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Thermococcus eurythermalis sp. nov., a conditional piezophilic, hyperthermophilic archaeon with a wide temperature range for growth, isolated from an oil-immersed chimney in the Guaymas Basin

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A conditional piezophilic, hyperthermophilic archaeon showing growth over a wide range of temperature, pH and pressure was isolated from an oil-immersed hydrothermal chimney at a depth of 2006.9 m in the Guaymas Basin. Enrichment and isolation of strain A501^T were performed at 80 °C at 0.1 MPa. Cells of isolate A501^T were irregular motile cocci with a polar tuft of flagella and generally 0.6–2.6 μm in diameter. Growth was detected over the range 50–100 °C (optimal growth at 85 °C) at atmospheric pressure and was observed at 102 °C at a pressure of 10 MPa. At 85 °C, growth was observed at a pressure of 0.1–70 MPa (optimum pressure 0.1 MPa–30 MPa), while at 95 °C, the pressure allowing growth ranged from 0.1 MPa to 50 MPa (optimum pressure 10 MPa). Cells of strain A501^T grew at pH 4–9 (optimum pH 7.0) and a NaCl concentration of 1.0–5.0 % (w/v) (optimum concentration 2.5 % NaCl). This isolate was an anaerobic chemo-organoheterotroph and was able to utilize yeast extract, peptone, tryptone and starch as the single carbon source for growth. Elemental sulfur and cysteine stimulated growth; however, these molecules were not necessary. The DNA G+C content of the complete genome was 53.47 mol%. The results of 16S rRNA gene sequence analysis indicated that strain A501^T belongs to the genus *Thermococcus*. There was no significant similarity between strain A501^T and the phylogenetically related species of the genus *Thermococcus* based on complete genome sequence alignments and calculation of the average nucleotide identity and the tetranucleotide signature frequency correlation coefficient. These results indicate that strain A501^T represents a novel species, *Thermococcus eurythermalis* sp. nov. The type strain is A501^T (=CGMCC 7834^T=JCM 30233^T).

A deep-sea hydrothermal vent is a likely environment for the discovery of extremophiles (Jørgensen & Boetius, 2007) and is ideal for studying multiple extreme adaptations of micro-organisms because of the unique physical conditions, including the combination of high temperature, high hydrostatic pressure, extreme pH, reducing power, toxic chemistry and steep physical-chemical gradients (Gorlas *et al.*, 2013). Many studies on the microbial diversity of the deep-sea hydrothermal vent ecosystem have shown that the euryarchaea group is abundant, including the often-cultured members of the order *Thermococcales* (Takai *et al.*, 2006). Moreover, species of the order *Thermococcales* are relatively abundant and a great diversity of species including thermophiles and hyperthermophiles have been isolated from various geothermally heated systems (Prieur, 2002). The order *Thermococcales* represents an archaeal order that contains only one family, *Thermococcaceae.* Three genera, *Thermococcus* (Zillig *et al.*, 1983), *Pyrococcus* (Fiala & Stetter, 1986) and *Palaeococcus* (Takai *et al.*, 2000), belong to this family. Members of the genus *Thermococcus* are organotrophic and anaerobic hyperthermophiles. Elemental sulfur is required for the growth of some species, and elemental sulfur stimulates growth significantly in other species. Twenty-nine species of the genus *Thermococcus* have been characterized to date according to the List of Prokaryotic Names with Standing in Nomenclature (LPSN; http://www.bacterio.net), and can be divided into two groups based on their respective DNA G+C content (Jolivet *et al.*, 2004); one group has a higher DNA G+C content of 50–60 %, and the other group has a lower DNA G+C content of 38–47 %. The temperature

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain A501^T is KJ616741. The accession numbers for the complete genome sequence of strain A501^T are CP008887 and CP008888.

Two supplementary figures and one supplementary table are available with the online Supplementary Material.

supporting growth of species of the genus *Thermococcus* ranges from 48 °C to greater than 100 °C, and the majority of species have a wide temperature range for growth of more than 30 °C. Compared with species of the genus *Pyrococcus*, the response to pressure in species of the genus *Thermococcus* is not well reported. *Thermococcus barophilus* was the first true piezophilic, hyperthermophilic archaeon described within the order *Thermococcales* (Marteinsson *et al.*, 1999).

In this study, strain A501^T was isolated from an oil-immersed chimney of a deep-sea hydrothermal vent in the Guaymas Basin and was characterized as a representative of a novel species of the genus *Thermococcus*. Strain A501^T was a conditional piezophilic, hyperthermophilic archaeon that could grow over wide ranges of temperature, pressure and pH.

Samples from the spire of a chimney, white-covered with oily areas, were collected by the submersible *Alvin* from a hydrothermal vent site at a depth of 2006.9 m in the Guaymas Basin [Gulf of California (27° 0.405′ N 110° 24.567′ W)] during a cruise on 14 November 2009. The preliminary temperature inside the samples was 229 °C. The samples were immediately transferred into sterile sample tubes, sealed with a lid and covered with aluminium foil; the samples were preserved at 4 °C. Members of the order *Thermococcales* are among the dominant micro-organisms in these samples, as shown by meta-genomic data (He *et al.*, 2013).

In the laboratory, the samples were used for enrichment cultures. These samples were incubated anaerobically at 80 °C for 7 days in YPS medium (Jolivet *et al.*, 2003). Positive enrichments were obtained. A coccoid bacterial strain was purified by the dilution-to-extinction method in YPS medium at 80 °C. A single colony was isolated with a rolling tube containing 1.2–1.5 % Gelrite in YPS medium; single colonies were obtained after 3 days. A single colony was cultured in liquid YPS medium at 80 °C. Both the dilution-to extinction method and the rolling tube method were repeated three times. The purified strain was obtained and named A501^T. For long-term storage, cryotubes containing strain A501^T with 5 % (v/v) DMSO (Sigma) as the cryoprotectant were frozen at −80 °C or in liquid nitrogen. The YPS medium was then replaced with *Thermococcales* Rich Medium (TRM; Zeng *et al.*, 2009) for better growth. Further investigations were carried out using this medium.

Using a light microscope (Nikon Eclipse 90i), cells of strain A501^T were examined and observed to be highly motile, irregular cocci occurring singly, in pairs, in lines or in aggregates. Transmission electron microscopy (Tecnai G2spirit Biotwin) revealed that the cells were 0.6–2.6 μm in diameter and 1.5–2 μm in diameter under optimal growth conditions; a polar tuft of flagella was observed (Fig. 1).

Strictly anaerobic TRM medium was used to determine the growth parameters of strain A501^T. TRM (5 ml) in anaerobic tubes was used at atmospheric pressure with N_2 as the gas phase, and 5 ml syringes with TRM were used at high hydrostatic pressures. The medium was inoculated with late exponential-phase cells at approximately 10^6 cells ml^{-1}. All of the samples were tested in duplicate, and uninoculated medium was used as a negative control. Growth was measured by determining the cell number, which was counted by microscopy or by flow cytometry (Marteinsson *et al.*, 1999).

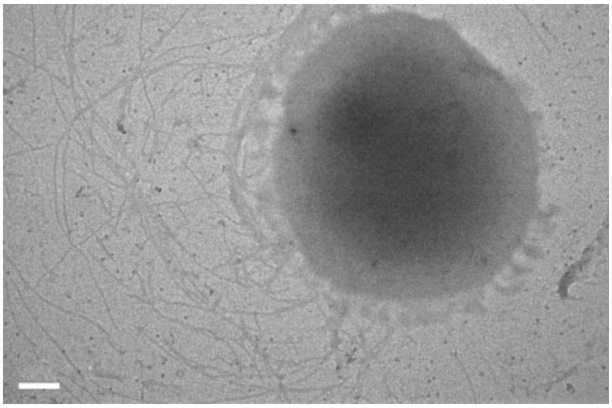

Fig. 1. Electron micrograph of a cell of strain A501^T with no staining. Bar, 200 nm.

Strain A501^T grew over the temperature range 50 °C to 100 °C at atmospheric pressure (Fig. S1a, available in the online Supplementary Material). Optimal growth occurred at 85 °C; no growth was observed at 102 °C. Cells lost the ability to divide after 12 h of cultivation at 102 °C, possibly because no growth occurred when the culture was inoculated back to fresh medium at 85 °C at atmospheric pressure. However, growth at 102 °C did occur at 10 MPa, and no growth was observed at 100 °C at the same pressure. At 10 MPa or 40 MPa, strain A501^T maintained the ability to divide, even at 112 °C, for at least 12 h; significant growth up to 10^8 cells ml^{-1} was observed after inoculating the culture back to fresh medium at 85 °C at atmospheric pressure. However, no growth occurred after inoculating a culture incubated at 115 °C for only 4 h back to fresh TRM at 85 °C and 0.1 MPa.

At 85 °C, growth was recorded over a pressure range from 0.1 MPa to 70 MPa (Fig. 2). The optimum pressure was 0.1–30 MPa because no significant difference was detected in the specific growth rate at these pressures. However, the growth yields at 10 MPa and 20 MPa were larger than those at 0.1 MPa and 30 MPa. No growth occurred at 80 MPa. At 95 °C, the pressure range narrowed to 0.1–50 MPa, and the optimum pressure was 10 MPa (Fig. 2). No growth occurred at 60 MPa. These results indicate that the isolate is a conditional piezophile.

The range of pH and NaCl concentration for growth were determined in TRM medium at 85 °C at atmospheric

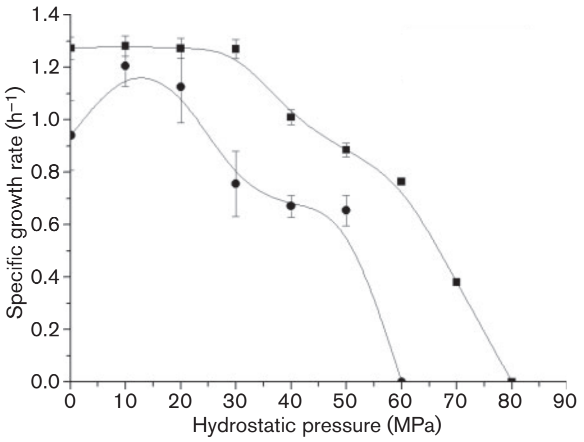

Fig. 2. Effects of the hydrostatic pressure and temperature on the specific growth rate of strain A501^T. Cells were grown in TRM medium at 85 °C (■) and 95 °C (●) at different hydrostatic pressures. The growth rates at and below 50 MPa were calculated by linear regression analysis along the exponential portion of the growth curves. For the operation of equipment used to test growth under higher pressures (>50 MPa), the growth rates were estimated as $\mu=(\ln N_2-\ln N_1)/(t_2-t_2)$, where N_2 and N_1 were the numbers of cells at the times (in h) t_2 and t_1, respectively. Error bars present the standard deviation shown when the results of more than two separate experiments were available.

pressure according to the method described by Marteinsson *et al.* (1999). Growth was detected from pH 4 to pH 9; optimal growth was observed at pH 7, and no growth was observed at pH 3.5 or pH 9.5 (Fig. S1b). Strain A501^T grew at concentrations of NaCl from 1.0 % to 5.0 % (w/v); optimal growth was observed at 2.5 % (w/v) NaCl, and no growth was observed at 0.5 % or 5.5 % (w/v) NaCl (Fig. S1c).

Under the conditions of optimal pH and NaCl concentration, 85 °C and atmospheric pressure, the doubling time of strain A501^T was approximately 48 min, and the growth yield reached greater than 10^8 cells ml^{-1}.

The medium used in the carbon source utilization tests was basal TRM (pH 7.0, no yeast extract or tryptone) with a single carbon source at a final concentration of 0.2 % (w/v) and supplemented with 0.002 % (w/v) yeast extract and elemental sulfur (10 g l^{-1}). The headspace gas in the tests was N_2. Single carbon sources used were as follows: yeast extract, peptone, tryptone, Casamino acids, glycogen, starch, citrate, lactate, acetate, formate, butyrate, propionate, pyruvate, succinate, cellobiose, trehalose, galactose, glucose, sucrose, fructose, cysteine, methionine, asparagine, histidine, lysine, tryptophan, tyrosine, phenylalanine and arginine.

All of the carbon source utilization tests were performed at 85 °C at atmospheric pressure and included inoculations with late exponential-phase cells (10^6 cells ml^{-1}). Growth was determined after 12 h and 24 h of incubation. For these tests, inoculated normal TRM was the positive control, and inoculated basal TRM was used as a negative control. No growth was observed in the inoculated basal TRM. All of the tests were performed in duplicate.

In tests of substrate utilization, yeast extract, tryptone and peptone supported rapid and significant growth of strain A501^T. Starch supported poor growth, with a maximum yield of only approximately 10^7 cells ml^{-1}. No obvious growth was observed with the other carbon sources under the test conditions.

Elemental sulfur and cysteine stimulated the growth of strain A501^T by three- to fourfold, although these molecules were not necessary for growth. Other sulfur substitutes, such as thiosulfate, polysulfide or methionine, could not stimulate growth.

Genomic DNA was prepared by the alkaline-SDS method. The entire genome of strain A501^T (unpublished) was sequenced by the paired-end strategy at Shenzhen Huada Genomics Institute (BGI; Shenzhen, China). Nearly 1340 Mb of reads was produced from a 500 bp paired-end library, and approximately 1013 Mb of reads was produced from a 5 kb paired-end library. The total of the reads covered the genome approximately 1105-fold. All of these reads were assembled by BGI, and 11 contigs (ranging from 3.5 kb to 543.3 kb) were generated. Gaps were closed and verified by PCR. The chromosome of strain A501^T was 2 122 535 bp and circular; the DNA G+C content was 53.47 mol%. A501^T possessed one 3.6 kb plasmid with 46.71 mol% DNA G+C content, which was considered as extra-chromosomal DNA.

The 16S rRNA genes (1488 bp) were predicted by the RNAmmer 1.2 Server on the basis of the entire genome sequence. BLAST results indicated that strain A501^T was a member of the genus *Thermococcus*. Similarity between A501^T and closely related species was greater than 98 %. The phylogenetic relationship based on 16S rRNA gene sequences of strain A501^T is shown in Fig. 3. The most closely related species are *Thermococcus kodakarensis* (99.1 % 16S rRNA gene sequence similarity) and *Thermococcus gammatolerans* (99.0 % similarity). Another phylogenetic tree based on the whole genome amino acid sequences was also reconstructed by using the CVTree tool (Xu & Hao, 2009), which is shown in Fig. S2.

Because of the high levels of similarity between species of the genus *Thermococcus*, quantitative DNA–DNA hybridizations were necessary. Because the two species most closely related to strain A501^T have complete genome sequences, in this study, quantitative DNA–DNA hybridizations were simulated by similarity analysis based on whole genome sequence alignments with the BLAST tool. The results of the alignments showed that the proportion of similar sequences between the genome sequence of strain A501^T and the other complete genome sequences of species of the genus *Thermococcus* available in GenBank was less than 50 %. The most similar species is *Thermococcus* sp. 4557, with 46 % coverage. *T. kodakarensis* KOD1 and *Thermococcus gammatolerans* EJ3, which represent the

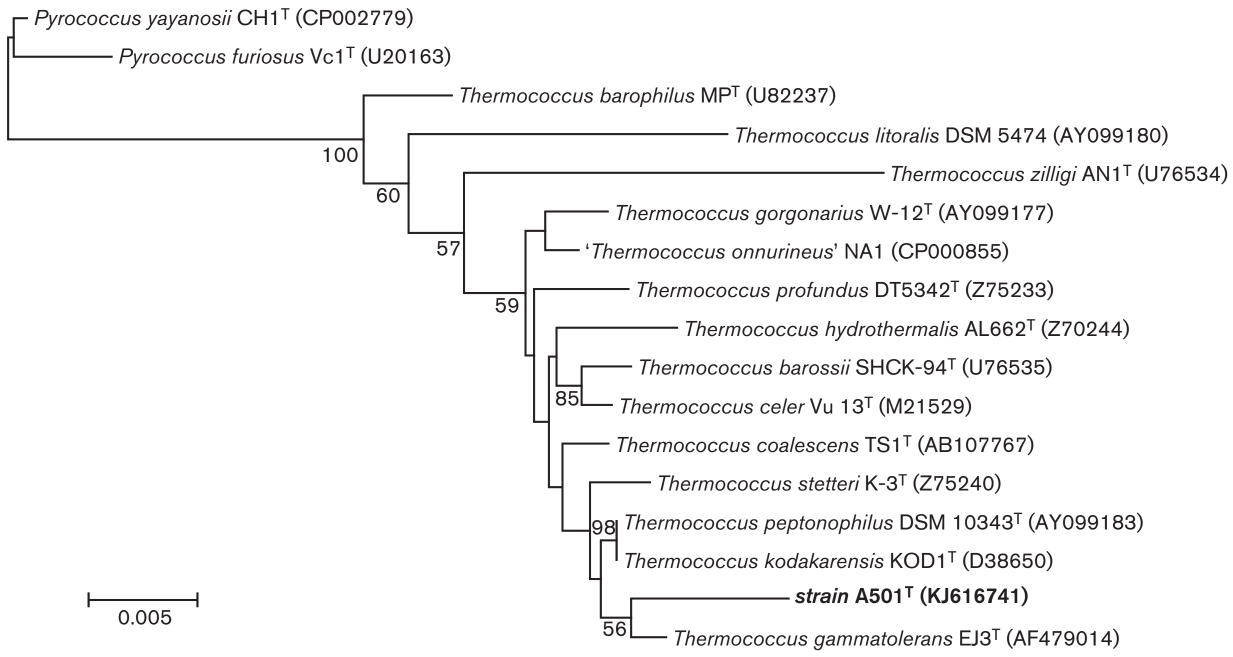

Fig. 3. Phylogenetic tree based on 16S rRNA sequences analysis (strain A501^T highlighted in bold type). The 16S rRNA gene sequences (1367 bp) of 15 species of the genus *Thermococcus* and two species of the genus *Pyrococcus* were aligned using the CLUSTAL W program (Thompson *et al.*, 1994). The distance matrix was calculated using the Kimura two-parameter method, and the tree was reconstructed using the neighbour-joining method (Saitou & Nei, 1987). Numbers at nodes indicate the absolute bootstrap values per 100 bootstraps performed; those greater than 50 % in 1000 replicates are shown. Bar, 0.005 substitutions per sequence position. GenBank accession numbers are given in parentheses.

most closely related species based on 16S rRNA gene sequences, both have 43 % similar sequences with an identity of 87 % and 90 %, respectively. For '*Thermococcus onnurineus*', *Thermococcus litoralis* and *T. barophilus*, the proportion of similar sequence in the genomes of their type strains was 33 %, 7 % and 5 %, respectively. All of the coverage values were below the threshold of 70 % DNA–DNA hybridization, which is generally accepted for delineation of a novel genomic species. Moreover, the average nucleotide identity (ANI) and the tetranucleotide signature frequency correlation coefficient (TETRA) between two genomes were calculated by JSpecies as a substitute for DNA–DNA hybridization (Richter & Rosselló-Móra, 2009). The results showed that all of the ANI and TETRA values between strain A501^T and related species were below the threshold values for delineating species, which are 95–96 % and 0.99, respectively (Table S1). Based on this method, the most similar species to strain A501^T was *T. gammatolerans*, with an ANI value of 79.87 % and a TETRA value of 0.97284. While the ANI and TETRA values when strain A501^T was compared with *T. kodakarensis* were 75.97 % and 0.95686. Thus, it is reasonable to suggest that the isolate A501^T represents a novel species.

Compared with phylogenetically related species of the genus *Thermococcus*, strain A501^T could be distinguished on the basis of different taxonomic parameters (Table 1). Strain A501^T was distinguishable from all of the related species based on its wide temperature range for growth at 0.1 MPa. The variation in the temperature range was up to 50 °C. Moreover, this strain differed from most of the other species in its pH range for growth, with *Thermococcus barossii* as the exception. However, the DNA G+C contents of *T. barossii* and strain A501^T are different. Strain A501^T was distinguishable from *T. kodakarensis* and *T. gammatolerans*, the two most closely related species based on the 16S rRNA sequencing analysis, by the optimum NaCl concentration for growth, substrate utilization and the function of elemental sulfur for growth.

On the basis of the phenotypic and genetic characteristics of strain A501^T, we report a novel species within the genus *Thermococcus*. We propose to name this species *Thermococcus eurythermalis* sp. nov. according to its wide temperature range for growth.

Description of *Thermococcus eurythermalis* sp. nov.

Thermococcus eurythermalis (eu.ry.ther.ma′lis. Gr. adj. *eurus* wide, broad; L. adj. *thermalis* thermal; N.L. masc. adj. *eurythermalis* with a broad temperature range).

Cells are irregular cocci (diameter 0.6–2.6 μm) and motile with a polar tuft of flagella. Growth occurs over the

Table 1. Characteristics of species of the genus *Thermococcus* that distinguish strain A501^T from its closest phylogenetic relatives

Taxa: 1, *Thermococcus eurythermalis* sp. nov. A501^T (this study); 2, *T. gammatolerans* (Jolivet *et al.*, 2003); 3, *T. kodakarensis* (Atomi *et al.*, 2004); 4, *T. peptonophilus* (González *et al.*, 1995); 5, *T. stetteri* (Miroshnichenko *et al.*, 1998); 6, *T. coalescens* (Kuwabara *et al.*, 2005); 7, *T. barossii* (Duffaud *et al.*, 1998); 8, *T. hydrothermalis* (Godfroy *et al.*, 1997); 9, *T. profundus* (Kobayashi *et al.*, 1994); 10, *T. gorgonarius* (Miroshnichenko *et al.*, 1998). +, Positive; −, negative; ND, no data; R, required; S, stimulatory.

Characteristic	1	2	3	4	5	6	7	8	9	10
Geographical origin	Guaymas Basin	Guaymas Basin	Kodakara Island, Kagoshima, Japan	Izu-Bonin forearc	Kraternaya cove, Ushishir archipelago, Northern Kurils	Suiyo Seamount, Izu-Bonin Arc	Juan de Fuca Ridge	East Pacific Rise	Middle Okinawa Trough	Whale Island, New Zealand
Sample type	Chimney, hydrothermal vent	Chimney, hydrothermal vent	Solfataric field	Hydrothermal vent	Marine solfataric field	Hydrothermal fluid	Rock fragment, hydrothermal vent	Rock suspensions, hydrothermal vent	Hydrothermal vent	Geothermal vent, tidal zone
Depth (m)	2007	2616	0	ND	ND	1380	ND	ND	1395	40
Motility	+	+	+	+	−	+	−	+	+	+
Size (μm)	0.6–2.6	0.6–1.4	ND	0.7–2	1–2	0.5–2	0.7–3.7	0.8–2	1–2	0.3–1.2
Temperature range for growth (°C)	50–100 (0.1 MPa)	55–95	60–100	60–100	55–94	57–90	60–92	55–100	50–90	68–95
Optimum temperature for growth (°C)	85	88	85	85–90	75	87	82.5	85	80	80–88
pH range for growth	4–9	4–8.5	5–9	4–8	5.7–7.2	5.2–8.7	4–9	3.5–9.5	4.5–8.5	5.8–8.5
Optimum pH for growth	7.0	6	6.5	6	6.5	6.5	6.5–7.5	6	ND	6.5–7.2
[NaCl] range for growth (%, w/v)	1–5	1–5	1–5	1–5	ND	1.5–4.5	1–4	2–8	1–6	1–6
Optimum [NaCl] for growth (%, w/v)	2.5	2	3	3	2.5	2.5	ND	3–4	ND	2–3.5
Piezophilic response	+	ND	ND	ND	+*	ND	ND	ND	ND	ND
Substrates utilized for growth										
Starch	Weakly	−	+	ND	+	−	ND	ND	+	ND
Sucrose	−	ND	+	−	ND	−	ND	ND	ND	ND
Maltose	−	−	ND	−	ND	−	ND	+	+	−
Pyruvate	−	−	+	−	ND	ND	ND	Weakly	+	Weakly
DNA G+C content (mol%)	53.5	51.3	52	52	50.2	53.9	60	58	52.5	50.6
Sulfur requirement	S	R	S/R	S	R	S	R	S	R	R
Doubling time under optimum conditions (min)	48	95	86	25	ND	24	35	90	50	ND

*Data from Canganella *et al.* (1997).

temperature range 50–100 °C (optimal growth at 85 °C) at 0.1 MPa and extends to 102 °C at 10 MPa. Hydrostatic pressure range for growth is 0.1–70 MPa (optimal growth at 0.1–30 MPa) at 85 °C and narrows to 0.1–50 MPa (optimal growth at 10 MPa) at 95 °C. Grows at pH 4–9 (optimal growth at pH 7.0) and with 1.0–5.0 % (w/v) NaCl (optimal growth with 2.5 % NaCl). Doubling time under optimal growth conditions is 48 min. Strictly anaerobe. Obligately chemo-organotroph. Yeast extract, tryptone, peptone and starch as single carbon sources can support growth, but starch supports very poor growth. Growth is stimulated by elemental sulfur and cysteine, but these molecules are not necessary.

The type strain, A501^{T} (=CGMCC 7834^{T}=JCM 30233^{T}), was isolated from the chimney sample of a deep-sea hydrothermal vent site at a depth of 2006.9 m; the vent was located in the Guaymas Basin (27° 0.405′ N 110° 24.567′ W).

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