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Spore-Forming Thermophilic Sulfate-Reducing Bacteria Isolated from North Sea Oil Field Waters

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Thermophilic sulfate-reducing bacteria were isolated from oil field waters from oil production platforms in the Norwegian sector of the North Sea. Spore-forming rods dominated in the enrichments when lactate, propionate, butyrate, or a mixture of aliphatic fatty acids (C_4 through C_6) was added as a carbon source and electron donor. Representative strains were isolated and characterized. The isolates grew autotrophically on H_2-CO_2 and heterotrophically on fatty acids such as formate, propionate, butyrate, caproate, valerate, pyruvate, and lactate and on alcohols such as methanol, ethanol, and propanol. Sulfate, sulfite, and thiosulfate but not nitrate could be used as an electron acceptor. The temperature range for growth was 43 to 78°C; the spores were extremely heat resistant and survived 131°C for 20 min. The optimum pH was 7.0. The isolates grew well in salt concentrations ranging from 0 to 800 mmol of NaCl per liter. Sulfite reductase P582 was present, but cytochrome *c* and desulfoviridin were not found. Electron micrographs revealed a gram-positive cell organization. The isolates were classified as a *Desulfotomaculum* sp. on the basis of spore formation, general physiological characteristics, and submicroscopic organization. To detect thermophilic spore-forming sulfate-reducing bacteria in oil field water, polyvalent antisera raised against antigens from two isolates were used. These bacteria were shown to be widespread in oil field water from different platforms. The origin of thermophilic sulfate-reducing bacteria in the pore water of oil reservoirs is discussed.

Sulfate-reducing bacteria (SRB) have caused problems in the various stages of oil production for almost a century. These bacteria reduce sulfate to hydrogen sulfide (H_2S), which causes corrosion of iron and steel alloys in oil wells and in the oil-processing system. Precipitation of sulfides in the oil reservoir may also reduce the permeability of the oil formation. The production of toxic H_2S may present a health hazard to the platform personnel and diminish oil quality by the souring of oil and gas (1, 9, 13, 22).

Water injection stimulates the productivity of many of the North Sea oil reservoirs, but a consequence may be an increased biogenic H_2S production. The mixture of seawater and in situ pore water provides favorable growth conditions for anaerobic bacterial communities, including SRB. Organic acids found in the pore water (2, 3, 6, 16) may be utilized by the SRB as carbon and energy sources, and seawater provides high concentrations of sulfate. However, the growth of SRB is limited by the physical conditions in the reservoir. The generation of H_2S at concentrations high enough to affect oil and gas production depends on favorable nutrients and physical conditions. The North Sea oil reservoirs, located 2 to 4 km below the sea level, are special geothermal habitats. The temperature varies from 60 to 120°C, and the pressure varies from 200 to 500 bar (20 to 50 MPa).

The majority of thermophilic SRB reported in the literature are isolated from geothermal habitats (1, 10, 17, 19, 25, 36), and their growth temperatures reflect the environment from which they were isolated. The optimum growth temperature for thermophilic eubacterial SRB ranges from 55 to 70°C, and the maximum growth temperature ranges from 70 to 85°C. The archaebacterial SRB *Archaeoglobus fulgidus* from thermal volcanic areas has a temperature optimum of 83°C and a maximum of 92°C (30).

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The origins of microbial populations in oil reservoirs have been discussed by several authors. The feasibility of microbes being injected into the reservoir by injection water was examined by Stott and Herbert (31). They concluded that the SRB isolated from North Sea water cannot grow in the reservoir unless the temperature falls below 45°C. Some authors suggest that SRB are indigenous inhabitants of oil reservoirs (1, 18, 22). Other authors claim that SRB are introduced into oil field waters as a result of exploration of the deposits (24).

We have isolated and characterized thermophilic SRB from North Sea oil field waters, i.e., produced waters comprising pure formation water or a mixture of formation water and injection water. Antibodies were raised against the isolated strains and used to determine the distribution of similar thermophilic SRB in water samples from different oil fields.

MATERIALS AND METHODS

Isolation and cultivation. Thermophilic SRB were enriched and isolated from oil field waters separated from crude oil. The waters originated from porous rock formations 2 to 4 km below the sea floor in the Norwegian sector of the North Sea. The water samples were taken from the production wellhead or from separators on the platform deck. Water from deeper formations in the reservoirs had the highest temperatures. The temperature at the sampling points varied between 60 and 85°C. On the platform, the wellhead had the highest temperature; it decreased further out in the processing system. The pH of the water after pressure release was 7.8.

For enrichment of SRB, water samples of 5 ml were added to 50 ml of the marine medium described by Widdel and Pfennig (35). Then 1 ml of trace element solution SL-10 (34) and 5 ml of vitamin solution (20) were added per liter of medium. The pH was adjusted to 7.1 with HCl or Na_2CO_3.

Acetate, propionate, or lactate was added aseptically to the medium from a sterile stock solution to a final concentration of 20 mmol/liter. Three consecutive transfers of the enrichment culture to the same medium were carried out. Pure cultures were isolated by using anaerobic gellan gum (Kelco Division of Merck and Co., San Diego, Calif.) in dilution series with an appropriate carbon source under N_2 and CO_2 in tubes. The incubation temperature was 60°C.

Chemical and other determinations. Bacterial morphology was investigated by using a phase-contrast microscope (Leitz Labolux K). For electron micrographs, cells were fixed with glutaraldehyde and embedded in Spurr low-viscosity resin (29) as described by Walther-Mauruschat et al. (33). Thin sections were contrasted with uranyl acetate (3%) and lead citrate and examined in a JEOL 100 CX electron microscope. The DNA was extracted and purified as described by Silhavy et al. (26, 27). The guanine-plus-cytosine content of the DNA was measured by thermal denaturation as described by De Ley (11) with DNA from *Escherichia coli* B type VIII DNA (Sigma D-2001) as a reference. The thermal denaturation profile was measured in a Shimadzu UV-240 UV-visible recording spectrophotometer.

The concentration of hydrogen sulfide was measured by the methylene blue method (7) or with copper sulfate (8). Acetate, volatile fatty acids, and alcohols were assayed by gas chromatography with a Hewlett-Packard HP 5890 gas chromatograph with a Carbowax 20 M column, flame ionization detector, and helium (30 ml/min) as carrier gas. Samples were acidified with formic acid and centrifuged before injection.

Test of heat resistance. A 10-ml suspension of endospores was added to a sterile 15-ml glass tube and gassed with N_2-CO_2 (80:20, vol/vol); then a rubber stopper and an aluminum cap were used to seal the tube. The tubes were then exposed to elevated temperatures for various time intervals. Overpressure was used at temperatures above 100°C. After the heat treatment, the spore suspension was transferred to fresh medium and incubated at 60°C.

Characterization of pigments. Frozen cells were suspended in 0.5 mmol of potassium phosphate buffer (pH 7.0) per liter and disrupted in a French pressure cell at 1,030 bar (103.0 MPa). Cell debris and metal sulfides were removed by centrifugation at 10,000 × *g* for 15 min. The extract was centrifuged at 130,000 × *g* for 30 min. The supernatant (cytoplasmic fraction) was tested for desulfoviridin by measuring its absorption spectrum and also applying the fluorescence test of Postgate (21). After the membrane pellet was washed once with 0.5 mmol of potassium phosphate buffer (pH 7.0) per liter, it was suspended in the same buffer containing 0.5% (vol/vol) Triton X-100 to extract pigments. Cytochromes of the *b* and *c* types in this extract and the carbon monoxide difference spectrum of the cytoplasmic fraction were measured as described by Widdel and Pfennig (35).

Serology. Polyvalent antisera were raised in rabbits against vegetative bacteria and spores of strains T93B and T90A. Fluorescence-labeled antibodies were then used to test the cross-reactivity among the different SRB strains as well as among other bacteria. Samples from oilfield water were filtered on 0.2-μm-pore-size filters (Nucleopore) and double stained with 4′,6-diamidino-2-phenylindole for total count of bacteria and fluorescein isothiocyanate-conjugated swine anti-rabbit antibodies for specific SRB staining (14). The stained preparations were examined with a Leitz Orthoplan microscope. Western immunoblotting was carried out as described by Burnette (4).

RESULTS

Enrichments and isolation. The oil field water contained a heterogenous population of bacteria when examined in the light microscope. Enrichments of thermophilic SRB were obtained with acetate, lactate, propionate, butyrate, or a mixture of valerate, caproate, and caprylate as carbon sources and electron donors. The enrichment cultures grew slowly; it took 3 to 6 weeks before H_2S could be detected in the culture, and occasionally 12 weeks of incubation was necessary. When H_2S was detected, enrichments were subcultured in the same medium. During three or four subsequent transfers, the population developed into a culture dominated by H_2S-producing, spore-forming bacteria. Although growth of the strains in enrichment cultures was very slow, pure cultures became dense after only 2 days of incubation in media with lactate or pyruvate.

Immunofluorescence studies showed that all H_2S-producing enrichment cultures from these water samples, regardless of the carbon and energy sources, contained morphologically similar bacteria that cross-reacted with antisera raised against T90A and T93B.

Characteristics of isolated strains. (i) Morphological properties. The cells of the isolated thermophilic SRB strains T93B and T90A were straight or curved rods, 0.6 to 1.0 μm by 2.0 to 3.0 μm, with rounded ends. Cells were usually single, but close to the maximum growth temperature they were sometimes arranged in chains. No flagella were observed with electron microscopy. Cells in the exponential phase stained gram negative. However, transmission electron micrographs revealed a gram-positive cell wall organization, similar to that observed for *Desulfotomaculum nigrificans* by Sleytr et al. (28) (Fig. 1B). Formation of endospores was observed in old cultures and in cultures exposed to high temperatures. Spores were spherical and paracentral and distended the cells (Fig. 1A). A 7-day-old colony embedded in gelrite gellan gum was white to yellow. The colonies were smooth and shaped like a discus.

(ii) Growth and nutritional properties. The ability to use different fatty acids and alcohols as carbon sources and electron donors was tested and compared with those of other thermophilic *Desulfotomaculum* species (Table 1). In a sulfate-free medium, strains T93B and T90A fermented pyruvate but not lactate, malate, fumarate, or fructose. With sulfate as an electron acceptor, the strains grew on butanol, propanol, valerate, butyrate, and lactate and produced H_2S to a concentration higher than 10 mmol/liter. When the bacteria grew on fatty acids, acetate accumulated in the medium. If the inoculum was taken from an old preculture, initiation of growth was favored by the addition of dithionite (0.2 mmol/liter, final concentration) as a strong reductant. D-Biotin was the only vitamin found to be essential for growth. However, better growth yield was observed when the complete vitamin solution described by Widdel et al. (34) was added.

Strains T93B and T90A grew at temperatures between 43 and 78°C, with optimal growth at 65°C (Fig. 2). Slow growth was observed at 43 and 78°C but not at 40 or 85°C. Growth occurred between pH 6.5 and 7.5 with an optimum at pH 7.0. Both strains grew at NaCl concentrations ranging from 0 to 1,200 mmol/liter. With both strains the growth yield (2×10^8 to 3×10^8 cells per ml), the H_2S production rate, and the final concentration of H_2S were highest with low salt con-

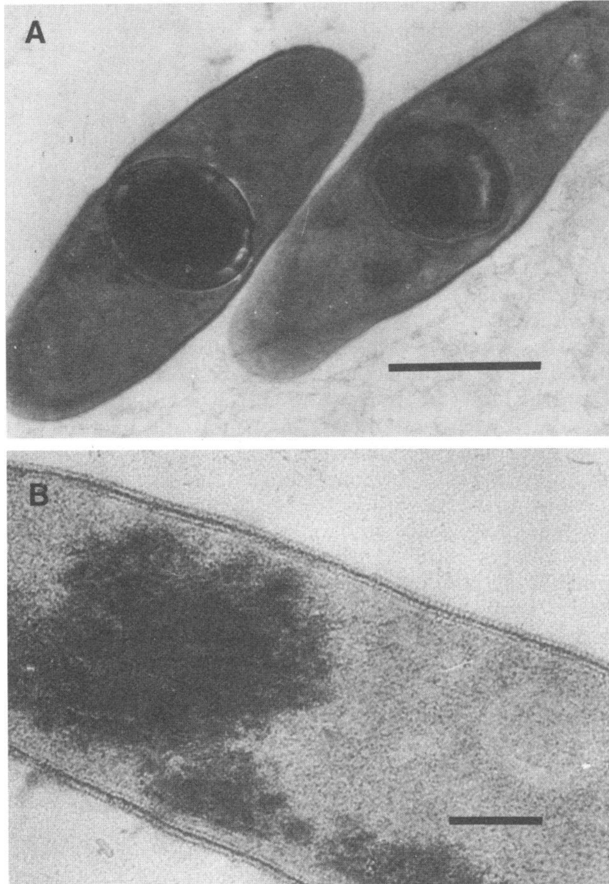

FIG. 1. Electron micrographs of the strain T93B. (A) Cells with spores from the stationary phase. Bar, 1 μm. (B) Thin section showing the gram-positive cell wall structure. Bar, 0.2 μm.

centrations (Fig. 3). During the first 8 days of incubation, no production of H_2S was observed in cultures containing 800, 1,000, or 1,200 mmol of NaCl per liter. However, after 18 days of incubation at these NaCl concentrations, the number of bacteria had increased two- to threefold and the H_2S concentration had increased to 5 to 7 mmol/liter.

Spores of strains T93B and T90A were highly heat resistant and were viable after exposure to 131°C for 20 min.

(iii) Pigments. Desulfoviridin was not detected in strains T93B and T90A. No type *c* cytochromes were found, but the Triton X-100 extract of the membrane fraction exhibited peaks at 558 nm, indicating the presence of type *b* cytochromes. The carbon monoxide difference spectrum of the cytoplasmic fraction exhibited peaks at 416, 542, and 594 nm and troughs at 435, 558, and 620 nm, similar to the spectrum of sulfite reductase P582 in *D. nigrificans* observed by Trudinger (32).

Serological identification. Antisera raised against strains T93B and T90A exhibited similar reactions. Both reacted with T93B and T90A in immunofluorescence tests and enzyme-linked immunosorbent assays. Western blots of whole cells showed that T93B and T90A had several common bands. However, at least three bands were specific for T90A with antisera raised against T93B and vice versa. Both sera reacted weakly with *D. nigrificans* and marine isolates of *Desulfovibrio desulfuricans* but not with *Desulfovibrio vulgaris*, *Desulfotomaculum acetoxidans*, *Desulfobacter propionicus*, *Thermodesulfobacterium mobile*, or a marine *Pseudomonas* sp.

Distribution of strains T93B and T90A. Strains of an immunological serotype similar to that of T93B and T90A were found in samples from three platforms of the Statfjord field (Statfjord A, B, and C), from two platforms of the Gullfaks field (Gullfaks A and B), from the Valhall field, and from the Ekofisk field. On the platforms Statfjord A, B, and C and Gullfaks A and B, the samples were taken on the wellhead of the production well before entering the separator system. Samples from Valhall and Ekofisk were collected at the end of the oil-processing system.

DISCUSSION

The physiological characterization showed that the isolated strains were very similar. Strains T93B and T90A were able to utilize the same spectrum of carbon sources and electron donors and showed similar responses to growth temperatures and salt concentrations. The two strains could be differentiated on the basis of their guanine-plus-cytosine contents and antigenic properties when whole cells were tested by Western blotting. The ability to form spores and the gram-positive cell wall type placed these isolates in the genus *Desulfotomaculum* (Table 1; Fig. 1). *D. nigrificans* has for several decades been the only thermophilic endospore-forming sulfate reducer (5). However, recently, three new *Desulfotomaculum* species have been described: *D. thermoacetoxidans* (17), *D. geothermicum* (10), and *D. kuznetsovii* (19).

Strains T93B and T90A could be distinguished from *D. nigrificans* by their higher optimum and maximum temperatures and by their ability to grow autotrophically on H_2-CO_2 and heterotrophically on methanol. Fructose was not utilized as carbon source, and the guanine-plus-cytosine content was higher than those in other *Desulfotomaculum* species (Table 1). It has been reported that high sulfide concentrations are inhibitory to *Desulfotomaculum* species and that growth ceases at H_2S concentrations around 4 mmol/liter (15). Our isolates grew and produced H_2S to a concentration of 13 to 18 mmol per liter on some substrates (Fig. 2).

The ability to grow autotrophically on H_2 and CO_2 is also found in the recently described species *D. thermoacetoxidans*, *D. geothermicum*, and *D. kuznetsovii*. Differentiation between these species and strains T93B and T90A can be made on the basis of acetate utilization (found in *D. thermoacetoxidans* and *D. kuznetsovii*) and growth on methanol (*D. kuznetsovii*, T93B, and T90A). The strains also differ with respect to their optimum and maximum growth temperatures and their guanine-plus-cytosine contents. Although the data given in Table 1 are sufficient to distinguish between T93B, T90A, and the previously described *Desulfotomaculum* species, the differences do not justify the addition of new species to the genus. On the contrary, the high content of guanine plus cytosine found in strain T90A indicates that we are dealing with a broad range of phenotypically similar strains and that DNA-DNA homology or 16S rRNA sequences for all of the above-mentioned species and strains should be examined before any species affiliation is made.

As shown by using the fluorescent-antibody technique with antibodies against T93B and T90A, the thermophilic spore-forming SRB dominated all our enrichment cultures on acetate, lactate, propionate, or butyrate or a mixture of valerate, caproate, and caprylate. These bacteria were de-

TABLE 1. Comparison of physiological and nutritional properties of the strains T93B and T90A with those of other thermophilic *Desulfotomaculum* strains[a]

Characteristic	Results for the indicated strain					
	D. nigrificans	*D. thermoacetoxidans*	*D. geothermicum*	*D. kuznetsovii*	T93B	T90A
Use of e^- donors						
H_2-CO_2	+[b]	+	+	+	+	+
Formate	+[b]	+	+	+	+	+
Acetate	−	+	−	+	−[c]	−[c]
Propionate	−	+	+	+	+	+
Pyruvate	+	+	NT	+	+	+
Lactate	+	+	+	+	+	+
Butyrate	−	+	+	+	+	+
Valerate	NT[d]	NT	NT	+	+	+
Caproate	NT	NT	+	+	+	+
Caprylate	NT	NT	NT	+	+	+
Methanol	−	NT	−	+	+[c]	+
Ethanol	+	−	+	+	+	+
Propanol	+[b]	+	NT	+	+	+
Butanol	+[b]	+	NT	+	+	+
Benzoate	−	−	−	−	−	−
Fructose	+	NT	+	−	−	−
Use of e^- acceptors						
SO_3^{2-}	+	−	+	+	+	+
$S_2O_3^{2-}$	+	+	NT	+	+	+
NO_3^-	−	−	−	−	−	−
Optimum temp (°C)	55	55–60	54	60–65	65	65
Maximum temp (°C)	70	65	56	85	78	78
G+C (mol% of DNA)	48.5–49.9	49.7	50.4	49	51	57
Gas vesicles	−	−	+	−	−	−

[a] Data from references 10, 15, 17, and 19.
[b] In the presence of 1 mmol of acetate per liter.
[c] Poorly utilized.
[d] NT, Not tested.

tected in the oil field water from all the platforms tested. Since the platforms are located in different oil fields in the North Sea, our findings indicate that thermophilic *Desulfotomaculum* species are common and widespread in oil field waters.

The physiological properties of strains T93B and T90A show that these bacteria are able to grow under the reservoir conditions. Strain T93B was tested for barotolerance in a reservoir simulator (23). The results showed that the strain produced H_2S at 78°C and 300 bar (30 MPa), conditions that are representative of large parts of the oil reservoirs. The presence of heat-resistant endospores would enable the bacteria to survive even in the higher-temperature regions of the reservoir.

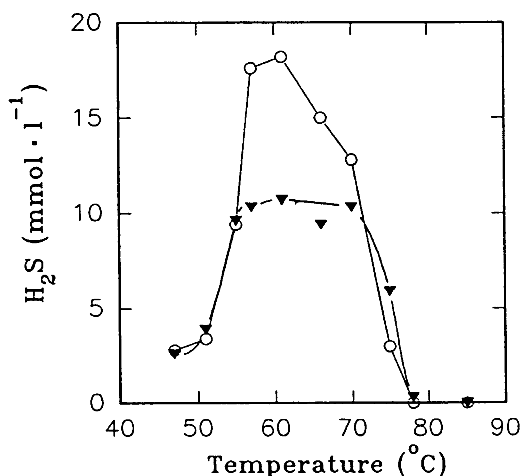

FIG. 2. Produced hydrogen sulfide after 1 week of incubation at different temperatures. Symbols: ○, T90A; ▼, T93B.

The bacteria are able to grow fermentatively on pyruvate and do not depend on sulfate for growth. This may be important, since the formation water contains little or no sulfate. The bacteria grew best at low NaCl concentrations; increasing salt concentrations inhibited growth and H_2S production. This may be an adaptation to reservoir conditions, since the salt concentration in formation water is often lower than that in seawater.

From the discussion in the literature about the origin of SRB in oil field waters (12, 18, 24, 31), three possible origins seem plausible. One is that the bacteria are injected and transported with the injection water. They develop in the reservoir and appear in the oil field water after breakthrough of injection water. The second possibility is that the area in the vicinity of a production well has been contaminated by SRB during drilling. The third possibility is that the bacteria were present in the reservoir before the exploitation of the oil field. They may have been deposited together with the original sediments and may have endured geological time, or they may have migrated through the aquifer into the reservoir from other locations.

Since T93B and T90A were isolated from the formation water before the breakthrough of injection water, the first hypothesis can be ruled out. This hypothesis would also

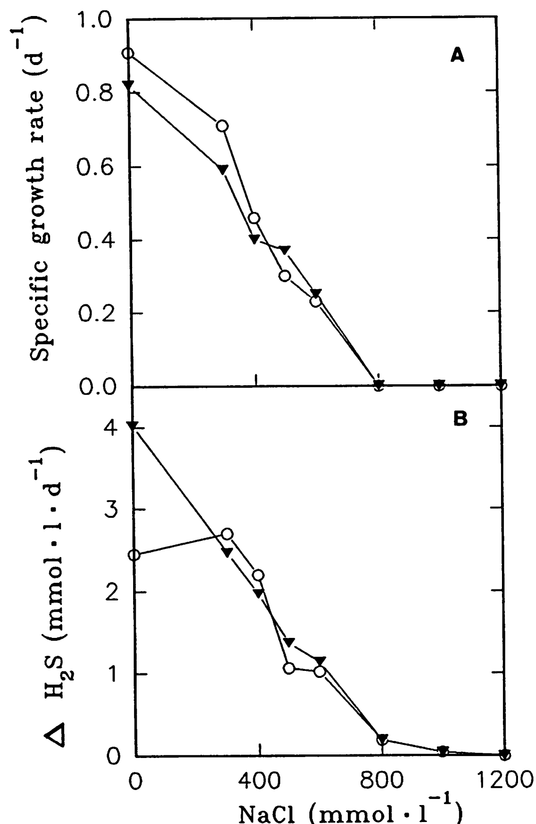

FIG. 3. Effect of the NaCl concentration in the growth medium on (A) the maximum specific growth rate (per day) and (B) the hydrogen sulfide production rate. Symbols: ○, T90A; ▼, T93B.

require the presence of thermophilic SRB in North Sea water, used as injection water. This possibility was evaluated by Stott and Herbert (31) and found to be unlikely unless the temperature falls below 45°C. This conclusion was based on the effects of elevated pressures and temperatures on growth, morphology, and metabolic activity of SRB isolated from the North Sea.

The introduction of bacteria by contaminated drilling mud or drilling equipment, as in the second hypothesis, cannot be ruled out. However, due to the large volumes of oil produced from these wells (one well usually produces thousands of cubic meters of oil per day), only a very high cell production can explain our experience of repeatedly subculturing thermophilic SRB from small volumes of oil field water. Such a high cell production is not likely to occur. During the period before formation water breakthrough, the contaminated area would contain a very low content of pore water. Hence, limited amounts of nutrients and electron donors are available, and no electron acceptor is available. T93B and T90A were isolated 3 and 5 years after the oil production had started. These fields had produced oil for more than a year before formation water appeared in the production well. The fatty acids found in formation waters cannot be fermented, and only after the injection of oil field chemicals may fermentable substrates become available. Therefore, it is not likely that any contamination can account for the amount of bacteria that we have observed.

The physiological properties of the SRB and their widespread occurrence in oil field waters suggest that these bacteria may be indigenous to the reservoir. Whatever their origin, growth of these bacteria in reservoirs represents a major cause of reservoir souring. After injection of seawater, sulfate becomes available as an electron acceptor in the reservoir. Furthermore, when T93B was cocultured with a thermophilic fermentative bacterium, the consortium was able to degrade carbohydrates like mono- and disaccharides with concomitant production of H_2S. Therefore, carbohydrates injected into the reservoir to improve the oil recovery may be potential substrates for thermophilic anaerobic communities. In the presence of sulfate, the thermophilic SRB are capable of utilizing a broad spectrum of carbon sources and electron donors, including the organic acids found in the formation water. Attention should therefore be paid to the possibility of increasing H_2S production by adding degradable chemicals to the reservoirs during the injection of seawater.

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