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Degradative Capacities and 16S rRNA-Targeted Whole-Cell Hybridization of Sulfate-Reducing Bacteria in an Anaerobic Enrichment Culture Utilizing Alkylbenzenes from Crude Oil

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A mesophilic sulfate-reducing enrichment culture growing anaerobically on crude oil was used as a model system to study which nutritional types of sulfate-reducing bacteria may develop on original petroleum constituents in oil wells, tanks, and pipelines. Chemical analysis of oil hydrocarbons during growth revealed depletion of toluene and *o*-xylene within 1 month and of *m*-xylene, *o*-ethyltoluene, *m*-ethyltoluene, *m*-propyltoluene, and *m*-isopropyltoluene within approximately 2 months. In anaerobic counting series, the highest numbers of CFU (6×10^6 to 8×10^6 CFU ml^{-1}) were obtained with toluene and benzoate. Almost the same numbers were obtained with lactate, a substrate often used for detection of the vibrio-shaped, incompletely oxidizing *Desulfovibrio* sp. In the present study, however, lactate yielded mostly colonies of oval to rod-shaped, completely oxidizing, sulfate-reducing bacteria which were able to grow slowly on toluene or crude oil. *Desulfovibrio* species were detected only at low numbers (3×10^5 CFU ml^{-1}). In agreement with this finding, a fluorescently labeled, 16S rRNA-targeted oligonucleotide probe described in the literature as specific for members of the *Desulfovibrionaceae* (suggested family) hybridized only with a small portion (<5%) of the cells in the enrichment culture. These results are consistent with the observation that known *Desulfovibrio* species do not utilize aromatic hydrocarbons, the predominant substrates in the enrichment culture. All known sulfate-reducing bacteria which utilize aromatic compounds belong to a separate branch, the *Desulfobacteriaceae* (suggested family). Most members of this family are complete oxidizers. For specific hybridization with members of this branch, the probe had to be modified by a nucleotide exchange. Indeed, this modified probe hybridized with more than 95% of the cells in the enrichment culture. The results show that completely oxidizing, alkylbenzene-utilizing sulfate-reducing bacteria rather than *Desulfovibrio* species have to be considered in attempts to understand the microbiology of sulfide production in oil wells, tanks, and pipelines when no electron donors other than the indigenous oil constituents are available.

Production of sulfide in oil field waters, a process which is often referred to as souring, has frequently been of concern (30, 42). Hydrogen sulfide may lead to (i) poisoning upon inhalation, (ii) contamination of oil and gas and thus an increase in their sulfur content, (iii) corrosion of pipelines and other containments made of steel, and (iv) conversion of various iron minerals to ferrous sulfide; the latter may plug oil-bearing strata and stabilize undesirable oil-water emulsions. At temperatures of less than approximately 100°C, hydrogen sulfide obviously originates from bacterial sulfate reduction. High cell densities of sulfate-reducing bacteria have been repeatedly observed in production waters (17, 37, 39, 54), and numerous isolates from oil fields have been characterized elsewhere (1, 10, 32, 38, 51, 53, 55, 56). More recently, archaeal sulfate reducers were detected in and isolated from oil wells (9, 28, 62). In addition, high fractionation of sulfur isotopes (with respect to parental sulfate) in preserved sulfide or in sulfur as an oxidation product of sulfide indicates that extensive bacterial sulfate reduction occurred in certain oil reservoirs (40, 60). During exploitation of oil fields, bacterial sulfate reduction is stimulated by injection of seawater (30, 42, 53), which contains as much as 28 mM sulfate. In reservoirs with temperatures of higher than approximately 100°C, sulfide may be of purely chemical origin (43).

Development of controlling measures against microbial sulfate reduction in oil fields requires knowledge of the involved sulfate-reducing bacteria and their in situ growth substrates. In accordance with the nutritional capacities of sulfate reducers isolated from oil fields, various compounds have been discussed to serve as electron donors for sulfate reduction. These include (i) hydrogen from geothermal processes (62), from corroding or cathodically protected iron (27, 71), and from anaerobic degradation of compounds added or formed during operation (17, 39); (ii) fatty acids from ancient maturation processes (8, 35) and in situ hydrous pyrolysis (14); (iii) polar organic by-products formed by aerobic bacteria during growth on hydrocarbons (12, 39); and (iv) hydrocarbons as the major oil constituents (1, 58).

In addition to classical counting and isolation techniques, immunological methods as well as analysis and hybridization of nucleic acids may provide information about the types of sulfate-reducing bacteria that occur in stratal water and production waters of oil fields. Staining of cells in production waters with fluorescently labeled antibodies revealed the presence

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of thermophilic sulfate reducers of the genera *Archaeoglobus* and *Thermodesulforhabdus* (41). Hybridization of labeled total DNA from oil field samples against that from a set of isolates (reverse sample genome probing) revealed the presence of *Desulfovibrio* species and unidentified species of sulfate-reducing bacteria (67–69). Furthermore, fragments of genes coding for hydrogenases were used for identification of *Desulfovibrio* species among oil field isolates (66). The most frequently applied methods involving nucleic acids for differentiation of sulfate-reducing bacteria in heterogeneous cultures and environmental samples are based on rRNA sequences. For instance, the specificities of probes designed for various groups of sulfate-reducing bacteria of the suggested families *Desulfovibrionaceae* and *Desulfobacteriaceae* (20, 72) were demonstrated by hybridization with rRNA extracted from pure cultures and from sediment samples (21). One of these probes, which was termed 804, hybridized with rRNA from five genera of the *Desulfobacteriaceae*, including *Desulfobacter* and *Desulfobacterium* (21). Analysis of 16S rRNA genes amplified from DNA that had been extracted from oil field production waters indicated the presence of various genera of the *Desulfovibrionaceae* and *Desulfobacteriaceae* (65). Nucleic acid probes have also been used to hybridize 16S rRNA in whole cells of sulfate-reducing bacteria. A fluorescently labeled probe termed SRB385 (3) allowed whole-cell detection of *Desulfovibrio* species in defined mixtures of eubacteria (3) and in biofilms (50). Cells of *Desulfobacter* species yielded a weak hybridization signal with this probe but gave a strong signal with another probe specifically designed for this genus (3, 50). In contrast to methods involving extracted nucleic acids, whole-cell hybridization has, to our knowledge, not been applied thus far to identify sulfate-reducing bacteria directly in samples from oil production waters.

In the present study, we used a previously established sulfate-reducing enrichment culture on crude oil (58) as a model system of bacterial habitats in which crude oil is the only potential source of organic substrates. This enrichment culture was recently shown to deplete crude oil of toluene, *o*-xylene, *m*-xylene, *o*-ethyltoluene, and *m*-ethyltoluene (58). In the present investigation, the enrichment culture is studied in more detail. (i) Consumption of particular alkylbenzenes is monitored over time to reveal possible substrate preferences. (ii) The major nutritional types of sulfate-reducing bacteria in the enrichment culture and their affiliation with known genealogic groups are examined by using classical counting techniques and 16S rRNA-targeted whole-cell hybridization, respectively. The results suggest that the enrichment culture on crude oil is dominated by sulfate-reducing bacteria belonging to the suggested family of the *Desulfobacteriaceae*; these mainly completely oxidizing, sulfate-reducing bacteria (72) have only rarely been considered thus far in microbiological investigations in oil fields.

MATERIALS AND METHODS

Sources of organisms. The mesophilic enrichment culture of sulfate-reducing bacteria growing with crude oil as the only source of organic substrates originated from the water phase of a North Sea oil tank, Wilhelmshaven, Germany (58). Further enrichment cultures were obtained from sulfide-rich sediments of the Guaymas Basin, Gulf of California, Mexico (58), and a small North Sea harbor near Wilhelmshaven, Germany. Pure cultures of sulfate-reducing and other bacteria used for whole-cell hybridization were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM), Braunschweig, Germany, and the American Type Culture Collection, Rockville, Md.

Media and conditions of cultivation. The enrichment culture was grown in defined, bicarbonate-buffered, sulfide-reduced mineral medium, essentially having the same salt composition as natural seawater (72), under a head space of N_2-CO_2 (90:10 [vol/vol]). Flat bottles (500 ml) with 400 ml of medium and 12 ml of crude oil were incubated horizontally to maximize the contact area between oil and medium and in this way facilitate substrate diffusion into the medium. The orifice sealed with a black rubber stopper was kept below the surface level of the medium to avoid adsorption of oil by the rubber (1, 46, 48). Anoxic crude oil was prepared and stored in a special flask provided with a side arm for gas supply of N_2 and an orifice sealed with Teflon-coated rubber (48). North Sea crude oil was kindly provided by J. Fischer, Wilhelmshaven, Germany.

Pure cultures of gram-negative sulfate-reducing bacteria (46, 72), *Desulfuromonas acetoxidans* (73), and *Pelobacter acetylenicus* (59) were grown in anoxic, bicarbonate-buffered, sulfide-reduced medium as described previously; according to the strain-specific salt requirements, either freshwater or saltwater medium as given in the corresponding literature was used. *Desulfotomaculum orientis* was grown with thioglycolate and ascorbate as reductants instead of sulfide (72). Defined organic substrates were as suggested in the references; *D. orientis* was grown with lactate. *Rhodospirillum rubrum* was grown in reductant-free medium with exclusion of oxygen on succinate and yeast extract (18). *Beggiatoa alba* was cultivated aerobically in liquid medium with acetate and yeast extract instead of nutrient broth (18). All other bacteria, *Halobacterium halobium* and *Saccharomyces cerevisiae*, were grown aerobically on agar plates with complex organic substrates as described elsewhere (6, 18).

For determination of cell numbers, a sample from the enrichment culture was consecutively diluted in steps of 1:10 with liquid anoxic medium under N_2-CO_2 (90:10 [vol/vol]) in butyl-rubber-sealed tubes (160 by 16 mm); transfers were made with N_2-flushed syringes. From each dilution step, 0.5 ml was then transferred to tubes containing approximately 10 ml of medium with molten agar, as used for the isolation of sulfate-reducing bacteria (72). Benzoate, cyclohexanecarboxylate, or caprylate (sodium salts) was added to the molten agar at final concentrations of 2 mM; acetate and lactate were added at final concentrations of 10 mM. The less-water-soluble substrates toluene (2% [vol/vol] in heptamethylnonane) and crude oil (undiluted) were added as a surface layer on top of the solidified agar (46), which in this case did not contain other organic substrates. The aromatic hydrocarbons were supplied to the cells in the agar by diffusion. The tubes were anoxically sealed under an atmosphere of N_2-CO_2 (90:10 [vol/vol]) and were incubated at 28°C.

Colonies for microscopic examination and further purification in subsequent agar dilutions were picked by means of finely drawn Pasteur pipettes (72).

For maintenance, the enrichment culture was incubated at 28°C for 6 weeks and then stored at 4°C for no longer than 8 weeks before subcultivation. No organic substrate other than crude oil was used for maintenance.

Chemical analyses. The amount of sulfide was determined by the methylene blue formation reaction in a colorimetric microassay as previously described (1).

Acetate and lactate were quantified by high-performance liquid chromatography on an ion exclusion column (300 by 7.8 mm; type WA1; Sarasep, Santa Clara, Calif.). The eluent was 0.05 mM H_2SO_4. The temperature of the column was 70°C, and the flow rate was 0.8 ml/min. The amounts of lactate (retention time, 6.7 min) and acetate (retention time, 9.7 min) were determined with a conductivity detector. The detection limit was 10 μM.

For oil analysis, samples were withdrawn from the oil phase of the enrichment culture through the stoppered orifice by means of an N_2-flushed syringe with a long (60-mm) needle. The oil samples were fractionated by medium-pressure liquid chromatography (49), and the aromatic hydrocarbon fraction was analyzed by gas chromatography as described elsewhere (58). In addition, for the separation of *m*-xylene and *p*-xylene, the aromatic fraction was analyzed on an FS-FFAP-CB-fused silica capillary column (50 m; inner diameter, 0.2 mm; film thickness, 0.22 μm; Chromatographie Service, Langerwehe, Germany) with a type 3700 gas chromatograph (Varian, Darmstadt, Germany). The temperature program was run from 70°C (5-min isotherm) to 200°C, at 3°C/min. The identification of *m*-propyltoluene was based on mass spectrometric data and comparison of relative gas chromatographic retention times with previously reported data (29). All other alkylbenzenes were identified by comparison of their gas chromatographic and mass spectrometric behavior with that of the authentic standards. Alkylbenzenes were quantified by determining their peak areas relative to that of naphthalene. Naphthalene yielded a prominent peak and belonged to those aromatic hydrocarbons which always exhibited the same ratio of peak areas before and after growth. Hence, naphthalene was considered an oil constituent that was not consumed under the given conditions (48).

The presence of desulfoviridin was tested by alkaline cell lysis under UV light (45).

Probing of whole cells. Oligodeoxynucleotide probes were synthesized by Biometra (Göttingen, Germany) with a DNA synthesizer (Applied Biosystems, Weiterstadt, Germany). The sequence of the previously described probe, SRB385 (3), was 5′-CGGCGTCGCTGCGTCAGG-3′ (complementary to 16S rRNA positions 402 to 385 [*Escherichia coli* numbering]). The sequence of the modified probe, termed SRB385Db, was 5′-CGGCGTTGCTGCGTCAGG-3′ (complementary to the same positions as those of probe SRB385). Control assays were carried out with a universal probe, EUB338, which detected all eubacteria (3), as well as with a nonsense probe, ANT. Probe EUB338 had the sequence 5′-GCTGCCTCCCGTAGGAGT-3′ (complementary to positions 355 to 338 [*E. coli* numbering]). The nonsense probe had the antisense sequence of probe SRB385. Probe 804, which had been applied to extracted rRNA (21), had the sequence 5′-CAACGTTTACTGCGTGGA-3′ (complementary to positions 804 to 821 [*E. coli* numbering]). All probes were labeled with fluorescein-5-isothiocyanate or tetramethylrhodamine-isocyanate.

The culture fluid was withdrawn from the pressurized (20 kPa), inverted bottles through the stoppers with a hypodermic needle. By this procedure, a transfer of oil and its interference with the staining procedure were avoided. The presently studied enrichment culture utilizing aromatic hydrocarbons grew homogeneously in the aqueous phase, and cells adhering to the oil phase were not detectable. Hence, the sample could be regarded as a representative cross section of the enrichment culture. The cells were washed with mineral medium and concentrated to an optical density at 660 nm of between 3 and 5. Cells from pure cultures for comparative hybridization experiments were washed in the respective media and concentrated in the same way.

The fixation and hybridization procedure was modified from that described by Amann et al. (4). For fixation, 1 volume of concentrated cell suspension was mixed with 3 volumes of 4% (wt/vol) paraformaldehyde in phosphate-buffered saline (PBS), and the mixture was incubated at 4°C for 5 h. PBS contained 20 g of NaCl and 3.0 g of $MgCl_2 \cdot 6H_2O$ (for marine bacteria) or 8.5 g of NaCl (for freshwater bacteria) per liter of 10 mM sodium phosphate buffer (pH 7.2). The cells were washed once with PBS to remove free paraformaldehyde, resuspended in PBS to yield a bacterial density of approximately 10^9 cells per ml, mixed with the same volume of absolute cold ethanol, and stored at −20°C for less than 4 weeks until hybridization. Hybridization was carried out on Teflon-covered slides with 10 glass surface windows (Cel-Line Associate, Newfield, N.J.) which were coated with gelatin [0.1% gelatin, 0.01% $KCr(SO_4)_2 \cdot 12H_2O$, in H_2O; air dried on the slide]. Aliquots of the fixed cell suspensions (2 to 5 μl) were spread on each of the gelatin-coated windows and air dried. The cells were then dehydrated by subsequent incubation in 50, 75, and 100% ethanol for 3 min (each step) at room temperature and were air dried. Each window was covered with 8 μl of hybridization buffer (1.8 mM NaCl, 0.2 M Tris-HCl [pH 7.2], 0.2% [wt/vol] sodium dodecyl sulfate, 30% [vol/vol] formamide) and 1 μl of oligonucleotide probe (25 ng). The compromise between signal intensity and stringency of hybridization was found to be optimal at a formamide concentration of 30% (vol/vol) in the hybridization buffer (formamide concentrations tested, 0, 10, 20, 25, 30, 40, 50, and 60% [vol/vol]). The slides were incubated for 2 to 5 h at 37°C in petri dishes which were sealed with Parafilm. To avoid drying of the sample, the petri dishes also contained a tissue soaked with hybridization buffer. Thereafter, the slides were incubated for 20 min in hybridization buffer at 37°C and then rinsed with distilled water to remove excess probe and salts. The slides were air dried in the dark. To prevent bleaching, 1 droplet of Citifluor (Citifluor, London, United Kingdom) was added to each window. The slides were sealed with a coverslip.

The hybridization result was examined with a fluorescence microscope (Axioplan; Zeiss, Oberkochen, Germany) equipped with a Peltier-cooled slow-scan charge-coupled device camera (Photometrics, Tucson, Ariz.) and a data system as previously described (50). The excitation wavelengths for the fluorescein-5-isothiocyanate- and the rhodamine-labeled probes were 489 and 541 nm, respectively; the emission wavelengths were 514 and 572 nm, respectively.

The fluorescence intensities of cells hybridized with labeled oligonucleotide probes were accurately measured as pixel intensities with a confocal laser scanning microscope (type LSM410; Zeiss) as described elsewhere (7, 26). The mean pixel intensity of single cells on an individual image was calculated with the software provided by Zeiss.

RESULTS

Enrichment of sulfate-reducing bacteria on crude oil. Enrichment studies with saline inocula (10% [vol/vol]) and crude oil were carried out to investigate if sulfate-reducing bacteria can in principle develop with oil constituents as sole electron donors and organic carbon sources. Mesophilic (28°C) sulfide-producing (≥10 mM) enrichment cultures on crude oil were obtained from every sample examined (North Sea oil tank, Guaymas Basin sediment, and coastal marine mud) within less than 8 weeks. A thermophilic (60°C) enrichment culture was obtained from Guaymas Basin sediment (58), but not from the other samples. Inoculated control media without oil did not produce sulfide from sulfate. With respect to the aspect of bacterial substrate utilization, such enrichment cultures may be regarded as model systems of anoxic oil-bearing strata, oil wells, pipelines, and tanks in which oil is the only potential source of organic substrates for sulfate-reducing bacteria. So far, none of the enrichment cultures with crude oil has been examined with respect to its whole bacterial composition; only one strain of a new thermophilic alkane-utilizing, sulfate-reducing eubacterium from Guaymas Basin sediment has been briefly characterized (58). In the mesophilic enrichment culture from the oil tank, bacteria grew homogeneously distributed in the aqueous phase (optical density at 660 nm, 0.15) and did not adhere to or emulsify the oil. Homogeneous growth in this culture was consistent with the observation that the somewhat water-soluble alkylbenzenes rather than the insoluble alkanes were utilized from the crude oil (58). Such homogeneous growth facilitates attempts to obtain a representative cross section of the bacterial population via samples from the aqueous phase. Hence, the enrichment culture from the oil tank appeared particularly suitable for application for the first time of both classical cell counting and molecular probing of whole cells in the analysis of bacterial populations growing on crude oil.

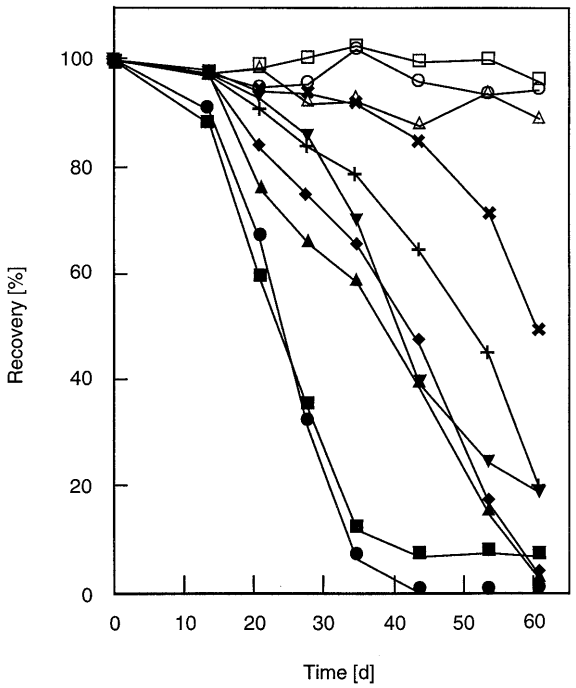

FIG. 1. Consumption of alkylbenzenes over time (d, days) in the oil-utilizing anaerobic enrichment culture. The consumption of alkylbenzenes at the given time points was calculated relative to their initial concentrations in the crude oil (recovery). Symbols for alkylbenzenes: ■, toluene; ●, *o*-xylene; ▲, *m*-xylene; ▼, *o*-ethyltoluene; ◆, *m*-ethyltoluene; +, *m*-propyltoluene; ✕, *m*-isopropyltoluene; □, ethylbenzene; ○, *p*-xylene; and △, *p*-ethyltoluene.

Selective utilization of alkylbenzenes from crude oil. The previous analysis of oil from the enrichment culture was carried out after the third transfer and revealed a sulfate-dependent consumption of toluene, *o*-xylene, *m*-xylene, *o*-ethyltoluene, and *m*-ethyltoluene (58). Possible preferences for alkylbenzenes during growth could not be recognized thus far because the oil in the initial study was analyzed only at the end of growth. Therefore, the time course of the consumption of individual alkylbenzenes from the oil was monitored in the present study (Fig. 1); at this state, the enrichment culture had been transferred eight times. Analysis of oil samples taken at intervals of 8 to 9 days revealed that toluene and *o*-xylene were consumed most rapidly. In comparison to the culture after the earlier transfers (58), the present subculture used not only C_1- to C_3-alkylbenzenes (toluene, xylenes, and ethyltoluenes) but also C_4-alkylbenzenes (*m*-propyltoluene and *m*-isopropyltoluene [*m*-cymene]). In agreement with former observations (58), no utilization of ethylbenzene, *p*-xylene, or *p*-ethyltoluene was detected.

Growth of the enrichment culture on crude oil was accelerated by incubation on a rotary shaker. Permanent shaking

shortened the incubation time required for full growth from 6 to 3 weeks (inoculum size, 5% [vol/vol]). This indicated that growth in the nonagitated culture was probably limited by the rate of substrate diffusion from the oil into the medium.

Subcultivation on individual aromatic hydrocarbons. Growth of the enrichment culture in subcultures with individual alkylbenzenes (each 2% [vol/vol] in heptamethylnonane as carrier phase [46]) confirmed the degradative capacities observed in the presence of crude oil. Toluene, *o*-xylene, and *m*-xylene yielded significant growth (optical density at 660 nm, 0.2) and net sulfide productions of 14, 13, and 7 mM, respectively, within 5 weeks (inoculum size, 5% [vol/vol]; incubation without shaking). *o*-Ethyltoluene and *m*-ethyltoluene were poorer growth substrates, yielding low cell densities (optical density at 660 nm, $\leq$0.1) and net sulfide productions of 4 and 2 mM, respectively, within 8 weeks. In contrast, neither growth nor sulfide formation occurred with ethylbenzene, *p*-xylene, or *p*-ethyltoluene.

Cell counting. The abundance of nutritional types of sulfate-reducing bacteria in the enrichment culture was determined in counting series with fatty acids and toluene as potential electron donors in oil fields (8, 17, 53, 58), with benzoate as a suitable substrate of various alkylbenzene-degrading anaerobes (11, 46, 47), and with crude oil. In counting series without added substrate, only small (diameter, $\leq$0.1 mm), frayed white colonies appeared, which probably grew on organic trace constituents of the agar; the abundance of these colonies corresponded to 10^5 CFU/ml. With any organic substrate added, distinct ochre-to-yellowish-brown, compact colonies (diameter, >0.1 mm) typical of sulfate-reducing bacteria (72) developed in addition. With substrates added as a surface layer (crude oil or toluene) to the agar tubes, the first three dilutions yielded pronounced zones of colony growth near the surface. At higher dilutions, colonies grew randomly distributed throughout the agar. The numbers of CFU obtained per ml of the enrichment culture were between approximately 6×10^6 and 8×10^6 with either crude oil, toluene, or benzoate and 2×10^6 with either acetate, caprylate, or cyclohexanecarboxylate. When well-separated pigmented colonies (20 colonies from agar tubes with oil, toluene, or benzoate) were transferred to liquid media with the corresponding substrate, growth with concomitant sulfide production occurred.

Since lactate is often employed for routine counting of sulfate-reducing bacteria (42, 45), cell counts were also determined with this substrate, and the development of colonies was examined in more detail. Lactate has been proven to be an excellent substrate for all *Desulfovibrio* species (45); however, it is also utilized by species of several other genera of sulfate-reducing bacteria (72). Indeed, at least two distinct populations of colonies developed with lactate. During the first week, relatively few big (diameter, approximately 1 mm) rapidly growing colonies appeared, corresponding to approximately 3×10^5 CFU ml^{-1} in the enrichment culture. Twelve such colonies were microscopically examined; they consisted exclusively of vibrio-shaped cells. Pure cultures obtained from this colony type (three strains studied) were desulfoviridin positive and not able to grow on crude oil, toluene, benzoate, butyrate, or acetate. Good growth occurred in 1 week on lactate, malate, ethanol, and H_2-CO_2 (80:20 [vol/vol], with 0.5 mM acetate as a carbon source for cell synthesis). The cultures formed 5.3 mM sulfide and 9.3 mM acetate when consuming 10.9 mM L-lactate, indicating incomplete oxidation (theoretical molar sulfide-to-lactate ratio, 1/2). These properties are characteristic of *Desulfovibrio* species. After a prolonged total incubation time of 3 to 4 weeks, smaller (diameter, approximately 0.2 mm) colonies appeared in high numbers, corresponding to approximately 6×10^6 CFU ml^{-1} in the enrichment culture. Again, 12 colonies of this type were microscopically examined; they contained exclusively oval to rod-shaped cells. Pure cultures obtained from this colony type (four strains studied) were desulfoviridin negative and were able to grow slowly on lactate, butyrate, toluene, and crude oil. They formed 11.5 mM sulfide when consuming 7.3 mM L-lactate, indicating complete oxidation (theoretical molar sulfide-to-lactate ratio, 3/2); in agreement with this, acetate formation was not detectable.

On any substrate including crude oil, the numbers of CFU were always significantly lower than the total cell density of 5×10^7 cells per ml, as determined by means of a counting chamber. Assuming that the utilizable substrates, viz., the alkylbenzenes, diffuse from the oil into the agar, in analogy to cultures with defined carrier phases (46), it must be concluded that only a portion of the cells in the enrichment culture formed colonies in the agar.

Probe design and whole-cell hybridization. Consumption of alkylbenzenes from oil (Fig. 1) and cell counts suggest that sulfate-reducing bacteria with the ability to degrade aromatic compounds were the dominant metabolic types in the enrichment culture. So far, all known sulfate-reducing bacteria degrading aromatic compounds have the capacity for complete oxidation of organic substrates (11, 46, 72). On the basis of 16S rRNA sequences, all non-spore-forming, completely oxidizing, sulfate-reducing bacteria belong to a cluster within the delta subdivision of the *Proteobacteria*; the cluster was tentatively termed *Desulfobacteriaceae* and opposed to *Desulfovibrionaceae* (20, 72). Therefore, we expected dominance of complete oxidizers and hence of members of the *Desulfobacteriaceae* in the enrichment culture. To verify this assumption, several described probes labeled with fluorescent dyes were tested against whole cells in hybridization assays.

A probe termed 804 which specifically hybridized with extracted DNA from several members of the *Desulfobacteriaceae* (21) did not yield a hybridization signal with whole cells of selected members of this family (*Desulfobacter latus*, *Desulfobacter postgatei*, *Desulfobacterium autotrophicum*, *Desulfobulbus propionicus*, and *Desulfococcus multivorans*) and the enrichment culture. In agreement with other reports (3, 50), probe SRB385 hybridized specifically with *Desulfovibrio*, *Desulfomicrobium*, and *Desulfobulbus* species, but not with complete oxidizers such as *Desulfobacter*, *Desulfobacterium*, *Desulfobacula*, *Desulfococcus*, and *Desulfonema* species (Table 1). As expected, this probe yielded distinctive hybridization signals only with a small portion of the cells in the enrichment culture (<5%; Fig. 2F). Sequence comparison between probe SRB385 and the target site on the 16S rRNA of most members of the *Desulfobacteriaceae* (Table 1) revealed a single base mismatch at position 396 (*E. coli* numbering). To achieve a complete match, cytosine at position 396 in probe SRB385 was substituted with thymine, yielding a probe which we termed SRB385Db. In contrast to probe SRB385, the modified probe, SRB385Db, hybridized with all tested members of the *Desulfobacteriaceae*, except for *Desulfobulbus* species, but not with *Desulfovibrio* species as the main representatives of the *Desulfovibrionaceae*. In the enrichment culture, more than 95% of the cells yielded a distinctive hybridization signal with probe SRB385Db (Fig. 2E). The signal intensity was lower than that in the case of the tested pure cultures, as can be expected for slowly growing bacteria such as those in the enrichment culture (5). Nevertheless, measurement of mean pixel intensities (7) clearly demonstrated the specificity of the hybridization in comparison with those of the other probes applied (Table 2). In control experiments, the universal bacterial (eubacterial) probe EUB338 hybridized with all reference strains and cells

TABLE 1. Target regions of 16S rRNA from various sulfate-reducing and other bacteria and results of whole-cell oligonucleotide probing

Bacterial species[a]	Oxidation[b]	Target sequence on the 16S rRNA[c]	Reference[d]	Fluorescence signal of probe[e]			
				SRB385	SRB385Db	EUB338	ANT
Desulfovibrionaceae							
Desulfovibrio africanus (DSM 2603)	I	5′-CCUGAC GCAGC**G**ACGC CG-3′	20				
Desulfovibrio desulfuricans (DSM 6949)	I	5′-CCUGAC GCAGC**G**ACGC CG-3′	44	+	−	+	−
Desulfovibrio gigas (DSM 1382)	I	5′-CCUNAC GCAGC**G**ACGC CG-3′	19	+	−	+	−
Desulfovibrio longus (DSM 6739)	NR	5′-NNNNAC GCAGC**G**ACGC CG-3′	32				
Desulfovibrio salexigens (DSM 2638)	I	5′-CCUGAC GCAGC**G**ACGC CA-3′	19	+	−	+	−
Desulfovibrio vulgaris (DSM 644)	I	5′-CCUNAC GCAGC**G**ACGC CG-3′	19	+	−	+	−
Desulfovibrio sp. strain El Agheila (DSM 1926)	NR	5′-CCCNAC GCAGC**G**ACGC CU-3′	20				
Desulfovibrio sp. strain Norway 4 (DSM 1741)[f]	NR	5′-CCUGAC GCAGC**N**ACGC CG-3′	20	+	−	+	−
Desulfobacteriaceae							
Sulfate reducing							
Desulfoarculus baarsii (DSM 2075)[g]	C	5′-CCUGAC GCAGC**C**AUGC CG-3′	19				
Desulfobacter curvatus (DSM 3379)	C	5′-CCUGAC GCAGC**G**ACGC CG-3′	19				
Desulfobacter hydrogenophilus (DSM 3380)	C	5′-CCUGAC GCAGC**A**ACGC CG-3′	19				
Desulfobacter latus (DSM 3381)	C	5′-CCNNAC GCAGC**A**AUGC CG-3′	19	−	+	+	−
Desulfobacter postgatei (DSM 2034)	C	5′-CCUGAC GCAGC**A**ACGC CG-3′	RDP	−	+	+	−
Desulfobacter sp. strain 4ac11 (DSM 2057)	C	5′-CCUGAC GCAGC**G**ACGC CG-3′	19				
Desulfobacterium autotrophicum (DSM 3382)	C	5′-CCUNAC GCAGC**A**ACGC CG-3′	19	−	+	+	−
Desulfobacterium niacini (DSM 2650)[h]	C	5′-CCUNAC GCAGC**A**ACGC NG-3′	19				
Desulfobacterium vacuolatum (DSM 3385)	C	5′-CCUGAC GCAGC**A**ACGC CG-3′	19	−	+	+	−
Desulfobacula toluolica (DSM 7467)	C[i]	5′-CCUGAC GCAGC**A**ACGC CG-3′	46	−	+	+	−
Desulfobotulus sapovorans (DSM 2055)[j]	I	5′-CCUGAC GCAGC**A**ACGC CG-3′	19	−	+	+	−
Desulfobulbus propionicus (DSM 2032)	I	5′-CCNNAC GCAGC**G**ACGC NG-3′	19	+	−	+	−
Desulfobulbus sp. strain 3pr10 (DSM 2058)	I	5′-CCUNAC GCAGC**G**ACGC CG-3′	19				
Desulfococcus multivorans (DSM 2059)	C	5′-CNUNAC GCAGC**A**ACGC CG-3′	19	−	+	+	−
Desulfomonile tiedjei (ATCC 49306)	NR	5′-UCUGAC GCAGC**A**ACGC CG-3′	22				
Desulfonema limicola (DSM 2076)	C	5′-CCUGAC GCAGC**A**ACGC CG-3′	25	−	+	+	−
Desulfonema magnum (DSM 2077)	C	5′-CCUGAC GCAGC**A**ACGC CG-3′	25	−	+	+	−
Desulfosarcina variabilis (DSM 2060)	C	5′-CCUGAC GCAGC**A**ACGC CG-3′	RDP				
Strain Hxd3 (DSM 6200)	C[k]	5′-CCUGAC GCAGC**A**ACGC CG-3′	2				
Nonsulfate reducing							
Desulfuromonas acetoxidans (DSM 684)	C[l]	5′-CCUGAC GCAGC**A**ACGC CG-3′	RDP	−	+	+	−
Myxococcus xanthus (DSM 435)	C[m]	5′-CCUGAC GCAGC**A**ACGC CG-3′	44	−	+	+	−
Pelobacter acetylenicus (DSM 3246)	I[n]	5′-CCUGAC GCAGC**A**ACGC CG-3′	24	−	+	+	−
Gram positive, spore forming							
Desulfotomaculum orientis (DSM 765)	I	5′-GGCGAC GAUCA**G**UAGC CG-3′	19	−	−	+	−
Other bacteria							
Bacillus subtilis (DSM 10)		5′-UCUGAC GGAGC**A**ACGC CG-3′	36	−	−	+	−
Beggiatoa alba (DSM 1416)		5′-CCUGAU CCAGC**A**AUGC CG-3′	63	−	−	+	−
Comamonas testosteroni (DSM 50244)[o]		5′-CCUGAU CCAGC**A**AUGC CG-3′	74	−	−	+	−
Corynebacterium variabilis (DSM 20132)		5′-CCUGAU GCAGC**G**ACGC CG-3′	15	−	−	+	−
Escherichia coli K-12 (DSM 498)		5′-CCUGAU GCAGC**C**AUGC CG-3′	61	−	−	+	−
Micrococcus luteus (ATCC 381)		5′-CCUNAU GCAGC**G**ACGC CG-3′	RDP				
Nocardioides simplex (DSM 20130)		5′-CCUGAU CCAGC**A**ACGC CG-3′	RDP	−	−	+	−
Rhodospirillum rubrum (DSM 467)		5′-CCUGAC GCAGC**C**AUGC CG-3′	31	−	−	+	−
Other organisms							
Halobacterium halobium (DSM 670)		5′-UGCGAU AAGGG**G**ACUC CG-3′	34	−	−	−	−
Saccharomyces cerevisiae (DSM 70449)		5′-GGGAGG UAGUG**A**CAAU AA-3′	57	−	−	−	−
Probe SRB385		3′-GGACTG CGTCG**C**TGCG GC-5′	3				
Target		5′-CCUGAC GCAGC**G**ACGC CG-3′					
Probe SRB385Db		3′-GGACTG CGTCG**T**TGCG GC-5′					
Target		5′-CCUGAC GCAGC**A**ACGC CG-3′					

[a] Collection numbers are in parentheses.
[b] Oxidation of organic substrates. C, complete; I, incomplete; NR, not reported. If not noted otherwise, data are taken from reference 72.
[c] Target sequences are complementary to positions 402 to 385 on the 16S rRNA (*E. coli* numbering). Sequence data are from the ribosomal RNA data base project (33) and from the European Bioinformatics database (23).
[d] References indicate original publication of 16S rRNA sequence and phylogenetic affiliation. RDP, available only from the ribosomal data base project (33).
[e] For descriptions of probes, see Materials and Methods.
[f] Future reclassification as *Desulfomicrobium baculatum* appears appropriate (72).
[g] Formerly described as *Desulfovibrio baarsii* (72).
[h] Formerly described as *Desulfococcus niacini* (72).
[i] Reference 46.
[j] Formerly described as *Desulfovibrio sapovorans* (72).
[k] Reference 1.
[l] Reference 73.
[m] Aerobic bacteria with terminal oxidation (52).
[n] Reference 59.
[o] Previously named *Pseudomonas testosteroni* (74).

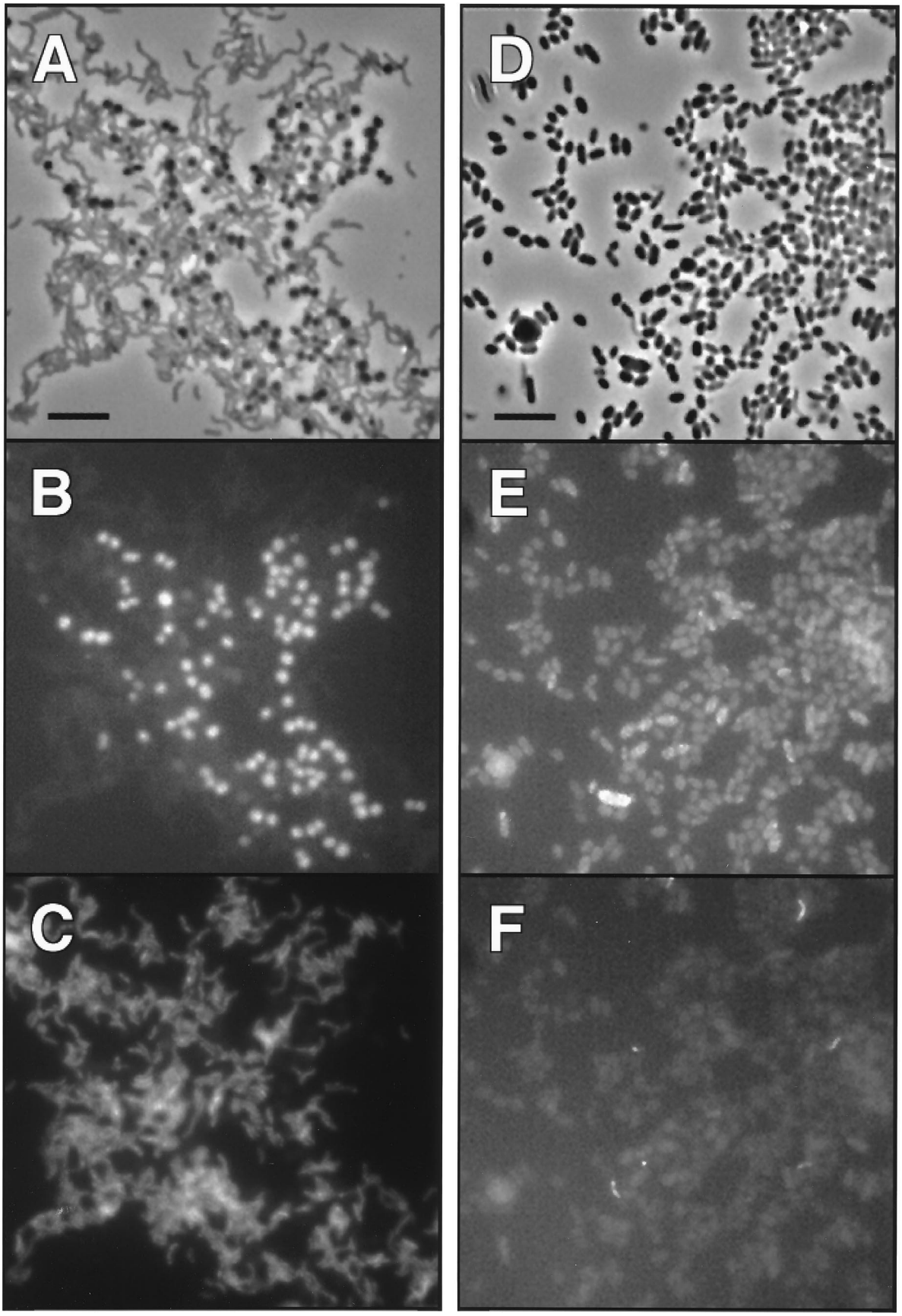
A
D
B
E
C
F

FIG. 2. Differentiation between *Desulfobacteriaceae* and *Desulfovibrionaceae* by whole-cell hybridization of reference organisms and the oil-utilizing sulfate-reducing enrichment culture. Whole-cell hybridization was carried out with the fluorescein-5-isothiocyanate-labeled probe SRB385Db (green fluorescence) and the rhodamine-labeled probe SRB385 (red fluorescence). Left panels are microscopic images of a mixture of pure cultures of the completely-oxidizing *Desulfococcus multivorans* (coccoid shape) and the incompletely oxidizing *Desulfovibrio desulfuricans* (vibrioid shape); right panels are microscopic images of the oil-utilizing enrichment culture. (A and D) Phase-contrast photomicrographs; (B and E) digital images after hybridization with probe SRB385Db and excitation at 489 nm; (C and F) digital images after hybridization with probe SRB385 and excitation at 541 nm. Bars, 10 μm.

of the enrichment culture. The antisense probe ANT yielded a much weaker signal with the reference strains and the enrichment culture. In conclusion, probes SRB385 and SRB385Db allowed a differentiation between members of the *Desulfovibrionaceae* (incomplete oxidizers) and *Desulfobacteriaceae* (mostly complete oxidizers) in defined cultures (Fig. 2A to C) and the enrichment culture of sulfate-reducing bacteria (Fig. 2D to F).

DISCUSSION

The presently studied enrichment culture with crude oil as the only source of organic substrates may be regarded as a laboratory model of the anaerobic microbial community that grows in moderately warm oil reservoirs, wells, tanks, and pipelines on indigenous substrates. The main difference between our laboratory culture and the in situ conditions (outside in the oil field) lies in the ion concentrations in the enrichment culture being constantly the same as those in seawater and in the growth conditions being optimized by the addition of ammonium, phosphate, vitamins, and high-sulfate concentrations to achieve appropriate growth times and cell densities for the analytical experiments. Even if under the presumably nonoptimal growth conditions in oil reservoirs and production plants the sulfate-reducing bacteria probably exhibit slower and poorer growth than in the enrichment culture, a utilization of alkylbenzenes from crude oil as in the model culture should in principle be possible also under in situ conditions. This assumption is supported by other studies showing selective depletion of alkylbenzenes in incubation experiments with nondegraded oils and sulfate-reducing bacteria from reservoir waters, as well as in oils from biodegraded reservoirs (16). Therefore, it may be concluded that alkylbenzenes as an important class of oil constituents belong to the major indigenous electron donors for sulfate reduction in moderately warm oil reservoirs and production plants (viz., under mesophilic conditions), as long as no further potential electron donors are provided; these may originate from added biodegradable chemicals (17) or from partial hydrocarbon oxidation by aerobic bacteria upon access of air (12, 39). Actually, toluene and xylenes together may account for as much as 3% of the oil constituents (64). Other quantitatively significant indigenous electron donors might be saturated hydrocarbons, as shown with a moderately thermophilic sulfate-reducing bacterium that grew by utilization of alkanes from crude oil (58). The possible role of mesophilic alkane-utilizing sulfate-reducing bacteria such as strain Hxd3 (1) in sulfide production has not been studied thus far in a model system. Extremely thermophilic sulfate-reducing *Archaea* that were detected in oil fields (9, 28, 62) have not been shown to utilize hydrocarbons.

TABLE 2. Relative mean pixel intensities of hybridization signals obtained with cells from the oil-utilizing enrichment culture

Probe	Mean pixel intensity[a]	Standard deviation
SRB385Db	83.8	6.2
SRB385	19.3	1.5
EUB338	69.1	1.7
ANT	23.6	2.9

[a] Measured by using a confocal laser-scanning microscope (LSM410; Zeiss). Five microscopical sections were analyzed to determine the fluorescence intensity of cells with each probe. The size of one section was 7,300 μm^2; the number of cells per section was ≥800.

The assumption of an anaerobic utilization of alkylbenzenes by sulfate-reducing bacteria in oil fields has to be reconciled with the observation that alkylbenzenes are still present in many oils. The preservation of anaerobically degradable constituents in oils over geological time in our opinion has several explanations. First, at many sites in the reservoir sulfate may have become the limiting factor for anaerobic oxidation processes, because of bacterial sulfate reduction during early diagenesis and oil maturation (40). Indeed, in many production waters, sulfate concentrations of less than 1 mM have been measured (8, 13, 41). Second, oil in reservoirs is trapped in the pores of rocks (64), which may impede diffusion of substrates and their accessibility by bacteria. Only during the process of oil extraction by water injection may conditions for bacterial growth become favorable by mixing processes, formation of emulsions, and new contact areas between oil and water. Third, sulfate-reducing bacteria that were formerly buried with the ancient sediments may have died off in the reservoirs because of unfavorable conditions (extreme temperature and salinity) at the deep sites of catagenesis and because of sulfate depletion. Only during secondary oil recovery are new bacteria introduced with the injected water. Upon cooling and dilution, the temperature and salinity, respectively, may become favorable for a variety of bacteria wider than that which could have developed before. If seawater is injected, high concentrations of the formerly depleted electron acceptor, sulfate, are also provided. The increase in sulfide production in oil field waters after the onset of operations may reflect the gradual spreading and growth of sulfate-reducing bacteria (17, 62). On the other hand, there are also assumptions that sulfate-reducing bacteria as well as other microorganisms in oil fields were deposited with the original sediments and survived for millions of years (28, 41, 53). In this case, the question would remain of how cells remained viable over such long periods. Survival appears unlikely in niches which are closed systems, viz., in which substrates are depleted after a while. A prerequisite for survival would be a minimal but more or less continuous supply of growth substrates to sustain viability over geological time, e.g., by slow hydrodynamic migration processes.

The high number of colonies obtained with toluene and benzoate confirmed that the ability to utilize aromatic compounds is a dominant physiological characteristic among the enriched cells. So far, sulfate-reducing bacteria in oil fields have often been counted and isolated with lactate (13, 37, 39, 42, 54), a component of standard media, which is particularly useful for rapid detection of *Desulfovibrio* species. *Desulfovibrio* species are effective scavengers of hydrogen (70); they may become abundant in oil fields if hydrogen is available, for instance from corroding or cathodically protected steel surfaces (27, 71) or from fermentative degradation of xanthan gum which is used in enhanced oil recovery (17). Cell counting and whole-cell probing in the oil-utilizing enrichment culture

revealed only a low percentage of *Desulfovibrio* species. This finding is in agreement with the experience that *Desulfovibrio* species are nutritionally relatively restricted and have never been reported to utilize aromatic compounds (72). In the presently studied enrichment culture, the *Desulfovibrio* species may have grown with low concentrations of organic compounds excreted by other cells or liberated upon cell lysis. However, *Desulfovibrio* species were not the only sulfate reducers that formed distinctive colonies in agar with lactate. Prolonged incubation of the agar tubes revealed high numbers of slowly growing, completely oxidizing sulfate-reducing bacteria able to grow on toluene and oil. Hence, even though lactate is not utilized by all completely oxidizing, sulfate-reducing bacteria (46, 72), long incubation of counting series with this substrate may allow detection of some of the species that utilize aromatic compounds in situ.

The newly applied probe SRB385Db allowed group-specific whole-cell detection for the first time of members of the *Desulfobacteriaceae*, a branch which besides a few incomplete oxidizers encompasses all completely oxidizing gram-negative, mesophilic sulfate-reducing bacteria. As expected from the target sequence, the probe also yielded a signal with the sulfur reducer *D. acetoxidans*, the fermentative bacterium *P. acetylenicus*, and the aerobe *Myxococcus xanthus*, which are all members of the delta subdivision of the *Proteobacteria*. Moreover, according to a search in the ribosomal data base (33), the probe would theoretically also react with *Bdellovibrio* species of the delta subdivision and with 8 *Campylobacter* species or strains, 3 *Chlorobium* species, 1 *Bacteroides* species, 2 *Leptospira* species, and 23 *Clostridium* species. The original probe, SRB385, would react with many actinomycetes. However, under the anoxic conditions in the oil-utilizing enrichment culture, which did not contain fermentable compounds, none of these non-sulfate-reducing bacteria is expected. Indeed, growth, sulfide production and anaerobic alkylbenzene utilization by the enriched bacterial population were strictly sulfate dependent (58).

For monitoring and investigation of sulfate-reducing bacteria in oil fields, alkylbenzene-degrading and other completely oxidizing species, viz., members of the *Desulfobacteriaceae*, will have to be considered in the future. However, for routine investigations in oil fields, the use of counting series with appropriate media and substrates appears presently easier to achieve than whole-cell hybridization with oligonucleotide probes. Even though counting series require incubation times of a few weeks, the costs for the technical expenditures are less than those for whole-cell hybridization. Recognition of potentially alkylbenzene-utilizing cells via the hybridization signal requires at least an epifluorescence microscope. Furthermore, conditions in oil wells, pipelines, and oil tanks are less defined than those in the presently studied, strictly anaerobic enrichment culture. Under incomplete exclusion of air and upon addition of chemicals such as xanthan gum to injection waters, aerobic as well as anaerobic fermentative bacteria are likely to develop and yield additional signals with the fluorescently labeled probes. In conclusion, at the present time 16S rRNA-based whole-cell hybridization appears practically useful in a selected number of scientific case studies rather than in routine monitoring of sulfate-reducing bacteria in oil fields.

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