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Application of Denaturing High-Performance Liquid Chromatography for Monitoring Sulfate-Reducing Bacteria in Oil Fields

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Sulfate-reducing bacteria (SRB) participate in microbially induced corrosion (MIC) of equipment and H_2S-driven reservoir souring in oil field sites. Successful management of industrial processes requires methods that allow robust monitoring of microbial communities. This study investigated the applicability of denaturing high-performance liquid chromatography (DHPLC) targeting the dissimilatory sulfite reductase ß-subunit (*dsrB*) gene for monitoring SRB communities in oil field samples from the North Sea, the United States, and Brazil. Fifteen of the 28 screened samples gave a positive result in real-time PCR assays, containing 9×10^1 to 6×10^5 *dsrB* gene copies ml^{-1}. DHPLC and denaturing gradient gel electrophoresis (DGGE) community profiles of the PCR-positive samples shared an overall similarity; both methods revealed the same samples to have the lowest and highest diversity. The SRB communities were diverse, and different *dsrB* compositions were detected at different geographical locations. The identified *dsrB* gene sequences belonged to several phylogenetic groups, such as *Desulfovibrio*, *Desulfococcus*, *Desulfomicrobium*, *Desulfobulbus*, *Desulfotignum*, *Desulfonatronovibrio*, and *Desulfonauticus*. DHPLC showed an advantage over DGGE in that the community profiles were very reproducible from run to run, and the resolved gene fragments could be collected using an automated fraction collector and sequenced without a further purification step. DGGE, on the other hand, included casting of gradient gels, and several rounds of rerunning, excising, and reamplification of bands were needed for successful sequencing. In summary, DHPLC proved to be a suitable tool for routine monitoring of the diversity of SRB communities in oil field samples.

Uncontrolled growth of microbes in oil field production systems may have severe negative impacts on productivity of the systems. Microbial activity may lead to increased frequency of equipment failure because of corrosion, elevated hydrogen sulfide (H_2S) concentrations, reservoir souring, formation of metal sulfide scales, filter plugging, loss of injectivity, and inefficient heat exchange (1).

Many of the microbes are indigenous to the oil-bearing deep subsurface environments, but oil production operations also introduce microbes to the system (2, 3). One of the most common and problematic groups of bacteria present in oil and gas field systems is sulfate-reducing bacteria (SRB) (4, 5). The activity of SRB is one of the most significant sources of H_2S in reservoir souring. The most frequently isolated mesophilic SRB from oil production waters belong to the genus *Desulfovibrio*, but members of the genera *Desulfacinum*, *Desulfobacter*, *Desulfobacterium*, *Desulfomicrobium*, and *Desulfotomaculum* have also been isolated (3). Thermophilic groups frequently detected from oil production waters include the bacterial genera *Thermodesulfobacterium* and *Thermodesulforhabdus* and the archaeal genus *Archaeoglobus* (6).

In order to effectively manage microbe-induced reservoir fouling, methods that allow robust monitoring of detrimental microbial communities are required. Currently, SRB in the oil field industry are mainly detected and quantified by using the culture-dependent most probable number (MPN) method which requires 28 days to obtain results (7). Furthermore, only a limited number of bacteria can be assessed by a culture-dependent approach (8). Direct culture-independent detection of SRB from community DNA using denaturing gradient gel electrophoresis (DGGE) or terminal restriction length polymorphism (TRFLP) analysis can significantly speed up microbial community profiling and process monitoring and simultaneously provide information about spatial and temporal changes in microbial community composition (9–12). However, these methods are labor-intensive, and gel-based analysis allows only a limited number of samples to be analyzed during the same assay under consistent conditions. As a result, new methods that allow automated and high-throughput analysis of process samples would be advantageous.

Denaturing high-performance liquid chromatography (DHPLC) is a method that has traditionally been used for DNA mutational analysis, especially for detection of single nucleotide polymorphisms, in medical applications. In recent years, the technique has been successfully applied to the study of bacterial diversity in various ecosystems, such as seawater (13), the gut (14), dairy products (15), soil, fermenter sludge and compost (16), sediments (17), and human infections (18–20), and to monitor fungal communities present in air (21), wood decay (22), cheese (23), and milk (24). Similar to DGGE, DHPLC can theoretically resolve DNA fragments with sequence and/or size differences. However, DHPLC is based on separation of partially heat-denatured DNA fragments by ion pair reverse-phase liquid chromatography instead of a denaturing chemical gradient. The DNA fragments are eluted from the column by an increasing gradient of acetonitrile, are detected by UV or fluorescence detector, and can be collected using an automated fraction collector for sequence analysis.

The aim of this study was to characterize SRB communities from oil field samples and to develop a DHPLC method for profiling SRB communities from oil field reservoirs.

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TABLE 1 Bacterial and archaeal strains used for method optimization and as reference strains

Phylogenetic cluster and species	Strain	Isolation source (type and location)	GenBank accession no.	GC content (%)	Fragment length (bp)	DHPLC migration (%)	DGGE migration (%)
Deltaproteobacteria/Desulfobacterales/Desulfobacteraceae							
Desulfobacter hydrogenophilus	DSM 3380	Marine mud, Italy	NA[a]	NA	NA	30	10
Desulfatiferula olefinivorans	DSM 18843/VTT E-103143	Oil-polluted sediment, France	DQ826725	61	381	61	72
Desulfobacter curvatus	DSM 3379^T/VTT E-001657^T	Marine mud, Italy	AF418199	52	350	NA	57
Desulfobacter vibrioformis	DSM 8776/VTT E-103147	Water-oil from oil platform, Norway	AJ250472	52	381	36	28
Desulfosarcina variabilis	DSM 2060^T/VTT E-001656^T	Marine black mud, France	AF191907	58	378	58	57
Deltaproteobacteria/Desulfovibrionales/Desulfovibrionaceae							
Desulfatibacillum alkenivorans	DSM 16219	Oil-polluted sediment, France	AY504426	59	377	54	57
Desulfovibrio vulgaris	DSM 644^T/VTT E-001447^T	Soil, UK	U16723	62	378	NA	57
Desulfovibrio alaskensis	DSM 16109/VTT E-103145	Gravel material from soured oil reservoir, USA	CP000112	61	353	NA	54
Desulfovibrio desulfuricans	DSM 17464/VTT E-103144	Oil well corrosion site, USA	AJ249777	61	378	57	54
Deltaproteobacteria/Desulfovibrionales/Desulfohalobiaceae							
Desulfonauticus autotrophicus	DSM 4206	Oil production water, Germany	NA	45	314	22	9
Deltaproteobacteria/Desulfovibrionales/Desulfomicrobiaceae							
Desulfomicrobium apsheronum	DSM 5918/VTT E-103146	Water, oil-bearing deposits, Russia	AF418188	59	393	58	59
Clostridia/Clostridiales/Peptococcaceae							
Desulfotomaculum geothermicum	DSM 3669/VTT E-061476^T	Anoxic geothermal ground water, France	AF273029	57[b]	NA	52, 53[c]	44
Archaea/Euryarchaeota/Archaeoglobi/Archaeoglobales/Archaeoglobaceae							
Archaeoglobus fulgidus	DSM 4304	Submarine hot spring, Italy	NC_000917	51	360	31	39

[a] NA, not available.
[b] The GC content is an estimate as the GenBank sequence entry does not cover the complete amplicon used in this study.
[c] The two values represent the two peaks in the chromatogram.

MATERIALS AND METHODS

Strains and environmental samples. Bacterial and archaeal strains or their DNA were obtained from the VTT Culture Collection (culturecollection.vtt.fi) or from the German Resource Centre for Biological Material (www.dsmz.de) (Table 1). In addition to these strains, *Desulfomicrobium macestii* DSM 4194 (VTT E-001444) was used in the DGGE ladder and quantitative PCR (qPCR) standards. The strains were chosen to represent microbial diversity detected at oil field sites or in marine environments. The strains were grown essentially as recommended by the culture collections. Injection water and samples of produced water (water recovered with the oil) ($n = 28$) were obtained from different oil field sites in the North Sea, the United States, and Brazil (Table 2). The water samples were filtered onto Sterivex-GP 0.22-μm-pore-size filter units (Millipore, Billerica, MA, USA) and shipped frozen to the laboratory.

DNA isolation. For the DNA analysis, Sterivex filter units containing the oil field samples were aseptically broken with a hammer, and the filters were cut into pieces (approximately 2.5 cm^2) with a sterile scalpel and placed with sterile forceps into the lysing tube of a DNA extraction kit. Total DNA from pure cultures and environmental samples was isolated with a FastDNA Spin kit for soil (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer's instructions, except that the cells were lysed for 2 min in a FastPrep-24 instrument (MP Biomedicals). For *Desulfobacter hydrogenophilus* (DSM 3380), *Desulfotomaculum geothermicum* (DSM 3669), and *Archaeoglobus fulgidus* (DSM 4304), a PowerSoil DNA extraction kit (MoBio Laboratories Inc., Carlsbad, CA, USA) was used according to the manufacturer's instructions, with the exception that the cells were lysed by bead beating with a Ribolyser (Hybaid) device for 30 s at 6 m s^{-1}.

Real-time quantitative PCR. Quantification of the *dsrB* copy number in the extracted DNA was performed using a LightCycler 480 instrument (Roche Diagnostics, Basel, Switzerland) and SYBR green-based detection for double-stranded DNA. An approximately 350-bp fragment of the *dsrB*

TABLE 2 Description of oilfield samples and SRB quantification results from qPCR of *dsr*B gene fragment

Sample location and code	Sample type and source	Sampling date (mo/yr)	Filtered vol (ml)	No. of *dsrB* gene copies/ml[a]	Sample selected for identification
North Sea					
MOB1	Injection water, well 1	01/2010	100	5×10^5	Yes
MOB2	Injection water, well 2	02/2010	100	4×10^4	Yes
MOB3	Injection water, well 2	04/2010	100	7×10^4	
MOB4	Injection water, well 3	05/2010	100	ND	
MOB5	Injection water, well 4	05/2010	100	ND	
MOB6-1	Produced water, well 5	01/2010	40	ND	
MOB6-2	Produced water, well 6	01/2010	40	ND	
MOB6-3	Produced water, well 7	01/2010	40	ND	
MOB6-4	Produced water, well 8	01/2010	40	ND	
MOB6-5	Produced water, well 9	01/2010	40	5×10^3	Yes
MOB6-6	Produced water, well 10	01/2010	40	ND	
MOB6-7	Produced water, well 11	01/2010	40	ND	
MOB6-8	Produced water, well 12	01/2010	40	ND	
MOB7	Injection water, well 13	11/2010	100	5×10^2	
MOB8	Produced water, well 13	12/2010	50	3×10^3	
MOB9	Produced water, well 13	12/2010	50	9×10^2	Yes
MOB10	Produced water, well 13	12/2010	50	1×10^3	
Brazil					
MOB11	Produced water, well 14	03/2011	1000	6×10^3	Yes
MOB12	Produced water, well 15	03/2011	1000	1×10^4	Yes
USA					
MOB13A	Produced water, well 16	07/2010	20	2×10^4	Yes
MOB13B	Produced water, well 16	07/2010	20	6×10^5	
MOB14	Produced water, well 17	11/2010	150	4×10^2	Yes
MOB15	Produced water, well 18	03/2011	100	ND	
MOB16	Produced water, well 19	03/2011	100	ND	
MOB20	Produced water, well 19	04/2011	20	ND	
MOB17	Produced water, well 20	03/2011	500	9×10^{1}*	Yes
MOB19	Produced water, well 20	04/2011	500	ND	
MOB18	Produced water, well 21	04/2011	500	8×10^2	Yes

[a] ND, not detected; *, extrapolated.

gene was amplified with primers dsr4R (5′-GTGTAGCAGTTACCGCA-3′) and DSRp2060F (5′-CAACATCGTYCAYACCCAGGG-3′) (11, 25). The amplification was done in a 20-μl reaction volume containing LightCycler 480 SYBR green I master mix (Roche Diagnostics), 0.5 μM each primer, and 2 μl of sample DNA. The amplification reaction program consisted of initial denaturation at 95°C for 5 min and 35 cycles with 10 s at 95°C (denaturation), 20 s at 57°C (annealing), and 20 s at 72°C (elongation). At the end of the run a melting curve analysis was performed from 65 to 97°C.

For preparation of standards for real-time PCR, the *dsrB* gene fragments from *Desulfovibrio desulfuricans* DSM 17464, *Desulfobacter vibrioformis* DSM 8776, and *Desulfomicrobium macestii* DSM 4194 were cloned into the pGEM-T vector (Promega, Madison, WI, USA) and transformed into *Escherichia coli* JM109 cells according to the manufacturer's instructions. The plasmids were purified with a QIAprep Spin Miniprep kit (Qiagen, Hilden, Germany), and the *dsrB* copy number of the plasmid preparations was calculated based on their DNA concentration, measured by a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). In every run, a dilution series of a mixture of these circular plasmids was incorporated as a standard curve. DNAs from *Pyrobaculum islandicum* DSM 4184 and *Allochromatium vinosum* DSM 180 were included as negative controls in each run (11). Each standard and sample were run as at least three replicates.

PCR amplification of *dsrB* gene fragments for DHPLC and DGGE. The same *dsrB* gene fragment as targeted in qPCR was amplified for community profiling by DHPLC and DGGE with primers dsr4R and DSRp2060F. In order to separate the *dsrB* gene fragments in DGGE, a 40-bp GC clamp was attached to the forward primer (5′-CGCCCGCCG CGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3′) (26). In the DHPLC analysis, gene fragments amplified both with and without the GC clamp were evaluated. The PCR amplification was performed in 50-μl reaction mixtures containing 1× DynaZyme II buffer (10 mM Tris-HCl, pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl, and 1% Triton X-100), 0.2 mM each deoxynucleoside triphosphate, 50 pmol of each primer, 1 U of Dynazyme II DNA polymerase (Finnzymes, Espoo, Finland), and 2 μl of template DNA. The PCR was performed in a MasterCycler thermal cycler (Eppendorf, Germany). The PCR program consisted of a 5-min initial denaturation at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, with a final elongation step at 72°C for 10 min. The amplification products were run in a 1.5% agarose gel and purified with a QIAquick PCR purification kit (Qiagen) according to the instructions of the manufacturer. The concentration of the purified PCR products was determined with a Nanodrop ND-1000 spectrophotometer (Thermo Scientific).

DHPLC. The DHPLC analysis was performed with a WAVE 4500 microbial analysis system composed of a DNASep cartridge, high-precision Peltier oven, quaternary gradient solvent delivery system, WAVE autosampler model 7200, and UV/visible light (UV/VIS) and fluorescence detectors (Transgenomic, Inc., Omaha, NE, USA). DHPLC analysis was optimized with individual and mixed *dsrB* gene products from pure cultures (Table 1) adjusted to a final concentration of 10 ng μl^{-1} by dilution in PCR-grade water. The separation of gene fragments was optimized by

varying the temperature (from 62°C to 70°C), acetonitrile gradient composition (from 46 to 70%), gradient rate (from 0.5% min^{-1} to 2% min^{-1}), and flow rate (from 0.3 ml min^{-1} to 1.2 ml min^{-1}). For pure cultures, a constant amount of 50 ng of each PCR amplification product was injected into the column, and for oil field samples 5 μl of undiluted amplification product was used. The amplification products were stained with WAVE Optimized HS staining solution during separation, and the elution of *dsrB* gene fragments was recorded with a fluorescence detector and visualized as chromatograms using Navigator software, version 3.0.0 (build 31) (Transgenomic, Inc.). All buffers and dyes were obtained from Transgenomic, Inc. Buffer A contained 0.1 M triethylammonium acetate (TEAA), and buffer B contained 0.1 M TEAA and 25% acetonitrile.

In the DHPLC analysis, the *dsrB* gene fragments were collected to chilled 96-well plates for sequencing by using an FCW 180 WAVE fragment collector (Transgenomic, Inc.) and the automated threshold function of the Navigator software (Transgenomic, Inc.). The unpurified fractions containing the collected peaks were then directly reamplified from the fractions with the corresponding primers under the same conditions as used in the original PCRs.

DGGE. DGGE analysis was performed with a DCode universal mutation detection system (Bio-Rad Laboratories GmbH, Germany). The PCR-amplified *dsrB* gene fragments containing the GC clamp were separated in a DGGE gel (160 mm by 160 mm by 1 mm) containing 8% acrylamide and 35 to 70% denaturing gradient (100% denaturing gradient contains 7 M urea and 40% formamide) in 0.5× TAE buffer (20 mM Tris, 10 mM acetate, 0.5 mM EDTA, pH 8.0) at 85 V and 60°C for 16 h. The DGGE gels were stained with SYBR green II (New England BioLabs, Ipswich, MA, USA) and imaged with a GelDoc imager (Bio-Rad, Hercules, CA, USA) under UV light. A sequence ladder consisting of PCR products from selected reference strains was run in parallel with the samples. The SRB ladder was a mixture of PCR products from *Desulfobacter hydrogenophilus* DSM 3380, *Desulfonauticus autotrophicus* DSM 4206, *Desulfobacter vibrioformis* DSM 8776, *Archaeoglobus fulgidus* DSM 4304, *Desulfotomaculum geothermicum* DSM 3669, *Desulfatiferula olefinivorans* DSM 18843, *Desulfovibrio desulfuricans* DSM 17464, *Desulfatibacillum alkenivorans* DSM 16219, *Desulfomicrobium macestii* DSM 4194, and *Desulfomicrobium apsheronum* DSM 5918 (Table 1, Fig. 1).

The bands corresponding to different *dsrB* gene fragments were excised from the DGGE gel using a Pasteur pipette and kept in 20 μl of PCR-grade water overnight at +4°C. The obtained gene fragments were then reamplified for sequencing with the corresponding primers under the same conditions as used in the original PCR. This procedure was repeated until a single band was obtained in the DGGE gel. The reamplification products were subsequently purified with a QIAquick PCR purification kit (Qiagen).

Sequencing. Sequencing of DHPLC fragments and DGGE bands was performed from both ends of the PCR amplicon with a BigDye Terminator, version 3.1, cycle sequencing kit (Applied Biosystems, California, USA) in an ABI Prism 310 genetic analyzer (Applied Biosystems);alternatively, samples were sent to Macrogen, Inc., for custom DNA sequencing using the EZ-purification service (Amsterdam, The Netherlands).

Community profiles and sequence analysis. For comparisons of the similarity of community profiles generated by DHPLC and DGGE, the chromatogram and gel data were transferred into BioNumerics, version 5.10, software (Applied Maths, Sint-Martens-Latem, Belgium). Pearson's curve-based correlations were calculated, and clustering was done with the unweighted pair group method with arithmetic mean (UPGMA). The Shannon-Wiener diversity index was calculated to compare DHPLC and DGGE profiles (27), and they were compared by one-way analysis of variance (ANOVA) using SPSS, version 19 (IBM, USA). The *dsrB* gene sequences were checked, assembled, and manually edited using the Geneious Pro software package (Biomatters, Inc., Auckland, New Zealand) or Kodon, version 3.61 (Applied Maths). The edited *dsrB* sequences (138 to 357 bp) were aligned to reference sequences (357 bp) using the MUSCLE (multiple sequence comparison by log expectation) alignment feature in Geneious Pro. The alignment was edited manually, and a maximum-likelihood phylogenetic tree was calculated on the nucleic acid sequence alignment using PhyLM (28) and a Jukes-Cantor substitution model (29). Bootstrap support for the nodes was calculated with 1,000 random repeats.

Nucleotide sequence accession numbers. The *dsrB* gene fragment nucleotide sequences of >200 bp were deposited in GenBank under the accession numbers KF269027 to KF269070.

RESULTS

SRB detection with quantitative real-time PCR. The SRB were detected with qPCR targeting the *dsrB* gene from 15 of the 28 water samples obtained from oil fields in the North Sea, the United States, and Brazil (Table 2). The qPCR assay was linear in the range of 2×10^2 to 2×10^7 *dsrB* copies per PCR. The *dsrB* gene copy numbers in the samples ranged from 10^2 to 10^5 ml^{-1}. Based on the qPCR results, 15 positive samples were selected for community analysis.

Optimization of the DHPLC protocol. Optimal conditions for the analysis of PCR-amplified *dsrB* gene fragments by DHPLC were determined with PCR amplicons from 12 sulfate-reducing bacterial strains and one archaeal strain (Table 1). Varying oven temperature, buffer flow rate, and strength and duration of the acetonitrile gradient showed that the best separation of *dsrB* gene fragments could be achieved by using an oven temperature of 65°C, a 54 to 70% acetonitrile gradient that changed 1.5% min^{-1}, and a 0.6-ml min^{-1} flow rate. The best peak separation was obtained with PCR amplicons containing the GC-rich clamp, while very poor separation was observed with gene fragments amplified without the GC clamp (data not shown).

Under the final optimized conditions, 10 strains out of the 13 tested yielded one or two major peaks in the chromatogram (Fig. 1). The first *dsrB* gene fragments to elute from the column 3 to 6 min after injection belonged to *Desulfobacter* and *Desulfonauticus* species as well as to *Archaeoglobus fulgidus*. These species had relatively low GC contents (45 to 52%) (Table 1). The gene fragments derived from *Desulfotomaculum*, *Desulfomicrobium*, *Desulfovibrio*, *Desulfatibacillum*, *Desulfatiferula*, and *Desulfosarcina* strains eluted 8 to 10 min after injection and were characterized by higher (57 to 61%) GC contents (Table 1). A significant ($P \leq 0.01$) positive correlation was observed between percent GC and retention time ($r = 0.97$). In general, the strains were distinguishable from each other based on the retention time of their corresponding *dsrB* amplicons. However, some strains had overlapping retention times. These included *Archaeoglobus fulgidus* DSM 4304 and *Desulfobacter hydrogenophilus* DSM 3380, *Desulfotomaculum geothermicum* DSM 3669 and *Desulfatibacillum alkenivorans* DSM 16219, and *Desulfosarcina variabilis* DSM 2060 and *Desulfomicrobium apsheronum* DSM 5918. The results were consistent across different runs, independent of whether the PCR-amplified gene fragments were injected to the column separately or as part of a mixture (data not shown). Three strains, *Desulfobacter curvatus* DSM 3379, *Desulfovibrio alaskensis* DSM 16109, and *Desulfovibrio vulgaris* DSM 644, could not be successfully separated by DHPLC. They produced smeared peaks in the chromatograms independent of different running parameters.

The separation of *dsrB* gene fragments in DHPLC was consistent with DGGE analysis (Fig. 1). The *dsrB* gene fragments showing low retention times on DHPLC were the ones to denature first in the DGGE analysis, whereas the ones possessing higher GC contents and retention times also migrated further in the denatur-

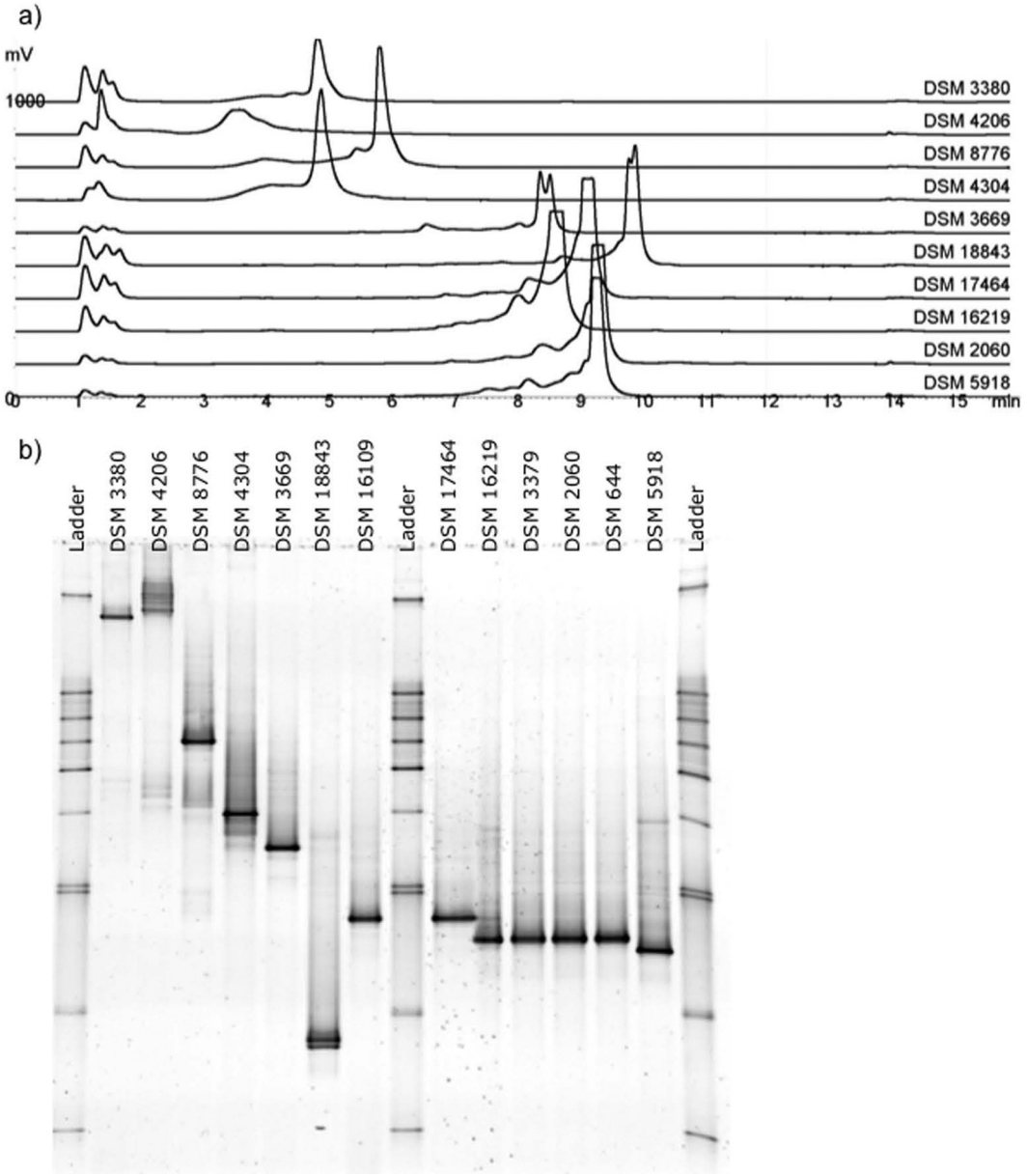

FIG 1 Separation of *dsrB* gene fragments PCR amplified from pure culture bacterial and archaeal cultures in a DHPLC chromatogram (a) and DGGE gel (b). DSM numbers are identified in Table 1.

ing gradient of the DGGE. However, among the *dsrB* gene fragments having lower GC contents, there were some discrepancies between the two methods in the order in which the *dsrB* fragments were denatured/eluted. For instance, the *dsrB* gene fragment PCR amplified from *Archaeoglobus fulgidus* DSM 4304 and *Desulfobacter hydrogenophilus* DSM 3380 eluted simultaneously in the DHPLC analysis but migrated far apart in the denaturing gel. In DGGE, strains *Desulfatibacillum alkenivorans* DSM 16219, *Desulfobacter curvatus* DSM 3379, *Desulfosarcina variabilis* DSM 2060, *Desulfovibrio vulgaris* DSM 644, and *Desulfomicrobium macestii* DSM 4194 could not be separated from each other, whereas *Desulfatibacillum alkenivorans* DSM 16219 and *Desulfosarcina variabilis* DSM 2060 were clearly distinguishable by DHPLC. Thus, the overall resolution was similar with both community profiling methods.

Community profiles of oil field samples. As shown in Fig. 2, DHPLC analysis of the *dsrB* gene profiles of the oil field samples was reproducible between different runs. Even though peak intensities varied across different runs, this had no effect on peak retention times. DHPLC and DGGE community profiles shared an overall similarity; samples with the lowest and highest diversity were detected by both methods (Fig. 3). The mean Shannon-Wiener diversity indexes were 1.17 (standard deviation [SD], 0.60) and 1.27 (SD, 0.36) for DHPLC and DGGE, respectively, and did not differ statistically significantly ($P \leq 0.1$) from each other. The clustering of SRB community profiles determined with DHPLC and DGGE shared an overall similarity with some exceptions (Fig. 4). The grouping followed the geographical location: samples MOB7 to MOB10 collected from the same North Sea site grouped together with both methods, as well as MOB2 and MOB3 from

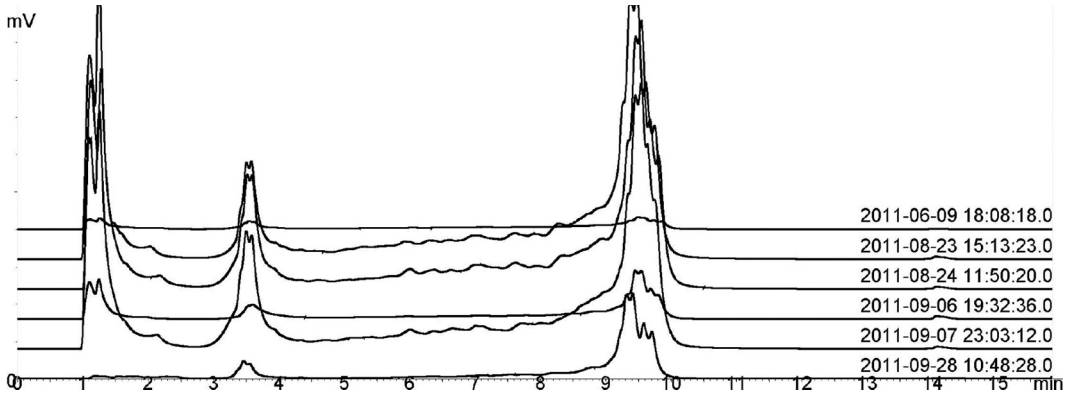

FIG 2 Replicate measurements of sample MOB3 in DHPLC. Samples are identified by date and time on the right side of the graph.

another North Sea site and MOB13A and -B from the same produced water in the United States.

One sample from each of these sample sets having similar profiles was selected for further analysis; i.e., altogether 10 samples were selected for identification of DHPLC fractions and DGGE bands by sequencing (Table 2). Sequencing of a total of 37 DHPLC fragments was attempted, and 19 of them were successfully sequenced without purification steps (51%) (Fig. 3). From DGGE gels 54 bands were excised, out of which 27 (50%) produced readable sequences after several reamplification and purification rounds.

Based on the phylogenetic analysis, the SRB found from the oil field water samples fell within several taxa (Fig. 5). The *dsrB* fragments amplified from North Sea water samples and detected by both DHPLC and DGGE were most closely related to *Archaeoglobus* (MOB1), *Desulfovibrio longus* (MOB2), and *Desulfotomaculum acetoxidans* (MOB2). In North Sea produced-water samples the fragments from DGGE and DHPLC were most similar to *Desulfovibrio aespoeensis* (MOB6-5) and *Archaeoglobus fulgidus* (MOB9). In addition, three other fragments closest to *Desulfotignum phosphitoxidans*, *Desulfovibrio aespoeensis*, and *Syntrophobacteraceae* were identified with DGGE. In the produced water from Brazil (MOB11) no fragments from DGGE could be identified, and all four fragments from DHPLC fell close to *Desulfovibrio longus* in the phylogenetic analysis. In the other sample from Brazil (MOB12), the four fragments identified with DHPLC and two identified by DGGE fell within the same cluster close to *Desulfococcus multivorans*. The DGGE analysis of the MOB12 sample showed three additional fragments belonging to *Desulfomicrobium* sp., *Desulfovibrio aespoeensis*, and *Desulfovibrio desulfuricans*. Produced-water sample MOB13 from the United States had the highest SRB diversity. Both methods identified *dsrB* fragments close to *Desulfovibrio* sp. In addition, a DHPLC fragment close to *Desulfococcus multivorans*, as well DGGE fragments close to *Desulfobulbus rhabdoformis*, *Desulfotignum phosphitoxidans*, and *Desulforhopalus singaporensis*, was identified. In another produced-water sample from the United States (MOB14), a fragment close to *Desulfovibrio aespoeensis* was detected by both methods. DGGE also showed additional fragments close to *Desulfobacter curvatus* and *Desulfovibrio zosterae*. The fragments identified from the well water samples MOB17 and MOB18 detected by both DHPLC and DGGE all fell within the same cluster close to *Desulfonatronovibrio hydrogenovorans*.

DISCUSSION

Uncontrolled microbial growth can have detrimental effects on production efficiency in oil production systems. SRB are one of the most common and problematic group of bacteria found in oil field systems (4, 5). These organisms are notoriously difficult to cultivate. MPN-based methods routinely applied in the oil and gas industry mainly reveal the easily cultivable species. Culture-independent methods for detecting and monitoring the presence and diversity of detrimental microbes are needed for enhanced process control, as well as for increasing knowledge about SRB ecology in oil fields. In this study, we evaluated the applicability of DHPLC to monitoring SRB in injection and produced waters from oil production sites in comparison with DGGE. To our knowledge, this is the first study in which DHPLC has been applied to SRB community profiling. The SRB were first detected by quantitative real-time PCR (qPCR). The analysis was based on the dissimilatory sulfite reductase (*dsr*) gene that encodes the enzyme catalyzing the conversion of sulfite to sulfide during sulfate reduction. Because this gene is required by all sulfate reducers, it has frequently been used as a functional marker both in qPCR and in community profiling by DGGE (9, 11, 30). Twenty-eight water samples were obtained from oil fields from very distinct geographic locations, 15 of which contained SRB based on qPCR quantification of *dsrB* gene fragments. These samples were analyzed by DHPLC and DGGE targeting the same functional gene (Table 2).

The qPCR assay targeting the *dsrB* fragment was shown to be applicable for the detection and quantification of SRB in environmental samples. The linear range of the assay, 2×10^2 to 2×10^7 *dsrB* copies per PCR, was well in the range found in other studies in which *dsrB* gene fragments were quantified (9, 30, 31). When qPCR is used, it should be borne in mind that the result is influenced by copy number, which may vary among species (Ribosomal RNA Operon Copy Number Database [http://rrndb.mmg.msu.edu/]). Bacteria exhibit great variation in 16S rRNA gene copy numbers, but the variation in the *dsrAB* copy number seems to be more restricted, which may make it a better candidate for quantitative applications. Notably *Desulfobulbus rhabdoformis*, *Desulfovibrio vulgaris*, *Desulfitobacterium hafniense*, and *Archaeoglobus fulgidus* have only a single copy of the *dsr* gene although more than one copy of *dsr* has been detected in some *Desulfovibrio* species (32).

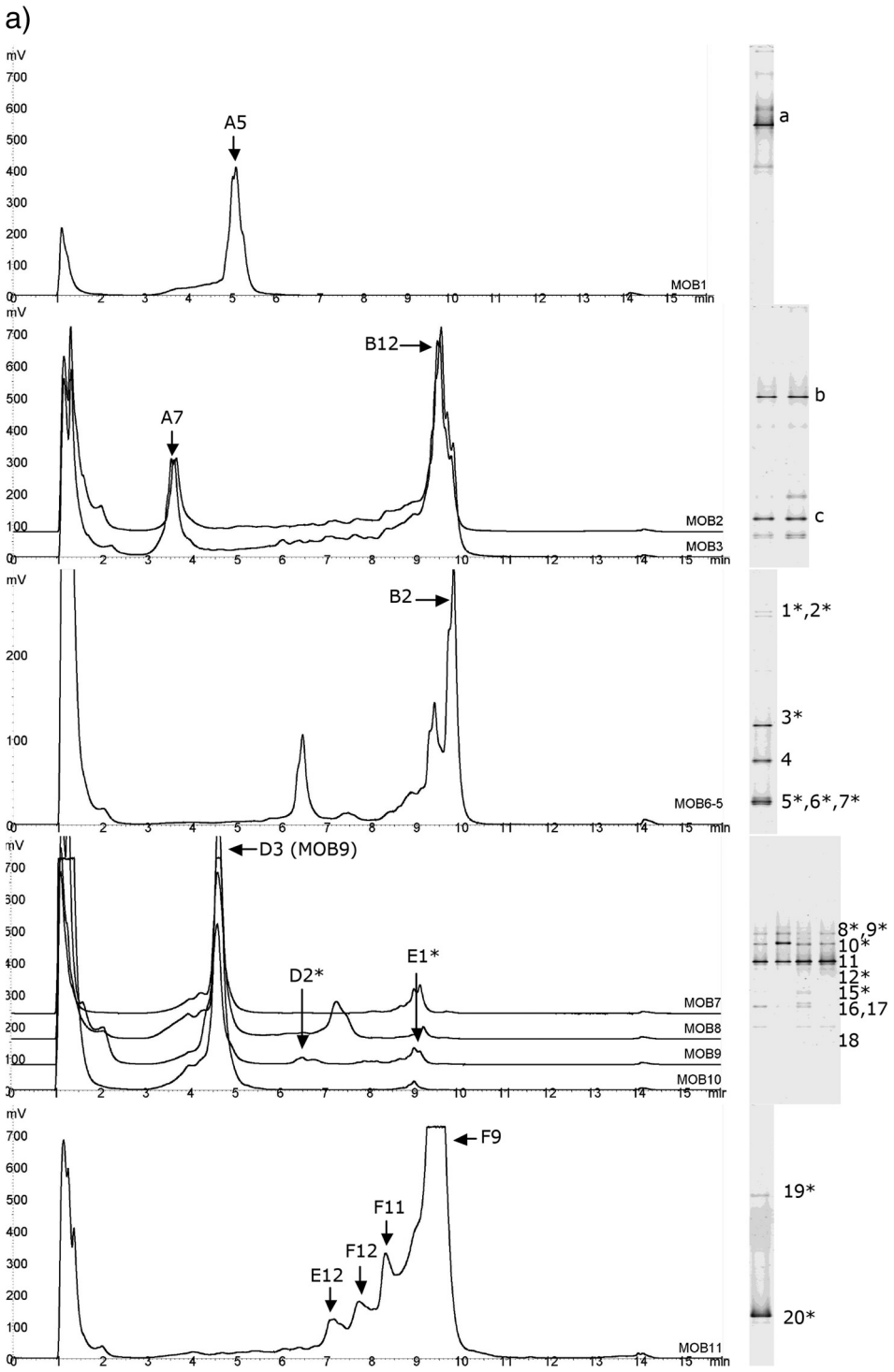

FIG 3 Community profiling of oil field samples. (a) Samples MOB1 to MOB11; (b) samples MOB12 to MOB18. DHPLC chromatograms are shown on the left, and DGGE gel lanes are on the right. Fragments collected from DHPLC or cut from DGGE gels and sequenced are marked with letters and numbers. *, sequencing was not successful; not included in the phylogenetic tree.

DHPLC and DGGE showed similar powers of discrimination with pure cultures of SRB when the *dsrB* gene fragment was targeted. The addition of the GC clamp was needed for discriminating genetic differences, as found also by Barlaan et al. (13). Elution of low-GC-content (45 to 52%) *dsrB* gene fragments before gene fragments with high GC contents (57 to 61%) in DHPLC showed that the separation was dependent on GC content, as in DGGE. A general agreement between the GC content and the behavior of gene fragments in DHPLC has also been reported in previous studies (13, 16, 33). Three SRB, however, produced irresolvable

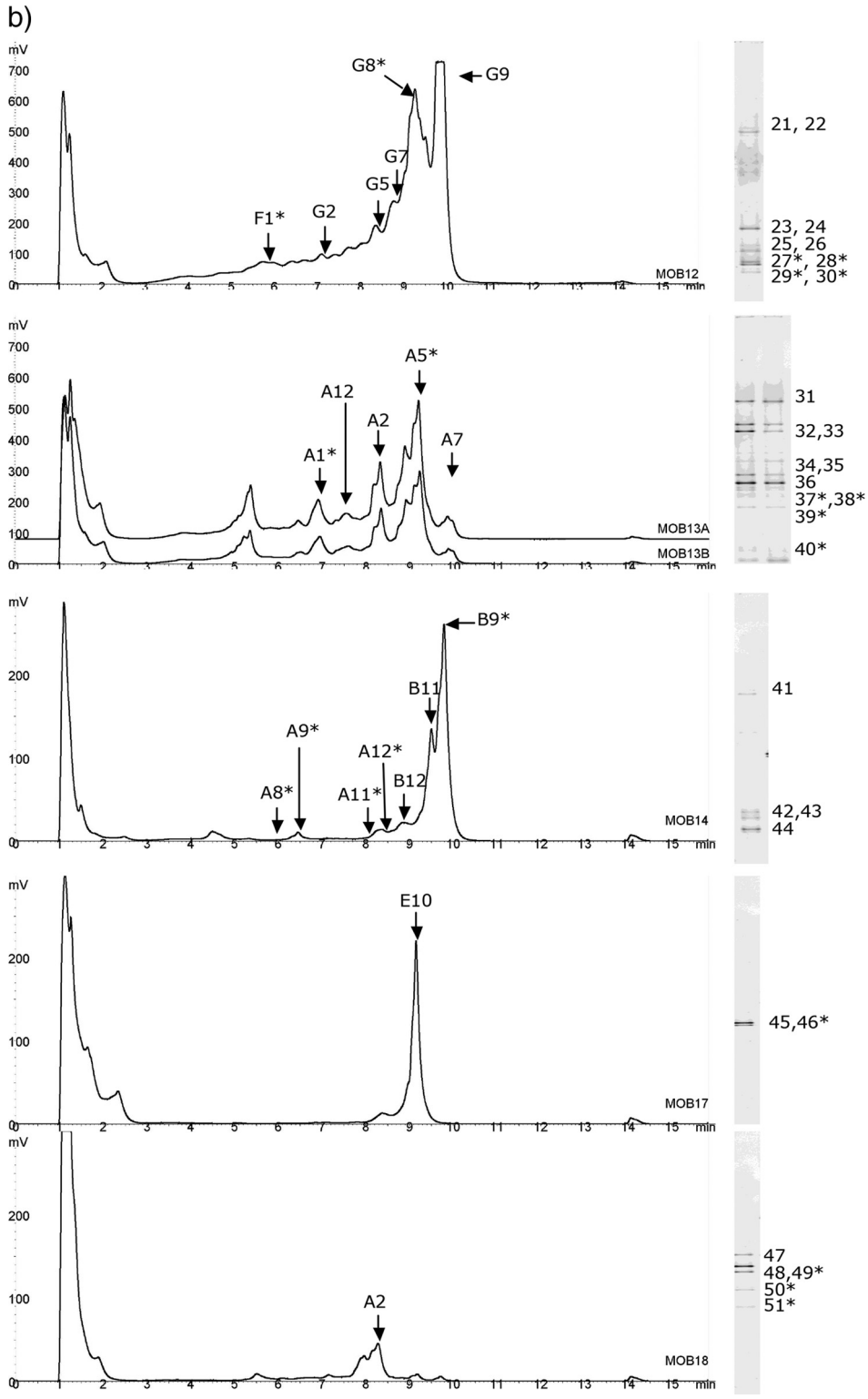

FIG 3 continued

smears in DHPLC which could not be explained by the GC contents of the amplicons (52 to 62%). Troedsson et al. (33) reported that the elution behavior of DNA fragments in DHPLC is correlated with their DNA helicity at the assay temperature.

DHPLC and DGGE gave, in general, similar results for SRB diversity in the oil field samples (Fig. 3 and 4). Similar DHPLC and DGGE profiles have also been obtained for intestinal bacterial communities by Goldenberg et al. (14) and for natural whey cul-

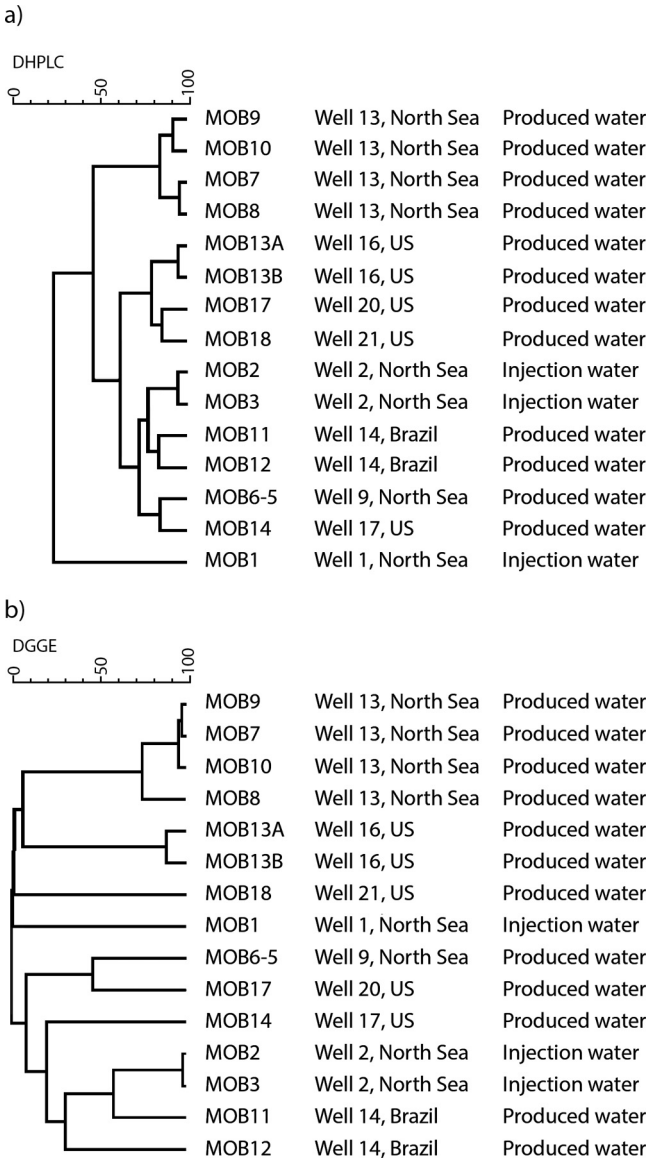

FIG 4 Cluster analysis of DHPLC (a) and DGGE (b) profiles generated from *dsrB* PCR-positive oil field samples. Pearson's correlation and UPGMA were used to construct the dendrograms.

tures by Ercolini et al. (15). In comparison to DGGE, DHPLC provided benefits in sample throughput, reproducibility, robustness, and flexibility. Analyzing a single sample by DHPLC took 16 min, making possible analysis of 90 samples in 24 h, whereas analysis by DGGE required 1.5 working days with a maximum capacity for analyzing 48 samples. The automated fraction collection in DHPLC successfully identified eluted gene fragments and allowed them to be collected for sequencing without a further purification step. In DGGE the preparation of gels is labor-intensive, and three to four rounds of excising the bands from the gels and reamplifying them were necessary in order to obtain readable sequences from them. The success rates of DNA sequencing were similar in DHPLC and DGGE, i.e., approximately 50%.

Our results showed that DHPLC is suited for studying the diversity of a specific group of organisms by targeting functional group-specific genes. In most bacterial DHPLC studies, this tech-

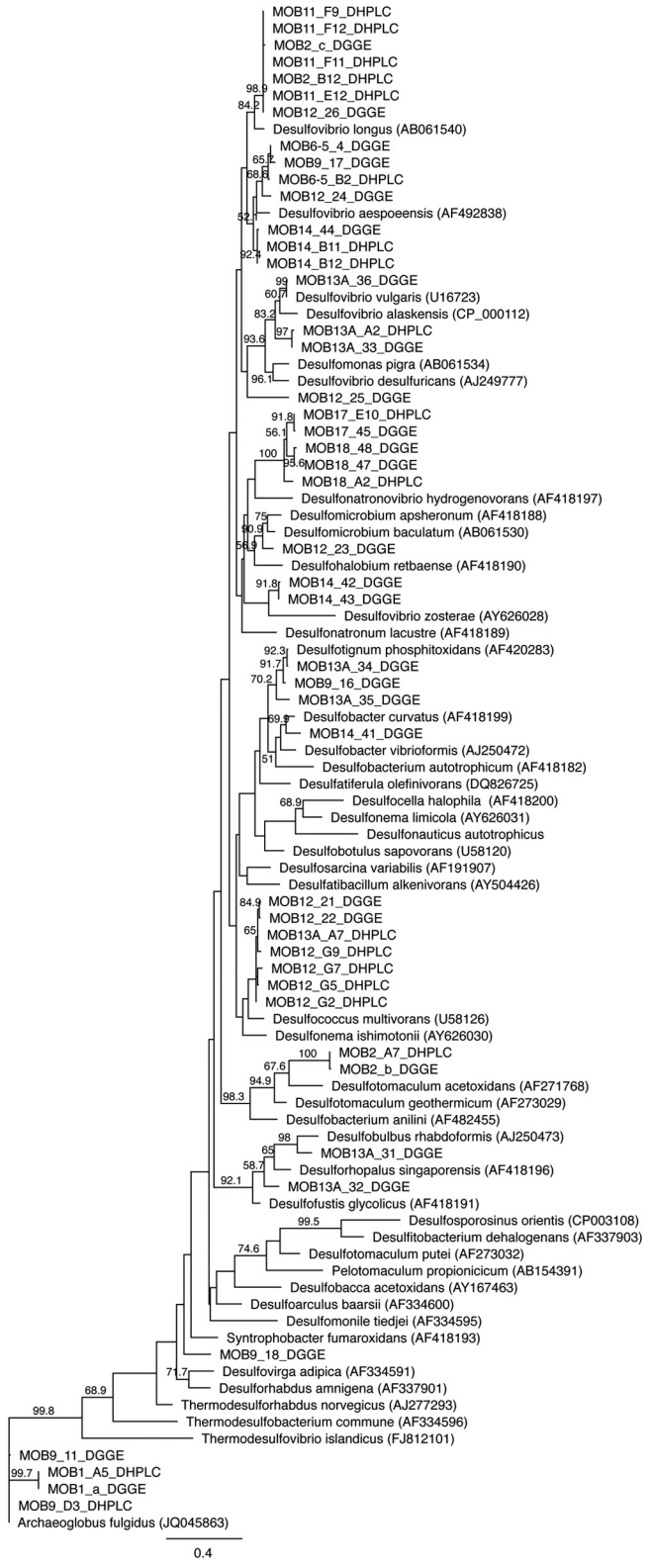

FIG 5 A maximum-likelihood phylogenetic tree of the *dsrB* sequences obtained by DHPLC and DGGE. Bootstrap probabilities (>50%) are indicated at branch nodes. Accession numbers are given in parentheses. Sequenced fragments are identified in Fig. 3.

nique has been used for 16S rRNA gene analysis (13–15). For SRBs, no 16S rRNA gene-based primer is available to detect all known SRB species. Wagner et al. (25) amplified the 1.9-kb *dsrAB* gene fragment with DSR1F and DSR4 primers, but later a shorter *dsrB* sequence was shown to be adequate for distinguishing between different species of SRB (11, 30). It is obvious that some *dsrB* fragments, despite their sequence divergence, may comigrate in both DHPLC and DGGE. Therefore, the DHPLC or DGGE profiles do not necessarily entirely reflect the true diversity in the field.

The SRB communities in the oil field samples were diverse, and the sequences identified belonged to several *dsrB* gene clusters (Fig. 5). *Desulfovibrio*-related sequences were the most common and were found from 7 of the 10 identified samples. Sequences close to *Desulfococcus*, *Desulfomicrobium*, *Desulfobulbus*, *Desulfotignum*, *Desulfonatronovibrio*, and *Desulfonauticus* were also detected. A wide range of *Desulfovibrionaceae*, *Desulfobacteraceae*, and *Desulfotomaculum*-related sequences have previously been found from oil field samples (34–38). *Archaeoglobus fulgidus*-like sequences were found from samples MOB1 and MOB9, which were both from the North Sea. *Archaeoglobus* sp. has previously been selectively enriched and immunomagnetically captured from three different platforms in the North Sea by Beeder et al. (6). Sample MOB7 was injection seawater, and samples MOB8 to MOB10 were produced waters from the same site, obtained 1 month after injection. The similarity of SRB profiles of these samples shows that the SRB injected into the well come back up, indicating that a continuous flow of SRB in the injection water to the reservoir may increase the risk of microbiological H_2S production, resulting in reservoir souring.

Care must be taken, however, in the interpretation of which species are indigenous in the oil field sites and which are introduced during reservoir development or sampling procedures. The aim of this study was not a systematic screening of oil field bacterial communities, for which the current sample set is not suitable. The sample set in this study included only water samples with planktonic bacteria even though the majority of bacteria in nature are attached to surfaces and form biofilms. For better understanding of the microbiology of oil reservoirs, improved sampling procedures of both planktonic and biofilm SRB communities would be needed (2, 39).

In this study, the DHPLC method was optimized and successfully applied for the profiling of SRB communities in oil field samples. Amplified *dsrB* fragments could be separated and collected by DHPLC. The results were consistent with DGGE analysis, which showed the applicability of the technique for studying the diversity of SRB based on *dsrB* gene sequence divergence. The advantage of DHPLC was that it provided a reproducible and automated method of analysis with a high sample throughput capability and flexibility, which are important for routine process monitoring in the oil sector. It is anticipated that the application described here also has broader applicability in the environmental diversity analysis of SRB.

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