Thermococcus nautili sp. nov., a hyperthermophilic archaeon isolated from a hydrothermal deep-sea vent

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Thermococcus nautili, strain 30-1T (formerly reported as Thermococcus nautilus), was isolated from a hydrothermal chimney sample collected from the East Pacific Rise at a depth of 2633 m on the ‘La chainette PP57’ area. Cells were motile, irregular cocci with a polar tuft of flagella (0.8–1.5 μm) and divided by constriction. The micro-organism grew optimally at 87.5 °C (range 55–95 °C), at pH 7 (range pH 4–9) and with 2 % NaCl (range 1–4 %). Doubling time was 64 min in Zillig’s broth medium under optimal conditions. Growth was strictly anaerobic. It grew preferentially in the presence of elemental sulfur or cystine, which are reduced to H2S, on complex organic substrates such as yeast extract, tryptone, peptone, Casamino acids and casein. Slow growth was observed on starch and pyruvate. Strain 30-1T was resistant to chloramphenicol and tetracyclin (at 100 μg ml−1) but sensitive to kanamycin and rifampicin. The G+C content of the genomic DNA was 54 mol%. Strain 30-1T harboured three plasmids named pTN1, pTN2 and pTN3 and produced membrane vesicles that incorporate pTN1 and pTN3. As determined by 16S rRNA gene sequence analysis, strain 30-1T is related most closely to Thermococcus sp. AM4 (99.3 % similarity) and Thermococcus gammatolerans DSM 15229T (99.2 %). DNA–DNA hybridization values (in silico) with these two closest relatives were below the threshold value of 70 % (33 % with Thermococcus sp. AM4 and 32 % with T. gammatolerans DSM 15229T) and confirmed that strain 30-1 represents a novel species. On the basis of the data presented, strain 30-1T is considered to represent a novel species of the genus Thermococcus, for which the name Thermococcus nautili sp. nov. is proposed. The type strain is 30-1T (=CNCM 4275=JCM 19601).

Since the discovery of anaerobic hyperthermophilic archaea by Karl Stetter and Wolfram Zillig, a number of studies have focused on their phylogeny, physiology, molecular biology and evolution (Zivanovic et al., 2002; Chaban et al., 2006; Brochier-Armanet et al., 2011; Farkas et al., 2013). These extremophilic micro-organisms were isolated from terrestrial hot springs, solfatara fields and marine hydrothermal vents. Among them, Thermococcales belong to the phylum Euryarchaeota. They are mainly obligate heterotrophs and use organic compounds as energy and carbon sources. Two members of the genus Thermococcus recently isolated are capable of lithotrophic growth on CO: ‘Thermococcus onnurineus’ and Thermococcus sp. AM4 (Sokolova et al., 2004; Lee et al., 2008). Commonly, Thermococcales exhibit better growth when they are cultivated in the presence of elemental sulfur, which is reduced to hydrogen sulfide (Zillig, 1992).

The order Thermococcales is composed of three genera: Pyrococcus, Thermococcus and Palaeococcus (Achenbach-Richter et al., 1988; Stetter, 1996; Takai & Sako, 1999). The genus Thermococcus was first described based on Thermococcus celer (Zillig et al., 1983) and, at the time of writing, comprises 34 recognized species. They are ubiquitously present in high-temperature environments such as hydrothermal deep-sea vents (Prieur, 2002). Only nine genomes of Thermococcus are available in public databases: Thermococcus kodakarensis (Fukui et al., 2005), ‘T. onnurineus’ (Lee et al., 2008), Thermococcus gammatolerans (Zivanovic et al., 2009), Thermococcus sibiricus (Mardanov et al., 2009), Thermococcus sp. 4557 (Wang et al., 2011), Thermococcus sp. CL1 (Jung et al., 2012), Thermococcus litoralis (Gardner et al., 2012), Thermococcus sp. AM4...
(Sokolova et al., 2004) and Thermococcus barophilus (Vannier et al., 2011).

The genetic tools for Thermococcales are already numerous and in constant progress. Genetic manipulation techniques have been mainly performed for T. kodakarensis (Leigh et al., 2011; Farkas et al., 2013) displaying natural competency. Gene disruption (Sato et al., 2005), gene insertion (Mueller et al., 2009), gene replacement on the chromosome (Santangelo et al., 2010), gene expression (Hirata et al., 2008), replication of shuttle vector pLC70 (Santangelo et al., 2008) and utilization of reporter genes (Santangelo et al., 2010) are now applicable for other species of the genus Thermococcus. Shuttle vectors pYS2 (Lucas et al., 2002) and pYS3 (Waeg et al., 2010), respectively used for Pyrococcus abyssi and Pyrococcus furiosus, are also available.

Thermococcales have been screened for the last 15 years to investigate the diversity of mobile genetic elements such as viruses and plasmids. Although an unexpected diversity of virus-like particles was revealed from Thermococcales enrichments (Geslin et al., 2003a), only two viruses, PAV1 (Pyrodictium abyssi virus 1) (Geslin et al., 2003b, 2007) and TPV1 (Thermococcus prieurii virus 1) (Gorlas et al., 2012), have been described from this order. However, about 40% of Thermococcales strains contain at least one extrachromosomal element (Benbouzid-Rollet et al., 1997; Frieur et al., 2004). To date, 15 plasmids have been described and sequenced from the genera Pyrococcus and Thermococcus and three of them are in the same strain: strain 30-1T (reviewed by Krupovic et al., 2013).

Since the isolation of strain 30-1T (Lepage et al., 2004), many studies have been performed on the extrachromosomal elements present, such as the rolling-circle plasmid pTN1 of 3.6 kb (Soler et al., 2007), which was used to construct shuttle vectors for T. kodakarensis (Santangelo et al., 2008), and the plasmid pTN2 of 13 kb (Soler et al., 2010, 2011), which was considered a member of a new plasmid family (Krupovic et al., 2013).

Notably, it has been recently shown that species of the genus Thermococcus produce extracellular membrane vesicles (MVs) which can transfer DNA (Soler et al., 2008; Gaudin et al., 2013a; Marguet et al., 2013). Interestingly, MVs produced by strain 30-1T harbour the genome of pTN1 and the genome of a defective virus named pTN3. It has been speculated that MVs can serve as vehicles for the transport of viral genome in the absence of viral infection (Gaudin et al., 2013b). These extracellular MVs could also facilitate horizontal gene transfer between hyperthermophiles and also allow the recombination between viral, plasmid and cellular genomes.

Strain 30-1T can therefore be considered as a model organism in studies of MVs. The whole genome was recently sequenced (unpublished data) and will soon be available (J. Oberto, personal communication).

Strain 30-1T was isolated from samples collected in 1999 from a hydrothermal chimney at the ‘La chainette’ area (12° 50.342’ N 103° 56.903’ W; 2633 m depth), located on the East Pacific Ocean Ridge, using the submersible Nautil during the AMISTAD cruise (Lepage et al., 2004). On board, samples of rock fragments were transferred to an anaerobic chamber and suspended in 5 ml of sterilized seawater reduced by addition of Na2S (0.1 mg L⁻¹) in Hungate tubes. Enrichment cultures were performed anaerobically under an atmosphere of N2 (Balch & Wolfe, 1976), by inoculating 500 μl of samples into Hungate tubes containing Zillig’s broth (ZB) medium and incubating at 90°C for 12–48 h as previously described (Lepage et al., 2004). Strain 30-1T was purified by isolation (three times) in ZB medium supplemented with 8% Gelrite gellan gum (Sigma-Aldrich) incubated in anaerobic jars under a CO2 (50 kPa)/H2 (20 kPa) atmosphere for 48 h at 80°C. Single colonies were picked up, suspended in 0.5 ml of reduced ZB medium and transferred into fresh liquid medium for 24 h at 90°C. The same conditions (described by Lepage et al., 2004) were used for the routine cultivation of strain 30-1T.

Thermococcus gournonarius DSM 10395T (Miroshnichenko et al., 1998), Thermococcus setteri DSM 5262T (Miroshnichenko et al., 1989), Thermococcus peptonophilus DSM 10343T (González et al., 1995), T. kodakarensis JCM 12380T (Atomi et al., 2004), Thermococcus guaymurasensis DSM 11113T (Canganella et al., 1998), T. gammatolerans DSM 15229T (Jolivet et al., 2003), Thermococcus fumicolans DSM 12820T (Godfroy et al., 1996), Thermococcus acidaminovorans DSM 11906T (Dirmeier et al., 1998), Thermococcus waotapuensis DSM 12768T (González et al., 1999), Thermococcus zillii DSM 2770T (Ronimus et al., 1997), Thermococcus pacificus DSM 10394T (Miroshnichenko et al., 1998), Thermococcus siculo DSM 12349T (Grote et al., 1999), Thermococcus celercrencs DSM 17994T (Kuwabara et al., 2007), Thermococcus profundus DSM 9503T (Kobayashi et al., 1994), Thermococcus thioreducens DSM 14981T (Pikuta et al., 2007), Thermococcus hydrothermalis CNCMI 1319T (Godfroy et al., 1997) and T. prieurii JCM 16307T (Gorlas et al., 2013) were used as reference strains.

The morphological characteristics of strain 30-1T were determined by phase-contrast microscopy (Nikon E600) and transmission electron microscopy (JEOL TEM CX II operating at 120 kV). For the latter, a sample droplet of 20 μl was spotted onto a Formvar-carbon grid (400 mesh; Euromex) and cells were negatively stained with 2% (w/v) uranyl acetate (Soler et al., 2008). Thin cell cross-sections were prepared as previously described (Marguet et al., 2013). Cells were irregular cocci with a diameter of 0.8–1.5 μm. Cells were present singly or in pairs (Fig. 1a). Cells were motile with the presence of a polar tuft of flagella (Fig. 1b). Thin sectioning revealed that cells were delimited by a typically thick cytoplasmic membrane (5 nm) covered by two protein layers separated by a periplasmic space (15 nm). The outer layer (S-layer) was covered by a dense network of lipopolysaccharides, with a hairy appearance (Fig. 1c). Cells produced a large number of MVs, some of which were covered by a dense S-layer, and nanotubes containing arrays of vesicles (Gaudin et al., 2013b; Marguet et al., 2013).
Growth was routinely measured by direct cell counting using a modified THOMA chamber (depth 0.1 mm) without fixation of cells. To determine the temperature for growth, cells were cultivated in Hungate tubes containing 6 mL of reduced ZB medium and using N₂ (100 %, 100 kPa) as headspace at temperatures ranging from (a) (b) (c)

**Fig. 1.** Micrographs of cells of strain 30-1T. (a, c) Thin sections of cells grown for 12 h in ZB medium. CM, cytoplasmic membrane; LPS, lipopolysaccharides; PS, periplasmic space; SL, S-layer. (b) Electron micrograph of negatively stained cells. Bars, 50 nm.

**Fig. 2.** Dendrogram showing the relationship between strain 30-1T and other Thermococcales. The topology shown corresponds to an unrooted tree reconstructed using the neighbour-joining algorithm with the modifications of Jukes and Cantor. Bootstrap values are indicated at branch nodes. Bar, 2 nt substitutions per 1000 nt.
from 50 to 95 °C. Strain 30-1T grew from 55 to 90 °C and the optimum temperature for growth was 87.5 °C (Fig. S1c, available in the online Supplementary Material). No growth was observed below 55 or above 90 °C. The optimal pH and NaCl concentration for growth were determined at 87.5 °C. To determine growth rates at different pH, ZB medium was modified by using the following buffers (Sigma), each at a concentration of 10 mM: pH 3–4, no buffer; pH 5–6, MES; pH 7, PIPES; pH 8–9, HEPES; and pH 10.0, no buffer. Growth of strain 30-1T was observed at pH 4–9 pH, with an optimum at pH 7 (Fig. S1a). No growth was observed at pH 3 or pH 10. To determine the optimal NaCl concentration for growth, cells were cultivated in ZB medium prepared with NaCl concentrations from 0 to 90 g l<sup>−1</sup> at 87.5 °C and pH 7. The strain grew with 10–40 g l<sup>−1</sup> with an optimum of 20 g l<sup>−1</sup> (Fig. S1b). No growth was observed in the absence of NaCl or above 50 g NaCl l<sup>−1</sup>. The effects of temperature, pH and salinity were determined in triplicate.

The doubling time measured for a fresh culture of strain 30-1T incubated under optimal conditions in ZB medium was 64 min.

Utilization of various individual substrates for growth was tested. Single substrates were added to a basal ZB medium supplemented with 0.02% yeast extract as growth factor. The following substrates were tested at a final concentration of 0.2% (w/v): yeast extract, peptone, tryptone, casein, starch, chitin, acetate, formate, pyruvate, cellulose, glucose, maltose, ethanol and Casamino acids. Unsupplemented medium was used as a negative control.

To determine the ability of the strain to grow in the absence of elemental sulfur, cells were cultivated in ZB medium without sulfur. Other single potential electron acceptors were tested and were added to sulfur-depleted media: L-cystine, thiosulfate (10 mM), sulfate (10 mM), sulfite (10 mM), fumarate (10 mM), nitrate (10 mM) and nitrite (10 mM).

The diverse media were all inoculated to a final concentration of 0.1% (v/v) (from a pre-culture with 10<sup>8</sup> cells ml<sup>−1</sup>) and incubated at 87.5 °C for 12 h. Results were considered positive when growth was observed after a transfer (10%, v/v, inoculum) on the same medium. All tests were performed in duplicate and growth was detected under a microscope (×100 magnification).

Significant growth was observed, under strictly anaerobic culture conditions, on complex carbon sources such as yeast extract, tryptone, peptone, Casamino acids and casein after three transfers in the same medium. Slow growth was observed on starch and pyruvate. Other growth substrates such as chitin, acetate, formate, cellulose, glucose, maltose and ethanol did not support growth of strain 30-1T. In summary, strain 30-1T is an obligate heterotroph that can use a variety of substrates as carbon and energy sources but is unable to grow in the presence of thiosulfate, sulfate, sulfite, fumarate, pyruvate, nitrate or nitrite as electron acceptors.

**Table 1. DNA–DNA hybridization data among members of the genus Thermococcus**

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<th>Distance</th>
<th>DDH (%)</th>
<th>DDH+/%</th>
<th>Distance</th>
<th>DDH (%)</th>
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<td>T. kodakarensis JCM 12380&lt;sup&gt;T&lt;/sup&gt;</td>
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<td>Thermococcus sp. AM4</td>
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<td>0.1533</td>
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<td>Strain 30-1T</td>
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<td>33.80</td>
<td>2.47</td>
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**Fig. 1. Summary of the phylogenetic position of strain 30-1T.**
Table 2. Characteristics that distinguish strain 30-1<sup>T</sup> from phylogenetically related species of the genus Thermococcus

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<tr>
<td>DNA G + C content (mol%)</td>
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<td>Presence of mobile elements</td>
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<td>Vesicles, pTN1, pTN2, pTP3</td>
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Strains: 1, 30-1<sup>T</sup> (data from this study); 2, T. gorgonarius DSM 10395<sup>T</sup> (Miroshnichenko et al., 1998); 3, T. stetteri DSM 5262<sup>T</sup> (Miroshnichenko et al., 1989); 4, T. peptonophilus DSM 10343<sup>T</sup> (González et al., 1995); 5, T. kodakarensis JCM 12380<sup>T</sup> (Atomi et al., 2004); 6, T. guaymasensis DSM 11113<sup>T</sup> (Canganella et al., 1998); 7, T. gammatolerans DSM 15229<sup>T</sup> (Jolivet et al., 2003); 8, T. funiculans DSM 12820<sup>T</sup> (Godfroy et al., 1996); 9, T. acidaminovorans DSM 11906<sup>T</sup> (Dirmeyer et al., 1998); 10, T. profundus DSM 9503<sup>T</sup> (Kobayashi et al., 1994); 11, T. thioreducens DSM 14981<sup>T</sup> (Pikuta et al., 2007); 12, T. waiotapuensis DSM 12768<sup>T</sup> (González et al., 1999); 13, T. zilligii DSM 2770<sup>T</sup> (Ronimus et al., 1997); 14, T. pacificus DSM 10394<sup>T</sup> (Miroshnichenko et al., 1998); 15, T. siculi DSM 12349<sup>T</sup> (Grote et al., 1999); 16, T. celericrescens DSM 17994<sup>T</sup> (Kuwabara et al., 2007); 17, T. hydrothermalis CNCMI 1319<sup>T</sup> (Godfroy et al., 1997); 18, T. prieurii JCM 16307<sup>T</sup> (Gorlas et al., 2013).

+ , Positive; –, negative; +/–, variable; S, stimulatory; R, required; ND, not determined.
acceptors. Elemental sulfur or cystine constitute an absolute requirement for growth.

Resistance to chloramphenicol, kanamycin, tetracyclin and rifampicin was tested at concentrations of 10, 25, 50 and 100 μg ml\(^{-1}\). Susceptibility to antibiotics was determined under standard conditions at 87.5 °C. Strain 30-1\(^{T}\) was resistant to chloramphenicol and tetracyclin. It was sensitive to rifampicin and growth was inhibited by kanamycin above 50 μg ml\(^{-1}\).

The DNA G + C content of strain 30-1\(^{T}\) was 54 mol% as determined using the whole genome sequence (J. Oberto, personal communication).

The 16S rRNA gene sequence was amplified by PCR as described by Lepage et al. (2004). This sequence (1440 bp) was first compared with those in available databases by using the BLAST program (Altschul et al., 1997). It was then aligned with its closest relatives by using the CLUSTAL W program (Thompson et al., 1994) with the software BioEdit (version 7.0.9.0). The phylogenetic tree was reconstructed with the neighbour-joining method with Jukes and Cantor (version 7.0.9.0). The phylogenetic tree was reconstructed with the neighbour-joining method with Jukes and Cantor corrections (Saitou & Nei, 1987) using the SEAVIEW program (Galtier et al., 1996). Bootstrapping was used to determine the robustness of the inferred topologies (1000 resamplings) (Fig. 2).

Phylogenetic analyses based on 16S rRNA gene sequences indicated that strain 30-1\(^{T}\) belongs to the order Thermococcales. Levels of 16S rRNA gene sequence similarity between strain 30-1\(^{T}\) and other Thermococcus species from the same cluster were as follows: Thermococcus sp. AM4, 99.3 %; T. gammadotolerans DSM 15229\(^{T}\), 99.2 %; T. guaymasensis DSM 11113\(^{T}\), 99 %; T. fumicolans DSM 12820\(^{T}\), 98.9 %; T. acidaminivorans DSM 11906\(^{T}\), 98 %. The status of strain 30-1\(^{T}\) with respect to its closest neighbours (T. gammadotolerans, Thermococcus sp. AM4) was assessed by DNA–DNA hybridization (DDH). In silico, DDH allows genome-to-genome distances between pairs of entirely sequenced genomes to be estimated in an easy and highly reliable way (Richter & Rosselló-Móra, 2009). The genome of strain 30-1\(^{T}\) was aligned, two-by-two by using a BLAST algorithm, with genomes of its closest neighbours and with the genome of T. kodakarenis JCM 12380\(^{T}\), which is considered as the outgroup in this study (Kent, 2002; Auch et al., 2010). Then, the set of high-scoring segment pairs between genomes was transformed into a single genome-to-genome distance value using a specific distance formula (Meier-Kolthoff et al., 2013). DDH values were also estimated with a generalized linear model using these distances values. DDH from the DNA of the strain 30-1\(^{T}\) gave the following levels of relatedness (Table 1): 33 % with Thermococcus sp. AM4, 32 % with T. gammadotolerans DSM 15229\(^{T}\) and 26 % with T. kodakarenis JCM 12380\(^{T}\). These values being less than 70 % confirmed that strain 30-1\(^{T}\) represents a novel species of the genus Thermococcus (Wayne et al., 1987).

In comparison with phylogenetically related species of the genus Thermococcus (Table 2), strain 30-1\(^{T}\) was distinguishable from T. gammadotolerans by its ability to grow on casein, starch and pyruvate. The genome of strain 30-1\(^{T}\) does not encode several of the enzymes, such as formate dehydrogenase, that allow T. gammadotolerans to grow anaerobically on formate (Kim et al., 2010) (Fig. S2) (Oberto, 2008; Despalins et al., 2011), as well as several of the enzymes that allow Thermococcus sp. AM4 to grow anaerobically on CO (Sokolova et al., 2004) (Fig. S3) (Oberto, 2008; Despalins et al., 2011). Strain 30-1\(^{T}\) can be also distinguished from other members of the Thermococcales by the presence of three mobile elements inside the cells (Fig. S4).

Based on the data presented, strain 30-1\(^{T}\) represents a novel species of the genus Thermococcus, for which we propose the name Thermococcus nautili sp. nov.

**Description of Thermococcus nautili sp. nov.**

Thermococcus nautili (nau’tii.li. N.L. gen. n. nautili referring to the submersible Nautili, which was used to collect samples from hydrothermal deep-sea vents).

Cells are irregular motile cocci (diameter 0.8–1.5 μm) with polar flagella. Obligately anaerobic. Elemental sulfur or cystine is necessary for growth. Does not use thiosulfate, sulfate, sulfite, fumarate, nitrate or nitrite as electron acceptors. Optimal growth occurs at 87.5 °C (range 55–90 °C), at pH 7 (range pH 4–9) and with 20 g NaCl l\(^{-1}\) (range 10–40 g l\(^{-1}\)). The doubling time is 64 min in ZB medium under optimal conditions. Obligate chemoorganotroph; growth occurs preferentially on complex substrates such as yeast extract, tryptone, peptone, Casamino acids and casein. Poor growth is observed on starch and pyruvate. Resistant to chloramphenicol and tetracyclin (100 μg ml\(^{-1}\)), but sensitive to kanamycin (50 μg ml\(^{-1}\)) and rifampicin.

The type strain, 30-1\(^{T}\) (=CNCM 4275\(^{T}\) =JCM 19601\(^{T}\)), was isolated from a deep-sea hydrothermal chimney located at the ‘La chaine’\(^{\prime}\) vent site at a depth of 2635 m in the East Pacific Ocean Ridge off the coast of Mexico. The DNA G+C content of the type strain is 54 mol%.

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