Petrotoga mobilis sp. nov., from a North Sea oil-production well

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Rod-shaped, thermophilic bacteria with a sheath-like outer structure ('toga') were isolated from hot oilfield water of a North Sea oil reservoir. One of the isolates, designated SJ95^T, is an obligately anaerobic, sheathed, Gram-negative, fermentative bacterium capable of reducing elemental sulfur to hydrogen sulfide and tolerating high salt concentrations. The optimum growth conditions for this isolate are 58–60 °C and pH 6.5–7.0 with 3–4% NaCl and 0.7% MgSO₄ · 7H₂O in the medium. Vitamins are required for growth. Growth is stimulated by yeast extract. Cells of strain SJ95^T vary in size from 1–2 to 40–50 µm in length and are motile with a subpolar flagellation. Cells grown on xylan have xylanase activity, presumably associated with the toga, and glucose isomerase activity was detected in xylose-grown cells. The DNA G+C content is 31 and 34 mol%, determined by the thermal denaturation and HPLC methods, respectively. Phylogenetically, strain SJ95^T is most closely related to *Petrotoga miotherma* with a 97.7% similarity level between their 16S rDNA sequences. The DNA–DNA reassociation value between the two DNAs was 35.6%. On the basis of differences in genotypic, phenotypic and immunological characteristics, strain SJ95^T (= DSM 10674^T) is proposed as the type strain of a new species, *Petrotoga mobilis*. It can be readily distinguished from *P. miotherma* by its motility.

Keywords: *Petrotoga mobilis* sp. nov., thermophile, oilfield water

INTRODUCTION

In recent years, several new members of the order *Thermotogales* have been isolated from volcanically and non-volcanically heated geothermal environments (Andrews & Patel, 1996; Fardeau *et al.*, 1997; Jeanthon *et al.*, 1995; Koch *et al.*, 1997). *Thermotogales* currently comprises five genera, *Thermotoga* (Huber *et al.*, 1986), *Thermosipho* (Huber *et al.*, 1989), *Fervidobacterium* (Huber *et al.*, 1990), *Geotoga* and *Petrotoga* (Davey *et al.*, 1993). All members of the *Thermotogales* group have a characteristic outer sheath-like structure ('toga'); they are thermophilic and hyperthermophilic prokaryotes representing an early divergence within the (eu)bacterial line of descent (Winker & Woese, 1991).

The North Sea oil reservoirs constitute a hot subterranean biosphere 1.2–6.0 km below the sea floor. Environmental parameters (temperature, pressure, pH, salinity, concentration of heavy metals, petroleum composition) vary widely from reservoir to reservoir. For instance, the temperature is 60–200 °C with an increase of 1 °C per 24–30 m increase in depth and the *in situ* pressure is 150–800 bar (15–80 MPa). Various physiological types of thermophilic prokaryotes have been isolated from petroleum reservoirs, e.g. sulfidogens (Beeder *et al.*, 1994, 1995; Nilsen *et al.*, 1996; Rees *et al.*, 1995; Rosnes *et al.*, 1991a), methanogens (Nilsen & Torsvik, 1996), and *Thermotogales* genera (Andrews & Patel, 1996; Davey *et al.*, 1993; Fardeau *et al.*, 1997; Jeanthon *et al.*, 1995; Stetter *et al.*, 1993).

The only *Petrotoga* sp. described so far is *Petrotoga miotherma*, which was isolated from petroleum reservoirs in Oklahoma and Texas, USA (Davey *et al.*, 1993). It is moderately thermophilic with an optimum growth temperature of 55 °C, non-motile, rod-shaped with a toga, obligately anaerobic, fermentative and

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capable of reducing elemental sulfur to hydrogen sulfide. *Petrotoga* differs from the other *Thermotogales* genera by its higher salt tolerance and 16S rRNA sequence, which shows it to be a distinct lineage within this order.

Organisms with a sheath-like outer structure have been isolated from North Sea oil wells. In this paper, the general characteristics and phylogenetic position of one of the isolates, strain SJ95^T, are described. Although it is phylogenetically closely related to *P. miotherma*, strain SJ95^T differs in terms of its genotypic, phenotypic and immunological properties. Therefore, strain SJ95^T is proposed as a new species, *Petrotoga mobilis* sp. nov.

METHODS

Organisms and source of inoculum. Strain SJ95^T was enriched from anoxic samples of production water taken from the water separator tanks on off-shore oil platforms. The pressure in the separators was 1–80 bar (0.1–8 MPa), the temperature was 70 °C and the pH was 7.8, measured after pressure release. *P. miotherma* (ATCC 51224^T) was obtained from the American Type Culture Collection (Rockville, MD, USA).

Enrichment, isolation and cultivation. Enrichment and routine cultivation were done in a medium containing the following components (l⁻¹): NaCl, 30.0 g; MgSO₄·7H₂O, 7.0 g; KCl, 0.34 g; NH₄Cl, 0.25 g; CaCl₂·2H₂O, 0.14 g; KH₂PO₄, 0.14 g; yeast extract (Difco), 0.2 g; 1 ml trace element solution SL-10 (Widdel *et al.*, 1983); and 0.5 ml resazurin (0.02 %). After autoclaving in a dispenser (Lien & Beeder, 1997), the hot medium was reduced with 4 ml 0.5 M Na₂S·9H₂O under argon gas. The pH was adjusted to 6.5 with 6 M HCl and 10 ml vitamin solution (Balch *et al.*, 1979) was added. The medium was dispensed into 50 or 200 ml serum bottles and Bellco tubes no. 2047 (Vineland) sealed with butyl rubber stoppers and aluminium crimp seals and a gas phase of argon (200 kPa). Before inoculation with 10 % inoculum, the substrates were added separately by syringe from anoxic stock solutions. The incubation temperature was 60 °C. Pure cultures were isolated by dilution series using the shake tube culture method (Widdel & Pfennig, 1984) with anoxic Gelrite gellan gum (final concentration 0.3 %, w/v; Kelco Division of Merck) as the gelling agent and starch (final concentration 0.5 %, w/v) as the substrate.

Microscopy. Cells were observed with a Labophot (Nikon) and a Vanox (Olympus) phase microscope to determine purity, morphology and Gram reaction, and to make photomicrographs (Pfennig & Wagner, 1986). Cells for electron microscopy were fixed in the medium with 1.5 % (v/v) glutaraldehyde for 30 min on ice. After washing with Tris/EDTA buffer (20 mM Tris, 1 mM EDTA, pH 6.8), a piece of carbon support film, about 10 nm thick, was floated on the surface of the sample. After attachment of the bacteria, the carbon film was washed with Tris/EDTA buffer and distilled water, picked up with 300 mesh copper grids and immediately blotted dry on filter paper. The grids were mounted on carbon conductivity tabs on stubs and, without further sputtering, observed in a Zeiss DSM982 Gemini at 1 kV, 10 µm aperture and 3 mm working distance using the in-lens detector.

Serology. Polyclonal antiserum was produced against strain

SJ95^T as described previously (Christensen *et al.*, 1992). Antigens were characterized by Western immunoblotting of SDS-soluble whole-cell extracts. SDS-PAGE of the whole-cell extracts was carried out as described by Laemmli (1970) with 12.5 and 4.5 % (w/v) polyacrylamide in the separation and stacking gels, respectively. The electrophoresis and subsequent immunoblotting were run as described previously (Christensen *et al.*, 1992).

Growth and metabolism. Growth (in Bellco tubes) was determined as an increase in OD₆₀₀. Hydrogen and carbon dioxide gases were determined with a Varian model 3300 GC equipped with a thermal conductivity detector and a Cromopack 45/60 molecular sieve 10'×1/8" (3.0 m by 3.2 mm) stainless steel column. The oven, injector and detector temperatures were 60, 125 and 125 °C, respectively, and the carrier gases for determination of hydrogen and carbon dioxide were argon and helium, respectively.

Short-chain fatty acids and alcohols were determined by HPLC using an Aminex HPX-87 column (300×7.5 mm; Bio-Rad; eluent, 40 mM H₂SO₄; column temperature, 65 °C; flow rate 0.6 ml min⁻¹) and a Refractive Index Detector 156 (Altex) at 35 °C. The injection volume was 100 µl.

Xylanase activity was determined photometrically by measuring the increase in reducing sugars released from birch wood xylan (Roth) using *p*-hydroxybenzoic acid hydrazide (PAHBAH) reagent (Lever, 1972). The standard incubation mixture contained: 1.8 ml 1 % birch wood xylan suspended in 50 mM potassium phosphate buffer, pH 6.5, and 0.2 ml enzyme solution. Standard incubation conditions were 5 h at 60 °C, after which the reaction was stopped by the addition of 3 ml PAHBAH reagent. After colour development for 5 min at 90–100 °C, A₄₁₀ was determined. In the enzyme blank, the enzyme was added after the addition of the PAHBAH reagent. Potassium phosphate buffer (50 mM) was used for the determination of the effect of pH (at 60 °C) and temperature (at pH 6.5) on enzyme activity. Xylanase activity was measured in a cell-free extract, intact cells and a culture filtrate of cells grown on xylan. These different fractions were obtained as follows. Samples of 20 ml culture were centrifuged at 10000 *g* for 10 min, and the supernatant was used as the culture filtrate. A suspension of intact cells (examined by microscopy) was made by suspending the pellet in 1 ml growth medium without a substrate. The cell-free extract was prepared from 0.5 ml cell suspension using ultrasonic disintegration followed by 10 min centrifugation at 10000 *g*. The activity of glucose isomerase was assayed as described by Lee *et al.* (1990).

DNA analyses. Genomic DNA for determination of the G+C content and DNA–DNA hybridization was isolated from 0.4–1.0 g frozen and thawed bacterial pellets. RNase (10 µl ml⁻¹) was added, and the mixture was incubated for 60 min at 65 °C. Thereafter, 0.5 ml 10 % (w/v) SDS and 50 µl (20 mg ml⁻¹) proteinase K were added followed by incubation at 37 °C for 1 h. After mixing the solution with 1.8 ml 5 M NaCl, 1.5 ml hexadecyltrimethyl ammonium bromide (Murray & Thompson, 1980) was added and the mixture was incubated at 65 °C for 20 min. An equal volume of chloroform:isoamylalcohol (24:1) was then added, and the mixture was centrifuged at 6000 *g* for 10 min. The water phase was transferred to another tube and 0.6 vol. 2-propanol was added. After 15 min, the DNA was collected and transferred to 1 ml 70 % ethanol and centrifuged for 5 min at 10000 *g*. The pellet was washed in 70 % ethanol and

finally dissolved in 1 ml 20 mM Tris/HCl buffer containing 1.0 mM EDTA. The G+C content of the DNA was measured by both the thermal denaturation method (De Ley, 1970), with *Escherichia coli* B type VIII DNA (no. D-2001; Sigma) as a reference, and the chemical method using HPLC to separate and quantify nucleosides (Mesbah *et al.*, 1989). For DNA-DNA hybridization studies, DNA was first subjected to hydroxyapatite chromatography (Cashion *et al.*, 1977); hybridization was carried out according to De Ley *et al.* (1970) with modifications using a Gilford System 2600 spectrophotometer equipped with a Gilford 2527-R thermoprogrammer and plotter. The DNA-DNA hybridization was carried out by M. Steffen at the DSMZ.

16S rDNA sequence determination and analysis. Genomic DNA extraction, PCR-mediated amplification of the 16S rDNA, and purification of PCR products were carried out using procedures described previously (Rainey *et al.*, 1996; Rainey & Stackebrandt, 1993). Purified PCR products were sequenced using the *Taq* DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems) as directed in the manufacturer's protocol. The Applied Biosystems 373A DNA Sequencer was used for electrophoresis of the sequence reaction products. The 16S rDNA sequence was manually aligned against *P. miotherma*, *Geotoga* and other *Thermotogales* representatives. Pairwise evolutionary distances were computed using the correction of Jukes & Cantor (1969). The least-squares distance method of De Soete (1983) was used in the construction of the phylogenetic dendrogram from distance matrices.

Nucleotide sequence accession numbers. The accession numbers of the reference strains used in the sequence comparison are as follows: *Aquifex pyrophilus*, M83548; *Fervidobacterium islandicum*, M59176; *Fervidobacterium nodosum*, M59177; *Geotoga petraea*, L10658; *Geotoga subterranea*, L10659; *Petrotoga miotherma*, L10657; *Thermosiphon africanus*, M24022; *Thermotoga maritima*, M21774; and *Thermotoga thermarum*, ARB8587.

RESULTS

Enrichment and isolation

Bacteria with an outer sheath (toga) were first observed in enrichment cultures of a thermophilic spore-forming *Desulfotomaculum* sp. This binary culture grown at 60 °C converted mono- and disaccharides to hydrogen sulfide, acetate, hydrogen and ethanol (Rosnes *et al.*, 1991b). Efforts were made to isolate the different members of the culture; *Desulfotomaculum* sp. was isolated by autoclaving the culture at 121 °C for 20 min, which killed the sheathed bacteria, but not the sporulators with extremely heat-resistant spores (Rosnes *et al.*, 1991a). Several dilution series were carried out in a glucose-containing medium without sulfate, but the spore-forming sulfate reducers ultimately appeared in all of the dilution tubes. However, successful enrichment of the sheathed bacteria was obtained by growing the binary culture in a mineral salt medium with starch and without sulfate. The enrichment was subcultured in this medium and during three subsequent transfers, the population developed into a culture dominated by rod-shaped bacteria with a sheath-like outer structure (toga). Several of these

Fig. 1. Morphology of strain SJ95^T. (a) Phase-contrast micrograph of cells. Bar, 10 µm. (b) Scanning electron micrograph of a cell with flagella. Bar, 2 µm.

bacteria were isolated in pure cultures. One of the isolates, strain SJ95^T, forming circular and whitish colonies, is described in this paper.

Table 1. Comparison of characteristics of strain SJ95^T and *P. miotherma*

Characteristic	<i>Petrotoga mobilis</i> SJ95 ^T	<i>Petrotoga miotherma</i>
Cell shape	Rod-shaped with toga	Rod-shaped with toga
Cell size (µm):		
Width	0.5–1.5	0.6
Length	1.0–50.0	2.0–7.5
No. of cells per sheath	1–24	1–5
Motility and flagellation	Motile, subpolar	Non-motile
Formation of spherical bodies	–	+
Optimum temperature (°C)	58–60	55
Optimum NaCl requirement (g l ⁻¹)	30–40	20
Growth factors requirements:		
Vitamins	+	ND
Yeast extract	(+)	+
Reduction of S ⁰ to H ₂ S	+	+
DNA G + C content (mol %)	31, 34*	32, 34–25, 40†
Substrates utilized:		
Xylan	+	–
Cellulose	–	–
Ribose	+	ND
Maltodextrin, glucose, lactose, sucrose	+	+
Galactose, maltose, xylose, cellobiose, fructose	+	+
Arabinose	+	+

* Values obtained by the thermal denaturation and HPLC methods, respectively.

† Values obtained by the thermal denaturation and HPLC methods and the HPLC value reported by Davey *et al.* (1993), respectively.

ND, Not determined.

Morphology

Cells of strain SJ95^T are rod-shaped and vary widely in size from small ones of only about 1 µm in length to those with a length of 40–50 µm. The width of the cells also varied (Fig. 1a; Table 1). A surrounding sheath was clearly visible under phase microscopy. The cells frequently appeared singly, in pairs or in chains within the sheath, sometimes with more than twenty small cells per sheath or with a single small cell within a long sheath which otherwise seemed to be empty. In contrast to *P. miotherma*, strain SJ95^T did not form enlarged spherical bodies in the stationary phase (Davey *et al.*, 1993). Gram staining was negative and no endospores were observed. However, motile cells were frequently observed and electron microscopy revealed some very long flagella (15–16 µm) and subpolar flagellation (Fig. 1b). The smaller single rods moved more rapidly than the longer single cells and pairs of rods which showed a slow flexing, fish-swimming motion. No twiddles, but a reversion of their swimming direction was observed.

Physiology

Strain SJ95^T is an obligate anaerobe that grows at temperatures between 40 and 65 °C (optimum at 58–60 °C). The pH range for growth was pH 5.5–8.5

(optimum at pH 6.5–7.0) (Table 1). The shortest doubling time was about 12 h. Growth was observed in 0.5–9.0% (w/v) NaCl and 0.1–0.8% (w/v) MgSO₄·7H₂O and was optimal at 3–4% NaCl and 0.7% MgSO₄·7H₂O. Several vitamins, e.g. folic acid, riboflavin, pantothenic acid, cobalamin, *p*-amino-benzoate and lipoic acid (200, 500, 500, 10, 500 and 500 µg l⁻¹, respectively) were required. Yeast extract (0.02%, w/v) stimulated growth.

The growth studies were done in the presence of vitamins, 0.02% final concentration yeast extract, and 0.5% final concentration substrate. Growth was observed on: starch, xylan, maltodextrin, maltose, cellobiose, sucrose, lactose, glucose, galactose, fructose, arabinose, xylose, ribose and rhamnose as carbon and energy sources. Highest yield was obtained with ribose, maltose and dextrin. Xylanase activity, optimum at 60–65 °C and pH 5.5–6.0, was detected in intact cells, in cell-free extract but not in the culture filtrate of cells grown on xylan. This indicates that the xylanase activity is located inside the toga as reported for amylases and xylanases from *Thermotoga* spp. (Sunna *et al.*, 1997). Glucose isomerase was detected in cell-free extracts of cells grown on xylose.

The following substrates were tested, but not utilized: cellulose, mannose, raffinose, Casamino acids, man-

Fig. 2. Protein pattern (a) and immunoblotting with anti-SJ95^T (b) after SDS-PAGE of SDS-soluble whole-cell extracts of strain SJ95^T (lane 1) and *P. miotherma* (lane 2). The positions of molecular mass standards (in kDa) are indicated on the left (standards in lane S).

nitro, inositol, methanol, ethanol, propanol, butanol, formate, acetate, butyrate, valerate, caproate, malate, lactate, fumarate and pyruvate.

The fermentation products detected in cultures growing on glucose were hydrogen gas, carbon dioxide, acetate and ethanol. As observed with *P. miotherma* (Davey *et al.*, 1993), no growth occurred with a headspace of 80% hydrogen and 20% carbon dioxide; this inhibition by hydrogen was eliminated by adding elemental sulfur or thiosulfate to the culture which caused production of hydrogen sulfide. Growth inhibition also occurred at low (0.1%, v/v) concentration of hydrogen in the headspace. Thiosulfate reduction increased cellular yields and growth rates of strain SJ95^T and *P. miotherma* (Fig. 3) similarly to that described by Ravot *et al.* (1995, 1996) for other members of the order *Thermotogales*.

Antibiotic sensitivity

Growth was inhibited at 10 µg ml⁻¹ final concentration of penicillin, ampicillin, vancomycin, chloramphenicol, rifampicin and streptomycin. The inhibition was irreversible since no viable cells were obtained after washing and resuspension in the growth medium. In

Fig. 3. Effect of thiosulfate on growth of strain SJ95^T (○, ●) and *P. miotherma* (□, ■). Cells were grown on maltodextrin (0.5%) at 58 °C with 20 mM final concentration of thiosulfate (□, ○). Controls (■, ●) were grown in the absence of thiosulfate.

contrast to the results of Davey *et al.* (1993), streptomycin (100 µg ml⁻¹) inhibited the growth of *P. miotherma*.

Whole-cell protein patterns and serology

Although *P. miotherma* and strain SJ95^T have proteins with the same electrophoretic behaviour in common, for example a protein with a molecular mass of about 97 kDa, strain SJ95^T contained specific protein bands (e.g. at 60, 38 and 19 kDa) that differentiate it from *P. miotherma* (Fig. 2a). The immunoblotting (Fig. 2b) using antiserum to SJ95^T demonstrated serological differences which discriminate strain SJ95^T from *P. miotherma*.

DNA base composition

The G + C content of the DNA of strain SJ95^T was 31.4 and 34.00 ± 0.10 mol% (*n* = 8), determined by the thermal denaturation and HPLC methods, respectively. The corresponding values found for DNA from *P. miotherma* were 32 and 34.25 ± 0.16 mol% (*n* = 7). In addition, it is noteworthy that neither DNA sample contained detectable 5-methylcytidine. The sensitivity of the chemical method for detecting this modified base in the DNA was >0.3% of the total DNA or 1.7% of the cytidine.

Phylogenetic analysis

A 16S rDNA sequence comprising 1473 nt between positions 32 and 1534 (*E. coli* positions) was determined for strain SJ95^T. A comparison of 1179 nt between positions 32 and 1357 (*E. coli* positions) of the 16S rDNA sequence of strain SJ95^T was made with eight representatives of the order *Thermotogales* and *Aquifex pyrophilus*. The resulting phylogenetic dendro-

Fig. 4. Phylogenetic dendrogram based on 16S rDNA sequence comparison indicating the position of *Petrotoga mobilis* (strain SJ95^T) within the radiation of the bacterial *Thermotogales* group. Bar, 10 nucleotide changes per 100 nucleotides.

gram (Fig. 4) shows strain SJ95^T to be closely related to the *Geotoga/Petrotoga* lineage within the *Thermotogales*. 16S rDNA similarity values between SJ95^T and members of the *Thermotogales* are 77.9–97.9%. The highest 16S rDNA sequence similarity in the comparison of the sequences of the strains shown in Fig. 4 is to *P. miotherma* at 97.9%. Direct pairwise comparison of the 16S rDNA sequences of strain SJ95^T and *P. miotherma* showed a similarity of 97.7%.

DNA–DNA homology

The DNA–DNA homology of strain SJ95^T and *P. miotherma* was examined; the hybridization value was 35.6%.

DISCUSSION

Strain SJ95^T was tentatively identified as a member of the *Thermotogales* mainly because of its rod-shaped cells with an outer sheath-like structure and its thermophilicity. This relationship was confirmed by 16S rDNA sequence analysis which showed that strain SJ95^T is most closely related to *P. miotherma*. Furthermore, strain SJ95^T shares several phenotypic properties with this species, e.g. moderate thermophilicity, a high salt tolerance, capability of fermenting a broad spectrum of carbohydrates, and a variable cell size (Table 1).

However, strain SJ95^T is significantly different from *P. miotherma*. Firstly, strain SJ95^T is motile with subpolar flagellation. The cells are longer (40–50 µm) than those of *P. miotherma* (7.5 µm) and they do not form enlarged spherical bodies in the stationary phase. Secondly, strain SJ95^T is able to grow on xylan with toga-associated xylanase activity. Thirdly, the protein profile and the antigen pattern of SDS-soluble whole-cell extracts are different in strain SJ95^T and *P. miotherma*. Fourthly, the level of 16S rDNA sequence similarity of 97.7% is sufficiently low to establish a new species (Stackebrandt & Goebel, 1994). Fifthly, the DNA base composition is slightly different according to results obtained using two different and unreliable methods, but differs by 6% according to the

reported values for *P. miotherma* (Davey *et al.*, 1993). Finally, the level of DNA–DNA hybridization of 35.6% between strain SJ95^T and *P. miotherma* is too low for these organisms to be considered as members of the same species. On the basis of these data, strain SJ95^T can be considered a new species of the genus *Petrotoga*, for which the name *Petrotoga mobilis* is proposed.

Description of *Petrotoga mobilis* sp. nov.

Petrotoga mobilis (mo'bi.lis. L. fem. adj. *mobilis* motile).

Rod-shaped cells with a sheath-like outer structure, Gram-negative, obligately anaerobic, heterotrophic, able to ferment a broad spectrum of carbohydrates including xylan. The cells vary in size (0.5–1.0 by 1.0–50 µm) and can be single, in pairs or in chains, sometimes with more than 20 cells within the sheath. Frequently motile with subpolar flagellation. Neither endospores nor enlarged spherical bodies in stationary phase are formed. Growth occurs at pH 5.5–8.5, 40–65 °C and at NaCl concentrations of 0.5–9.0% NaCl (optimum at 3–4%). Several vitamins are required and yeast extract stimulates growth. The DNA G + C content is 31 and 34 mol%, as determined by thermal denaturation and HPLC methods, respectively. Isolated from hot oilfield water from an oil reservoir in the North Sea. The type strain is SJ95^T (= DSM 10674^T).

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