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Methanococcus thermolithotrophicus Isolated from North Sea Oil Field Reservoir Water

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Methanococcus thermolithotrophicus **ST22 was isolated from produced water of a North Sea oil field, on mineral medium with H_2-CO_2 as the sole source of carbon and energy. The isolate grew at 17 to 62°C, with an optimum at 60°C. The pH range was 4.9 to 9.8, with optimal growth at pH 5.1 to 5.9; these characteristics reflected its habitat. Strain ST22 was quickly identified and distinguished from the type strain by immunoblotting.**

Hot oil reservoirs are potential habitats for thermophilic methanogens. However, only a few studies of thermophilic methanogens from oil reservoirs have been reported. From seawater-flooded oil wells in California, rod-shaped, thermophilic marine methanogens were isolated (24). *Methanobacterium thermoaggregans* (6) and *Methanobacterium thermoalcaliphilum* (9) were isolated from produced oil field waters from previous USSR territory. The type strain of *Methanococcus thermolithotrophicus*, SN 1, is the only strain of this species known until now (29) and was isolated from geothermally heated sea sediments in Italy (15). This study reports the isolation and characterization of *M. thermolithotrophicus* ST22, originating from oil field reservoir water collected at the wellhead of a backflooded reperforated injection well on an oil platform at the Statfjord field in the North Sea. The reservoir had been flooded with seawater for several years. The water samples were a mixture of injected anaerobic seawater (78%) and formation water (in situ porewater) (22%) which was allowed to back flow out of the injection wellhead. The samples were taken after continuous backflooding for 26 h at a rate of 250 m^3/h. The water originated from the Upper Brent Group, which is a porous rock formation located 2.6 km below the sea floor. Initially, this formation had a temperature of 85°C and a pressure of 30 MPa. The formation water had a salinity of 2.5% (wt/vol). The in situ pH of the Statfjord reservoir is estimated to be 5.0 to 5.5. Because of the injection of cold seawater, the reservoir area close to the injector is cooled down and a temperature gradient is formed. The temperature of the backflooded water was 39°C. After pressure release, the pH was 7.1. Samples were taken anaerobically and brought to the laboratory without temperature control.

The enrichment medium was composed as described by Möller-Zinkhan et al. (23) but modified by omitting peptone and yeast extract and exchanging K_2HPO_4 with H_2KPO_4. One milliliter of trace element solution (31) and 5 ml of vitamin solution (25) were added per liter of medium. The pH was adjusted to 6.8 with HCl or Na_2CO_3. For enrichment of thermophilic methanogens, H_2-CO_2 (80:20, vol/vol; 0.2 MPa) and acetate (100 mM, final concentration) were used as substrates. N_2-CO_2 (80:20, vol/vol) was used as the headspace gas for the enrichment cultures on acetate. Incubation temperatures were 60, 80, and 92°C. The growth medium used throughout these studies was the enrichment medium described above modified by omitting Na_2SO_4 and vitamins. The pH was adjusted to 5.7. Cysteine-HCl was added to a final concentration of 0.025% (wt/vol) prior to inoculation. Cysteine-HCl could be substituted by dithionite as a reducing agent. Pure cultures were isolated in a dilution series by using the shake tube culture method (30) with anaerobic Gelrite gellan gum (Kelco Division of Merck and Co., San Diego, Calif.) as a gelling agent. Strain ST22 was isolated at 60°C under an atmosphere of H_2-CO_2 (80:20, vol/vol; 0.2 MPa). This isolate gave pale yellowish, smooth, circular colonies. At 60, 80, and 92°C, methane production was detected in enrichment cultures with acetate as the substrate. Repeated efforts to isolate methanogenic pure cultures growing on acetate failed.

Unless otherwise noted, strain ST22 was cultured at 60°C in 100-ml serum bottles containing 35 ml of medium under an H_2-CO_2 atmosphere (80:20, vol/vol; 0.2 MPa). The pH experiments were performed with an H_2-CO_2 atmosphere of 70:30 (vol/vol) (0.2 MPa) to prevent rapid change in the buffer capacity of the medium by the consumption of CO_2 during growth, with a subsequent increase of pH in the medium. Initial pH was measured immediately after inoculation. *M. thermolithotrophicus* SN 1 (DSM 2095) from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM), Göttingen, Germany, was cultured as described by Huber et al. (15).

Cells from the late logarithmic growth phase were tested for susceptibility by use of detergents and hypertonic conditions (7). Gram staining was performed by the Hucker method (11). Methane in the gas phase was assayed as described previously (5). Polyclonal antiserum against strain ST22 (anti-ST22) was produced as described previously (8). Antigens were characterized by Western immunoblotting of sodium dodecyl sulfate (SDS)-soluble whole-cell extracts (5). Cell morphology was investigated by using a phase-contrast microscope (Labolux K; Leitz) and by examining negatively stained preparations in a JEOL 100 S electron microscope. Grids coated with Formvar-carbon (Balzers AG) and rendered hydrophilic by glow discharge were used for negative staining with 1% (wt/vol) uranyl acetate (pH 4.2).

Sequence analysis of the genes coding for 16S rRNA (rDNA) was performed by DSM. Genomic DNA extraction, PCR-mediated amplification of the 16S rDNA, and purification of PCR products were carried out as described previously (2, 26, 27). The purified PCR products were sequenced by

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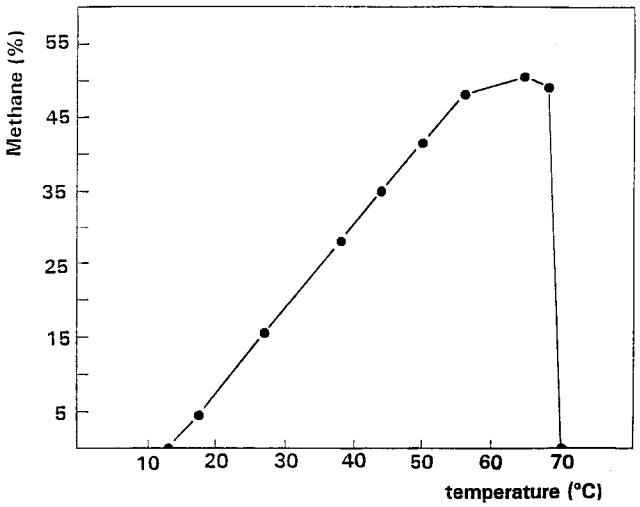

FIG. 1. Effect of incubation temperature on methane production in strain ST22. Methane in the headspace was measured after 5 days of incubation.

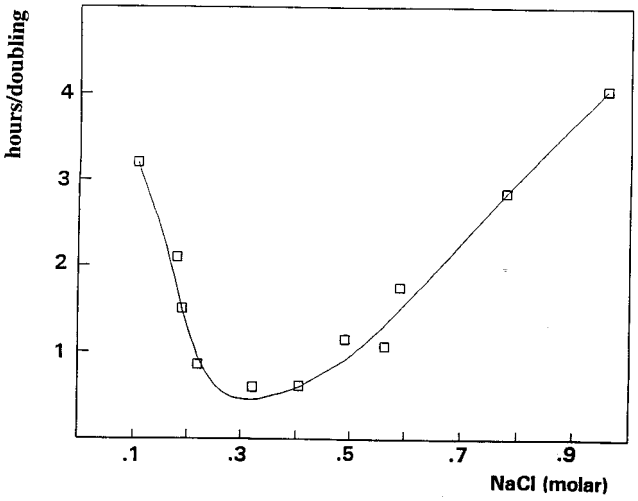

FIG. 2. Effect of NaCl concentration on growth of strain ST22.

using the *Taq* Dye-Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, Calif.) as directed in the manufacturer's protocol. Sequencing reaction products were electrophoresed with an Applied Biosystems 373A DNA sequencer. The 16S rDNA sequence was manually aligned with representative sequences of members of the domain *Archaea*. Pairwise evolutionary distances were computed by using the correction of Jukes and Cantor (18). The least-squares distance method of DeSoete (10) was used in the construction of the phylogenetic dendrogram from distance matrices. The G+C content of the DNA was measured by thermal denaturation (21) with *M. thermolithotrophicus* SN 1 and *Escherichia coli* B type VIII DNA (no. D-2001; Sigma) as references. DNA was isolated as described by Marmur (22).

Stock culture of strain ST22 was transferred into the growth medium each day. For long-term preservation, the strain was grown overnight, repressurized, and stored at room temperature, after which it can serve as an inoculum for at least 2 years. When stored at 8°C, strain ST22 was viable for several months. Strain ST22 is deposited at DSM under number DSM 8766.

Cells of strain ST22 were regular to irregular coccoids, occurring singly and in pairs. The average diameter was 1.0 to 1.2 μm. The cells stained gram negative. They had a tumbling motion, with flagella inserted in several areas on the cell surfaces. H_2-CO_2 and formate were metabolized. Acetate, methanol, methylamines, isopropanol plus CO_2, ethanol plus CO_2, and dimethyl sulfide were not metabolized. Vitamins and growth factors were not required for growth. The isolate grew successfully in medium without trace element solution added for at least eight transfers with 10% inoculum. This indicates that strain ST22 requires only very low concentrations of trace elements. Addition of crude oil to the medium did not inhibit or stimulate growth on formate. Strain ST22 grew on H_2 plus CO_2 at temperatures between 17 and 62°C, with optimal growth at 60°C; no growth was observed at 15 or 68°C. However, methane production was observed at 15 and 68°C (Fig. 1), indicating that methane production was not coupled to growth at these temperatures. Growth and methane production occurred between initial pHs 4.9 and 9.8, with an optimum at pH 5.1 to 5.9. At initial pHs 4.9 and 5.1, a prolonged lag phase of 12 h was observed. No growth at pH 4.7 was measured. Growth was observed in medium with 100 to 1,600 mM NaCl, with optimum growth at 250 to 400 mM NaCl (Fig. 2). No growth was measured at 70 and 1,800 mM NaCl. The shortest generation time measured was 32 min under optimum growth conditions (60°C, initial pH 5.7, and 300 mM NaCl). Penicillin G and kanamycin (200 μg/ml) did not inhibit growth.

Strain ST22 was susceptible to lysis under hypertonic conditions; a soft cell pellet lysed immediately when suspended in distilled water but not in the growth medium. The isolate was susceptible to lysis by SDS, indicating a proteinaceous cell wall (7), a general characteristic of the genus *Methanococcus* (13, 29).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of SDS-soluble whole-cell extracts revealed that strain ST22 and *M. thermolithotrophicus* SN 1 had very similar protein patterns (Fig. 3A). *M. thermolithotrophicus* SN 1 showed five protein bands with molecular masses of 30, 43, 44, 55, and 58 kDa that were absent from or present at much lower levels in strain ST22. Immunoblotting with anti-ST22 showed that strain ST22 and *M. thermolithotrophicus* SN 1 had several common antigen bands (Fig. 3B). However, significant differences which make it possible to distinguish between them were evident at approximately 21, 35, 40, 61, 66, 72, and 77 kDa.

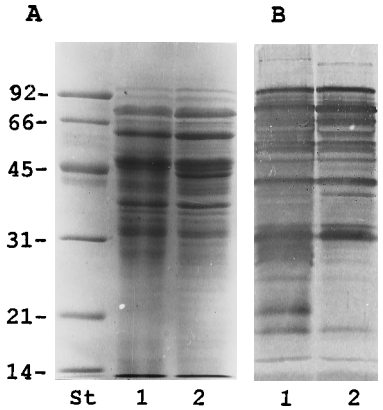

FIG. 3. Protein patterns (A) and antigen patterns with anti-ST22 (B) after SDS-PAGE of SDS-soluble whole-cell extracts of strain ST22 (lanes 1) and *M. thermolithotrophicus* SN 1 (lanes 2). Lane St, molecular mass standard, with values (in kilodaltons) indicated on the left.

The G+C content of the DNA of strain ST22 was 32 mol%. The 16S rDNA sequence of strain ST22 from around position 400 to position 1390 was determined. In this stretch, the 16S rDNA sequence was identical to the published sequence of *M. thermolithotrophicus*, showing that isolate ST22 is a strain of this species. However, phenotypically, strain ST22 differs from the type strain SN 1 in several aspects. While strain ST22 grows between 17 and 62°C, the type strain grows between 30 and 70°C (15). The pH optimum of strain ST22 is 5.1 to 5.9, compared with 6.5 to 7.5 for strain SN 1 (15). Strain ST22 could grow at an initial pH of 4.9, while strain SN 1 was unable to grow at pHs below 6. These differences reflect the ecological habitat from which strain ST22 was isolated.

Strain ST22 may have been introduced into the oil reservoir during seawater injection. Anaerobic conditions have been observed within a large crustacean fecal pellet found attached to a particle of marine snow in pelagic seawater (1). It is also known that hyperthermophiles can survive in cold seawater (16). Thus, strain ST22 may have survived in anaerobic microniches in open North Sea water. This implies that strain ST22 has been transported with ocean streams from its original hot habitat to the Statfjord oil well from which it was isolated. Produced formation water from a less thermophilic North Sea oil reservoir (60 to 62°C) discharged into the North Sea could be the source of strain ST22. It is also possible that strain ST22 is indigenous to the Statfjord reservoir. Other microorganisms have also been isolated from areas with temperatures higher than their maximum growth temperature (17, 33). Strain ST22 needs a temperature lower than that of the reservoir for growth. However, the injection of seawater cools the formation to a temperature that allows the organism to grow. In our study, methanogenesis was detected also in enrichment cultures at 80 and 92°C, showing the presence of methanogens able to grow at reservoir temperatures.

Strain ST22 could grow at an initial pH of 4.9. This correlates well with the in situ pH in the reservoir before exploration. However, it could also grow at alkaline conditions, as in seawater. A pH gradient from 5 to 8 is created in the reservoir during seawater injection. Strain ST22 is able to grow throughout this pH range.

Strain ST22 can grow only on H_2-CO_2 and formate. In the reservoir, H_2 (12) as well as CO_2 (3) can be produced by geothermal reactions. Formic acid is not reported as a component of formation waters (20). Acetate is present in North Sea oil field reservoirs at concentrations up to 20 mM (4). Methane production was detected in enrichment cultures with acetate as the substrate at 60, 80, and 92°C. However, we were not able to obtain pure cultures from these enrichments. The reason might be that acetate is degraded indirectly by a coculture, rather than directly by acetoclastic methanogens. This has also been indicated by studies of methanogenesis in other hot oil field reservoirs (6, 9). Cocultures consisting of acetoclastic acetogens and autotrophic methanogens performing interspecies hydrogen transfer have been described previously (19, 34). Aliphatic carboxylic acids with up to eight carbon atoms are present in North Sea oil formation waters (4), and several genera of thermophilic sulfate reducers have been isolated from these waters (5, 8, 28). Syntrophic sulfate reducers catabolizing these organic acids in the absence of sulfate might therefore be another source of H_2 in this environment. This process has been detected in mesophilic fermenters (14, 32). Thus, if H_2 is available as an energy source, the reservoir area close to the injector is a suitable habitat for growth of strain ST22.

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