Thermococcus thioeducens 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°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 thioeducens* 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* (Marteinsson *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 thioeducens* 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-20P^T 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-20P^T 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-20P^T. The colonies of strain OGL-20P^T 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-20P^T to be irregular, motile cocci with a diameter of 0.7–1.7 μ m. 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 (Genesis 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,

Fig. 1. TEM image of a negatively stained cell of strain OGL-20P^T with a single coiled flagellum. Bar, 0.5 μ m.

3.0, 5.0, 7.0 or 10.0 % (w/v) NaCl. The NaCl requirement was studied using a modified medium in which NaHCO₃ was replaced with K₂CO₃, and Na₂S was replaced with K₂S. Growth of strain OGL-20P^T was observed in the temperature range 55–95 °C, with an optimum between 83 and 85 °C. It survived for 30 min at 101 °C, but incubation at 103 °C for 2 h killed the cells. Growth of strain OGL-20P^T was observed within the pH range 5.0–8.5, with an optimum pH of 7.0. It also grew within an NaCl concentration range of 1–5 % with an optimum of 3 %. No growth was observed for NaCl concentrations below 0.5 or above 7 %. The doubling time, measured by direct cell counting under a phase-contrast microscope, for a fresh culture of OGL-20P^T incubated under optimal conditions was 30 min.

Strain OGL-20P^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⁻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): Na₂SO₄, 20; Na₂SO₃, 5; Na₂S₂O₃·H₂O, 10; NaNO₃, 10; Fe(OH)₃, 100; S⁰, 300. An amorphous FeOOH suspension (iron gel) was prepared by neutralizing a 0.4 M solution of FeCl₃ 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^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⁻¹. Substrate utilization was tested by cultivating strain OGL-20P^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, 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).

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 H₂SO₄ 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 CO₂ were measured in the gas phase during growth of OGL-20P^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⁻¹ (chloramphenicol, rifampicin) or 250 µg ml⁻¹ (ampicillin, tetracycline, kanamycin and gentamicin). Before incubation at 83 °C, antibiotic-containing cultures were pre-incubated at 37 °C for 12 h. Strain OGL-20P^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^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^T, along with part of the 23S rRNA gene and the spacer region, was selectively amplified with the following primers: 5'-TCCGGTTGATCCTGCCGG-3' (forward) and 5'-CTTTCCTGCGGGTACTAAG-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 °C initial denaturation, followed by 30 cycles of 45 s denaturation at 95 °C, 45 s annealing at 57 °C and 4 min extension at 72 °C, with a final extension step at 72 °C 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^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 tRNA^{Ala} gene and part of the 23S rRNA gene, was obtained after amplification of strain OGL-20P^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^T to the 11 closest species

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 nt.

was constructed, based on 1400 common nucleotide sites (Fig. 2). Pairwise distances between the OGL-20P^T 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-20P^T were as follows: *Thermococcus celer* JCM 8558^T, 14 %; *Thermococcus barossii* ATCC BAA-1085^T, 17 %; *Thermococcus hydrothermalis* AL662^T, 16 %; *Thermococcus kodakarensis* ATCC BAA-918^T, 5 %; *Thermococcus profundus* ATCC 51592^T, 4 %; *Thermococcus acidaminovorans* DSM 11906^T, 5 %; *Thermococcus stetteri* DSM 5262^T, 4 %; *Thermococcus peptonophilus* ATCC 700098^T, 5 %; *Thermococcus gorgonarius* ATCC 700654^T, 5 %; *Thermococcus coalescens* JCM 12540^T, 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-20P^T was also isolated from a deep-sea ecosystem, characterized by high pressure (230 atm), localized 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 invertebrates (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-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 *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-20P^T is included in the clade represented by the type species of the genus *T. celer*. Comparison of strain OGL-20P^T 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-20P^T and the closest species are shown in Table 1.

On the basis of comparative morphological, physiological and genomic data, we conclude that strain OGL-20P^T

Table 1. Comparison of characters of strain OGL-20P^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. thioeducens* OGL-20P^T (this study); 2, *T. coalescens* JCM 12540^T (Kuwabara *et al.*, 2005); 3, *T. celer* ATCC 35543^T (Zillig *et al.*, 1983); 4, *T. barossii* DSM 9535^T (Duffaud *et al.*, 1998); 5, *T. hydrothermalis* AL662^T (Godfroy *et al.*, 1997); 6, *T. kodakarensis* ATCC BAA-918^T (Atomi *et al.*, 2004); 7, *T. profundus* ATCC 51592^T (Kobayashi *et al.*, 1994); 8, *T. acidaminovorans* DSM 11906^T (Dirmeier *et al.*, 1998); 9, *T. stetteri* DSM 5262^T (Miroshnichenko *et al.*, 1989); 10, *T. peptonophilus* ATCC 700098^T (González *et al.*, 1995); 11, *T. gorgonarius* ATCC 700654^T (Miroshnichenko *et al.*, 1998).

Character	1	2	3	4	5	6	7	8	9	10	11
G + C content (mol%)	52.9	53.9	56.6	60.0	58.0	52	52.5	49	50	52	50.6
Motility/flagellation	Motile/ monotrichous	Motile/tuft 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 ⁰ requirement	Obligate	−§	Facultative	Obligate	Facultative	Facultative	Obligate	Facultative	Obligate	Facultative	Obligate
Optimum pH (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)
Optimum NaCl, % (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)
Optimum T, °C (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	+
Methylpropionate	−	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, Da	1.23 × 10 ⁹	ND	1.24 × 10 ⁹	1.18 × 10 ⁹	ND	ND	ND	ND	ND	ND	ND
Rifampicin resistance	−	−	+	−	+	ND	−	ND	−	−	−

*All strains hydrolyze 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 thioeducens* sp. nov. is proposed.

Description of *Thermococcus thioeducens* sp. nov.

Thermococcus thioeducens (thi.o.re.du'cens. Gr. n. *thion* sulfur, L. part. adj. *reducens* reducing, N.L. part. adj. *thioeducens* reducing sulfur).

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, sulfite, sulfate, Fe(III) 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 a 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-20P^T (=JCM 12859^T=DSM 14981^T=ATCC BAA-394^T) 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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