Thermococcus thioreducens sp. nov., a novel hyperthermophilic, obligately sulfur-reducing archaeon from a deep-sea hydrothermal vent

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A hyperthermophilic, sulfur-reducing, organo-heterotrophic archaeon, strain OGL-20P^T, was isolated from "black smoker" chimney material from the Rainbow hydrothermal vent site on the Mid-Atlantic Ridge (36.2 ^{o}N, 33.9 ^{o}W). The cells of strain OGL-20P^T have an irregular coccoid shape and are motile with a single flagellum. Growth was observed within the pH range 5.0–8.5 (optimum pH 7.0), NaCl concentration range 1-5 % (w/v) (optimum 3 %), and temperature range 55-94 ^{o}C (optimum 83-85 ^{o}C). The novel isolate is strictly anaerobic and obligately dependent upon elemental sulfur as an electron acceptor, but it does not reduce sulfate, sulfite, thiosulfate, iron (III) or nitrate. Proteolysis products (peptone, bacto-tryptone, casamino-acids, and yeast extract) are utilized as substrates during sulfur-reduction. Strain OGL-20P^T is resistant to ampicillin, chloramphenicol, kanamycin, and gentamycin, but sensitive to tetracycline and rifampicin. The G+C content of DNA is 52.9 mol%. The 16S rRNA gene sequence analysis revealed that strain OGL-20P^T is closely related to *Thermococcus coalescens* and related species, but no significant homology by DNA-DNA hybridization was observed between those species and the new isolate. On the basis of physiological and molecular properties of the new isolate, we conclude that strain OGL-20P^T represents a new separate species within the genus *Thermococcus*, and propose the name *Thermococcus thioreducens* sp. nov. The type strain is OGL-20P^T (= ATCC BAA-394^T = JCM 12859^T = DSM 14981^T).

The Gen Bank accession number for the 16S rRNA gene sequence of strain

47 OGL-20P ^{T} is AF 394925.

48

49 The genus *Thermococcus* was created in 1983, and currently 25 species have been
50 validly published. All members of this genus are characterized by a thermophilic
51 nature, anaerobiosis with sulfur-type respiration and sometimes sulfur stimulation for
52 fermentation (Zillig, 1992; Zillig & Reysenbach, 2002). The typical ecological systems
53 for the habitat of *Thermococcus* species include geothermal springs (volcanic
54 fumaroles, geysers, and deep-sea hydrothermal vents), deep subsurface biosphere such
55 as deep crustal rocks and aquifers and high-temperature oil wells (Stetter *et al.,* 1993;
56 Takahata *et al.,* 2000; Miroshnichenko *et al.,* 2001). Most species of the genus
57 *Thermococcus* are marine and have an optimum NaCl concentration of about 3 %
58 (w/v), but there are also fresh-water species, e.g. *T. zilligii* (Ronimus *et al.,* 1997) and
59 *T. waiotapuensis* (González *et al*, 1999). Most members of the *Thermococcus* genus
60 grow optimally at neutral or slightly acidic pH, and only *T. alkaliphilus* is capable of
61 growth at pH 10.5 with optimum around 9.0 (Keller *et al.,* 1995). The minimum
62 temperature for growth of *Thermococcus* is 50 ^{o}C and the maximum is about 95 ^{o}C as
63 for *T. celer, T. litoralis,* and *T. fumicolans* (Zillig *et al.,* 1983; Neuner *et al.,* 1990;
64 Godfroy *et al.,* 1996). Many species of the genus *Thermococcus* have been isolated
65 from deep-sea hydrothermal vents with environmental pressures in excess of 200
66 atmospheres. Obligate dependence upon pressure was determined at 95-100 ^{o}C for *T.*
67 *barophilus* (Marteinsson *et al.,* 1999). The most radioresistant hyperthermophilic
68 archaeon, *T. gammatolerans*, is capable of surviving 30 kGy γ–ray irradiation (Jolivet
69 *et al.,* 2003). Most species of the genus *Thermococcus* are sulfur reducing organisms,

however, Slobodkin *et al.* (1999) reported dissimilatory reduction of Fe(III) by *Thermococcus* sp.T642. In this article we describe a novel hyperthermophilic archaeon *Thermococcus thioreducens* sp. nov., which is an obligate sulfur-reducer, and was isolated from the Rainbow deep-sea hydrothermal vent site in the Mid-Atlantic Ridge.

"Black Smoker" chimney material samples were collected in October 1999 from 2,300 meter depth in the Rainbow hydrothermal vent field (36.2 ^{o}N; 33.9 ^{o}W) about 800 km southwest of the Azores on the Azorean segment of the Mid-Atlantic Ridge. Remote manipulators (on the *Mir* submersible launched from the Russian oceanographic research vessel *Akademik Mstislav Keldysh*) were used to place the samples on a collection tray for return to the surface. After a brief exposure to the ambient atmosphere during the submersible recovery out of the water, the samples were hermetically sealed in sterile vessels with screw caps and maintained at 4 ^{o}C in an insulated cooler during transport to the Astrobiology Laboratory of the NASA, Marshall Space Flight Center. Strain OGL-20P^{T} was isolated from a sample of black colored fine-grained sand and mud (neutral pH, 3 % (w/v) salinity) that contained chimney debris material and organic sediments.

The enrichment, isolation, and cultivation of the new isolate were performed in a liquid medium under a highly purified 100 % nitrogen atmosphere. The basal medium contained g l^{-1}: KH_2PO_4 , 0.3; $MgCl_2 \cdot 6H_2O$, 0.1; KCl, 0.3; NH_4Cl, 1.0; $NaHCO_3$, 0.2; $CaSO_4 \cdot 7H_2O$, 0.005; NaCl, 30.0; $Na_2S \cdot 9H_2O$, 0.4; yeast extract, 0.5; sulfur powder, 10.0, peptone, 5.0, and resazurin, 0.001. The medium was supplemented with 2 ml of vitamin solution (Wolin *et al.,* 1963) and 1 ml of trace element solution as described

93 earlier (Pikuta *et al.*, 2000). The final pH^{22C} of the medium after autoclaving was 7.2-
94 7.4.
95 Unless otherwise noted, enrichment and pure cultures were grown in 10 ml of medium
96 in Hungate tubes under one atmosphere of N_2 (100 %). All transfers and samplings of
97 cultures were performed with sterile syringes. The medium was sterilized at 121 ^{o}C for
98 60 min and after adding sulfur to the tubes under an atmosphere of 100 % nitrogen an
99 additional sterilization was performed at 110 ^{o}C for 30 min. All incubations for
100 physiology description were carried out at 83 ^{o}C. One half gram of sample L-20 was
101 injected into the medium and incubated for 24 h. A pure culture of strain OGL-$20P^{T}$
102 was obtained after repeated serial dilutions. The culture on the 10^{-9} dilution with the
103 monotypic morphology was chosen for the following "roll-tube" serial dilutions
104 purification. Growth of colonies occurred after 2-3 days incubation on 3 % (w/v) Difco
105 agar in Hungate tubes at 70 ^{o}C. One colony on the 10^{-8} dilution tube was chosen for
106 consequent purification and designated as strain OGL-$20P^{T}$. The colonies of strain
107 OGL-$20P^{T}$ on the surface of the agar were whitish-cream in color, glossy and shining,
108 with a round shape (~1.5 mm diameter), irregular cleaved edges and convex with
109 denser raised conic center. In deep agar, colonies had a convex-convex lenticular
110 shape.
111 Phase-contrast microscopy revealed the cells of strain OGL-$20P^{T}$ were irregular,
112 motile cocci with diameter 0.7 to 1.7 μm. Some of the time the cells looked as
113 diplococci or conglomerates of 10-15 cells. Transmission Electron Microscopy was
114 carried out using a JEOL TEM 100 CX II operating at 80 kV. Negative staining was

115 performed using a uranyl acetate procedure as described previously (Pikuta *et al.*,
116 2003). TEM images showed the presence of a single flagellum (Fig. 1).
117 Culture growth was measured by direct cell counting under a phase-contrast
118 microscope (Fisher Micromaster, USA), by measuring sulfide produced from sulfur in
119 the process of growth (Truper & Schlegel, 1964), or by estimating an increase in
120 optical density at 595 nm (Genesis 5; Spectronic Instruments, USA). The pH of the
121 medium was adjusted to defined values with sterile stock solutions of 6 N HCl or 6 N
122 NaOH under a flow of N_2 and measured using a pH meter (model 230 Aplus, Orion,
123 USA) calibrated at 22 ^{o}C. All measurements were performed after cooling the culture
124 samples to room temperature. The temperature range for growth was determined in the
125 liquid medium at pH 7.3. The effect of NaCl concentration on growth was determined
126 in the liquid medium containing 0.0, 0.5, 1.0, 2.0, 3.0, 5.0, 7.0, and 10.0 % (w/v) NaCl.
127 NaCl requirement was studied using a modified medium, in which $NaHCO_3$ was
128 replaced with K_2CO_3 and Na_2S was replaced with K_2S. Growth of strain OGL-20P^T
129 was observed in the temperature range of 55 to 95 ^{o}C, with optimum between 83 and
130 85 ^{o}C. Strain OGL-20P^T survived during 30 minutes at 101 ^{o}C, but incubation at 103
131 ^{o}C during 2 h killed the cells. Growth of strain OGL-20P^T was observed within the pH
132 range of 5.0-8.5, with optimum pH at 7.0; within NaCl concentration range of 1 to 5 %
133 (w/v) with optimum of 3 % (w/v). No growth was detected for NaCl concentrations
134 below 0.5 % or above 7 % (w/v). The doubling time measured by direct cell counting
135 under a phase-contrast microscope for a fresh culture of OGL-20P^T incubated at
136 optimal conditions was 30 minutes.

Strain OGL-20P^T was found to be strictly anaerobic. The catalase activity, which was tested as described by Smilbert & Krieg (1994), showed negative reaction. The utilization of various electron acceptors was studied in a medium containing peptone (5g l^{-1}) as an electron donor. Electron acceptors were added in the form of autoclaved or filter-sterilized stock solutions. The final concentrations of electron acceptors were the following (mM): Na_2SO_4, 20; Na_2SO_3, 5; $Na_2S_2O_3 * 5H_2O$, 10; $NaNO_3$, 10; $Fe(OH)_3$, 100; and S^o, 300. Amorphous FeOOH suspension (iron gel) was prepared by neutralizing a 0.4 M solution of $FeCl_3$ to pH 7 by 10 N NaOH as described previously (Lovley & Phillips, 1986). Only elemental sulfur was used as an electron acceptor, which resulted in the production of hydrogen sulfide (15-20 mM). No growth was observed in the absence of sulfur on all tested substrates.

The ability of the new archaeon to utilize various substrates was tested by using the liquid medium supplemented with autoclaved or filter-sterilized substrates to a final concentration of 5 g l^{-1}. The substrate utilization was tested by cultivation of strain OGL-20P^T during 1-6 days on different substrates and growth was detected under a microscope and by measurement of hydrogen sulfide. Growth was observed on proteolysis products: peptone, bactotryptone, casamino acids, and yeast extract. No growth was observed in the presence of glucose, fructose, maltose, sucrose, *D*-mannitol, glycerol, methanol, ethanol, butyrate, propionate, acetate, formate, lactate, pyruvate, citrate, and separate amino acids (*L*- and *D*- leucine, *L*- and *D*-methionine, *L*- and *D*- histidine HCl, *L*- cysteine, *L*- proline, *L*- lysine, *L*- cystine, glycine, *L*-glutamine, *L*- alanine, *L*- serine, *L*- tyrosine, *L*- phenylalanine, *L*- valine, *L*-isoleucine, *L*- tryptophan, *L*- arginine).

End products of sulfur respiration in the liquid phase were determined by HPLC. Separation was done on Aminex HPX-87H (BioRad) column with 5 mM H_2SO_4 as the mobile phase. Gases were measured with a gas chromatograph 3700 (Varian) equipped with Porapak Q column and TCD detector. Nitrogen was used as the gas carrier. Acetate (2.1 mM) and ethanol (3.7 mM) were detected in the liquid phase as minor end products. Hydrogen sulfide (more than 20 mM) and traces of hydrogen and CO_2 were measured in the gas phase during the growth of OGL-20P^T.

Antibiotic susceptibility was determined by transferring an exponentially growing culture into the basal medium containing filter-sterilized antibiotics at a concentration of 100 μg ml^{-1} (chloramphenicol, rifampin) or 250 μg ml^{-1} (ampicillin, tetracycline, kanomycin, and gentamycin). Before incubation at 83 ^oC, antibiotic-containing cultures were pre-incubated at 37 ^oC for 12 h. Strain OGL-20P^T was resistant to ampicillin, gentamycin, kanamycin and chloramphenicol (growth without changes of morphology and motility), but was sensitive to tetracycline and rifampin**.**

Genomic DNA was isolated through a standard phenol / chloroform extraction followed by ethanol precipitation (Sambrook *et al.*,1989). The G+C content of DNA was determined by HPLC (Mesbah *et al.,* 1989). Details of the procedure were described previously (Hoover *et al.,* 2003). The result reported was the mean of two determinations for each of two degradations of the archaeal DNA. The G+C content of the genomic DNA of strain OGL-20P^T was 57.2 ± 0.2 mol% (mean ± SD, n = 6).

The 16S rRNA gene of strain OGL-20P^T, along with a part of 23S rRNA gene and the spacer region, was selectively amplified with the following primers: 5'-TCCGGTTGATCCTGCCGG-3' (forward) and

5’-CTTTTCCTGCGGGTACTAAG-3' (reverse). PCR was performed with 30 pmol of each primer in a 50 µl volume, using 2 U ThermalAce DNA polymerase (Invitrogen, USA) in the provided buffer. The thermal cycling profile was as follows: 3 min at 95^{o}C initial denaturation, followed by 30 cycles of 45 s denaturation at 95 ^{o}C, 45 sec annealing at 57 ^{o}C and 4 min extension at 72 ^{o}C, with a final extension step at 72 ^{o}C during 15 min. The amplified fragment was extracted from a 1.5 % agarose gel using the Qiaquick extraction kit (Qiagen, USA), and then subcloned using the Zero Blunt TOPO PCR Cloning kit (Invitrogen, USA). Six clones were sequenced in both directions using the dye terminator Ampli*Taq* FS cycle sequencing kit (Applied Biosystems, USA) with both vector-based primers and primers specific to 16S internal sequence (designed by us).

The 16S rRNA sequence of strain OGL-20P^{T} was aligned with closely related sequences found in GenBank after a BLAST search (Altschul *et al.*, 1990), using ClustalW (Thompson *et al*. 1994). Pairwise distances were computed with MEGA version 3.1 (Kumar *et al*., 2004) using the Jukes-Cantor model (Jukes & Cantor, 1969). An unrooted phylogenetic tree was constructed with the same MEGA program using the Neighbor-Joining method (Saitou & Nei, 1987).

A sequence covering 1885 nucleotides, including most (1452) of the 16S rRNA gene, the tRNA^{Ala} gene and a part of the 23S rRNA gene, was obtained after amplification of strain OGL-20P^{T} DNA. The 16S rRNA gene sequence corresponds to positions 37-1496 of the *Pyrococcus furiosus* 16S rRNA sequence (accession number U20163) used as a reference. A BLAST search against the Genbank database revealed a high similarity (> 97 %) with sequences from the *Thermococcus* genus. A phylogenetic

dendrogram showing the relationship of strain OGL-20P^T to the 11 closest species was constructed, based on 1400 common nucleotide sites (Fig. 2). Pairwise distances between the OGL-20P^T sequence and its closest neighbors were 0.003, 0.006, 0.006 and 0.007 for *T. coalescens*, *T. celer*, *T. hydrothermalis* and *T. barossii* respectively based on the same 1400 nucleotide sites. The sequence of the 16S rRNA gene of strain OGL-20P^T was deposited in GenBank under accession number AF394925.

Homologies of genomic DNA between the new isolate and the phylogenetically closest *Thermococcus* species were determined as described previously (Pikuta *et al.*, 2006). The DNA-DNA hybridization values with labeled DNA from strain OGL-20P^T were as follows: *T. celer* JCM 8558^T: 14 %, *T. barossii* ATCC BAA-1085^T: 17 %, *T. hydrothermalis* AL662^T: 16 %, *T. kodakaraensis* ATCC BAA-918^T: 5 %, *T. profundus* ATCC 51592^T: 4 %, *T. acidaminovorans* DSM 11906^T: 5 %, *T. stetteri* DSM 5262^T: 4 %, *T. peptonophilus* ATCC 700098^T: 5 %, *T. gorgonarius* ATCC 700654^T: 5 %, *T. coalescens* JCM 12540^T: 13 %, and '*T. radiotolerans*' JCM 11826^T: 18 %.

Almost half of the *Thermococcus* species were isolated from deep-sea hydrothermal vents with high pressure conditions (200-350 atmospheres), located in different parts of the world (Kobayashi *et al.*,1994; Huber *et al.*,1995; Godfroy *et al.*,1996; Godfroy *et al.*,1997; Canganella *et al.*,1998; Duffaud *et al.*,1998; Grote *et al.*,1999). Strain OGL-20P^T was also isolated from a deep-sea ecosystem, characterized by high pressure (230 atmospheres), localized high temperatures (300 to 400 ^oC within the Black Smoker vents), and very high thermal gradients, (temperature drops to 2 ^oC a

few centimeters away from the "chimney"). As the "smoke" condenses above the vent, the precipitated minerals are also spread around the nearby ocean floor; black color pyrites (FeS) surrounding Black Smokers is a result of interaction of sulfide with iron; orange color is a result of a trivalent iron (Fe^{+3}) appearance. Unpigmented invertebrates (shrimps, crabs, and worms) represent multicellular organisms in the ecosystem. Their energy source is partially provided by the metabolism of microorganisms (in our laboratory the cells with morphology similar to the new archaeon were found in the intestines of shrimps with a strong smell of sulfur.)

Strain OGL-20P^T is a hyperthermophilic, heterotrophic, sulfur-dependent, coccoid archaeon inhabiting a deep-sea hydrothermal system in the Mid-Atlantic Ridge. In line with those properties, comparison of the 16S rRNA gene places the strain in a clade of the euryarchaeotic order, the order *Thermococcales*, and most related to the genus *Thermococcus.* Currently, the genus *Thermococcus* contains 25 validly published species, which are separated into two major clades represented by *T. celer* and *T. litoralis*, and two independent lineages of *T. barophilus* and *T. atlanticus*. The separation of the two major clades is also supported by the DNA base composition. The strain OGL-20P^T is included in the clade represented by *T. celer*. Comparison of strain OGL-20P^T with closest neighbors on the phylogenetic tree showed a 16S rRNA sequence difference of less than 1 %. However, the DNA-DNA- hybridization showed less than 20 % similarity with them. Phenotypic and genotypic differences between strain OGL-20P^T and the closest species are shown in comparative table1.

On the basis of comparative data about morphology, physiology and genomic characteristics we conclude that strain OGL-20P^T represents a separate taxon on the

252 species level. The name *Thermococcus thioreducens* sp. nov., is proposed for the new
253 species.
254
255 **Description of *Thermococcus thioreducens* sp. nov.**
256
257 *Thermococcus thioreducens* (thi.o.re.du'cens. Gr. n. *thion* sulfur, L. part. adj.
258 *reducens* reducing, N.L. part. adj. *thioreducens* reducing sulfur).
259
260 Cells are irregular cocci with 0.7- 1.8 μm diameter, motile by single flagellum.
261 Heterotroph, strict anaerobe. Obligately dependent from elemental sulfur. Catalase
262 negative. Grows on peptone, bacto-tryptone, casamino acids, yeast extract as electron
263 donors. No growth on *D*-glucose, fructose, maltose, sucrose, *D*-mannitol, glycerol,
264 methanol, ethanol, butyrate, propionate, acetate, formate, lactate, pyruvate, citrate and
265 amino acids. Thiosulfate, sulfite, sulfate, iron (III) or nitrate cannot support growth as
266 electron acceptors. Cells are hyperthermophiles growing between 55 ^{o}C and 94 ^{o}C
267 with optimum at 83- 85 ^{o}C, and pH range 5.0 - 8.5 (optimum 7.0), and with NaCl
268 concentration (w/v) range 1 - 5 % (optimum 3 %) The doubling time is 30 min. The
269 main end product of growth with peptone and sulfur is H_2S (more than 20 mM);
270 minor end products are: CO_2, H_2 (0.05 mM), acetate (2 mM), ethanol (3.7 mM).
271 Sensitive to tetracycline and rifampin**.** The G+C content of DNA is 52.9 mol%
272 (HPLC).

Source of isolation: deep sea "Black Smoker" chimney debris in mud at Rainbow hydrothermal vent site at 2,300 meters depth in Atlantic Ocean off the coast of the Azores.

Type strain: *Thermococcus thioreducens* OGL-20P^T (= JCM 12859^T = DSM 14981^T = ATCC BAA-394^T).

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REFERENCES

Atomi, H. Fukui, T., Kanai, T., MorikAWA, M. & Imanaka, T. (2004). Description of *Thermococcus kodakaraensis* sp. nov., a well studied hyperthermophilic archaeon previously reported as *Pyrococcus* sp. KOD1. *Archaea* **1**, 263-267.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403-410.

Canganella, F., Jones, W. J., Gambacorta, A. & Antranikian, G. (1998). *Thermococcus guaymasensis* sp. nov. and *Thermococcus aggregans* sp. nov., two novel thermophilic archaea isolated from the Guaymas Basin hydrothermal vent site. *Int J Syst Bacteriol* **48**, 1181-1185.

Dirmeier, R., Keller, M., Hafenbradl, D., Braun, F. J., Rachel, R., Burggraf, S. & Stetter, K. O. (1998). *Thermococcus acidaminovorans* sp. nov., a new hyperthermophilic alkaliphilic archaeon growing on amino acids. *Extremophiles* **2**, 109-114.

Duffaud, G. D., d`Hennezel, O. B., Peek, A. S., Reysenbach, A. -L. & Kelly, R. M. (1998). Isolation and characterization of *Thermococcus barossii,* sp. nov., a hyperthermophilic Archaeon isolated from a hydrothermal vent flange formation. *Syst Appl Microbiol* **21**, 40-49.

Godfroy, A., Meunier, J. -R., Guézennec, J., Lesongeur, F., Raguénès, G., Rimbault, A. & Barbier, G. (1996). *Thermococcus fumicolans* sp. nov., a new hyperthermophilic archaeon isolated from a deep-sea hydrothermal vent in the North Fiji Basin. *Int J Syst Bacteriol* **46**, 1113-1119.

Godfroy, A., Lesongeur, F., Raguénès, G., Quérellou, J., Antoine, E., Meunier, J. –R., Guézennec, J. & Barbier, G. (1997). *Thermococcus hydrothermalis* sp. nov., a new hyperthermophilic archaeon isolated from a deep-sea hydrothermal vent. *Int J Syst Bacteriol* **47**, 622-626.

González, J. M., Kato, C. & Horikoshi, K. (1995). *Thermococcus peptonophilus* sp. nov., a fast-growing, extremely thermophilic archaebacterium isolated from deep-sea hydrothermal vents. *Arch Microbiol* **164**, 159-164.

Grote, R., Li, L., Tamaoka, J., Kato, C., Horikoshi, K. & Antranikian, G. (1999). *Thermococcus siculi* sp. nov., a novel hyperthermophilic archaeon isolated from a deep-sea hydrothermal vent at the Mid-Okinawa Trough. *Extremophiles* **3**, 55-62.

Huber, R., Stöhr, J., Hohenhaus, S., Rachel, R., Burggraf, S., Jannasch, H. W. & Stetter, K. O. (1995). *Thermococcus chitonophagus* sp. nov., a novel, chitin-degrading, hyperthermophilic archaeum from a deep-sea hydrothermal vent environment. *Arch Microbiol* **164**, 255-264.

Hoover, R.B., Pikuta, E.V., Bej, A.K., Marsic, D., Whitman, W.B., Tang, J. & Krader, P. (2003). *Spirochaeta americana* sp. nov., a new haloalkaliphilic, obligately anaerobic spirochaete isolated from soda Mono Lake in California. *Int J Syst Evol Microbiol* **53**, 815-821.

Jolivet, E., L'haridon, S., Corre, E., Forterre, P. & Prieur, D. (2003). *Thermococcus gammatolerans* sp. nov., a hyperthermophilic archaeon from deep-sea hydrothermal vent that resists ionizing radiation. *Int J Syst Evol Microbiol* **53**, 847-851.

Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In Mammalian Protein Metabolism, vol 3, pp 21-132. Edited by H. N. Munro. New York. Academic Press.

Keller, M., Braun, F. -J., Dirmeier, R., Hafenbradl, D., Burggraf, S., Rachel, R. & Stetter, K.O. (1995). *Thermococcus alcaliphilus* sp.nov., a new hyperthermophilic archaeum growing on polysulfide at alkaline pH. *Arch Microbiol* **164**, 390-395.

Kobayashi, T., Kwak, Y. S., Akiba, T., Kudo, T. & Horikoshi, K. (1994). *Thermococcus profundus* sp. nov., a new hyperthermophilic archaeon isolated from a deep-sea hydrothermal vent. *Syst Appl Microbiol* **17**, 232-236.

Kumar, S., Tamura, K. & Nei, M. (2004). MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment, *Brief Bioinform* **5**, 150-163.

Kuwabara, T., Minaba, M., Iwayama, Y., Inouye, I., Nakashima, M., Marumo, K., Maruyama, A., Sugai, A., Itoh, T., Ishibashi, J., Urabe, T. & Kamekura, M. (2005). *Thermococcus coalescens* sp. nov., a cell-fusing hyperthermophilic archaeon from Suiyo Seamount. *Int J Syst Evol Microbiol* **55**, 2507-2514.

Lovley, D. R. & Phillips, E.J.P. (1986). Organic matter mineralization with reduction of ferric iron in anaerobic sediments. *Appl Environ Microbiology* **51**, 683-689.

Marteinsson, V. T., Birrien, J. -L., Reysenbach, A. -L., Vernet, M., Marie, D., Gambacorta, A., Messner, P., Sleytr, U. B. & Prieur, D. (1999). *Thermococcus barophilus* sp. nov., a new barophilic and hyperthermophilic archaeon isolated under hydrostatic pressure from a deep-sea hydrothermal vent. *Int J Syst Bacteriol* **49**, 351-359.

Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159-167.

Miroshnichenko, M. L., Bonch-Osmolovskaya, E. A. , Neuner, A., Kostrikina, N. A., Chernyh, N. A. & Alekseev, V. A. (1998). *Thermococcus stetteri* sp. nov., a new extremely thermophilic marine sulfur-metabolizing archaebacterium. *Appl Microbiol* **12**, 257-262.

Miroshnichenko, M. L., Gongadze, G. M., Rainey, F. A., Kostyukova, A. S., Lysenko, A. M., Chernyh, N. A. & Bonch-Osmolovskaya, E. A. (1998). *Thermococcus gorgonarius* sp. nov. and *Thermococcus pacificus* sp. nov.: heterotrophic extremely thermophilic archaea from New Zealand submarine hot vents. *Int J Syst Evol Microbiol* **48**, 23-29.

Miroshnichenko, M. L., Hippe, H., Stackebrandt E., Kostrikina, N. A., Chernyh, N. A., Jeanthon, C., Nazina, T. N., Belyaev, S. S. & Bonch-Osmolovskaya, E. A. (2001). Isolation and characterization of *Thermococcus sibiricus* sp. nov. from a Western Siberia high-temperature oil reservoir. *Extremophiles* **5,** 85-91.

Neuner, A., Jannasch H. W., Belkin S. & Stetter, K.O. (1990). *Thermococcus litoralis* sp. nov.: a new species of extremely thermophilic marine archaebacteria. *Arch Microbiol* **153**: 205-207.

Pikuta, E. V., Lysenko, A. M., Suzina, N, Osipov, G.A., Kuznetsov, B., Tourova. T., Akimenko, V. K. & Laurinavichus, K. S. (2000). *Desulfotomaculum alkaliphilum* sp. nov., a new alkaliphilic, moderately thermophilic, sulfate-reducing bacterium. *Int J Syst Evol Microbiol* **50**, 25-33.

Pikuta, E. V., Hoover, R. B., Bej, A. K., Marsic, D., Detkova, E. N., Whitman, W. B. & Krader, P. (2003). *Tindallia californiensis* sp. nov., a new anaerobic, haloalkaliphilic, spore-forming acetogen isolated from Mono Lake in California. *Extremophiles* **7**, 327-334.

Pikuta, E. V., Itoh, T., Krader, P., Tang, J., Whitman, W. B., and Hoover, R. B. (2006). *Anaerovirgula multivorans* gen. nov., sp. nov., a novel spore-forming, alkaliphilic anaerobe isolated from Owens Lake, California. *Int J Syst Evol Microbiol* **53**, 2623-2629.

Ronimus, R. S., Reysenbach, A. -L., Musgrave, D. R. & Morgan, H. W. (1997). The phylogenetic position of the *Thermococcus* isolate AN1 based on 16S rRNA gene sequence analysis: a proposal that AN1 represents a new species, *Thermococcus zilligii* sp. nov. *Arch Microbiol* **168**, 245-248.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406-425.

Sambrook, J., Fritch, E. F. & Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Slobodkin, A. I., Jeanthon, C., L'Haridon, S., Nazina, T., Miroshnichenko, M., & Bonch-Osmolovskaya, E. (1999). Dissimilatory reduction of Fe(III) by thermophilic bacteria and archaea in deep subsurface petroleum reservoirs of Western Siberia. *Curr Microbiol* **39,** 99-102.

Smilbert, R. M. & Krieg, N. R. (1994). Phenotypic characterization. In *Methods for general and molecular microbiology*, pp. 611-654. Edited by R. G. Gerhardt, Murray

E., Wood W. A., & Krieg N. R. American Society for Microbiology: Washington, D.C.

Stetter, K. O., Huber, R., Blochl, E., Kurr, M., Eden, R. D., Fielder, M., Cash, H. & Vance, I. (1993). Hyperthermophilic archaea are thriving in deep North Sea and Alaskan oil reservoirs. *Nature* **365**, 743-745.

Takahata, Y., Nishijima, M., Hoaki, T. & Maruyama, T. (2000). Distribution and physiological characteristics of hyperthermophiles in the Kubiki oil reservoir in Niigata, Japan. *Appl Environ Microbiol* **66**,73-79.

Thompson, J.D., Higgins, D.G. & Gibson, T.J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673-4680.

Trüper, H. G. & Schlegel, H. G. (1964). Sulfur metabolism in *Thiorhodaceae*: I. Quantitative Measurements on growing cells of *Chromatium okenii*. Antonie van Leeuwenhoek **30**, 225-238.

Wolin, E. A., Wolin, M. J., & Wolfe, R. S. (1963). Formation of methane by bacterial extracts, *J. Biol. Chem.* **238,** 2882-2886.

Zillig, W., Holz, I., Janekovic, D., Schäfer, W. & Reiter, W. D. (1983). The Archebacterium *Thermococcus celer* represents, a novel genus within the Thermophilic branch of the Archaebacteria. *Syst Appl Microbiol* **4**, 88-94.

Zillig, W. (1992). The order Thermococcales. In: *The Prokaryotes*. 2nd edn., Vol 1, pp. 702-706. Edited by A. Balows, H. Truper, M. Dworkin, W. Hander, & K.H. Schleifer. New York: Springer-Verlag Inc.

Zillig, W. & Reysenbach A. –L. (2002). Class IV. Thermococci *class nov*. In Bergey's Manual of Systematic Bacteriology, 2nd edn, vol. 1. pp. 342-346. Edited by D. R. Boone & R. W. Castenholz: New York: Springer.

FIGURES

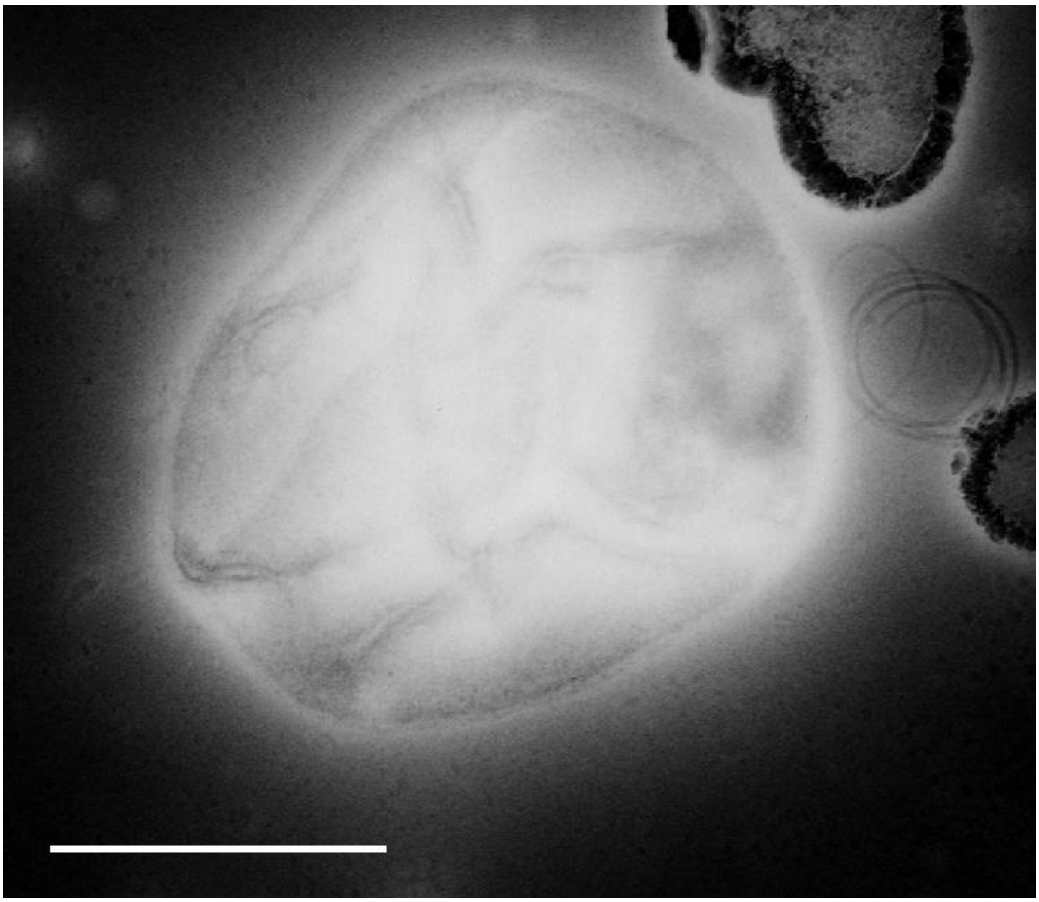

Fig. 1. TEM image by negative staining of cell of strain OGL-20P^T with single coiled flagellum. Bar = 0.5 μm.

Fig. 2. Unrooted phylogenetic tree indicating the position of strain OGL-20P^T among closest taxa. Numbers at the nodes are bootstrap values obtained from 1000 replicates. Nucleotide sequence accession numbers are shown in parentheses. Scale bar indicates 1 inferred nucleotide substitution per 1000 nucleotides.

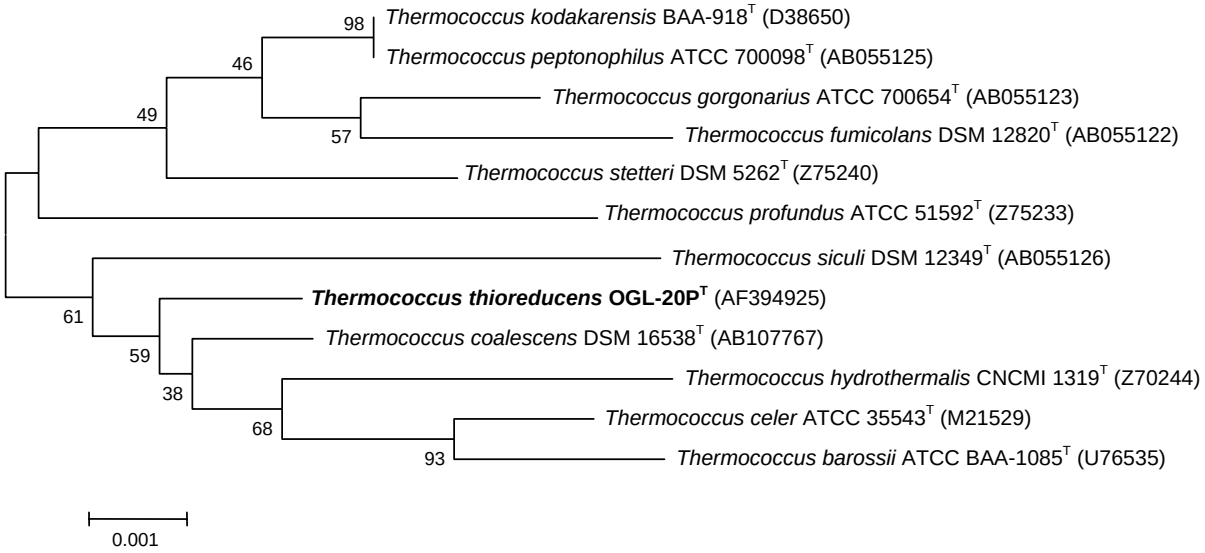

Table 1. Comparative table for strain OGL-20P^T and phylogenetically closest species.

Species: / Features:	*T. thioreducens* OGL-20P^T (present work)	*T. coalescens* JCM 12540^T (Kuwabara *et al.*, 2005)	*T. celer* ATCC 35543^T (Zillig *et al.*,1983)	*T. barossii* DSM 9535^T (Duffaud *et al.*,1998)	*T. hydrothermalis* AL662^T (Godfroy *et al.*,1997)	*T. kodakaraensis* ATCC BAA-918^T (Atomi *et al.*, 2004)	*T. profundus* ATCC51592^T (Kobayashi *et al.*, 1994)	*T. acidaminovorans* DSM 11906^T (Dirmeier *et al.*, 1998)	*T. stetteri* DSM 5262^T (Miroshnichenko *et al.*, 1989)	*T. peptonophilus* ATCC 700098^T (Gonzalez *et al.*, 1995)	*T. gorgonarius* ATCC 700654^T (Miroshnichenko *et al.*, 1998)
G+C, mol%	52.9	53.9	56.6	60.0	58.0	52	52.5	49	50	52	50.6
Motility/ Flagellation	motile/ monotrichous	motile/tuff of flagella	motile/polar polytrichous	non-motile	motile/ monotrichous	motile/ polar tuft of flagella	motile/polar tuft of flagella	motile/tuft of flagella	non-motile	motile/	motile/polar flagella
S^o requirement	obligate	-$^{\$}$	facultative	obligate	facultative	facultative	obligate	facultative	obligate	facultative	obligate
pH opt. (range)	7.0 (5.0-8.5)	6.5 (5.2-8.7)	5.8	6.5-7.5 (3-9)	6.0	6.5 (5-9)	4.5-8.5	9.0 (5.0-9.5)	6.5 (5.7-7.2)	6 (4-8)	6.5-7.2 (5.8-8.5)
NaCl, % opt.(range)	3.0 (1.0-5.0)	2.5 (1.5-4.5)	3.8-4.0 (1-4)	2.0 (1.0-4.0)	2-3	3 (1-5)	2-4 (1-6)	2-3 (1-6)	2.5 (1-4)	3 (1-5)	2.0-3.5 (1-5)
T, °C opt. (range)	83-85 (55-95)	87 (57-90)	88 (50-94)	82.5 (60-94)	85 (55-100)	85 (60-100)	80 (50-90)	85 (56-93)	76 (55-94)	85-90 (60-100)	80-88 (68-95)
Substrates:*											
Proteolysis products**	+	+	+	+	+	+	+	+	+	+	+
Starch	-	-	ND	ND	-	+	+	-	+	ND	-
Pyruvate	-	ND	ND	ND	(+)	+	+	ND	ND	-	(+)
End products:											
H_2	+	ND	+	+	+	+	+	ND	ND	-	+
CO	+	ND	-	+	-	ND	+	ND	ND	+	+
CO_2	+	ND	+	+	+	ND	+	+	+	+	+
Acetate	+	ND	ND	ND	+	ND	ND	ND	+	ND	+
Lactate	-	ND	ND	ND	+	ND	ND	ND	ND	ND	ND
***Iso*-butyrate**	-	ND	ND	ND	+	ND	ND	ND	+	ND	+
Propionate	-	ND	ND	ND	-***	ND	ND	ND	-	ND	+
***Methyl*-propionate**	-	ND	ND	ND	+	ND	ND	ND	-	ND	-
***Iso*-valerate**	-	ND	ND	ND	-	ND	ND	ND	+	ND	+
t_D, min	30	ND	50	33	90	ND	50	120	72	25	ND
Genome size, Dalton	1.23 x 10^9	ND	1.24 x 10^9	1.18 x 10^9	ND	ND	ND	ND	ND	ND	ND
Rifampin resistance	-	-	+	-	+	ND	-	ND	-	-	-

"+" - positive test; "-" – negative test; "(+)" – weak growth; ND – no data. Genome size was detected as described previously (Hoover *et al*., 2003). $^{\$}$ - stimulatory only.
* - all strains hydrolize casein & starch, but not chitin; **- hydrolysed proteins or peptides (peptone, yeast extract, meat extract etc.); ***- produces *2-methyl*-propionate, *3-methyl-thio*-propanoate & propanoate.