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

Elena V. Pikuta,1 Damien Marsic,2 Takashi Itoh,3 Asim K. Bej,4 Jane Tang,5 William B. Whitman,6 Joseph D. Ng,2 Owen K. Garriott7 and Richard B. Hoover1

Correspondence
Elena V. Pikuta
elenapikuta@hotmail.com
1Astrobiology Laboratory, NASA/NSSTC, VP62, 320 Sparkman Dr., Huntsville, AL 35805, USA
2Laboratory for Structural Biology, Department of Biological Sciences, The University of Alabama in Huntsville, MSB 221, Huntsville, AL 35899, USA
3Japan Collection of Microorganisms, RIKEN BioResource Center, 2-1 Hirosawa, Wako-shi, Saitama 351-0198, Japan
4Department of Biology, University of Alabama at Birmingham, Birmingham, AL 35294, USA
5United States Department of Agriculture, Monitoring Programs Office, 8609 Sudley Rd., Suite 206, Manassas, VA 20110, USA
6Department of Microbiology, University of Georgia, Athens, GA 30602-2605, USA
7Department of Biological Sciences, UAH, Huntsville, AL 35899, USA

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°N, 33.9°W). The cells of strain OGL-20P T have an irregular coccoid shape and are motile with a single flagellum. Growth was observed within a pH range of 5.0–8.5 (optimum pH 7.0), an NaCl concentration range of 1–5 % (w/v) (optimum 3 %) and a temperature range of 55–94 °C (optimum 83–85 °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, Fe(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 gentamicin, but sensitive to tetracycline and rifampicin. The G + C content of the 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, for which we propose the name Thermococcus thioreducens sp. nov. The type strain is OGL-20P T (≡ JCM 12859 T = DSM 14981 T = ATCC BAA-394 T).

The genus Thermococcus was created in 1983, and currently 26 species have been validly published. All members of this genus are characterized by a thermophilic nature, anaerobiosis with sulfur-type respiration and sometimes sulfur stimulation for fermentation (Zillig, 1992; Zillig & Reysenbach, 2002). The typical ecological systems for the habitat of Thermococcus species include geothermal springs (volcanic fumaroles, geysers and deep-sea hydrothermal vents), deep subsurface biosphere, such as deep crustal rocks, and aquifers and high-temperature oil wells (Stetter et al., 1993; Takahata et al., 2000; Miroshnichenko et al., 2001). Most species of the genus are marine and have an optimum NaCl concentration for growth of about 3 % (w/v), but there are also freshwater species, e.g. Thermococcus zilligii (Ronimus et al., 1997) and Thermococcus waiotapuensis (González et al., 1999). Most grow optimally at neutral or slightly acidic pH, but only Thermococcus alcaliphilus is capable of growth at pH 10.5 with an optimum around 9.0 (Keller et al., 1995). Most species

Abbreviation: TEM, transmission electron microscopy.

The GenBank accession number for the 16S rRNA gene sequence of strain OGL-20P T is AF394925.
have a minimum temperature for growth around 50 °C and the maximum around 95–100 °C, e.g. Thermococcus celer, Thermococcus litoralis and Thermococcus fumicolans (Zillig et al., 1983; Neuner et al., 1990; Godfroy et al., 1996). Many species have been isolated from deep-sea hydrothermal vents with environmental pressures in excess of 200 atm. Obligate dependence upon pressure at 95–100 °C has been determined for Thermococcus barophilus (Martineisson et al., 1999). The most radio-resistant hyperthermophilic archaeon, Thermococcus gammatolerans, is capable of surviving 30 kGy γ-ray irradiation (Jolivet et al., 2003). Most species of the genus 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 2300 m depth in the Rainbow hydrothermal vent field (36.2°N, 33.9°W) about 800 km south-west 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 recovery of the submersible from the water, the samples were hermetically sealed in sterile vessels with screw caps and maintained at 4 °C in an insulated cooler during transport to the Astrobiology Laboratory of the NASA Marshall Space Flight Center, Huntsville, AL, USA. Strain OGL-20PT was isolated from a sample of black-coloured, 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 liquid medium under a highly purified 100 % nitrogen atmosphere. The basal medium contained (g l−1): KH2PO4, 0.3; MgCl2.6H2O, 0.1; KCl, 0.3; NH4Cl, 1.0; NaHCO3, 0.2; CaSO4.7H2O, 0.005; NaCl, 30.0; Na2S.9H2O, 0.4; yeast extract, 0.5; sulfur powder, 10.0; peptone, 5.0; resazurin, 0.001. The medium was supplemented with 2 ml vitamin solution (Wolin et al., 1963) and 1 ml trace element solution as described previously (Pikuta et al., 2000). The final pH of the medium after autoclaving was 7.2–7.4 at 22 °C.

Unless otherwise noted, enrichment and pure cultures were grown in 10 ml medium in Hungate tubes under 100 % N2 (1 atm). All transfers and samplings of cultures were performed with sterile syringes. The medium was sterilized at 121 °C for 60 min and, after adding sulfur to the tubes under flow of 100 % N2, an additional sterilization was performed at 110 °C for 30 min. All incubations for physiological tests were carried out at 83 °C. One half gram of sample L-20 was injected into the medium and incubated for 24 h. A pure culture of strain OGL-20PT was obtained after repeated serial dilutions. A culture with monotypic morphology on the 10−9 dilution plate was chosen for roll-tube serial dilution purification. Growth of colonies occurred after 2–3 days incubation on 3 % (w/v) Difco agar with sulfur powder in Hungate tubes at 70 °C. One colony from the 10−8 dilution roll tube was chosen for subsequent purification and was designated strain OGL-20PT. The colonies of strain OGL-20PT on the surface of the agar were whitish-cream in colour, glossy and shining, with a round shape (~1.5 mm diam.) and irregular cleaved edges, and convex with a denser raised conic centre. In deep agar, colonies had a convex/convex lenticular shape.

Phase-contrast microscopy revealed the cells of strain OGL-20PT to be irregular, motile cocci with a diameter of 0.7–1.7 μm. Occasionally, some of the cells appeared as diplococci or conglomerates of 10–15 cells. Transmission electron microscopy (TEM) was carried out using a JEOL TEM 100 CX II operating at 80 kV. Negative staining was performed using a uranyl acetate procedure as described previously (Pikuta et al., 2003). TEM images showed the presence of a single flagellum (Fig. 1).

Culture growth was measured by direct cell counting under a phase-contrast microscope (Fisher Micromaster) by measuring sulfide produced from sulfur (Trüper & Schlegel, 1964) or by estimating the increase in optical density at 595 nm (Genesys 5; Spectronic Instruments). The pH of the medium was adjusted to defined values with sterile stock solutions of 6 M HCl or 6 M NaOH under a flow of N2 and measured using a pH meter (model 230 Aplus; Orion) calibrated at 22 °C. All measurements were performed after cooling the culture samples to room temperature. The temperature range for growth was determined in liquid medium at pH 7.3. The effect of NaCl concentration on growth was determined in liquid medium containing 0.0, 0.5, 1.0, 2.0, 3.0 and 4.0 % w/v NaCl. Cultures were incubated for 1 week at 83 °C. Growth was visually determined by an increase in turbidity of the medium after being counted in a hemacytometer. Growth was also measured by estimating the increase in optical density at 595 nm (Genesys 5; Spectronic Instruments). The pH of the medium was maintained at 7.3.

![Fig. 1. TEM image of a negatively stained cell of strain OGL-20PT with a single coiled flagellum. Bar, 0.5 μm.](http://www.microbiologyresearch.org/1613)
Strain OGL-20P\textsuperscript{T} was found to be strictly anaerobic. Catalase activity, which was tested as described by Smibert & Krieg (1994), showed a negative reaction. The utilization of various electron acceptors was studied in a medium containing peptone (5 g l\textsuperscript{-1}) as electron donor. Electron acceptors were added in the form of autoclaved or filter-sterilized stock solutions. The final concentrations of electron acceptors were as follows (mM): Na\textsubscript{2}SO\textsubscript{4}, 20; Na\textsubscript{2}SO\textsubscript{3}, 5; Na\textsubscript{2}S\textsubscript{2}O\textsubscript{3}, 10; NaNO\textsubscript{3}, 10; Fe(OH)\textsubscript{3}, 100; S\textsubscript{0}, 300. An amorphous FeOOH suspension (iron gel) was prepared by neutralizing a 0.4 M solution of FeCl\textsubscript{3} to pH 7 by using 10 M NaOH as described by 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 strain OGL-20P\textsuperscript{T} to utilize various substrates was tested by using liquid medium supplemented with autoclaved or filter-sterilized substrates at a final concentration of 5 g l\textsuperscript{-1}. Substrate utilization was tested by cultivating strain OGL-20P\textsuperscript{T} for 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, bacitryptone, 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).

Metabolic end products in the liquid phase were determined by HPLC. Separation was done on an Aminex HPX-87H (Bio-Rad) column with 5 mM H\textsubscript{2}SO\textsubscript{4} as the mobile phase. Gases were measured with a gas chromatograph 3700 (Varian) equipped with a Porapak Q column and a 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 (> 20 mM) and traces of hydrogen and CO\textsubscript{2} were measured in the gas phase during growth of OGL-20P\textsuperscript{T}.

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

Genomic DNA was isolated by 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 G + C content of the genomic DNA of strain OGL-20P\textsuperscript{T} was 57.2 ± 0.2 mol% (mean of two determinations for each of two degradations of the DNA ±SD, n = 4).

The 16S rRNA gene of strain OGL-20P\textsuperscript{T}, along with part of the 23S rRNA gene and the spacer region, was selectively amplified with the following primers: 5′-TCCGGTTGAT-CCTGCCGG-3′ (forward) and 5′-CTTTTCCTGCGGTAGCTAAAG-3′ (reverse). PCR was performed with 30 pmol each primer in a 50 µl volume, using 2 U ThermalAce DNA polymerase (Invitrogen) in the buffer provided. The thermal cycling profile was as follows: 3 min at 95 ºC initial denaturation, followed by 30 cycles of 45 s denaturation at 95 ºC, 45 s annealing at 57 ºC and 4 min extension at 72 ºC, with a final extension step at 72 ºC for 15 min. The amplified fragment was extracted from a 1.5 % agarose gel using the Qiaquick extraction kit (Qiagen), and then subcloned using the Zero Blunt TOPO PCR Cloning kit (Invitrogen). Six clones were sequenced in both directions using the Dye Terminator AmpliTaq FS Cycle Sequencing kit (Applied Biosystems) with both vector-based primers and primers specific to the 16S internal sequence (designed by ourselves).

The 16S rRNA sequence of strain OGL-20P\textsuperscript{T} was aligned with closely related sequences found in GenBank after a BLAST search (Altschul et al., 1990), using CLUSTAL W (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 neighbour-joining method (Saitou & Nei, 1987).

A sequence covering 1885 nt, including most (1452 nt) of the 16S rRNA gene, the rRNA\textsuperscript{Ala} gene and part of the 23S rRNA gene, was obtained after amplification of strain OGL-20P\textsuperscript{T} DNA. The 16S rRNA gene sequence corresponded to positions 37–1496 of the Pyrococcus furiosus 16S rRNA sequence (accession no. U20163) used as a reference. A BLAST search against the GenBank database revealed a high level of similarity (> 97 %) with sequences from other Thermococcus species. A phylogenetic dendrogram showing the relationship of strain OGL-20P\textsuperscript{T} to the 11 closest species.
was constructed, based on 1400 common nucleotide sites (Fig. 2). Pairwise distances between the OGL-20PT sequence and its closest neighbours were 0.003, 0.006, 0.006 and 0.007 for Thermococcus coalescens, Thermococcus celer, Thermococcus hydrothermalis and Thermococcus barossii, respectively, based on the same 1400 nucleotide sites.

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 labelled DNA from strain OGL-20PT were as follows: Thermococcus celer JCM 8558T, 14%; Thermococcus barossii ATCC BAA-1085T, 17%; Thermococcus hydrothermalis AL662T, 16%; Thermococcus kodakarenensis ATCC BAA-918T, 5%; Thermococcus profundus ATCC 51592T, 4%; Thermococcus acidaminovorans DSM 11906T, 5%; Thermococcus stetteri DSM 5262T, 4%; Thermococcus peptonophilus ATCC 700098T, 5%; Thermococcus gorgonarius ATCC 700654T, 5%; Thermococcus coalescens JCM 12540T, 13%; and ‘Thermococcus radiotolerans’ JCM 11826, 18%.

Almost half of the known Thermococcus species have been isolated from deep-sea hydrothermal vents with high pressure conditions (200–350 atm) located in different parts of the world (Kobayashi et al., 1994; Huber et al., 1995; Godfroy et al., 1996, 1997; Canganella et al., 1998; Duffaud et al., 1998; Grote et al., 1999). Strain OGL-20PT was also isolated from a deep-sea ecosystem, characterized by high pressure (230 atm), high temperatures (300 to 400 °C within black smoker vents) and very high thermal gradients, (temperature drops to 2 °C a few centimetres away from the chimney). As the hydrothermal fluid condenses above the vent, the precipitated minerals are also spread around the nearby ocean floor; black pyrites (FeS) surrounding black smokers is a result of the interaction of sulfide with iron and the orange colour is a result of Fe³⁺. Since it is a mantellic rather than a basaltic substrate, the fluid chemistry of the Rainbow site is completely original as fluids are depleted in sulfide but enriched in both methane and hydrogen (Charlou et al., 2002). Unpigmented invertibrates (shrimps, crabs and worms) represent multicellular organisms in the ecosystem; their energy source is partially provided by the metabolism of micro-organisms (in our laboratory, cells with a morphology similar to the new archaeon were found in shrimps intestines that had a strong smell of sulfur).

Strain OGL-20PT is a hyperthermophilic, heterotrophic, sulfur-dependent, cocoid 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 Thermococcales, and most closely related to the genus Thermococcus. Currently, the genus Thermococcus contains 26 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 DNA base composition. Strain OGL-20PT is included in the clade represented by the type species of the genus T. celer. Comparison of strain OGL-20PT with its closest neighbours on the phylogenetic tree showed a 16S rRNA sequence difference of less than 1%; however, DNA–DNA hybridization showed less than 20% similarity. Phenotypic and genotypic differences between strain OGL-20PT and the closest species are shown in Table 1.

On the basis of comparative morphological, physiological and genomic data, we conclude that strain OGL-20PT...
Table 1. Comparison of characters of strain OGL-20PT\(^T\) and its phylogenetically closest species

+ Positive test; −, negative test; (+), weak growth; ND, no data. Genome size was detected as described previously (Hoover et al., 2003). 1, T. thioreducers OGL-20PT (this study); 2, T. coalescens JCM 12540T (Kuwabara et al., 2005); 3, T. celer ATCC 35543T (Zillig et al., 1983); 4, T. barossii DSM 9535T (Duffaud et al., 1998); 5, T. hydrothermalis AL662T (Godfroy et al., 1997); 6, T. kodakarensis ATCC BAA-918T (Atomi et al., 2004); 7, T. profundus ATCC 35543T (Kobayashi et al., 1994); 8, T. acidaminovorans DSM 11906T (Dirmeier et al., 1998); 9, T. stetteri DSM 5262T (Miroshnichenko et al., 1989); 10, T. peptonophilus ATCC 700098T (Gonza´lez et al., 1995); 11, T. gorgonarius ATCC 700654T (Miroshnichenko et al., 1998).

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<td>1.23 × 10(^9)</td>
<td>ND</td>
<td>1.24 × 10(^9)</td>
<td>1.18 × 10(^9)</td>
<td>ND</td>
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<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Rifampicin resistance</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>−</td>
<td>ND</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

\(^*\)All strains hydrolize casein, but not chitin.

\(†\)Hydrolysed proteins or peptides (peptone, yeast extract, meat extract, etc.).

\(§\)Produces 2-methylpropionate, 3-methylthiopropionate.

\(§\)Stimulatory only.
represents a separate taxon at the species level for which the name Thermococcus thioreducens sp. nov. is proposed.

**Description of Thermococcus thioreducens sp. nov.**


Cells are irregular cocci with a diameter of 0.7–1.8 μm, motile by a single flagellum. Heterotrophic, strictly anaerobic. Obligately dependent upon elemental sulfur. Catalase-negative. Grows with peptone, bacto-tryptone, Casamino acids and yeast extract as electron donors, but no growth on D-glucose, fructose, maltose, sucrose, D-mannitol, glycerol, methanol, ethanol, butyrate, propionate, acetate, formate, lactate, pyruvate, citrate or amino acids. Thiosulfate, sulfate, sulfite, Fe(II) and nitrate cannot support growth as electron acceptors. Cells are hyperthermophilic, growing between 55 and 94 °C with an optimum at 83–85 °C, and in a pH range of 5.0–8.5 (optimum 7.0) with an NaCl concentration range of 1–5 % (w/v) (optimum 3 %). Doubling time is 30 min. The main end product of growth with peptone and sulfur is H₂S (more than 20 mM); minor end products are CO₂, H₂ (0.05 mM), acetate (2 mM) and ethanol (3.7 mM). Sensitive to tetracycline and rifampicin. The G+C content of the DNA is 52.9 mol% (HPLC).

Type strain is strain OGL-20PT (=JCM 12859T =DSM 14981T =ATCC BAA-394T) isolated from deep-sea black smoker chimney debris in sediment mud at the Rainbow hydrothermal vent site at a depth of 2300 m in the Atlantic Ocean off the coast of the Azores.

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