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Distribution of Thermophilic Marine Sulfate Reducers in North Sea Oil Field Waters and Oil Reservoirs

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The distribution of thermophilic marine sulfate reducers in produced oil reservoir waters from the Gullfaks oil field in the Norwegian sector of the North Sea was investigated by using enrichment cultures and genus-specific fluorescent antibodies produced against the genera *Archaeoglobus*, *Desulfotomaculum*, and *Thermodesulforhabdus*. The thermophilic marine sulfate reducers in this environment could mainly be classified as species belonging to the genera *Archaeoglobus* and *Thermodesulforhabdus*. In addition, some unidentified sulfate reducers were present. Culturable thermophilic *Desulfotomaculum* strains were not detected. Specific strains of thermophilic sulfate reducers inhabited different parts of the oil reservoir. No correlation between the duration of seawater injection and the numbers of thermophilic sulfate reducers in the produced waters was observed. Neither was there any correlation between the concentration of hydrogen sulfide and the numbers of thermophilic sulfate reducers. The results indicate that thermophilic and hyperthermophilic sulfate reducers are indigenous to North Sea oil field reservoirs and that they belong to a deep subterranean biosphere.

North Sea oil reservoirs are subterranean habitats situated 1.2 to 6 km below the sea floor, with pressures from 15 to 80 MPa and temperatures from 60 to 200°C. They are naturally heated because of the depth of the reservoir. The reservoirs contain formation water (in situ pore water) with organic acids at concentrations up to 20 mM (3). The concentration of sulfate is between 0 and 0.6 mM. Offshore oil fields are commonly flooded with anoxic seawater to enhance oil recovery. It has been argued that injection of seawater may stimulate growth of thermophilic sulfate reducers because high concentrations of sulfate are introduced with the injection water (9). The biogenic production of H_2S causes corrosion of iron and steel alloys in the oil wells and in the oil- and gas-processing system. Restrictions are often set on the H_2S content in the export gas, which may demand considerable investments. Microbial growth and precipitation of sulfides in the oil reservoir may also reduce the permeability of the oil formation (2, 11, 12, 15, 16, 20, 33). H_2S may also represent a health hazard to the platform personnel (21).

Increasing numbers of sulfate-reducing bacteria and archaea have been identified and isolated from oil field waters (2, 5, 6, 8, 9, 24, 27, 32, 34–36, 41). From North Sea oil field waters, representatives of four genera of marine thermophilic sulfate reducers have been isolated. *Desulfotomaculum* spp. were isolated from Statfjord oil reservoirs (27, 34). *Archaeoglobus fulgidus* 7324 was isolated from Veslefrikk oil field waters and also detected at the Gullfaks field (5). *A. fulgidus*, *Archaeoglobus profundus* and a novel species of *Archaeoglobus* were found in reservoir fluid from the Thistle platform (41). *Desulfacinum infernum* was isolated from British oil field water (32), while *Thermodesulforhabdus norvegicus* was isolated from the Norwegian Gullfaks oil field (6). Thermophilic sulfate reducers able to use hydrocarbons in crude oil directly under strictly anoxic conditions have been described previously (37). However, the sulfate reducers isolated from oil reservoirs do not degrade hydrocarbons but rather degrade organic acids present in the reservoirs (5, 6, 27, 32, 34).

In most cases, water samples from different parts of the reservoir are collected at the platform deck from a common sample point as close to the production wellhead as possible. The following question then arises: Do the observed thermophilic sulfate reducers grow only in the production facilities on the platform and in the production tubings, or do they actually grow in the reservoir? The criterion which we have used to evaluate this question is that if they grow in production facilities, then all samples from one platform should contain identical strains; however, various strains of microorganisms in the different samples would indicate the presence of different microbial communities in different parts of the reservoir.

Traditionally, identification and quantification of sulfate reducers in oil field water samples have been based on enrichment and most-probable-number dilution series (4, 14, 43). DNA probes have also been used for identification of mesophilic sulfate reducers in oil field waters (44, 45). Few articles about the serology of thermophilic sulfate reducers have been published, and they all deal with the immunological characterization of specific sulfate reducers by the immunoblotting technique (5, 8, 27). However, serological studies of mesophilic sulfate reducers have been performed (1, 26, 28, 29, 38, 39). Fluorescent-antibody (FA) techniques have previously been successfully used to detect various bacterial species in different environments (7, 13, 19).

In this study we use the FA technique to detect thermophilic, marine sulfate reducers directly in North Sea oil field water samples and in primary enrichments from the same samples. The aims of this study were to examine the distribution of three genera of thermophilic marine sulfate reducers (*Archaeoglobus*, *Desulfotomaculum*, and *Thermodesulforhabdus*), to see if there were any correlation between the time to seawater breakthrough and duration of seawater injection and the distribution and numbers of thermophilic marine sulfate reducers, and to determine whether the activity of the thermophilic sulfate reducers in the produced water could account for the H_2S content of the production fluids.

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TABLE 1. Reservoir conditions in oil production wells

Well[a]	Formation[b]	Block	Seawater breakthrough (mo)[c]	Total flooding period (mo)	Water (%)[d]	Seawater (%)[e]	SO_4^{2-} in formation water (mg liter^{-1})	H_2S (mg liter^{-1}) in:	
								Water	Gas
A13	LB	G1	2	75	66	45	60	6	8
A17	LB	H1	7	67	67	85	20	13	220
A17	LB	H1	7	72	67	80	20	ND[f]	160
A19	N	G2	9	70	80	90	15	3	15
A39	LB	H1	7	15	42	70	20	ND	1
B5	LB	H5	5	64	75	50	15	1.5	18
B7	LB	H2	11	62	60	85	20	11	40
B9	T	G5	38	62	61	57	30	ND	3
B9	T	G5	38	64	ND	57	30	1	7
B9	T	G5	38	68	ND	57	30	ND	11
B11	LB	H5	20	51	62	40	15	1.5	20
B11	LB	H5	20	56	65	40	15	ND	11
B11	LB	H5	20	58	ND	40	15	ND	8
B11	LB	H5	20	64	ND	40	15	ND	30
C14	S	K2	8	23	ND	80	55	ND	4
C19	S	I4	—[g]	17	ND	0	40	ND	<0.5

[a] Duplication of wells indicates that samplings were done at several different times.
[b] LB, Lower Brent; N, Ness; T, Tarbert; S, Statfjord.
[c] Time from start of injection to seawater breakthrough.
[d] Total water content of production fluid.
[e] Seawater content of produced water.
[f] ND, not determined.
[g] No seawater breakthrough.

MATERIALS AND METHODS

Reservoir conditions. Three different platforms (A, B, and C) constitute the Gullfaks oil field. This field has a very complex fault pattern that intersects and divides the reservoir into many unconnected fault blocks (30). The field consists of four separate formations: the Brent Group, Cook, Statfjord, and Lomvi. Their unique chemical and physical conditions are the result of their different geological histories. The Brent Group is subdivided into the Broom, Lower Brent, Ness, and Tarbert formations, found on separate fault blocks. These formations had initial temperatures of 71 to 74°C and pressures of 26 to 29 MPa prior to oil exploration. All oil field water samples collected at the Gullfaks A and B platforms originated from the Brent Group (Table 1). Samples from the Gullfaks C platform originated from the Statfjord formation, which had an initial temperature and pressure of 83 to 86°C and 28 to 30 MPa, respectively (Table 1).

To maintain the reservoir pressure during oil production, 3,000 to 7,000 m^3 of seawater is injected into each well on the Gullfaks field every day. The concentration of hydrogen sulfide in Gullfaks formation water is less than 1 mg/liter. After seawater breakthrough, the well produced a mixture of formation water and seawater together with the oil. The concentration of hydrogen sulfide stayed at a constant low level for at least one pore volume after seawater breakthrough, followed by gradual increase (42). By injection of seawater, the reservoir gradually cools down. As a consequence, the temperatures of the produced water from wells A17, A39, and B5 were 8, 2, and 9°C lower than the initial temperatures of these wells, respectively. The temperature of the produced waters from the other wells did not decrease.

Sampling and sample sources. Oil field waters were collected anaerobically on the platform decks from a special sample separator and transported onshore without temperature control. In most cases, samples taken from different wells on the same platform were tapped off from the same sample separator. Each sample of 100 ml of produced water was used for direct counts and for enrichment of thermophilic sulfate reducers. Sample C19 was taken before seawater breakthrough and consisted of pure formation water. All other samples were mixtures of formation water and injected seawater (Table 1). For each sample, the water cut, i.e., the fraction of water in produced fluids, and the seawater content were determined.

Enrichment. For enrichment of thermophilic sulfate reducers, 5-ml water samples were added to 50 ml of marine medium (48), modified as described by Beeder et al. (5). The enrichment cultures with acetate and lactate as the substrate (final concentration, 20 mM) were incubated at 60 and 80°C. Growth and sulfide production were recorded after 1, 2, and 3 months of incubation. All bottles and rubber stoppers were autoclaved twice at 140°C to kill thermoresistant *Desulfotomaculum* endospores (34).

Preparation of antisera. Polyclonal antisera were raised in rabbits against *Desulfotomaculum* sp. strain T93B (anti-T93B), *A. fulgidus* 7324 (anti-7324), and *T. norvegicus* A8444 (anti-A8444) as described previously (5, 6, 34).

FA-DAPI double staining. Identification and enumeration of thermophilic sulfate reducers both directly in oil field water samples and in primary enrichment cultures were done by a double-staining technique (18). By this method, microorganisms collected on membrane filters were stained by combining indirect fluorescein isothiocyanate (FITC)-labelled antibodies (FA) and DAPI (4′,6-diamidino-2-phenylindole). DAPI stains DNA specifically (31), thereby enabling the total number of bacteria and the number of specific antibody-labelled bacteria on the same filter to be counted. By filtering 5 ml of a oil field water sample and examining 50 fields of view on the filter, the detection limit was 100 cells per ml.

Produced water samples were preserved with 2% formaldehyde overnight at 8°C.

Enrichment cultures and pure cultures were centrifuged at 13,000 × *g* for 4 min (Biofuge 13; Heraeus Sepatech). The pellet was resuspended and preserved in phosphate-buffered saline (10 mM Na_2HPO_4 and 10 mM NaH_2PO_4 in 0.85% NaCl [pH 7.2]) with 2% formaldehyde and kept at 8°C until examined. The preserved samples were filtered through prestained polycarbonate filters (17) with a diameter of 25 mm and a pore size of 0.2 μm (Nuclepore Polycarbonate; Costar) and thereafter washed with filter-sterile phosphate-buffered saline (pH 7.4).

Cross-reactivity. Cross-reactivity tests were carried out to verify the specificities of the antisera. The sulfate reducers included in these tests are listed in Table 2.

Microscopy. Filter preparations were examined with a Microphot microscope (Nikon Corporation, Tokyo, Japan) with a Plan-APO 100/1.40 oil immersion objective. A Nikon UV1-A, with excitation filter EX365/10, dichroic mirror DM400, and barrier filter BA400, was used for viewing DAPI staining, and a Nikon B-2A filter block with excitation filter EX450-490, dichroic mirror DM510, and barrier filter BA520 was used for viewing FITC staining. By interchanging the two filter packages, both FA and DAPI staining could be observed in the same field of view. Only cells exhibiting both a clear halo when examined with the filter package for FITC and a blue color with the filter package for DAPI were scored as positive. The cells reacting with the antibodies always exhibited a morphology similar to that of the homologous strain.

Chemical determination. Hydrogen sulfide production was detected with copper sulfate (10).

RESULTS

Specificity of antisera and effect of chloroform treatment. The antisera reacted strongly, i.e., made a clear halo, in reaction with their homologous strains (Fig. 1). They were genus specific, i.e., cross-reacted with thermophilic sulfate reducers from the same genus but not with strains belonging to the other genera tested (Table 2). Anti-T93B also reacted with a mesophilic *Desulfotomaculum* strain.

In order to reduce the background fluorescence, the oil field water samples were treated with chloroform to extract traces of

TABLE 2. Cross-reactivity tests of mesophilic and thermophilic sulfate reducers

Sulfate reducer	Source[a]	Growth temp (°C)	Reference	Cross-reactivity to[b]:		
				Anti-7324	Anti-T93B	Anti-A8444
Archaeoglobus fulgidus						
7324[c]	DSM 8774	80	5	+	−	−
Z	DSM 4139	80	48	+	−	−
Desulfotomaculum thermocisternum ST90[c]	DSM 10259	60	27	−	+	−
Desulfotomaculum sp. strain T93B[c]	DSM 8775	60	34	−	+	−
Desulfotomaculum nigrificans type strain	DSM 574	60	46	−	+	−
Desulfotomaculum kuznetsovii VKM B-1805	DSM 6115	60	25	−	+	−
Thermodesulforhabdus norvegicus A8444[c]	DSM 9990	60	6	−	−	+
Thermodesulfobacterium mobile GFA1[c]	DSM 8975	60	8	−	−	−
Desulfotomaculum acetoxidans VKM B-1644	DSM 771	30	47	−	+	−
Desulfovibrio desulfuricans						
4303[c]	IM	30	26	−	−	−
9301[c]	IM	30	26	−	−	−
Desulfobulbus sp. strain M16[c]	DSM 8777	30		−	−	−
Desulfobacter sp. strain B54[c]	DSM 8776	30		−	−	−

[a] DSM, Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany; IM, Department of Microbiology, University of Bergen, Bergen, Norway.
[b] +, cross-reactivity; −, no cross-reactivity.
[c] Isolated from North Sea oil field water at Department of Microbiology, University of Bergen.

crude oil before the FA samples were prepared. Formaldehyde-preserved cells of *A. fulgidus* 7324, *Desulfotomaculum* sp. strain T93B, and *T. norvegicus* A8444 were treated with chloroform and double-stained as described above. The chloroform treatment had no effect on the specificity of the sera. It did not reduce the number of positive cells or the intensity of the halo.

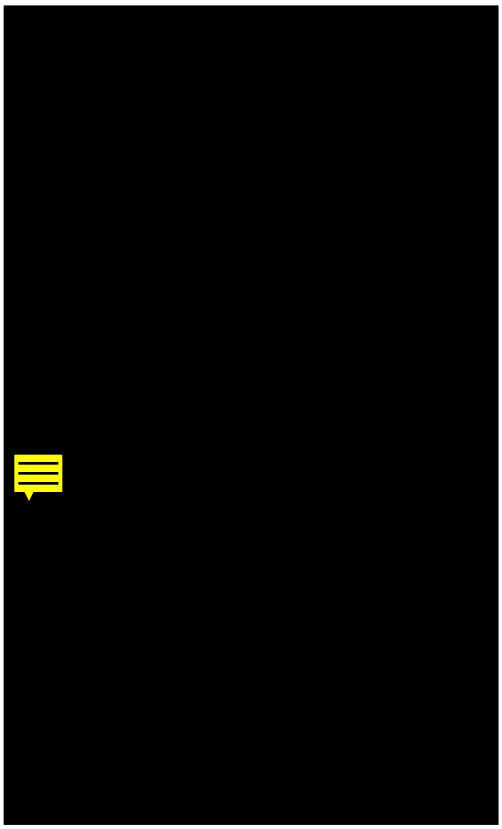

FIG. 1. A mixture of *T. norvegicus* A8444 and *Desulfotomaculum* sp. strain T93B double-stained with DAPI (A) and FA anti-A8444 (B) and examined in the same field of view. Only *T. norvegicus* exhibited a halo when examined with the filter package for FITC.

Total number of cells. In well C19, sampled before seawater breakthrough, the total number of cells was 1×10^5/ml. After seawater breakthrough, the number of cells varied from 1×10^4 to 5×10^5/ml (Table 3). The total number of cells increased in well A17, decreased in well B9, and increased to a maximum in well B11 during the observation period (Table 3).

Direct FA counts of thermophilic sulfate reducers. In 9 of 16 samples, the number of thermophilic sulfate reducers was below the detection limit (100 cells per ml). However, in some samples as many as 2×10^4 sulfate reducers per ml were found (Table 3). *Archaeoglobus* strains were detected in four of the samples (Table 3), and in well A39, all the cells counted were *Archaeoglobus* strains. *Thermodesulforhabdus* strains were detected in five samples. In well B11 (November 1994), all the bacteria detected were strains of this genus. *Desulfotomaculum*

TABLE 3. Direct counts of sulfate reducers in produced oil field water samples

Well	Sampling date	FA counts ml^{-1}, 10^2			Total counts (10^4)
		Anti-7324	Anti-A8444	Anti-T93B	
A13	October 1994	—[a]	—	—	1
A17	May 1994	ND[b]	—	ND	2
	October 1994	—	—	—	5
A19	October 1994	—	—	—	1
A39	May 1994	200	—	—	2
B5	June 1994	8	10	—	40
B7	June 1994	—	—	200	20
B9	June 1994	—	—	—	50
	November 1994	—	—	—	1
	March 1995	28	5	—	1
B11	April 1994	—	—	—	2
	September 1994	100	4	—	40
	November 1994	—	200	—	2
	March 1995	—	—	—	1
C14	October 1994	—	—	—	9
C19	February 1995	—	—	—	10

[a] —, below the detection limit (<100 cells ml^{-1}).
[b] ND, not determined.

TABLE 4. Enrichment cultures of sulfate reducers in produced oil field water samples

Well	Sampling date	Production of sulfide in enrichment culture with carbon source and incubation temp indicated[a]						
		Acetate, 60°C			Lactate			
					60°C			80°C
		Archaeoglobus spp.	*Thermodesulfo-rhabdus* sp.	Unidentified sulfate reducer[b]	*Archaeoglobus* spp.	*Thermodesulfo-rhabdus* sp.	Unidentified sulfate reducer[b]	*Archaeoglobus* spp.
A13	October 1994	−	−	−	−	−	−	−
A17	May 1994	−	−	−	+	−	−	−
	October 1994	−	−	−	+	+	−	−
A19	October 1994	+	+	−	−	−	−	−
A39	May 1994	−	−	−	+	+	−	+
B5	June 1994	+	−	−	−	+	−	+
B7	June 1994	−	+	−	+	+	−	+
B9	June 1994	−	+	−	−	−	+	+
	November 1994	−	−	−	+	−	−	+
	March 1995	−	−	−	−	−	+	+
B11	April 1994	−	−	+	+	−	−	−
	September 1994	−	−	+	+	−	−	+
	November 1994	−	−	−	+	−	−	+
	March 1995	−	−	−	−	−	−	+
C14	October 1994	−	−	−	−	−	+	+
C19	February 1995	−	−	+	+	−	+	+

[a] −, no sulfide produced; +, sulfide produced.
[b] See the text for an explanation.

spp. were found only in well B7, where it made up 10% of the total number of cells.

FA studies of thermophilic enrichment cultures. As can be seen from the data in Table 4, 15 of 16 samples contained culturable thermophilic sulfate reducers. In enrichment cultures from well A13, sulfide production was not detected. *Archaeoglobus* strains were detected in 11 enrichment cultures, and *Thermodesulforhabdus* strains were detected in 6 enrichment cultures at 60°C. Growth of *Desulfotomaculum* spp. was not detected in any of the enrichment cultures (Table 4) at any temperature.

The *Archaeoglobus* strains isolated from different wells on the same platform were not identical. For instance, well B11, sampled in April 1994, contained *Archaeoglobus* strains able to grow at 60°C but not at 80°C; well B11 samples in March 1995 contained *Archaeoglobus* strains able to grow at 80°C but not at 60°C. Well B5 contained *Archaeoglobus* strains able to grow at 60°C on acetate but not on lactate, and well B7 contained *Archaeoglobus* strains able to grow at 60°C on lactate but not on acetate (Table 3). Enrichments of *Archaeoglobus* strains were obtained with lactate as a substrate at 80°C in many, but not all, wells (e.g., no positive enrichment cultures from wells A13, A17, and, A19).

Rod-shaped sulfate reducers that did not react with the FITC-labelled antibodies were observed in some of the enrichment cultures at 60°C, with acetate or lactate as the substrate. No endospores were observed in these enrichment cultures. These sulfate reducers are termed unidentified in Table 4, meaning that they are immunologically and morphologically different from the genera *Archaeoglobus*, *Desulfotomaculum*, and *Thermodesulforhabdus*. From the well without seawater breakthrough (C19), an unidentified strain of sulfate reducer was detected in the enrichment culture, in addition to *Archaeoglobus* organisms (Table 4).

No positive enrichment cultures were obtained at 80°C with acetate as the substrate (data not shown).

DISCUSSION

For practical operational reasons, the majority of the wells on the same platform were sampled with the same sample separator. In order to prevent contaminations, the sample separator was rinsed by flushing several volumes of each sample. If the sample separator, wellhead, and well tubing were sources of contamination, then samples originating from different wells on the same platform would be contaminated by identical microbial strains. No such common strains were detected. The *Archaeoglobus* strains isolated from different wells on the same platform were not identical. This finding confirms that *Archaeoglobus* organisms are not a general contaminant on the platforms and that each sample was unique and may reflect the distribution of thermophilic sulfate reducers in the different fault blocks of the reservoir formations.

The antibodies were genus specific. Anti-T93B reacted with the mesophilic *D. acetoxidans*, indicating that this serum does not differentiate between mesophilic and thermophilic *Desulfotomaculum* species. In direct FA counts, only one oil field water sample gave a positive score with anti-T93B. Since we were not able to detect growth of thermophilic *Desulfotomaculum* strains in the enrichment cultures, we conclude that the *Desulfotomaculum* strain detected by direct counts was either a nonculturable thermophilic *Desulfotomaculum* strain or a mesophilic strain originating from the injected seawater. Earlier investigations have shown that enrichments of Gullfaks formation water sampled before seawater breakthrough was dominated by culturable thermophilic *Desulfotomaculum* strains (8, 34). These findings were not confirmed in the present study. In this study, however, only one well (C19) was sampled before seawater breakthrough, and this sample did not contain culturable thermophilic *Desulfotomaculum* strains.

One might expect that breakthrough of seawater would be followed by an increase in the number of thermophilic sulfate reducers in produced water. However, this possibility was not supported by the results from the examination of well B11. There was no correlation between the number of sulfate re-

ducers and the concentration of hydrogen sulfide in the production fluids. Well A17 had the highest concentration of hydrogen sulfide, but we were unable to detect sulfate reducers from this well using the FA technique. However, low numbers of *Thermodesulforhabdus* and *Archaeoglobus* organisms were present, as determined by their presence in the enrichment cultures. The number of sulfate reducers in produced waters does not necessarily reflect the processes taking place in the reservoir where the sulfate reducers most likely grow in a biofilm. An increase in the number of sessile sulfate reducers in the reservoir may not lead to an increase in the number of sulfate reducers in the water phase collected at the platform deck. However, growth of sessile thermophilic sulfate reducers may explain the increase in the concentration of hydrogen sulfide in the production fluids after seawater breakthrough (42).

Most of the samples contained more than one type of sulfate reducer. *Archaeoglobus* and *Thermodesulforhabdus* species dominated the enrichment cultures at 60°C. In addition, growth of unidentified sulfate reducers was detected. The diversity of strains belonging to the genera *Archaeoglobus* and *Thermodesulforhabdus* in these samples indicates that different strains inhabit different parts of the reservoir. *Thermodesulforhabdus* strains were detected in samples from Gullfaks A and B but not from Gullfaks C. This is in accordance with the temperature maximum for *T. norvegicus* (74°C), which is below the initial temperature of the Statfjord formation, where samples from Gullfaks C originated.

In all sulfide-producing enrichments at 80°C, *Archaeoglobus* strains were detected. This is in agreement with the results of Stetter and coworkers (41), who found *Archaeoglobus* strains in all the samples examined from hot oil wells at the Thistle platform. The genus *Archaeoglobus* is defined as a hyperthermophile (40), i.e., organisms with optimal growth at temperatures above 80°C (22). However, *A. fulgidus* 7324 has optimal growth at 76°C (5). Samples from wells A17 (May 1994), A17 (October 1994), and A19 and B11 (April 1994) in this study contained *Archaeoglobus* strains that were able to grow at 60°C but not at 80°C. These results indicate that North Sea oil field waters also contain *Archaeoglobus* strains that are not true hyperthermophiles.

Stetter et al. (41) found it likely that hyperthermophiles have entered oil field reservoirs with injected seawater partly because all reservoirs studied have been flooded with seawater. In the present study, we detected thermophilic sulfate reducers from pure formation water, i.e., *Archaeoglobus* strains and an unidentified thermophilic sulfate reducer (sample C19). *A. fulgidus* has previously been detected in pure formation water from the Gullfaks field (5) and later was also isolated from pure formation water originating from a French oil field (23). *Desulfotomaculum* sp. strain T93B and *Desulfotomaculum thermocisternum* were isolated from reservoir water sampled before seawater breakthrough, which consisted therefore of pure formation water, indicating that oil reservoirs are their natural habitat (27, 34). *Desulfotomaculum nigrificans* has been isolated from pure formation water from a Russian oil field (35). *Desulfacinum infernum* (32) and *T. norvegicus* (6) were both isolated from North Sea oil field waters. These genera have not yet been found anywhere else.

Desulfotomaculum strains are reported to be active and to produce hydrogen sulfide at 30 MPa and 80°C, conditions that are representative of many North Sea oil reservoirs (33). *A. fulgidus* TF2 can also grow under the extreme physical conditions found in North Sea oil field reservoirs (41). Both *Desulfotomaculum* and *Archaeoglobus* strains should therefore be able to inhabit such reservoirs.

The facts that hyperthermophiles are able to survive in cold seawater and that several of the reservoir species are identical to those found in submarine hot vents (41) do not exclude the possibility that they can be indigenous to North Sea oil field reservoirs as well. A plausible explanation would be that they all belong to a common deep subterranean biosphere.

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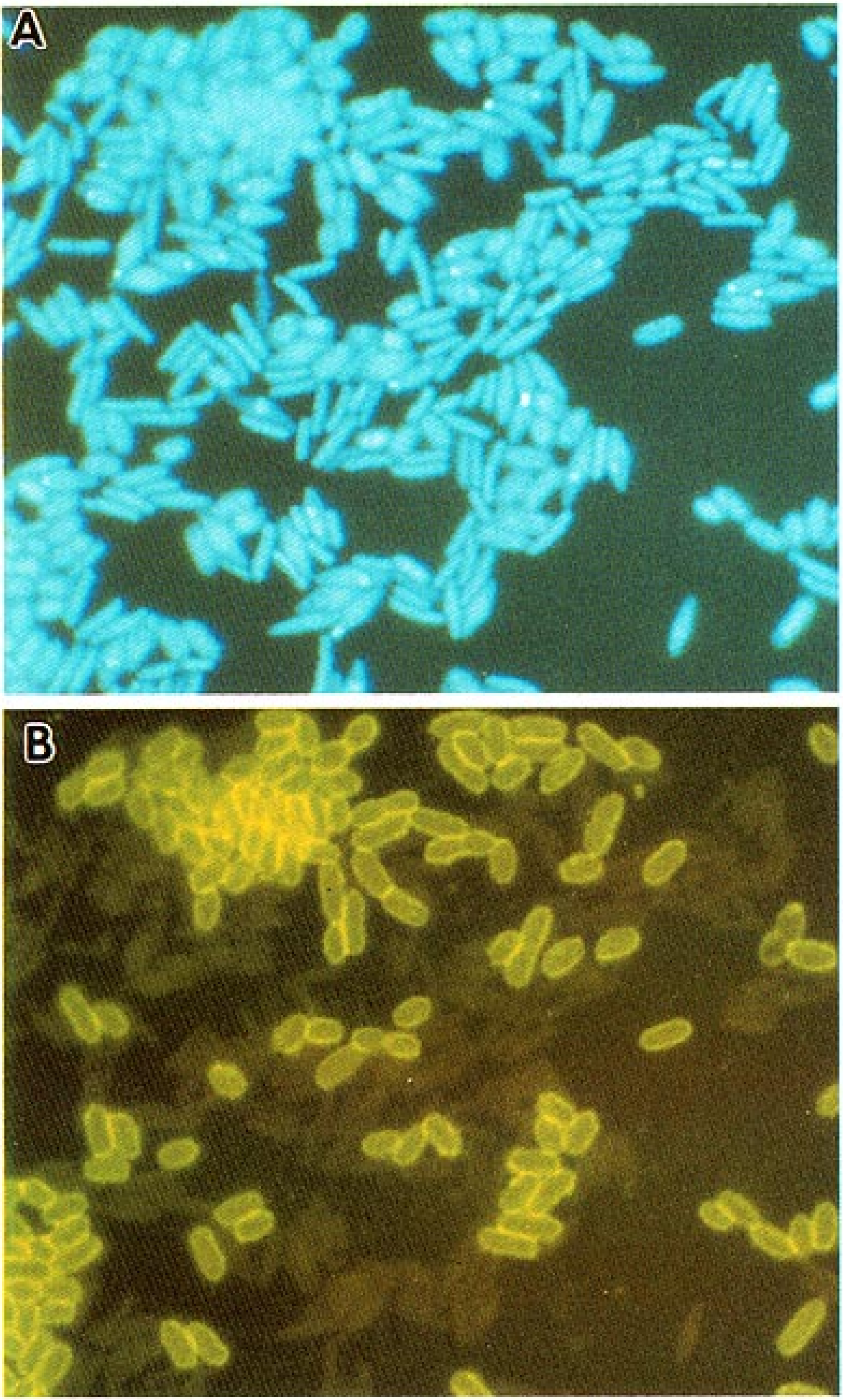
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