Extremophiles (2001) 5:85–91
Digital Object Identifier (DOI) 10.1007/s007920100175

ORIGINAL PAPER

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Isolation and characterization of *Thermococcus sibiricus* sp. nov. from a Western Siberia high-temperature oil reservoir

Received: May 8, 2000 / Accepted: November 28, 2000 / Published online: April 5, 2001

Abstract Anaerobic organotrophic hyperthermophilic Archaea were isolated from five of eight samples from oil wells of the Samotlor oil reservoir (depth, 1,799–2,287 m; temperature, 60°–84°C). Three strains were isolated in pure cultures and characterized phylogenetically on the basis of comparison of the 16S rRNA gene sequences. All strains belonged to a new species of the genus *Thermococcus*, with *Thermococcus litoralis, Thermococcus aggregans, Thermococcus fumicolans*, and *Thermococcus alcaliphilus* being the nearest relatives (range of sequence similarity, 97.2%–98.8%). Strain MM 739 was studied in detail. The new isolate grew on peptides but not on carbohydrates. Elemental sulfur had a stimulatory effect on growth. The temperature range for growth was between 40° and 88°C, with the optimum at 78°C; the pH range was 5.8 to 9.0, with the optimum around 7.3; and the salinity range was 0.5% to 7.0%, with the optimum at 1.8%–2.0%. The doubling time at optimal growth conditions was about 43 min. The G+C content of the DNA was 38.4 mol%. The DNA–DNA relatedness between strain MM 739 and *T. litoralis* was 27%; between strain MM 739 and *T. aggregans*, it was 22%. Based on the phenotypic and genomic differences with known *Thermococcus* species, the new species *Thermococcus sibiricus* is proposed. The isolation of a hyperthermophilic archaeum from a deep subsurface environment, significantly remote from shallow or abyssal marine hot vents, indicates the existence of a subterranean biosphere inhabited by indigenous hyperthermophilic biota.

Key words Deep subsurface environment · Hyperthermophilic Archaea · *Thermococcus sibiricus*

Communicated by G. Antranikian

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Introduction

Hyperthermophilic Archaea comprise a phylogenetically and metabolically diverse group of prokaryotes, being represented by members of both Crenarchaeota and Euryarchaeota and including organisms with lithotrophic–organotrophic and aerobic–anaerobic types of metabolism (Stetter 1996). Representatives of the order *Thermococcales* form the most numerous group to date. Two genera, *Thermococcus* (Zillig et al. 1983) and *Pyrococcus* (Fiala and Stetter 1986), include at present 23 species: 4 species belong to the genus *Pyrococcus* and 19 represent the genus *Thermococcus*. The main phenotypic differentiation between the two genera is the optimal growth temperature, which is between 95° and 100°C for members of the genus *Pyrococcus* and between 80° and 90°C for those of the genus *Thermococcus*. Representatives of the *Thermococcales* have coccoid cells with or without flagella; they are obligate anaerobes with a fermentative metabolism that grow on peptides, polysaccharides, or sugars and reduce elemental sulfur to avoid the formation of molecular hydrogen, which inhibits growth in closed vessels (Schönheit and Schäfer 1995).

Most *Thermococcales* are neutrophiles, growing optimally at pH 6.0–7.0; two species of the genus *Thermococcus, Thermococcus alcaliphilus* (Keller et al. 1995) and *Thermo-*

coccus acidaminovorans (Dirmeier et al. 1998), exhibit optimum growth at pH 9.0. *Thermococcales* are common inhabitants of submarine hot vents of both shallow water (Kostyukova et al. 1999) and the deep sea (Prieur et al. 1995). Two species, *Thermococcus zilligii* (Ronimus et al. 1997) and *Thermococcus waiotapuensis* (Gonzáles et al. 1999), were isolated from terrestrial hot springs in New Zealand and require a lower concentration of salt than marine species. There is also evidence of their presence in the deep subsurface biosphere: offshore oil wells (Stetter et al. 1993; Grassia et al. 1996; Orphan et al. 2000; Takahata et al. 2000) and the Paris Basin (L'Haridon et al. 1995). We report here on the isolation of a new species of the genus *Thermococcus* from a Western Siberia high-temperature oil reservoir, for which we propose the name *Thermococcus sibiricus*.

Materials and methods

Sampling

Samples were taken during October 1997 and June 1998 at the Samotlor oil reservoir, Nizhnevartovsk, Western Siberia. Samples of stratal fluid were taken from oil-bearing horizons located at depths of 1,800 to 2,350 m. The temperature of stratal fluids in upper horizons was about 60°C; that of the deepest (Jurassic) fluids was about 84°C (Table 1). The stratal fluid was a fine emulsion of oil in the formation water, the content of the latter varying from 40% to 99% (v/v). Samples of stratal fluid were taken directly from the production wellheads in 500-ml serum bottles that were immediately sealed with rubber stoppers and screw caps and transported to the laboratory at ambient temperature. The water phase, after the separation from oil, was used for further investigations. The main characteristics of samples are given in Table 1 . Formation water of four oil wells (13044, 642, 692, 39636) located in upper horizons had lower salinity (7–20 g l^{-1}). The other four oil wells (706b, 735b, 739, 12597), located in deeper horizons with a higher temperature, had higher water salinity (24–52 g l^{-1}). The pH of the water varied from 7.0 to 7.5. Chemical analyses of water were undertaken according to Reznikov et al. (1970).

Enrichment and isolation of hyperthermophilic Archaea

For the enrichment and isolation of hyperthermophilic Archaea, the following medium was used (mg l^{-1}): KCl, 330; NH_4Cl, 330; KH_2PO_4, 330; $MgCl_2 \cdot 6H_2O$, 330; $CaCl_2 \cdot 2H_2O$, 330; NaCl, 18,000; $Na_2S \cdot 9H_2O$, 500; peptone, 5,000; yeast extract (Difco), 100; elemental sulfur, 10,000; resazurin, 2. Trace elements (Kevbrin and Zavarzin 1992) and vitamins (Wolin et al. 1963) were added as 1 ml l^{-1}. The pH of the medium was adjusted to 6.8 at room temperature. Anaerobically prepared medium was dispensed in 15-ml Hungate tubes; the headspace (5 ml) was filled with an oxygen-free mixture of N_2/CO_2 (8:2, v/v). Tubes were inoculated with 0.1 ml formation water and incubated at 85°C. Pure cultures of hyperthermophilic Archaea were obtained by serial 10-fold dilutions.

Morphology and ultrastructure studies

The morphology of the new isolates was examined by phase-contrast light microscopy. The ultrastructure was studied on a JEM-100 electron microscope, the samples being prepared as described elsewhere (Bonch-Osmolovskaya et al. 1990).

Physiology and metabolic studies

Microbial growth was monitored by direct cell counting under the light microscope. The influence of temperature, pH, and salinity on the growth of the new isolate was determined by the generation time, pH being adjusted at room temperature. Possible substrates were added to the same medium as used for enrichment, but devoid of peptone, in a concentration of 5,000 mg l^{-1}. Organic acids were added as their sodium salts. Lithotrophic growth was assessed under

Table 1. Characteristics of sampling sites in Samotlor oil reservoir, Western Siberia

Well code	Depth (m)	Temperature (°C)	pH	Salinity (g l^{-1})	Ca^{2+} (g l^{-1})	Mg^{2+} (g l^{-1})	Cl^- (g l^{-1})	HCO_3^- (g l^{-1})	Fe total (g l^{-1})	$K^+ + Na^+$ (g l^{-1})
13044	1,799	60	7.30	7.73	0.38	0.13	4.24	0.66	0.011	2.31
642	1,850	60	7.35	20.51	0.48	0.03	12.05	0.55	nd	7.40
692	1,900	60	7.50	12.05	0.28	0	6.91	0.51	nd	4.35
39636	2,090	70	7.35	9.10	0.40	0.05	5.36	0.27	0.002	3.02
706b	2,300	72	7.00	39.78	0.80	0.06	23.43	0.97	nd	14.52
735b	2,287	84	7.25	32.85	1.00	0.32	19.32	1.04	0.07	11.2
739	2,350	84	7.30	24.80	0.781	0	14.89	0.27	nd	8.86
12597	2,350	84	7.30	52.31	0.80	0	14.89	0.27	nd	19.55

nd, no data

a H_2/CO_2 (8:2) gas mixture. Metabolic products were detected using the methods described by Miroshnichenko et al. (1994).

Genomic DNA studies

Determination of the G+C content of the genomic DNA and DNA–DNA hybridization were performed according to the protocols described previously (Miroshnichenko et al. 1998).

16S rDNA sequence determination and phylogenetic analysis

Genomic DNA extraction, polymerase chain reaction-mediated (PCR-mediated) amplification of the 16S rDNA, and sequencing of the PCR products were performed as described previously (Rainey et al.1996), except that archaeal-specific PCR primers were used for DNA amplification and sequencing reactions (Barns et al. 1994). The sequence reactions were analyzed by electrophoresis using a model 373A automatic DNA sequencer (Applied Biosystems, Foster City, CA, USA). The 16S rDNA sequences were manually aligned with those of members of *Thermococcus* and related organisms deposited in the 16S rDNA databank of the DSMZ. Evolutionary distances were calculated by the method of Jukes and Cantor (1969). Phylogenetic dendrograms were reconstructed using the treeing algorithm of De Soete (1983) and Felsenstein (1993).

Nucleotide sequence accession numbers

Accession numbers for sequences used in the construction of the phylogenetic tree are *Thermococcus acidaminovorans* DSM 11906^T (Y15935), *Thermococcus aggregans* DSM 10587^T (Y08384), *Thermococcus barophilus* DSM 11836^T (U82237), *"Thermococcus barossii"* DSM 9535 (U76534), *Thermococcus celer* DSM 2476^T (M21529), *Thermococcus chitonophagus* DSM 10152^T (X99570), *Thermococcus fumicolans* CIP 104680^T (Z70250), *Thermococcus gorgonarius* DSM 10395^T (Y16226), *Thermococcus guaymasensis* DSM 11113^T (Y08385), *Thermococcus hydrothermalis* AL662^T (Z70244), *Thermococcus litoralis* DSM 5473^T (Z70252), *Thermococcus pacificus* DSM 10394^T (Y16227), *Thermococcus peptonophilus* DSM 10343^T (D37983), *Thermococcus profundus* DSM 9503^T (Z75233), *Thermococcus siculi* DSM 12349^T (AB010893), *Thermococcus stetteri* DSM 5262^T (Z75240), and *Thermococcus zilligii* DSM 2770^T (U76534). The sequence of *Pyrococcus woesei* DSM 3773^T is available from the Ribosomal Data Project (RDP) (Maidak et al. 1996). The sequence of strain MM 739 has been deposited in the EMBL database under the accession number AJ 238992. Partial sequences of *T. siculi* DSM 12349^T, *T. peptonophilus* DSM 10343^T, and *T. alcaliphilus* DSM 10332^T obtained during this work were deposited in the EMBL database under the accession numbers AJ 298870, AJ 298871, and AJ 298872, respectively.

Results

Isolation of anaerobic hyperthermophilic Archaea from a Siberian high-temperature oil reservoir

Samples of formation water from eight oil-producing wells (see Table 1) were used for the inoculation of peptone-sulfur medium. After 1–2 days of incubation at 85°C, five samples, i.e., 642, 692, 739, 13044, and 39636, produced abundant growth of irregular coccoid cells. Three cultures were purified by serial dilution, and the new isolates were designated as strains MM 739, MM 642, and MM 39636. Strain MM 739 was chosen for a detailed characterization.

Morphology and ultrastructure of the new isolate

Cells of isolate MM 739 are irregular nonmotile cocci, 0.5–1 μm, without flagella (Fig. 1a). Thin sections revealed a cell envelope consisting of a cytoplasmic membrane and one layer of subunits (Fig. 1b).

Physiology of growth

Isolate MM 739 is an obligate anaerobe, as no growth was observed without prereduction of the growth medium. It is an obligate organotroph, utilizing peptone, yeast extract, beef extract, and soya bean extract. The presence of elemental sulfur in the medium was not obligatory but stimulated growth. No growth was observed on starch, pyruvate, glucose, acetate, methanol, ethanol, lactate, or H_2/CO_2 mixture (8:2, v/v) in either the presence or the absence of sulfur. H_2S, CO_2, acetate, isobutyrate, and isovalerate were the main metabolic products when peptone was used as the substrate.

Growth of isolate MM 739 was observed in the temperature range from 40° to 88°C with the optimum at 78°C. No growth was detected below 37° or above 91°C. The pH range of growth was from 5.8 from 9.0 with the optimum at 7.5. No growth was observed at pH 5.6 or 9.5. Isolate MM 739 grew in the salinity range from 5 to 70 g l^{-1} NaCl, with no growth at 2 or 75 g l^{-1}. Optimal growth was observed at 18–20 g l^{-1} NaCl. At optimal conditions (78°C, pH 7.5, 18 g l^{-1} NaCl), the generation time was 43 min.

Determination of G+C content of the DNA, DNA–DNA cross-hybridization, and phylogenetic analysis

The G+C content of the genomic DNA of isolate MM 739 determined by melting point analysis of DNA was found to be 38.4 ± 0.5 mol%. The DNA–DNA cross-hybridization

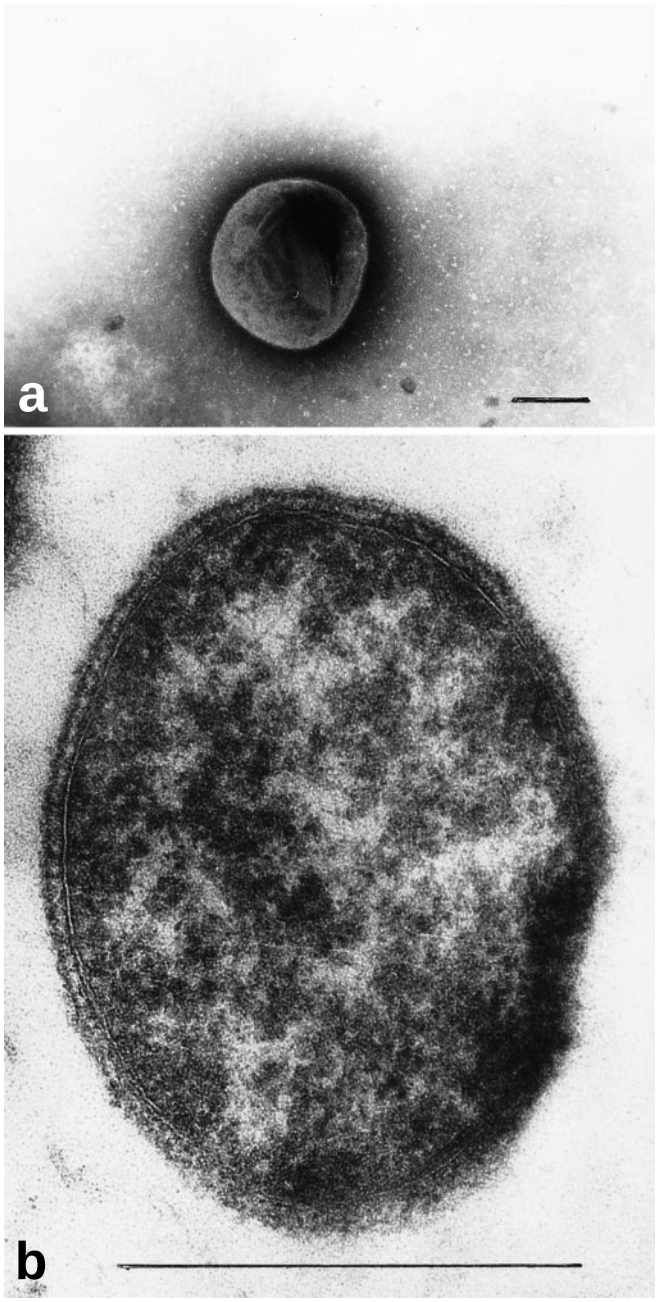

Fig. 1a, b. Whole-cell preparations (**a**) and thin sections (**b**) of isolate MM 739 cells. *Bar* 0.5 μm

reaction between isolate MM 739 and *T. litoralis* DSM 5474^T showed 27% relatedness; between MM 739 and *T. aggregans* DSM 10587^T, it was 22%.

A continuous stretch of 1,305 nucleotides was determined for the 16S rDNA of strain MM 739, ranging from positions 5′ 18 to 3′ 1,361 (*Escherichia coli* sequence; Brosius et al. 1981). Similarity values were calculated for this strain and members of the genus *Thermococcus* for which 16S rDNA sequences have been deposited. Strain MM 739 shared the highest binary 16S rDNA similarity values with *T. aggregans* DSM 10587^T, *T. alcaliphilus* DSM 10332^T, *T. fumicolans* CIP 104680^T, and *T. litoralis* DSM 5473^T (98.8%–97.2% similarity). All other *Thermococcus* species were more distantly related. To improve the 16S rDNA database of *Thermococcus* type strains, the almost complete 16S rDNA sequence of *T. alcaliphilus* DSM 10332^T was determined; several gaps in the deposited sequences of *T. peptonophilus* DSM 10343^T and *T. siculi* DSM 12349^T were closed. The topology of dendrograms based upon maximum-likelihood (not shown) and neighbor-joining algorithms was similar, clustering strain MM 739 with "*T. litoralis*," *T. fumicolans, T. alcaliphilus*, and *T. aggregans*, showing an approximately equidistant relationship to these species (Fig. 2). Only a few branching points, including the strain cluster containing strain MM 739, were supported by high bootstrap values.

Analysis of a 370-nucleotide-long stretch of 16SrDNA (covering a region between positions 97 and 469 [*E. coli* numbering]) indicates that isolates MM 642 and MM 39636 share 100% sequence similarity with strain MM 739. These isolates may therefore be considered as strains of the same species.

Discussion

A large number of thermophilic and hyperthermophilic organisms have been isolated from oil wells operating with the original formation water or after injection of outside water. Bacteria isolated from terrestrial or marine oil fields include mainly species of the genera *Thermotoga, Geotoga, Petrotoga, Desulfotomaculum,* and *Thermoanaerobacter* (Davey et al. 1993; Fardeau et al. 1993, 1997; Jeanthon et al. 1995; Ravot et al. 1995; Nilsen et al. 1996; Lien et al. 1998; Orphan et al. 2000, Takahata et al. 2000). Hyperthermophilic Archaea, first discovered in terrestrial and submarine hot vents, were afterwards found in deep subsurface environments. In an offshore oil production platform in the North Sea, hyperthermophilic Archaea were found and identified as *Archaeoglobus fulgidus, Archaeoglobus profundus*, "*Archaeoglobus lithotrophicus*," *Thermococcus celer*, *Thermococcus litoralis*, and *Pyrococcus* sp. (Stetter et al. 1993; Beeder et al. 1994). These organisms were considered to represent the indigenous microbiota; another possibility was that hyperthermophiles were introduced with the injection of ocean water. The latter assumption could be supported by the evidence of the presence of viable hyperthermophilic cells in cold seawater (Stetter et al. 1993). The isolation of *Archaeoglobus fulgidus* and *Thermococcus litoralis* from a continental oil well (Paris Basin) was interpreted as indicating the indigenous origin of hyperthermophilic Archaea in the deep subsurface biosphere (L'Haridon et al. 1995).

The Samotlor high-temperature oil reservoir is located in Western Siberia, in the middle of the Eurasian continent, and is far remote from both the ocean and volcanic areas. Isolate MM 739 was obtained from the formation water

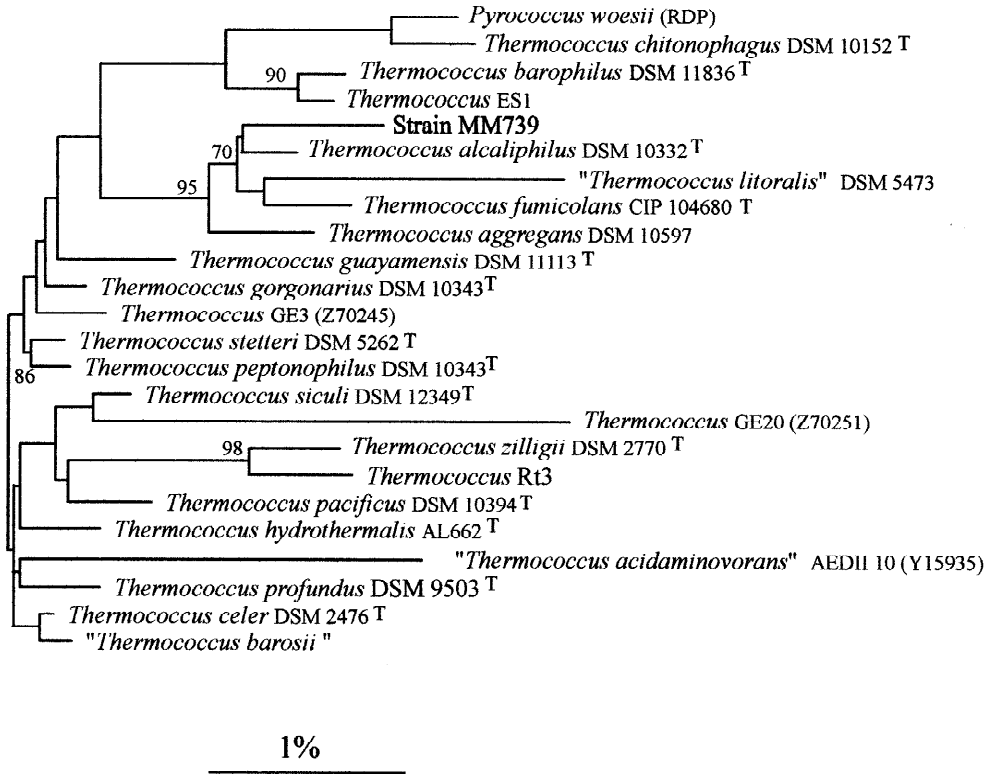

Fig. 2. Phylogenetic position of strain MM 739 within the radiation of *Thermococcus* species, as derived from neighbor-joining analysis of 16S rDNA sequences. Bootstrap values of 500 resamplings (>70%) are indicated at branch points. *Bar* indicates one substitution per 100 bases

sample of a Jurassic horizon located at a depth of 2,350 m. Temperature, pH, and salinity characteristics for growth are in good correlation with the natural conditions at the sampling site (see Table 1). Hyperthermophilic Archaea were also found at four other wells of the same oil reservoir. Well 39636 is also located at a high-temperature oil-bearing Jurassic horizon, whereas three other samples originated from wells with a lower temperature at the depth range 1,800–1,900 m flooded with injection water. The presence of hyperthermophilic Archaea, at least in Jurassic horizons that were never flooded, might be explained by their indigenous origin. These organisms could have been deposited with the original sediment and have survived over geologic time by metabolizing buried organic matter.

Phenotypically, hyperthermophilic archaeal isolates obtained from Siberian oil wells resemble representatives of the genus *Thermococcus*: they are obligately anaerobic thermophiles with a fermentative metabolism, capable of growth on complex organic substrates, and are stimulated by the presence of elemental sulfur. The unusual feature of isolate MM 739 is its growth under extremely wide ranges of temperature, pH, and salinity. Other deep subsurface isolates, such as *Thermococcus* sp. obtained from the Kubiki oil reservoir, also had a low minimum growth temperature, 46°–47°C. Recently, organotrophic hyperthermophilic Archaea were isolated from the hydrothermal plumes of deep-sea hot vents (Summit and Baross 1999) and were believed to be the indigenous inhabitants of the deep subsea floor biosphere. All these isolates had a lower temperature limit of growth at 45°C, thus resembling isolates obtained from the oil wells in this characteristic.

Comparison of 16S rRNA sequences revealed that all three subterranean isolates belong to the same species. The level of similarity of 16S rRNA gene sequences between isolate MM 739 and its four closest relatives, i.e., *T. alcaliphilus* (Keller et al. 1995), *T. aggregans* (Canganella et al. 1998), *T. fumicolans* (Godfroy et al. 1996), and *T. litoralis* (Neuner et al. 1990), was 97.2%–97.8%. The optimum and range of temperature, pH, and salinity for growth of isolate MM 739 are clearly distinct from those of its phylogenetic neighbors (Table 2). In contrast to *T. aggregans*, isolate MM 793 grew only on peptides, but not with starch, sugars, or pyruvate. *T. alcaliphilus* has a much higher pH range and optimum for growth than isolate MM 739. During growth on proteinaceous substrates, isolate MM 739 produced only acetate, isobutyrate, and isovalerate as the fermentation products, whereas *T. aggregans* and *T. fumicolans* also formed propionate or lactate. Unlike *T. fumicolans* and *T. alcaliphilus,* the isolate MM 739 was nonmotile. The G+C content of DNA of isolate MM 739 is 38.4 mol%, which is closest to that of *T. litoralis* (38%–40 mol%), whereas *T. aggregans, T. alcaliphilus*, and *T. fumicolans* have a higher G+C content (42, 42.4, and 54.5 mol%, respectively). DNA–DNA cross-hybridization between isolate MM 739 and *T. litoralis* showed 27% relatedness, whereas between MM 739 and *T. aggregans* it was even less, 22%. Based on these results, we propose for the novel isolates obtained from Siberian oil wells the name

Table 2. Comparison of characteristics of isolate MM 739 and closely related *Thermococcus* species

Characteristic	MM 739	*T. aggregans*	*T. alcaliphilus*	*T. fumicolans*	*T. litoralis*
Temperature (°C)					
Min	40	60	56	73	55
Opt	78	88	85	85–90	85
Max	88	94	90	103	98
pH:					
Min	5.8	5.6	6.5	4.5	4.0
Opt	7.8	7.0	9.0	8.0	6.0
Max	9.0	7.9	10.5	9.5	8.0
NaCl (g l–1)					
Min	5	10	10	10	18
Opt	18–20	25	20–30	20–40	25
Max	70	30	60	80	65
Motility	–	–	+	+	–
Substrates					
Peptides	+	+	+	+	+
Polysaccharides	–	Starch	–	–	Starch
Sugars	–	Maltose, dextrose	–	Maltose	Maltose
Sulfur requirement	Fac., stim.	Fac., stim.	Fac., stim.	Fac., stim.	Fac., stim.
Products of fermentation on proteinaceous substrates	Acetate *i*-Butyrate *i*-Valerate	Acetate Propionate *i*-Butyrate *i*-Valerate	Acetate Propionate *i*-Butyrate *i*-Valerate	Acetate Lactate Propionate Phenylacetate 2-Methylpropionate	nr
Doubling time (min) at optimal conditions	43	nr	90	120	50
G+C of DNA (mol%)	38.4	42	42.4	54.5	38–40

Fac., facultative; stim., stimulation; nr, not reported

Thermococcus sibiricus sp. nov. with strain MM 739, DSM 12597^T, as the type strain.

Description of *Thermococcus sibiricus* sp. nov.

Thermococcus sibiricus sp. nov. (si.bi'ri.cus N.L. masc. adj. *sibiricus*, originating from Siberia). Cells are nonmotile irregular cocci 0.5–1.0 μm in diameter. Anaerobe. Organotroph, fermenting peptides. No growth was found on sugars, polysaccharides, organic acids, alcohols, or molecular hydrogen. Elemental sulfur stimulates fermentative growth on peptides in closed vessels. Hyperthermophile, growing in the temperature range from 40° to 88°C, with an optimum at 78°C. Neutrophile, growing in the pH range 5.8 to 9.0 with an optimum at 7.5. Adapted to marine salinity, 5–70 g l^{-1} NaCl, with the optimum at 18–20 g l^{-1}. G+C content of DNA, 38.4 mol%.

Isolated from formation water of high-temperature Samotlor oil reservoir, Western Siberia.

Type strain MM 739 was deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) GmbH, Germany, under DSM 12597.

Acknowledgments This work was supported by INTAS grant N 96-1341, and by the program "Biodiversity" of the Russian Federation Ministry of Science and Technology. The authors express their gratitude to K.L. Matveev, Geological Department of Nizhnevartovskneft' Oil/Gas Production Company, for his help with the organization of field research and for the geologic information provided.

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