and around the world, respectively (5,6).

During the iodine recovery process, sulfuric acid and sodium hypochlorite are added to the formation water so that iodide ions are oxidized to molecular iodine ($2I^- + NaClO + H_2SO_4 \rightarrow I_2 + Na^+ + Cl^- + SO_4^{2-} + H_2O$). After the recovery process, the formation water (referred to as waste fluid) is reinjected into aquifers through reinjection wells to alleviate subsidence problems. Because the waste fluid should contain a high concentration of sulfate, this injection may affect the microbial communities in deep sedimentary aquifers. Although aquifers formed in sedimentary environments are known to be major habitats for terrestrial subsurface environments (7), diversity and compositions of microorganisms involved in sulfur cycle is still poorly characterized. Understanding natural-gas-reservoir microbiology is also important from the perspective of natural gas and iodine development because sulfate-containing fluid injection may lead to reservoir souring, i.e., an increase of sulfide concentration in the production water by microbial activities, which may cause pipeline corrosion (8). Lim et al. (9) reported that the introduction of sulfate-amended waste fluid can cause bioclogging and the corrosion of the surface gas infrastructures possibly due to the hydrogen sulfide produced via microbial sulfate reduction. However, its effect on the microbial

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https://doi.org/10.1016/j.jbiosc.2018.06.013

community in deep subsurface gas-bearing aquifers has not been explored.

In this study, we examined the microbial communities involved in sulfur cycling in gas-bearing aquifers subjected to the injection of sulfate-containing waste fluid by performing molecular analyses, specifically 454 pyrosequencing of 16S rRNA genes and Sanger sequencing of dissimilatory (bi)sulfite reductase (*dsr*) genes, coupled with geochemical analyses. The formation water was collected from gas-bearing aquifers through gas production wells, namely, GOT1, GOT2, GOT3, GOT4, GOT5 and AOY3 (4). The data from these samples were comparatively examined with respect to distance from the reinjection wells (Fig. 1).

MATERIALS AND METHODS

The formation water samples were collected from the aquifers at 6 production wells, namely, GOT1, GOT2, GOT3, GOT4, GOT5 and AOY3 (Fig. 1), each of which was screened as being somewhere between 180 and 930 m deep. The samples for environmental sequencing, geochemical and sulfur isotopic analyses were collected at the same time. The DNAs extracted from the water samples in this study for the bacterial 16S rRNA and *dsrAB* gene analyses were the same as those in Katayama et al. (4) for the archaeal 16S rRNA gene analysis, in which 4 L of water samples was used to DNA extraction. The aquifers consist of repeating sequences of turbidite (alternating beds of sandstone and mudstone) in the Kokumoto, Umegase, Otadai and Kiwada formations of the Plio-Pleistocene Kazusa group (1). The electrical logging data revealed the lateral continuity of each sand layer with a slight dip (3°) toward the west-northwest (1), indicating that the sand aquifers are continuously distributed in the studied area.

The waste fluid was collected from a tank from which it is pumped to reinjection wells located <700 m from GOT1, GOT2, GOT4 and GOT5, <1.3 km from GOT3 and <8 km from AOY3 (Fig. 1). Approximately 100 kL of waste fluid per day per reinjection well had been continuously reinjected into the aquifer for more than 40 years. Through these reinjection wells, waste fluid is reinjected into the aquifers that are also the targets of gas-associated water production from GOT1-5. The meteoric water was collected from a well (approximately 8 m deep) located near the GOT1 well (<50 m). These water samples (2 L) were subjected to geochemical analysis.

Geochemical analysis The chemical composition of the waste fluid samples was measured as described by Katayama et al. (4).

Stable isotopic analysis of dissolved sulfate in the formation water, waste fluid and meteoric water samples was performed immediately after sample collection. Sulfate was extracted through cation-exchange chromatography and concentrated by evaporation. A $BaCl_2$ solution was then added to form a barium sulfate precipitate, which was wrapped in a tin capsule containing V_2O_5 powder and subjected to sulfur isotope ratio measurement using an elemental analyzer/isotopic ratio mass spectrometer (IsoPrime EA; GV Instruments, Manchester, UK). The sulfur isotopic composition is expressed in the standard δ-notation given by $\delta^{34}S = (R_{\text{sample}}/R_{\text{standard}} - 1) \times 1000$, where R is $^{34}S/^{32}S$. Values are expressed in

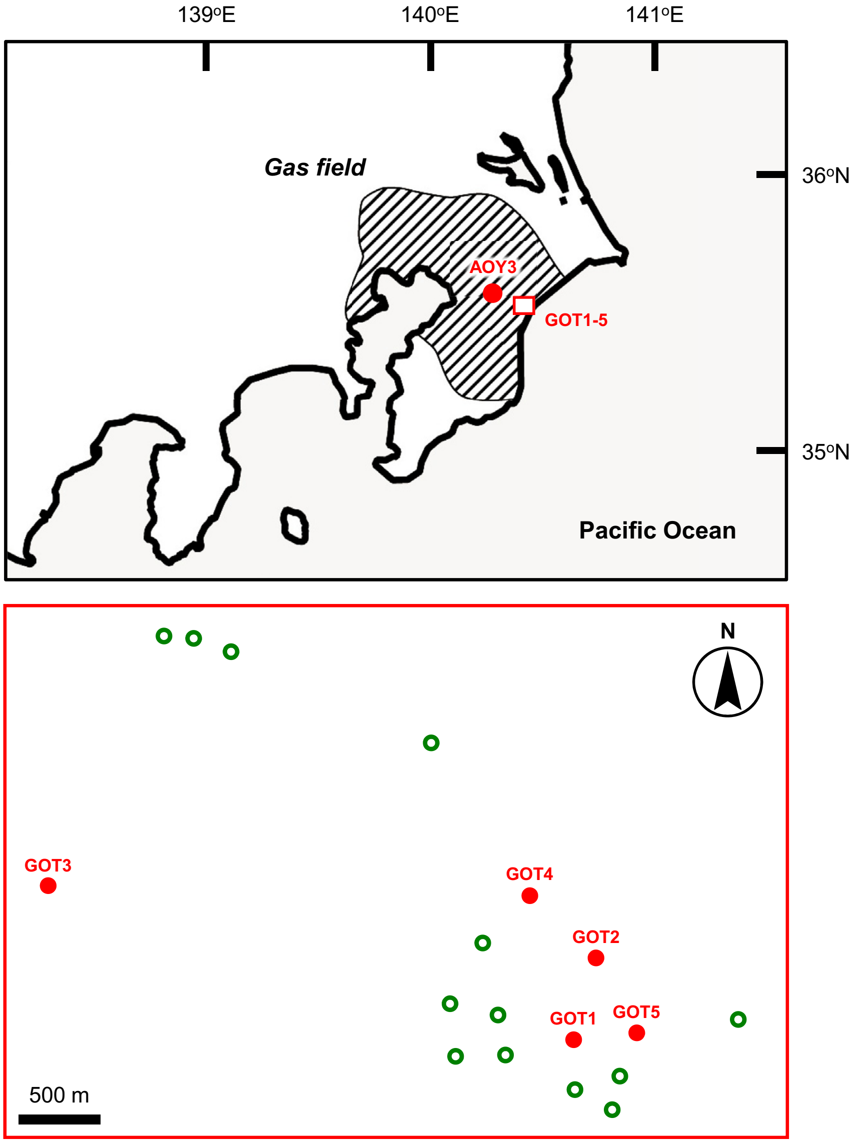

FIG. 1. Maps of the study site and geographical relationships between the sampled production wells (closed red circle) and reinjection wells (open green circle). The distribution of the gas field is adapted from Yazaki (38).

permille (‰) deviation from the Vienna Canyon Diablo Troilite (VCDT) standard, and the analytical reproducibility is estimated to be ±1‰ (10).

Quantitative analysis of the 16S rRNA and *dsrA* genes Bacterial and archaeal 16S rRNA genes were quantified by real-time PCR with the quenching probe method (11) using Titanium Taq DNA polymerase (Takara, Otsu, Japan) and a Rotor-Gene Q real-time cycler (Qiagen, Valencia, CA, USA). The sequences of the primers and probes used in this study are described in Table S1. The cycling conditions were initiated with 93 °C for 2 min, followed by 50 cycles of 93 °C for 15 s, 61 °C for 20 s and 72 °C for 25 s. The partial sequence of *dsrA* gene (approximately 1300 bp) was quantified by real-time PCR with the SYBR green method using SYBR Premix Ex-Taq II (Takara). The length of amplified *dsrAB* genes was approximately 200 bp. The cycling conditions were initiated with 95 °C for 30 s, followed by 50 cycles of 95 °C for 5 s, 60 °C for 10 s and 72 °C for 20 s. Ten-fold serial dilutions of the target PCR products of *Escherichia coli* K12 (ATCC 10798) (for the bacterial 16S rRNA gene), *Methanobacterium bryantii* M.o.H. (ATCC 33272) (for the archaeal 16S rRNA gene) and *Desulfovibrio desulfuricans* subsp. *desulfuricans* (NBRC 13699) (for the *dsrA* gene) were also amplified to calculate gene copy numbers.

454 pyrosequencing of bacterial 16S rRNA amplicons The V3 and V4 regions of the bacterial 16S rRNA genes were amplified using Phusion High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA, USA), Bac805R primer (Table S1) fused to 454-specific adaptor A containing 6-bp barcode sequences and Bac338F primer (Table S1) fused to adaptor B. The cycling conditions were initiated with 98 °C for 2 min, followed by 30–35 cycles of 98 °C for 10 s, 50 °C for 20 s and 72 °C for 30 s, and a final extension period of 7 min at 72 °C. The products of five replicate PCRs for each sample were pooled and purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). Pyrosequencing then was performed using a 454 Life Sciences GS FLX Titanium platform (Roche, Basel, Switzerland) at Hokkaido System Science Co., Ltd. (Sapporo, Japan).

Cloning and Sanger sequencing of the *dsrAB* genes The partial sequence of *dsrAB* genes (approximately 2500 bp) was amplified with AmpliTaq Gold 360 DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA) using the primer pair Dsr619AF and Dsr1905BR (12) (Table S1). The length of amplified *dsrAB* genes was approximately 1300 bp. We used the dsrAB genes for this phylogenetic analysis, but not the dsrA gene, because of their longer sequence length of the formers. The cycling conditions were initiated with 95 °C for 10 min, followed by 30–35 cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 90 s, and a final extension period of 7 min at 72 °C. The PCR products were purified via agarose gel electrophoresis using the Wizard SV Gel and PCR Clean-Up System, cloned into the pCR4-TOPO vector (Thermo Fisher Scientific), transformed and sequenced with the dideoxynucleotide chain-termination method using BigDye terminator reagents (Thermo Fisher Scientific) and an automated sequence analyzer (3730 DNA Analyzer, Thermo Fisher Scientific) according to the manufacturer's instructions.

Sequence analysis The 454 pyrosequencing reads, including the archaeal 16S rRNA gene pyrosequencing reads (DRA001051), which were previously sequenced from the same DNA samples as those for bacterial 16S rRNA gene sequences (4), were analyzed using Mothur ver. 28 (13) as described previously (4). The quality-filtered sequences were classified using a Bayesian classifier based on the Silva taxonomy SSU Ref NR 99 release 123 dataset (14) with a confidence threshold of 80%. The putative sulfate-reducing and sulfur-oxidizing bacteria and anaerobic methanotrophic archaea (ANME) were searched based on this taxonomic information.

The Sanger sequences of the *dsrAB* gene were translated *in silico* to their corresponding amino acid sequences, which were then aligned using ClustalW in MEGA 6 (15). Nucleotide sequences with >97% sequence similarity were treated as an Operational Taxonomic Unit (OTU). Neighbor-joining and maximum-likelihood trees were constructing using MEGA and TREEFINDER (16), respectively.

The 454 pyrosequencing data for the bacterial 16S rRNA genes were submitted to the DNA Data Bank of Japan (DDBJ) Sequence Read Archive database under accession no. DRA002430. The *dsrAB* gene sequences were deposited into GenBank/The European Molecular Biology Laboratory (EMBL)/DDBJ under accession nos. AB855991 to AB856022.

RESULTS

Water geochemistry The geochemical characteristics of the waste fluid analyzed in this study are shown in Table S2, together with those of the formation water that had been previously analyzed (4). The concentrations of sulfate, nitrate and nitrite in the waste fluid samples were approximately 50- to 500-fold higher than those in the formation water samples. The redox potential of the waste fluid was also much higher than that of the formation water.

To specify the source of sulfate in the formation water, we measured the stable sulfur isotope ratios of the dissolved sulfate ($\delta^{34}S\text{-}SO_4^{2-}$) in the formation water, waste fluid, and meteoric water. We could not obtain data for the formation water from GOT1, GOT2, GOT3, GOT4 and AOY3 due to its low sulfate concentrations and/or the presence of organic matter, which inhibited the measurements. The $\delta^{34}S$ value of the formation water from GOT5 was −3.8‰, which was almost identical to that of the waste fluid (−3.4‰) but markedly different from that of the meteoric water in this area (+10.5‰) and the surface seawater (approximately +20‰) (17).

Microbial population The copy numbers of the bacterial and archaeal 16S rRNA genes and the *dsrA* genes in the formation water

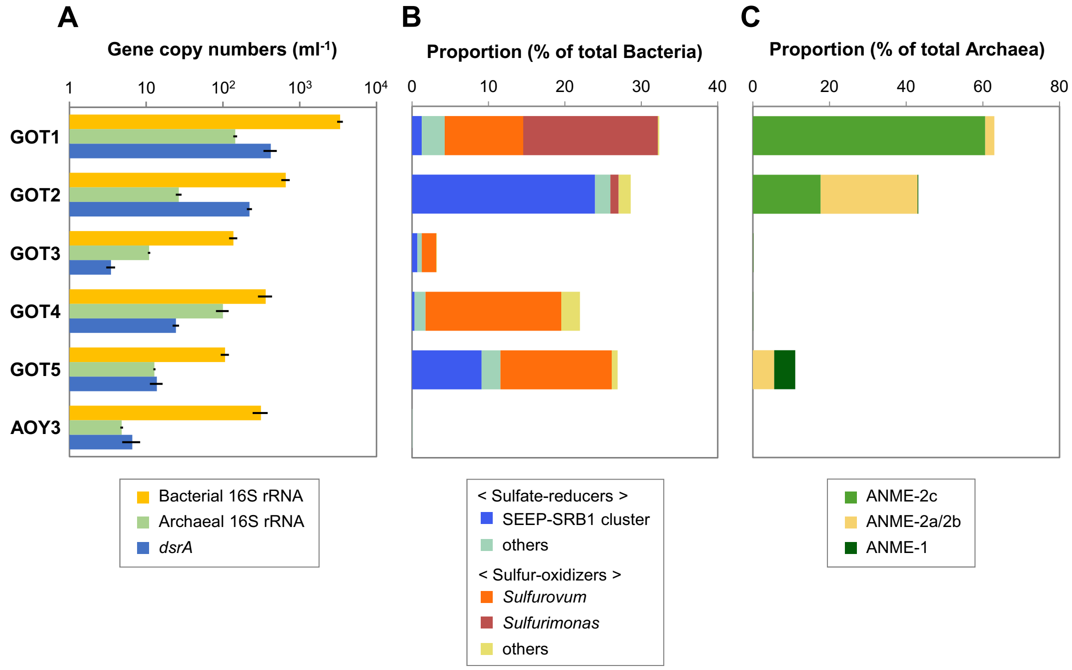

FIG. 2. Enumeration of the 16S rRNA and *dsrA* gene copy numbers (A) and the distributions of the sulfate-reducing and sulfur-oxidizing bacteria (B) and anaerobic methanotrophic archaea (C) based on 16S rRNA gene amplicon sequencing of the formation water samples.

samples were measured using quantitative real-time PCR (Fig. 2A). In all samples, the bacterial 16S rRNA gene copy numbers were higher than those for archaea. The *dsrA* gene copy numbers for GOT3 and AOY3 were <10 gene copies/ml, whereas the other 4 GOT samples were 10-10^2 gene copies/ml.

Microbial community compositions The bacterial community structure in the formation water samples was determined via 454 pyrosequencing of the 16S rRNA gene amplicons. After quality filtering, pyrosequencing yielded 1202-5223 reads per sample. At a cutoff value of 97% similarity, a total of 2078 OTUs representing an average Good's coverage of 92%. The dominant phyla or classes ($>10\%$ of the total sequences in at least one sample) included Epsilon-, Delta-, Alpha-, Gamma-, and Betaproteobacteria, Actinobacteria, 'Atribacteria' lineage JS1 and 'Omnitrophica' (formally called candidate division OP3) (Fig. S1). In these sequences, putative sulfate-reducing and sulfur-oxidizing bacteria were found (Fig. 2B). The relative abundances of putative sulfate-reducing plus sulfur-oxidizing bacteria in GOT3 and AOY3 were lower than those in the other 4 GOT samples. The sulfate reducers were assigned to Deltaproteobacteria. Sulfate reducers in other taxonomic groups, such as Nitrospirae, Clostridia and Thermodesulfobacteria (18), were not detected. The majority belong to the SEEP-SRB1 cluster in the family Desulfobacteraceae. The SEEP-SRB1 cluster is commonly detected in methane seep sediments and has been identified as the dominant partner of anaerobic methanotrophs, the ANME-2 group (19).

The putative sulfur oxidizers were assigned to Epsilon-, Gamma- and Betaproteobacteria. Other known sulfur oxidizers, such as green and purple sulfur bacteria, were not detected. The predominant taxa were the genera *Sulfurovum* and *Sulfurimonas* in the class Epsilonproteobacteria (Fig. 2B), both of which were originally isolated from marine environments and grow chemolithoautotrophically via the oxidation of reduced sulfur compounds with oxygen, nitrate or nitrite as an electron acceptor (20,21). The 16S rRNA gene sequences related to sulfate reducers and sulfur oxidizers, such as Desulfobacteraceae and *Sulfurimonas*, were also, but rarely, recovered from a coastal aquifer, in which sulfate-containing seawater intrusion occurred (22). On the other hand, the predominance of sulfur oxidizing *Sulfurimonas* or *Sulfurovum* was observed in an aquifer of low salinity and high sulfide contents (23).

Because the sulfate-reducing partners of ANME groups were recovered from the formation water samples as a major population, we further analyzed the diversity of the ANME groups (Fig. 2C). We used the archaeal pyrosequencing reads of the 16S rRNA gene amplicons that were previously sequenced from the same DNA sample, as we did in the bacterial 16S rRNA and *dsrAB* gene analyses (4). These sequences were assigned to the ANME-2c, ANME-2a/b and ANME-1 groups. The ANME-2c group, the most abundant taxon in the studied aquifers, was previously reported to occur mostly in marine environments (24). In other aquifer environments, the ANME-1 and ANME-2a groups have also been, albeit rarely, detected, whereas the ANME-2c, the most predominant group of archaeal communities in the studied samples, has never been detected (24). The high proportions of the ANME-2 group and its sulfate-reducing partner, namely, the SEEP-SRB1 cluster, suggest the occurrence of the anaerobic oxidation of methane (AOM) coupled to sulfate reduction.

***dsrAB* gene diversity** The clone library analysis of the dissimilatory sulfite reductase gene (*dsrAB*) revealed that more phylogenetically diverse sulfate reducers were recovered compared with those deduced from the 16S rRNA gene analysis (Fig. 3). The PCR amplicons of the *dsrAB* gene were not obtained from the GOT3 and AOY3 samples, although *dsrA* gene copies were successfully quantified. This may be partially due to the differences between the primers used for the PCR amplification of the *dsrA* and *dsrAB* genes. The majority of the DsrAB amino acid sequences were assigned to Desulfobacteraceae, Desulfobulbaceae, the deeply branching clades (namely, Group IV (25) and Group V (26)), the *Desulfobacterium anilini* group and Desulfovibrionaceae. The Cluster B sequences in Desulfobacteraceae, which accounted for $>20\%$ of the clones in all samples, are frequently found in marine sediments (27,28) and are suggested to stem from sulfate reducers involved in the AOM (27). Approximately 10–35% of the clones in the GOT1, GOT2 and GOT4 samples were affiliated with the deeply branching clades, which have been frequently recovered from deep marine sediments (25,27,28).

DISCUSSION

In this study, the effect of sulfate-amended fluid injection on subsurface microbial communities was assessed based on the distance from reinjection wells because sulfate-containing fluid should diffuse horizontally along the bedding planes in gas-bearing aquifers and become diluted with the original formation water, which is depleted in sulfate. Among the six wells at which formation water samples were collected in this study, the AOY3 well is located furthest from the reinjection wells, the GOT3 well is located second furthest, and the other 4 GOT wells are located within the dense area of reinjection wells (Fig. 1).

The δ^{34}S value is useful to specify the source of sulfate in the studied samples because the sulfur used in Japanese industries is characterized by a low δ^{34}S value due to its manufacture from crude oil or base-metal sulfides and is different from that in natural sulfate (29). In fact, the δ^{34}S value in sulfate was clearly different between the waste fluid and meteoric water collected in this study. Although the δ^{34}S value could be determined only for the GOT5 sample, the isotopic data clearly indicate that the sulfate in this formation water is derived mostly from the waste fluid and that the water sample is therefore suitable for the aim of this study.

The data from the molecular gene analyses were consistent with our predictions. The absolute or relative abundances of microorganisms involved in sulfur cycling, such as sulfate-reducing, sulfur-oxidizing and anaerobic methanotrophic microbes, were lower in the GOT3 and AOY3 samples compared to those in the other 4 GOT samples, including GOT5, which suggests that the sulfate-containing waste fluid injection drives microbial sulfur cycling in the aquifers, and this effect was much lower in the aquifers located more than 1.3 km from the reinjection wells. Because the level of redox potential and the concentrations of sulfate, nitrate and nitrite in the waste fluid sample turned out to be high, the injection of waste fluid containing sulfate, oxygen, nitrate and nitrite might stimulate not only sulfate-reducing bacteria, but also sulfur-oxidizing bacteria, which can utilize oxygen, nitrate or nitrite as an electron acceptor, as described above, in the aquifers. The high concentrations of oxygen, nitrate and nitrite in the waste fluid sample may be caused by exposure to air and nitrification activity during the transportation of the produced formation water. Indeed, 16S rRNA gene sequences related to ammonia-oxidizing *Nitrosomonas* were recovered from the waste fluid sample (GenBank/EMBL/DDBJ accession no. LC383457).

Our previous study showing a low microbial population and methanogenic activity rate in the studied aquifers suggests the limited availability of carbon and energy sources other than carbon dioxide and methane (4). This situation would favor sulfate reduction coupled to AOM over sulfate reduction coupled to the oxidation of organic matter and chemolithoautotrophic sulfur oxidation over heterotrophic sulfur oxidation when sulfate is supplied via waste fluid injection. In hydrocarbon seep sediments,

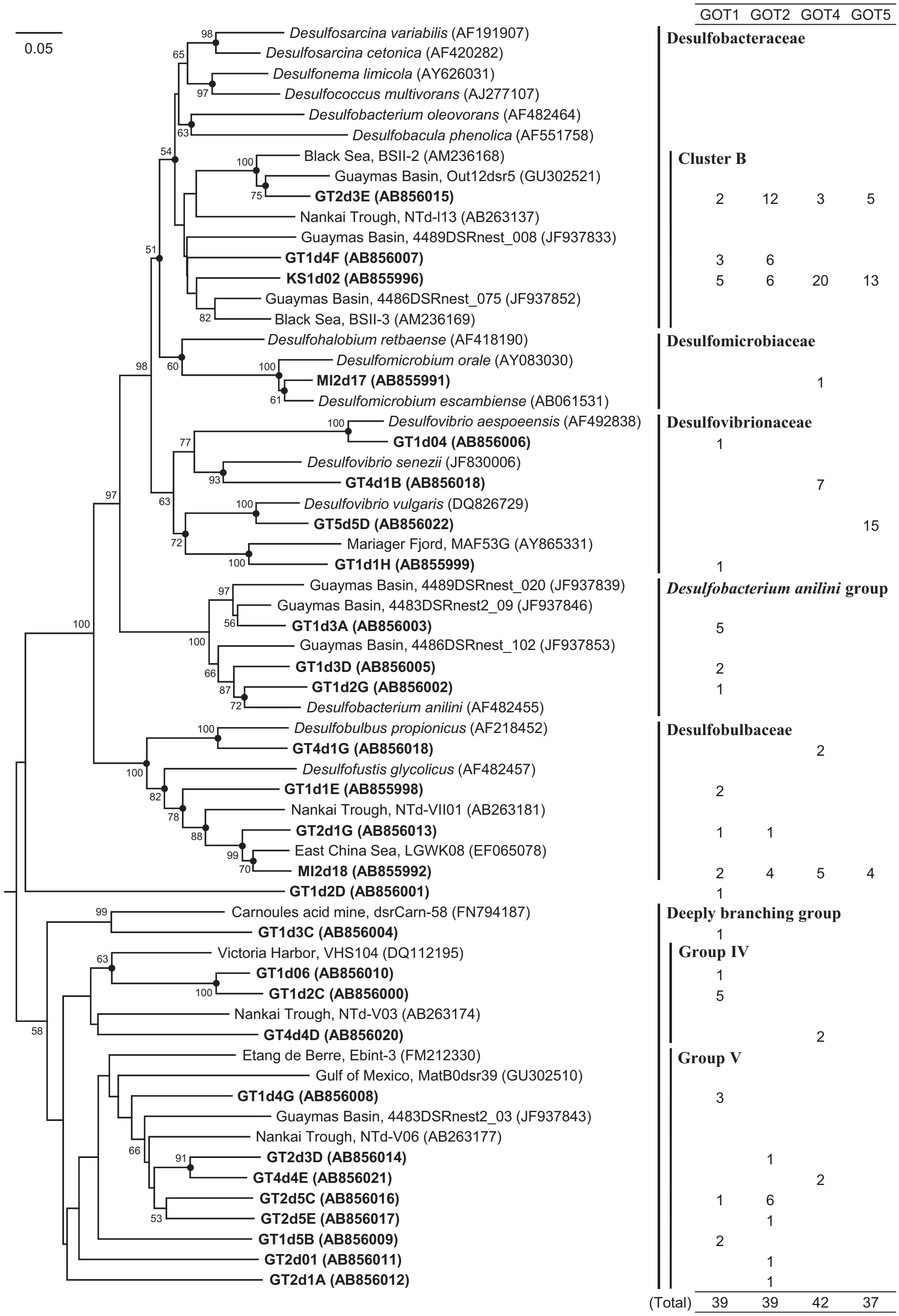

FIG. 3. Neighbor-joining tree based on the translated partial amino acid sequences of the *dsrAB* genes from the formation water (bold) and related sequences. The number of sequences in each OTU in each water sample is indicated in the right column. Filled circles indicate branches that were also found in the maximum-likelihood tree. *Thermodesulfovibrio islandicus* (AF334599) was used as an outgroup (not shown). The scale bar represents 0.05 amino acid substitutions per position.

sulfate reduction is fueled by the oxidation of hydrocarbons, making the reaction proceed faster than in the case of AOM (30). The 16S rRNA gene sequencing analysis indicated that microorganisms responsible for this hydrocarbon degradation in seep sediments belong to the *Desulfosarcina*/*Desulfococcus* clade (31), which was not detected in the studied aquifers. The adaptation of chemolithoautotrophic sulfur oxidizers to organic carbon-limited habitats was previously proposed based on their carbon fixation ability (32,33). Sulfate reducing and anaerobic methanotrophic microorganisms predominantly detected in this study were minor or absent in other aquifer environments, suggesting that the injection of waste fluid provided indigenous microbial communities in deep sedimentary aquifers with unique environmental conditions.

The marine origin of the phylogenetic taxa recovered in this study is consistent with the depositional environments of the aquifers, i.e., deep-sea environments. Previous studies have reported that the molecular signature of relict microorganisms derived from past depositional environments was maintained in buried muddy sediments with low porosity (34–36). Fredrickson et al. (36) suggested that the low permeability of subsurface muddy sediments limits the transport of substrates and microbial activities, providing conditions for the long-term preservation of microbial communities. On the other hand, high-permeable sandy sediments were not previously considered as such conditions because microorganisms are assumed to be relatively active in sand layers (7). This study is the first to show that microbial communities can also be preserved in sandy sediments that form high-permeability aquifers since their deposition in the Plio-Pleistocene period, providing novel insights into long-term survival of microorganisms in deep biosphere.

Recently, Sunaba et al. (8) reported that the corrosion of well tubing was observed in the surface part of reinjection wells in this gas field, which they suggested to be due to microbial sulfide production induced by the addition of sulfate to the waste fluid. Our findings tell us how far the effects of sulfate-containing fluid injection extend. This new information is meaningful for the further development of natural gas and iodine resources in Japan because injection wells of sulfate-containing fluid occur in many areas in this gas field. Furthermore, the ability of sulfur oxidizing microorganisms to consume dissolved sulfides is potentially useful to alleviate infrastructure corrosion (37). Thus, the occurrence of putative sulfur oxidizers found in this study are also important in this context and should be further investigated in future studies.

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jbiosc.2018.06.013.

ACKNOWLEDGMENTS

This work was supported by a grant from the Japan Oil, Gas and Metals National Corporation (JOGMEC).

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