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Characterization of 16S rRNA Genes from Oil Field Microbial Communities Indicates the Presence of a Variety of Sulfate-Reducing, Fermentative, and Sulfide-Oxidizing Bacteria

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Oil field bacteria were characterized by cloning and sequencing of PCR-amplified 16S rRNA genes. A variety of gram-negative, sulfate-reducing bacteria was detected (16 members of the family *Desulfovibrionaceae* and 8 members of the family *Desulfobacteriaceae*). In contrast, a much more limited number of anaerobic, fermentative, or acetogenic bacteria was found (one *Clostridium* sp., one *Eubacterium* sp., and one *Synergistes* sp.). Potential sulfide oxidizers and/or microaerophiles (*Thiomicrospira*, *Arcobacter*, *Campylobacter*, and *Oceanospirillum* spp.) were also detected. The first two were prominently amplified from uncultured production water DNA and represented 28 and 47% of all clones, respectively. Growth on media containing sulfide as the electron donor and nitrate as the electron acceptor and designed for the isolation of *Thiomicrospira* spp. gave only significant enrichment of the *Campylobacter* sp., which was shown to be present in different western Canadian oil fields. This newly discovered sulfide oxidizer may provide a vital link in the oil field sulfur cycle by reoxidizing sulfide formed by microbial sulfate or sulfur reduction.

Oil fields, especially those in which oil is produced by water injection, harbor an anaerobic microbial community in which sulfate-reducing bacteria (SRB) are prominent (4). The source of these bacteria, i.e., whether they are indigenous or introduced with the injection water, has not yet been resolved. Stetter et al. (23) have recently proposed that thermophilic archaea present in hydrothermal vents can survive in cold seawater and infect distantly located oil fields that have resident temperatures of ca. 100°C and that are injected with large volumes of seawater. Both environments contained the thermophilic, sulfate-reducing archaea *Archaeoglobus fulgidus* and *Archaeoglobus profundus*. Similarity between the organisms in the hydrothermal vent and oil field environments was inferred from genomic dot blot DNA hybridization studies (7, 23). We have made extensive use of a reverse genomic dot blot DNA hybridization procedure (reverse sample genome probing) to document the diversity of SRB in oil fields in western Canada harboring mesophilic microbial communities (28, 30, 31). This work has resulted in the isolation of a large number of SRB standards, defined as species with little or no cross-hybridization of their chromosomal DNAs in dot blots under stringent conditions. Many of these species, especially isolates using lactate as the electron donor for sulfate reduction, could be identified as *Desulfovibrio* spp. with the help of a specific gene probe derived from the genes for [NiFe] hydrogenase (27). The large genomic diversity in the genus *Desulfovibrio* has been corroborated by the sequencing of 16S rRNA genes. Pairs of sequences often shared degrees of similarity below the minimum required for classification in the same genus (5). In this paper, we report 16S rRNA sequences for many of the oil field SRB standards that we had previously isolated, allowing their identification in terms of the most closely related species in the Ribosomal Database Project (RDP) data bank of 16S rRNA sequences. Shotgun analysis of DNAs isolated from enrichment cultures or directly isolated from production waters indicated the presence of bacteria not previously identified in oil fields. The data contribute to our understanding of the origin and evolution of oil field bacteria and to the elucidation of microbial interactions important for the anaerobic, microbial degradation of oil.

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MATERIALS AND METHODS

Molecular biology reagents. Deoxyoligonucleotides EUB338 and UNIV907-R (positions 338 to 356 and 907 to 926 of the *Escherichia coli* 16S rRNA sequence [2]) were modified by the addition, respectively, of a *Bam*HI and a *Hin*dIII recognition sequence at their 5′ ends and were used for PCR amplification of 16S rRNA genes. These deoxyoligonucleotides were obtained from the University Core DNA Services of the University of Calgary, which also provided the random hexamers. Restriction and modification enzymes, *Taq* and Klenow polymerase, and cloning and sequencing vectors M13mp18 and M13mp19 (34), as well as a T7 polymerase dideoxy sequencing kit, were obtained from Pharmacia. Radioisotopes [α-^{32}P]dCTP (3,000 Ci/mmol; 10 mCi/ml) and [γ-^{32}P]ATP (3,000 Ci/mmol; 10 mCi/ml) were from ICN, while α-^{35}S-dATP (400 Ci/mmol; 10 mCi/ml) was from Amersham. These radioisotopes were used for random hexamer labeling (30), oligonucleotide end labeling (14), and dideoxynucleotide sequencing, respectively.

Source of DNA preparations. All DNA preparations originated from shallow (600-m-deep) oil fields in western Canada with a resident temperature of approximately 25°C and from which oil is produced by water flooding. Samples were taken from production units above ground in previous work (28, 30, 31). Production units, consisting of a free water knockout unit and a water plant to separate produced water and oil, had water production rates of 200 to 1,200 m^3/day. The produced water, which was reinjected into the reservoir, was quite saline (20 to 70 g of NaCl per liter). Total community DNAs isolated from 1-liter production water samples were the same as those described earlier (28). DNA preparations WW20WP, WW6FWKO, WW6WP, WW1WP, WW14FWKO, and WW14WP isolated in week 7 of the previous study were used.

DNA preparations from liquid enrichment cultures and from cultures of colony-purified (CP) SRB standards generated previously (30, 31) in media with either lactate, ethanol, benzoate, decanoate, propionate, or acetate as the electron donor and carbon source were also analyzed. The names of the SRB standards were created according to the electron donor and carbon source used for their first isolation and an identifying number (31).

All DNAs were dissolved in TE (10 mM Tris-HCl, 0.1 mM EDTA [pH 8]).

PCR, shotgun cloning, and sequencing. The strategy followed was similar to that described elsewhere (6). Small-subunit rRNA genes from 34 different DNA

TABLE 1. Distribution of 16S rRNA sequences among DNA preparations

DNA[a]	Status[b]	No. of DNA preparations[c]	Total no. of sequences obtained	Distribution[d]
CVO	CP	1	4	ARCO29 (4)
*Lac*3	CP	1	15	DVIB12 (15)
*Lac*4	CP	1	12	DVIB128 (12)
*Lac*5	CP	1	12	DVIB77 (9), DVIB85 (3)
*Lac*6	CP	1	20	DVIB57 (20)
*Lac*7	CP	1	10	DVIB104 (5), DVIB115 (2), EUBA7 (3)
*Lac*10	CP	1	12	DVIB137 (9), DVIB43 (3)
*Lac*15	CP	1	11	DVIB104 (11)
*Lac*17	CP	1	7	DVIB116 (7)
*Lac*21	CP	1	15	DVIB103 (13), DVIB116 (2)
*Lac*23	CP	1	5	SYNE4 (4), DVIB115 (1)
*Lac*8	LC	1	8	DVIB33 (5), DVIB104 (2), DVIB148 (1)
*Lac*12	LC	1	9	DVIB115 (9)
*Lac*24	LC	1	5	DVIB149 (5)
*Lac*26	LC	1	5	DVIB115 (5)
*Eth*3	LC	2	16	DVIB16 (12), ARCO4 (2), DVIB33 (1), EUBA7 (1)
*Ben*1	LC	2	24	THIO1 (8), DBAC28 (4), DBAC25 (3), THIO2 (3), ARCO4 (2), EUBA7 (2), DVIB16 (1), OCEA3 (1)
*Ben*5	LC	1	3	DVIB33 (1), DVIB44 (1), EUBA7 (1)
*Dec*3	LC	1	7	DVIB12 (4), DVIB104 (2), DBAC24 (1)
*Dec*9	LC	1	4	DVIB12 (3), CLOS1 (1)
*Pro*4	LC	2	16	DBUL31 (11), EUBA7 (4), THIO1 (1)
*Pro*5	LC	2	7	EUBA7 (4), DBUL35 (3)
*Pro*10	LC	2	23	DBUL8 (22), DVIB104 (1)
*Pro*12	LC	2	7	DCOC4 (7)
*Ace*1	LC	1	24	DBAC9 (13), THIO1 (6), DVIB115 (4), EUBA7 (1)
*Ace*3	LC	1	12	DBAC14 (10), EUBA7 (2)
*Ace*5	LC	1	12	THIO1 (11), DVIB57 (1)
WW6WP	TC	1	47	ARCO4 (22), THIO1 (13), OCEA2 (7), OCEA8 (3), OCEA7 (2)

[a] CVO is DNA from an isolate purified on medium 295 as indicated in the text. SRB standards *Lac*3 through *Ace*5 are as described before (30, 31). Total community DNA preparation WW6WP is the same as that described in reference 28.
[b] CP, colony purified; LC, liquid enrichment culture; TC, total community DNA.
[c] Number of DNA preparations from which clones for sequencing were obtained.
[d] The numbers in parentheses indicate the distribution of the 36 16S rRNA sequences identified in Table 2. The ranking is in order of decreasing frequency of observation.

preparations were amplified (Table 1). The typical reaction mixture included 10 μl of template DNA (200 ng), 10 μl of 10× *Taq* buffer (500 mM KCl, 15 mM $MgCl_2$, 100 mM Tris-HCl [pH 9]), 5 μl of $MgCl_2$ (50 mM), 5 μl of deoxynucleoside triphosphates (10 mM each), 2 μl of each of the primers (EUB338 and UNIV907-R [50 pmol/μl]), 0.5 μl of *Taq* polymerase (5 U/μl), and 67.5 μl of water. The typical reaction conditions were 30 cycles of 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C. Following amplification, the PCR product was purified by electrophoresis through low-gelling-temperature agarose, digested with restriction endonucleases *Bam*HI and *Hin*dIII, and ligated to the replicative forms of M13mp18 and M13mp19 (34). Single-stranded DNAs prepared from recombinant phage plaques were sequenced by the dideoxy method of Sanger et al. (21), with the universal sequencing primer GTAAAACGACGGCCAGT or primers targeted to conserved regions of the 16S rDNA insert (positions 516 to 530 or 690 to 697 of the *E. coli* sequence) being used.

Filter hybridization. Heat-denatured double-stranded DNAs (PCR products or chromosomal DNAs) or single-stranded M13 DNAs were spotted in 2-μl volumes of defined concentration on Hybond-N membrane filters and were covalently linked by UV irradiation (30). Double-stranded DNAs were labeled by using Klenow polymerase, [α-^{32}P]dCTP, and random hexamers as primers (30). After being boiled, the labeled probe was hybridized to a dot blot at 68°C, with the high-stringency protocol being used (14, 29). Deoxyoligonucleotides were end labeled with [γ-^{32}P]ATP and polynucleotide kinase (14). Labeled deoxyoligonucleotide probes were incubated with dot blots in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.2) (14) at selected temperatures in the range from 45 to 68°C.

Data analysis. Nucleic acid sequence data for overlapping sequences were assembled with the programs of Staden (22). Ranked listings of best matches were generated by comparing each assembled sequence with those in the RDP database with the program SIMILARITY_RANK (13). The best matches were then retrieved and used for construction of a phylogenetic tree with phylogenetic analysis using parsimony software (24).

Enrichment and colony purification of sulfide-oxidizing bacteria. Production water samples were collected under strictly anaerobic conditions. After shipment of the samples, bottles were stored in an anaerobic chamber (Coy Laboratory Products Inc., Ann Arbor, Mich.). Medium preparation and incubation of liquid enrichment cultures and plates were all performed anaerobically. Colonies of sulfide-oxidizing bacteria were obtained by plating enrichments on agar-containing medium 295 for the cultivation of *Thiobacillus denitrificans* (8). A single colony was picked and further purified by several passages through a series of serial dilutions in medium containing sulfide as the electron donor for nitrate reduction. The resulting isolate was designated CVO.

Nucleotide sequence accession numbers. The sequence data generated for this study have been deposited in GenBank under accession numbers U46505 to U46540.

RESULTS

Data collection, assembly, and comparison. PCR of 34 different DNA preparations (Table 1) with primers EUB338 and UNIV907-R gave a 560- to 580-bp PCR product in all cases. Clones obtained by the ligation of this PCR product to the M13 vectors and transfection of competent *E. coli* were then sequenced. Multiple clones were analyzed for each DNA preparation (Table 1). Sequence assembly gave 36 assembled sequences, most corresponding to the full length of the PCR product (Table 2). These sequences were then used to search the RDP database with the program SIMILARITY_RANK (13). The database of all 36 assembled sequences was appended to the RDP database prior to the initiation of the search. Appending the 36-sequence database allowed the similarity of the query sequence to all other sequences obtained in this study and to those in the RDP database to be determined.

A survey of the best matches for all 36 sequences is shown in Table 2. High similarities ($S_{ab} > 0.8$) were found for ARCO4 (*Arcobacter nitrifigilis*), DBAC9 (*Desulfobacter* sp. strain 3ac10),

TABLE 2. Survey of the best matches of all 36 assembled sequences

Name of the assembled sequence[a]	Length (nucleotides)[b]	Organism with best matching sequence[c]	S_{ab} coefficient[d]
ARCO4	517	*Arcobacter nitrofigilis*	0.890
ARCO29	505	*Campylobacter* sp.	0.596
CLOS1	209	*Clostridium ramosum* 113-I	0.512
DBAC9	544	*Desulfobacter* sp. strain 3ac10	0.812
DBAC14	544	*Desulfobacter hydrogenophilus* AcRS1	0.805
DBAC24	249	*Desulfosarcina variabilis* Montpellier	0.663
DBAC25	136	*Desulfobotulum sapovorans* Lindhorst	0.550
DBAC28	191	*Desulfobacula toluolica* (Tol2)	0.690
DBUL8	546	*Desulfobulbus* sp. strain 3pr10	0.528
DBUL31	547	*Desulfobulbus* sp. strain 3pr10	0.579
DBUL35	546	*Desulfobulbus* sp. strain 3pr10	0.569
DCOC4	548	*Desulfococcus multivorans* Goettingen	0.805
DVIB12	542	*Desulfovibrio* sp. strain PT-2	0.597
DVIB16	541	*Desulfovibrio salexigens*	0.821
DVIB33	304	*Desulfovibrio salexigens*	0.586
DVIB43	537	*Desulfovibrio longus* SEBR 2582	0.525
DVIB44	183	*Desulfovibrio longus* SEBR 2582	0.682
DVIB57	538	*Desulfovibrio* sp. strain PT-2	0.563
DVIB77	541	*Desulfovibrio* sp. strain PT-2	0.607
DVIB85	166	*Desulfovibrio* sp. strain PT-2	0.633
DVIB103	544	*Desulfovibrio gigas*	0.748
DVIB104	542	*Desulfomicrobium escambium*	0.663
DVIB115	540	*Desulfomicrobium escambium*	0.843
DVIB116	540	*Desulfovibrio desulfuricans*	0.693
DVIB128	539	*Desulfovibrio* sp. strain PT-2	0.598
DVIB137	545	*Desulfovibrio longus* SEBR 2582	0.718
DVIB148	281	*Desulfomicrobium escambium*	0.697
DVIB149	542	*Desulfovibrio* sp. strain PT-2	0.635
EUBA7	513	*Eubacterium limosum*	0.745
OCEA2	543	*Oceanospirillum jannaschii*	0.607
OCEA3	104	*Halovibrio variabilis*	0.653
OCEA7	406	*Oceanospirillum multiglobuliferum*	0.583
OCEA8	542	*Oceanospirillum jannaschii*	0.627
SYNE4	518	*Synergistes jonesii* 78-1	0.607
THIO1	545	*Thiomicrospira* sp. strain L12	0.813
THIO2	221	*Thyasira flexuosa* gill symbiont	0.583

[a] First four letters of the best match and an identifying number.
[b] Length of the assembled sequence after the removal of primer sequences.
[c] The best matching sequence was found with SIMILARITY_RANK (13).
[d] Similarity coefficients for query and matching sequences (13).

DBAC14 (*Desulfobacter hydrogenophilus* AcRS1), DCOC4 (*Desulfococcus multivorans* Goettingen), DVIB16 (*Desulfovibrio salexigens*), and THIO1 (*Thiomicrospira* sp. strain L12). S_{ab} values are not directly related to percent identity. High S_{ab} values indicate the presence of large, uninterrupted stretches of sequence identity between the query and database sequences (13). Percentages of identity calculated for all pairs of DVIB sequences listed in Table 2 ranged from 80 to 94%, whereas S_{ab} values ranged from 0.4 to 0.7. The 16S rRNA sequences obtained for oil field bacteria of the superfamily *Desulfovibrionaceae* are as diverse as those present in the RDP database (5).

Phylogenetic placement of oil field bacteria. A tree indicating the phylogenetic relationships of oil field bacteria and their RDP homologs is shown in Fig. 1. The sequences found for oil field bacteria in this study can be classified into four groups, which represent in part the different functions that these bacteria may catalyze in the oil field environment. The group of sulfide oxidizers and microaerophiles (Fig. 1) is novel, and members of the indicated genera have not been previously described as components of the oil field microbial community. Surprisingly, 16S rRNA sequences recovered from a directly extracted oil field DNA were totally dominated by this group and gave 22 ARCO4, 13 THIO1, 7 OCEA2, 3 OCEA8, and 2 OCEA7 sequences (WW6WP) (Table 1). Preliminary data for 33 clones from another directly extracted oil field DNA indicated the presence of 24 ARCO4 and no THIO1 sequences. ARCO4, with *A. nitrofigilis* as the RDP homolog (Table 2), is thus most frequently amplified from uncultured oil field DNA.

Isolation of anoxic sulfide oxidizer CVO. The sulfide oxidizers and microaerophiles, suggested to be present by 16S rRNA sequencing, can possibly be isolated on media for the RDP homologs. THIO1 sequences were first detected in enrichment cultures on media with benzoate or acetate as the carbon source and electron donor for sulfate reduction (*Ben*1, *Ace*1, and *Ace*5 [Table 1]). Selective enrichment of THIO1 was attempted by inoculation of these primary enrichments into media for *Thiomicrospira denitrificans* (10) and *Thiobacillus denitrificans* (8). These media, which provide energy for growth from the oxidation of sulfide (or thiosulfate) by nitrate, failed to enrich THIO1. This failure is shown by hybridization with THIO1-specific probe P84 (^{32}P-TATTAGCAACTAACCTTT CCTCACAATTGAAAGT, corresponding to positions 433 to 466 of the *E. coli* 16S rRNA sequence). PCR products obtained for DNAs from sulfide-nitrate enrichments did not hybridize with labeled P84 (Fig. 2C, spots 3, 4, 6, 8, and 9), whereas PCR products from a primary enrichment (Fig. 2C, spot 16) and from some total community DNAs (Fig. 2C, spots 20 to 23 but not 24 and 25) were positive. Nevertheless, enrichment and plating of samples from production waters from an oil field in Saskatchewan on sulfide-nitrate media did give a CP isolate that was designated CVO. Under an electron microscope, CVO appeared to be coccobacilli approximately 0.5 μm in length (data not shown). When chromosomal DNA from CP CVO was labeled by the random hexamer procedure and used as a probe, strong hybridizations with itself (Fig. 2A, spot 18), with DNAs from other CP sulfide oxidizers (Fig. 2A, spots 17 and 19), and with DNAs from some sulfide-nitrate enrichments (Fig. 2A, spots 3, 4, 6, 8, and 9) were observed. Sequencing of amplified and cloned 16S rDNAs and database comparisons indicated that CVO is more closely related to the *Campylobacter* sp. than to the *Thiobacillus* sp. or *Thiomicrospira denitrificans* (Fig. 1 and Table 2 [ARCO29]). Further confirmation that the hybridizations between chromosomal DNA from CVO and the sulfide-nitrate enrichments in Fig. 2A are due to the presence of the *Campylobacter* sp. was obtained by probing PCR products with ARCO29-specific oligonucleotide probe P97 (^{32}P-CACCTGACTTGATATCC, corresponding to positions 433 to 466 of the *E. coli* 16S rRNA sequence). As can be seen in Fig. 2B, PCR products from sulfide-nitrate enrichment DNAs (spots 3, 4, 6, 8, and 9) hybridized positively with P97, while that from the DNA in spot 7 did not hybridize. Hybridization of these same PCR products with labeled EUB338 as a positive control indicates that the DNA in spot 7 did give a PCR product (Fig. 2D) but that the DNAs in spots 2 and 5 (Fig. 2B to D) did not. The P97 probe did not react with the PCR product from *Thiomicrospira denitrificans* ATCC 33889 (Fig. 2B, spot 1), as was expected on the basis of the known 16S rRNA sequences. PCR products generated directly from production water DNAs (Fig. 2B, spots 20 to 25) and from one of the primary benzoate-sulfate enrichment DNAs (Fig. 2B, spot 16) used to generate the sulfide-nitrate enrichments also failed to hybridize with P97. Thus, a CVO-positive PCR product is only obtained with universal primers if the organism is first enriched on sulfide-nitrate medium, indicating that its *rrnA* genes are not efficiently amplified in competition with those from other bacteria. Hybridization of the same

FIG. 1. Phylogenetic placement of 16S rRNA sequences from oil field bacteria. Only sequences longer than 400 nucleotides were used for the analysis and are compared with those of RDP homologs (Table 2). TD3 and Tol2 are SRB that use hydrocarbons as electron donors for sulfate reduction (18, 19). Nucleotides 364 to 905 of the *E. coli* sequence served as the outgroup for rooting the tree. The sequences were aligned according to the method of Woese et al. (33). The numbers on the branches are the results of 100 bootstrap replicates and indicate the frequency with which the sequences grouped together in the way shown (24).

filters in Fig. 2B and C with ARCO4-specific labeled deoxyoligonucleotide P98 (^{32}P-CTTCCAACTTATCTATC) gave only hybridization with the PCR products obtained for the total community DNAs (data not shown). This result is in agreement with the frequent recovery of ARCO4 clones from these DNAs, as discussed above, and indicates that ARCO4, like THIO1, may not be culturable on the media employed.

DISCUSSION

The objectives of the current study were to characterize previously isolated SRB standards (28, 30, 31) by 16S rRNA sequencing in terms of the most closely related RDP homologs and to extend our knowledge of the oil field microbial community by randomly searching for novel clones in total community DNAs. Our longer-term goal is to isolate these novel microorganisms and add their chromosomal DNAs to our oil field master filter, which can then serve to analyze free-floating (planktonic) and attached (biofilm) microbial communities in the oil field environment in increasing detail. The SRB standards used were two types: CP or liquid culture enriched (LC) (Table 1). We analyzed multiple clones by 16S rRNA sequencing for both CP and LC standards (Table 1). Obtaining pure isolates of anaerobes can be difficult, and the analysis of multiple clones is a way to assess purity.

Colony purification on media in which lactate was the electron donor for sulfate reduction selects for members of the *Desulfovibrionaceae*, a superfamily that includes all *Desulfovibrio* and *Desulfomicrobium* spp. (5, 32). These species oxidize lactate incompletely to form acetate, CO_2, and H_2S. Ethanol and hydrogen are also common electron donors (26). Members of this family have not been found to use crude oil components as an electron donor. TD3, an SRB capable of the oxidation of aliphatic oil components, branches off from the *Desulfovibrionaceae* lineage (Fig. 1) (19). The clonal homogeneity of CP standards *Lac*3 (DVIB12), *Lac*4 (DVIB128), *Lac*6 (DVIB57), *Lac*15 (DVIB104), and *Lac*17 (DVIB116) confirms their purity. *Lac*3, *Lac*4, and *Lac*6 are not identical, despite sharing the same RDP homolog with low sequence similarity (*Desulfovibrio* sp. strain LT2 [S_{ab} = 0.5 to 0.6]) (Table 2). Their pairwise S_{ab} values were also only 0.52 to 0.58, and their genomic DNAs did not cross-hybridize. Evidently, the collection of 16S rRNA sequences in the RDP database is still too incomplete to allow identification at the species level. Some CP standards gave mixed 16S rRNA sequences, e.g., *Lac*5 (DVIB77 and DVIB85), *Lac*10 (DVIB137 and DVIB43), and *Lac*21 (DVIB103 and DVIB116), while *Lac*23 gave, surprisingly, SYNE4 as the most frequent clone. Further work is required to determine whether the SRB represented by these sequences can be separated by plating techniques. Of the LC standards, *Lac*12 and

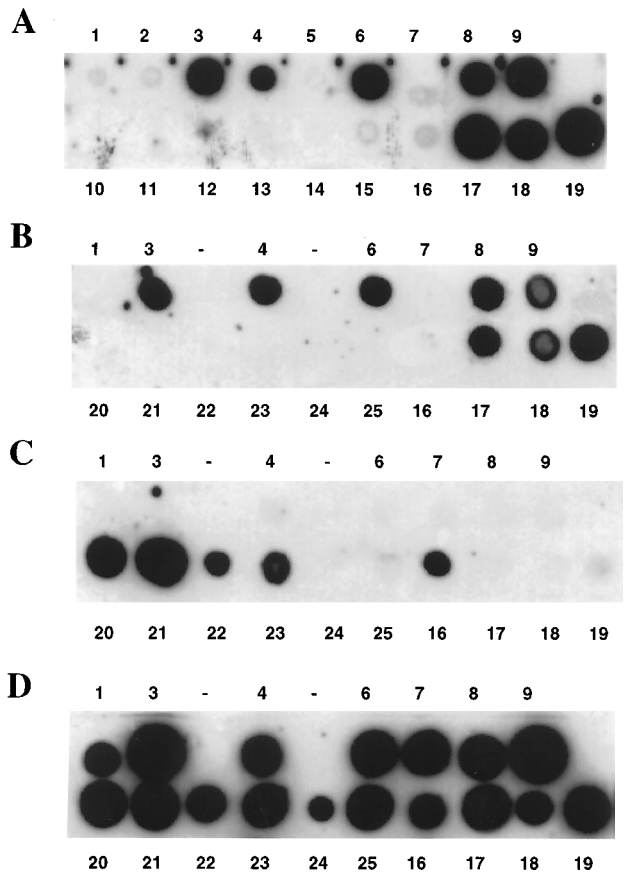

FIG. 2. Detection of *Campylobacter*-like sulfide-oxidizing bacterium CVO and of *Thiomicrospira*-like bacterium THIO1. (A) The following chromosomal DNAs were denatured and spotted on the filter: 1, *Thiomicrospira denitrificans* ATCC 33889; 2 to 13, DNAs from sulfide-nitrate enrichments (benzoate-sulfate and acetate-sulfate enrichments obtained from a variety of oil field samples and identified as *Ben*1 or *Ace*5 by chromosomal DNA hybridization were used as inocula); 14, *Lac*6; 15, *Lac*15; 16, *Ben*1; 17 to 19, DNAs for three isolates colony purified on sulfide-nitrate medium (CV1, CVO, and CVE, respectively). The filter was hybridized with labeled chromosomal DNA from isolate CVO. (B) PCR products of chromosomal DNAs, generated with primers EUB338 and UNIV907-R, were denatured and spotted on the filter. The filter was hybridized with CVO-specific, end-labeled deoxyoligonucleotide P97. The PCR products were from the same chromosomal DNAs as those described above for panel A, except for positions 20 to 25, which were PCR products derived from total community DNAs WW20WP, WW6FWKO, WW6WP, WW1WP, WW14FWKO, and WW14WP, respectively. (C) The same filter as that in panel B was hybridized with THIO1-specific, end-labeled deoxyoligonucleotide P84. (D) The same filter as that in panel B was hybridized with end-labeled EUB338 as a positive control.

*Lac*26 (both DVIB115) and *Lac*24 (DVIB149) appeared homogeneous. *Lac*12 and *Lac*26 are genomically distinct, despite having nearly identical 16S rRNA sequences. The 16S rRNA sequence data indicate the presence of at least 16 different members of the family *Desulfovibrionaceae* in western Canadian oil fields (DVIB12 to DVIB149) (Table 2). This diversity of 16S rRNA sequences (Fig. 1) agrees with the genomic diversity inferred previously by reverse sample genome probing (28, 30, 31). Several of the RDP homologs (*Desulfovibrio gigas* and *D. salexigens*) have been isolated from saline environments similar to those in the oil fields from which our samples originated (Table 2). One RDP homolog, *Desulfovibrio longus*, was actually isolated from an oil well in France (12).

The members of the family *Desulfovibrionaceae* oxidize their organic substrates by using sulfate as the electron acceptor for CO_2 and H_2S (32). Aromatic and aliphatic lower-chain fatty acids (benzoate, butyrate, propionate, and acetate) are common electron donors, while strain Tol2 (*Desulfobacula toluolica*) oxidizes toluene (18). LC standards grown on benzoate, decanoate, propionate, or acetate as the carbon source and electron donor for sulfate reduction (*Ben*1 to *Ace*5) gave 16S rRNA sequences that belonged to this superfamily (Table 1). *Pro*4 and *Pro*10 gave primarily DBUL31 and DBUL8 sequences. Their RDP database homolog, a *Desulfobulbus* sp., uses propionate as the carbon source and electron donor for sulfate reduction (32). LC standard *Pro*12 gave exclusively DCOC4 sequences. Its RDP homolog, *D. multivorans*, can use propionate and, as the name implies, a variety of other substrates (32). LC standards *Ace*1 and *Ace*3 gave DBAC9 and DBAC14 with a *Desulfobacter* sp. as the RDP homolog, as this genus specializes in the use of acetate as the electron donor for sulfate reduction. In total, eight different representatives of the superfamily *Desulfobacteriaceae* were found by 16S rDNA sequencing, confirming that these organisms are important components of the oil field community (4). DBAC28 has Tol2 (*D. toluolica*) as the RDP homolog (Table 2). DBAC28 was part of a diverse, stable enrichment culture on benzoate (*Ben*1) and may also be able to use toluene (Table 1).

EUBA7, SYNE4, and CLOS1 have RDP homologs among the anaerobic, fermentative, and/or acetogenic, low G+C, gram-positive bacteria (17). *Eubacterium limosum* can demethoxylate such aromatic substrates as 3,4,5-trimethoxybenzoate (3) or gasoline condensates (16), with methanol, acetate, and hydrogen being produced. *Synergistes jonesii* is an anaerobic rumen bacterium capable of fermenting toxic pyridine diols, with acetate and propionate being produced (1).

ARCO4, ARCO29, OCEA2, OCEA3, OCEA7, OCEA8, THIO1, and THIO2 are a polyphyletic group, with similarity to members from the gamma (*Oceanospirillum* and *Thiomicrospira* spp.) and delta (*A. nitrofigilis* and *Campylobacter* spp.) subdivisions of the proteobacteria. Literature data indicate that the RDP database homologs grow generally only under microaerophilic or anaerobic conditions. *Oceanospirillum* spp. are marine spirilla requiring NaCl for growth (20), which may explain their presence in saline oil field production waters. *Thiomicrospira denitrificans* has the capacity to oxidize reduced sulfur compounds (e.g., sulfide) by using nitrate as an electron acceptor (10), while free-living *Campylobacter* spp. have shown ability to use fermentatively produced hydrogen for the reduction of nitrate, thiosulfate, elemental sulfur, or oxygen (11). *A. nitrofigilis* was isolated from the roots of a salt marsh plant, *Spartina alterniflora* (15). *A. nitrofigilis* was originally described as a nitrogen-fixing, microaerophilic *Campylobacter* sp. and was later reclassified (25). Our finding that CVO (ARCO29 with a *Campylobacter* sp. as the RDP homolog [Table 2]) is capable of anaerobic sulfide oxidation indicates that organisms in this group contribute to sulfide recycling.

A model indicating the interdependence of the bacterial groups identified is shown in Fig. 3. The members of the families *Desulfovibrionaceae* and *Desulfobacteriaceae* present in oil fields use hydrocarbons, organic acids, or hydrogen as electron donors for sulfate reduction. Organic acids may be formed by such fermentative or acetogenic community members as EUBA7 that release methanol from ethers or esters. The abundance of electron donors, particularly hydrocarbons, in the oil field environment will result in the rapid conversion of the limited pool of oxidized forms of inorganic sulfur to sulfide. The sulfide-oxidizing bacteria may use nitrate or oxygen, which reach the oil-bearing formation through diffusion or convection from surface layers ($\Phi_{NO_3^-}$ and Φ_{O_2}) (Fig. 3), for the reoxidation of sulfide. The magnitude of these fluxes may be rate

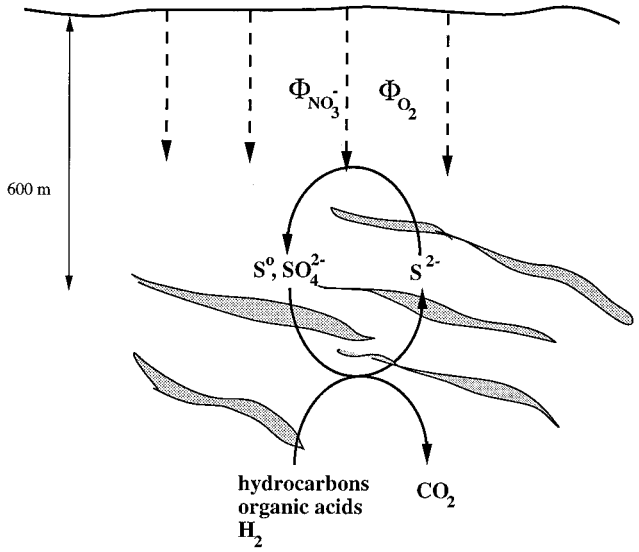

FIG. 3. Model for the functioning of the oil field microbial community derived from the current study. Hydrocarbon degradation is primarily catalyzed by sulfate reducers and is limited by the availability of oxidized forms of inorganic sulfur. *Campylobacter* spp., such as CVO, that are widely distributed in oil fields (Fig. 2) reoxidize sulfide. The flux of nitrate and oxygen ($\Phi_{NO_3^-}$ and Φ_{O_2}) is targeted towards sulfide reoxidation and is rate limiting to the activity of the microbial community.

limiting for microbial activity in parts of the oil-bearing subsurface. These parts are essentially anaerobic, which is corroborated by the wide variety of SRB, fermenters, and acetogens found by culturing or 16S rRNA analysis. The flux of nitrate or oxygen may thus be used primarily for the oxidation of sulfide, not for the oxidation of hydrocarbons. Bacteria using oxygen or nitrate directly for hydrocarbon degradation are thought to be absent or much reduced in numbers in the anaerobic, oil-bearing subsurface in this model (Fig. 3). They are found in the more aerobic surface layers. Water injection, a commonly used engineering practice for oil production, stimulates microbial activity by replenishing the pool of oxidized forms of inorganic sulfur through subsurface stirring.

The possibility of using nitrate amendment for the removal of sulfide from production waters or produced oil has been suggested (9). Our results show that the bacteria capable of catalyzing this process are naturally present in the oil field community, but the model (Fig. 3) suggests that any artificial increase of the nitrate flux may lead to an undesirable boost in oil field microbial activity.

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