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Thermococcus piezophilus sp. nov., a novel hyperthermophilic and piezophilic archaeon with a broad pressure range for growth, isolated from a deepest hydrothermal vent at the Mid-Cayman Rise ⭑

Dalmasso Cécile [1, 2, 3], Oger Philippe [4], Selva Gwendoline [1, 2, 3], Courtine Damien [1, 2, 3], L'Haridon Stéphane [1, 2, 3], Garlaschelli Alexandre [1, 2, 3], Roussel Erwan [1, 2, 3], Miyazaki Junichi [5], Reveillaud Julie [1, 2, 3], Jebbar Mohamed [1, 2, 3], Takai Ken [5], Maignien Lois [1, 2, 3], Alain Karine [1, 2, 3, *]

[1] Université de Bretagne Occidentale (UBO, UEB), Institut Universitaire Européen de la Mer (IUEM) − UMR 6197, Laboratoire de Microbiologie des Environnements Extrêmes (LM2E), Place Nicolas Copernic, F-29280 Plouzané, France
[2] CNRS, IUEM − UMR 6197, Laboratoire de Microbiologie des Environnements Extrêmes (LM2E), Place Nicolas Copernic, F-29280 Plouzané, France
[3] Ifremer, UMR 6197, Laboratoire de Microbiologie des Environnements Extrêmes (LM2E), Technopôle Pointe du diable, F-29280 Plouzané, France
[4] Université de Lyon, INSA Lyon, CNRS UMR 5240, 11 Avenue Jean Capelle, F-69621 Villeurbanne, France
[5] Department of Subsurface Geobiological Analysis and Research (D-SUGAR), Japan Agency for Marine-Earth Science and Technology (JAMSTEC), 2-15 Natsushima-cho, Yokosuka 237-0061, Japan

* Corresponding author : Karine Alain, email address : Karine.Alain@univ-brest.fr

⭑ *Note*: The EMBL/GenBank/DDBJ 16S rRNA gene sequence accession number of strain CDGS^T is LN 878294. Accession number of genome sequence of strain CDGS^T is CP015520.

Abstract :

A novel strictly anaerobic, hyperthermophilic archaeon, designated strain CDGS^T, was isolated from a deep-sea hydrothermal vent in the Cayman Trough at 4964 m water depth. The novel isolate is obligate anaerobe and grows chemoorganoheterotrophically with stimulation of growth by sulphur containing compounds. Its growth is optimal at 75 °C, pH 6.0 and under a pressure of 50 MPa. It possesses the broadest hydrostatic pressure range for growth that has ever been described for a microorganism. Its genomic DNA G + C content is 51.11 mol%. The novel isolate belongs to the genus *Thermococcus*. Phylogenetic analyses indicated that it is most closely related to *Thermococcus barossii* DSM17882^T based on its 16S rRNA gene sequence, and to '*Thermococcus onnurineus*' NA1 based on its whole genome sequence. The average nucleotide identity scores with these strains are 77.66% for *T. barossii* and 84.84% for '*T. onnurineus*', respectively.

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Based on the draft whole genome sequence and phenotypic characteristics, strain CDGS^T is suggested to be separated into a novel species within the genus *Thermococcus*, with proposed name *Thermococcus piezophilus* (type strain CDGS^T = ATCC TSD-33^T = UBOCC 3296^T).

Keywords : *Thermococcus*, piezophile, hydrothermal vent, Cayman Trough

Introduction

Deep-sea hydrothermal vents are characterized by steep physical-chemical gradients between hot reduced streams (up to 450°C) and cold (2-4°C) with neutral pH surrounding seawater. These ecosystems are ideal to search for poly-extremophilic microorganisms with unique combination of extreme growth conditions. Since 1977 and the discovery of black smokers, numerous thermophilic and hyperthermophilic microorganisms have been isolated from deep-sea vents [8,17]. Among them, only four *Bacteria* and eleven *Archaea* are piezophiles or obligate piezophiles, e.g. requirering high hydrostatic pressure for growth [12].

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Molecular inventories of the microbial diversity and culture-based approaches have repeatedly demonstrated that *Thermococcales* are common inhabitants of the hot area of the hydrothermal ecosystem [1]. The order *Thermococcales* encompasses three genera: *Thermococcus* [27], *Pyrococcus* [7] and *Palaeococcus* [23]. Representatives of the genus *Thermococcus* are all hyperthermophilic chemoorganoheterotrophs fermenting notably peptides and polysaccharides and reducing sulphur species. There are also two *Thermococcus* isolates able to grow lithotrophically by carboxydotrophy [22].

Currently, thirty two species of *Thermococcus* have been fully characterized with validly published names (source: http://www.bacterio.net/). Only 15 genomes of *Thermococcus* are available in public databases.

In this study, a novel *Thermococcus* isolate, referenced as strain CDGS^T, was isolated from the deepest known deep-sea hydrothermal vent (the Beebe vent field, so-called Piccard). Its physiology and its genome were analyzed, and showed a wide physiological tolerance for the novel isolate to hydrostatic pressure as well as its taxonomic separation into a new species.

Materials and Methods

In June 2013, chimney fragments were collected by the DSV *Shinkai 6500* from the Beebe vent field (18°32.7881‘ N, 81°43.11844‘ W) in the Cayman Trough, at a depth of 4964 m, during an expedition (YK13-05) of the oceanographic cruise ―QUELLE2013‖ using RV *Yokosuka*. Chimney fragments were collected in small boxes and lifted up to the surface on the DSV. Once onboard, subsamples were immediately transferred anaerobically into sterile Schott vials, and stored at 4 °C.

Samples were then transferred into anaerobic medium (DSM 141 modified medium) for enrichment cultures, under an atmosphere of H_2/CO_2 (80/20, 2 atm) and incubated at 60°C, as described in Supplementary Material (SM). Isolation was performed by three repeated

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streaking on plates, at 70°C, onto modified Ravot medium (see SM). After six days of incubation, a single white colony was picked and referenced as strain $CDGS^T$.

Morphological characteristics of the cells were determined by light microscopy (BX60, Olympus) and scanning electron microscopy (SEM) (Quanta 200, FEI).

The physiological characterization (substrate utilization, antibiotic resistance, etc.) of the novel isolate was carried out in triplicates in modified Ravot medium at 75°C unless otherwise noted, and is described in detail in SM. The temperature range for growth of the novel isolate was examined within a range of 55-95 °C with intervals of 2-5 °C. The pH range was checked within values 4.0-9.0. Salt tolerance was tested within range of 0-8% (w/v) NaCl. The pressure range (0.1, 20, 35, 50, 65, 70, 90, 105, 120, 125, 130 and 135 MPa) was tested into high-pressure high-temperature reactors. Growth yields and growth rates were measured, cellular activity was monitored (ATPmetry), morphology was visualized by SEM and sub-cultivations were done. Respiration and fermentation end products were identified by ionic chromatography.

The genomic DNA of the novel isolate was extracted with the kit Genomic-tip 20G and Genomic DNA buffer set (Qiagen®) for wholegenome shotgun sequencing using the PacBio technology (one large insert library + two smartcells) (Duke University, Durham, USA). In addition, genomic DNA was purified with phenol-chloroform, and sequenced on a 318 Chip and the HiQ chemistry on an Ion Torrent PGM platform (IGFL, ENSL, Lyon, France) to correct and close-up the genome. The genome was assembled into one contig from the PacBio reads using the HGAP assembler included in a local installation of the PacBio SMRT portal (V. 2.3.0). The complete genome sequence was corrected by mapping assembly of the IonTorrent reads on the PacBio contig using MIRA 4 and the Newbler 2.8 suite of programs, and then manually curated.

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Annotation and comparative genomics were performed on the MicroScope Microbial Genome Annotation and Analysis Platform (MaGe) at the Genoscope [23,25]. Average nucleotide identity (ANI) scores were calculated using the JSpeciesWS [19], and the EzGenome program (http://www.ezbiocloud.net/ezgenome/ani) with defaults parameters. *In silico* DNA-DNA hybridization (DDH) estimations were calculated using the genome-to-genome distance calculator (GGDC2.0) [2,3]. Dot plot analyses of self-similarity between strain genomes were performed using the Nucmer V3.1 program, with default parameters.

16S rRNA gene sequence phylogenetic analyses were performed using Geneious version 6.1.6 (MUSCLE program for the alignment and manual refining). Phylogenetic reconstructions were made on the basis of evolutionary distance (neighbor-joining method with Jukes and Cantor corrections) [20] and maximum-likelihood (PHYML) [10], with 1000 bootstrap replications. Ribosomal protein sequence phylogenetic analyses were performed using PHYML. Ribosomal proteins were identified in complete genomes and aligned individually with MUSCLE. Alignments were optimized manually before concatenation. The phylogenetic reconstruction was done using PHYML excluding invariable sites from the analysis, using the LG model and a model-given amino acid equilibrium frequencies. The robustness of the inferred topology was assessed with 1000 bootstrap replications.

Pairwise 16S rRNA sequence similarity was calculated using the global alignment algorithm implemented at the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/) [13].

Results & discussion

The ultraslow spreading Mid-Cayman Rise hosts the deepest known hydrothermal vent field, the Beebe vent field, at ~ 5000 m water depth [9]. This active vent field is characterized

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by end-member fluids showing acidic pH (3.2), high temperature (close to 400°C), low methane content, and high concentrations of hydrogen (>20 mM) and sulphide [18]. The novel isolate CDGS^T was isolated from this site.

Cells of strain CDGS^T were motile irregular cocci of 0.8 to 1.7 μm in diameter (average 1.1 ± 0.3, *n*=18) (Fig. S1). They produced membrane vesicles and connected to other cells with nanotubes.

At atmospheric pressure, the novel isolate grew at temperatures between 60 and 90 °C (Fig. 1) and with an optimal temperature of 75 °C. It was neutrophilic, growing from pH 5.5 to 9.0, with a clear optimum at pH 6.0 (Fig. 1). Its growth was optimal at the salinity of seawater (30 g/L NaCl), and within a range of 20-60 g/L NaCl (Fig. 1). Interestingly, the novel isolate was found to be piezophilic with an optimum pressure for growth at 50 MPa (Table S1). Under optimal growth conditions (75°C, pH 6.0, 30 g/L NaCl, 50 MPa; with L-cystine), the doubling time of the novel isolate was 92 minutes.

The novel isolate was found to grow on complex organic substrates by fermentation without sulphur-containing compounds, but growth was stimulated by these sulphur species added to detoxify the excess of produced hydrogen. Growth was clearly stimulated by L-cystine, elemental sulphur (flower or colloidal) or polysulphur, that were all reduced to hydrogen sulphide. Best growth was observed on complex peptide carbon sources such as yeast extract, tryptone, peptone, beef extract and casamino acids in the presence of sulphur inorganic species. Slow growth was also observed on casein, D(+)-galactose, D(+)-glucose, D(+)-mannose, xylan, succinate, glutamate and L-glycin in the presence of sulphur. Unlike *T. onnurineus*, we did not observe growth on formate as sole carbon and energy source, which is not surprising since the genome of strain CDGS^T lacks a formate transporter. Growth by

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carboxydotrophy was not tested since CooS and CooF, the two central proteins in the microbial carbon monoxide metabolism, are also absent from the genome sequence of strain CDGS^{T}. The novel isolate was unable to reduce thiosulphate, sulphate, sulphite, nitrate and nitrite, and its growth was inhibited by oxygen. Metabolic end products are listed in Supplementary Material.

Strain CDGS^{T} was resistant to penicillin, kanamycin, chloramphenicol, streptomycin and vancomycin at 100 µg/mL, and it was sensitive to simvastatin.

The final genome assembly comprised one circularized contig of 1,928,919 bp, with an average DNA G+C content of 51.11 mol%. As for other *Thermococcales*, there is one copy of the 16S-23S operon, two copies of 5S rRNAs and 45 tRNA genes. The genome could encode 2418 coding sequences (CDS) and 15 miscellaneous RNA. It had a protein coding density of 87.71 %. About 50 % of all genes could be classified into Cluster of Orthologous Groups (COG) of functions. Among the identified CDS, 15% shared no homology with any previously reported sequences and 37% were homologs of previously reported genes of unknown function.

Levels of 16S rRNA gene sequence similarity with the closest relatives *Thermococcus barossii* DSM9535^{T} and ‗*Thermococcus onnurineus*' str. NA1 were 99.31 % and 99.30 %, respectively (Fig. S4). To further elucidate the taxonomic position of strain CDGS^{T}, (i) a phylogeny was constructed to compare concatenated ribosomal protein sequences from all available genomic sequences of *Thermococcus* species (Fig. 2), (ii) *in silico* DNA-DNA hybridizations were performed and (iii) average nucleotide identity (ANI) scores were calculated. The ANI scores between strain CDGS^{T} and ‗*T. onnurineus'* str. NA1 on one hand, and strain CDGS^{T} and *T. barossii* DSM9535^{T} on the other hand, were, respectively 84.84 % and 77.66 % with EzGenome (and 84.73 % and 76.75 % with JSpeciesWS) (Fig. 2), which are below the 95-96% cut-off value for species delineation by this approach [19]. The digital DNA-

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DNA hybridization values (identities / total length) between strain CDGS^T and (i) _T. onnurineus' str. NA1 and (ii) *T. barossii* DSM9535^T were (i) 50.50 % and (ii) 21.70 % respectively, which are far below the threshold value (70%) for prokaryotic species differentiation [26]. These whole-genome analyses indicated that strain CDGS^T represents a novel species that is most closely related to _*Thermococcus onnurineus'* strain NA1 (Fig. 2). Dot plot analyses of the regional self-similarity of these two genomes showed that the synteny was highly conserved between the two species with only few inversions (Fig. S3).

In comparison with phylogenetically related species of the genus *Thermococcus* (Table 1), strain CDGS^T was distinguishable from its closest relatives by its genomic DNA G+C content, its substrate preferences, its capacity to grow without sulphur species, its optimal temperature and pH for growth, and its piezophilic trait and wide pressure range for growth. A comparative genomic study of *Thermococcus* species showed that strain CDGS^T shared only one fifth of its proteins with other type strains of *Thermococcus* species with publicly available genomes, and onehalf with its closest relative _*T. onnurineus*' NA1 (Fig. 2). The main differences in genetic content between these two strains were observed in COG categories related to amino acid transport and metabolism, carbohydrate transport and metabolism, signal transduction mechanisms and groups of general function prediction or unknown functions (Table S2, Fig. 2). Of note, while both CDGS^T and *Thermococcus onnurineus'* NA1 possess all the proteins required for organotrophic growth on peptides, amino acids, or sugars, CDGS's genome lacks some CDS necessary to
grow by carboxydotrophy or formate oxidation.

In view of all above mentioned phylo-phenetic distinctive features, we propose that the isolate CDGS^T should be assigned as the type strain of a novel species, for which the name *Thermococcus piezophilus* sp. nov. is proposed.

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As stated above, the novel isolate is piezophilic. Its optimal pressure for growth is 50 MPa, the hydrostatic pressure of its natural habitat, as repeatedly examined using multiple proxies (lag phase duration, growth rate, growth yield) (Table S1). Strain $CDGS^T$ grew well under pressures up to 105 MPa at the optimal growth temperature of 75 °C (Fig. 1, Fig. S2). Within 105-125 MPa, growth yield was very low but reproducible. Above 125 MPa, the growth yield was negligible. Intracellular ATP (iATP) content per cell at 120 MPa was similar to the optimal 50 MPa pressure (Table S3), that was consistent with observations of cell growth at this pressure. At 125 and 130 MPa, iATP was ca. 10 times higher indicating a higher activity of cell metabolism, and probably the activation of cell stress responses. After 38h of incubation at 135 MPa, we observed only a fewer decrease in cell concentration, and iATP content was close to the detection limit, suggesting no biomass growth had occurred at 135 MPa (Fig. S2, Table S3). No visible alterations of cell walls or morphological changes were observed. To further confirm growth, maintenance or survival under these extreme high-pressure conditions (125-135 MPa), we performed subcultures at atmospheric pressure. We were able to get growth for cells from all pressure conditions tested, confirming that strain $CDGS^T$ cells survived at least 38h at 135 MPa. Interestingly, at atmospheric pressure, iATP content was higher than at the optimal pressure for growth (Table S3), which may reflect the activation of stress response mechanisms. The induction of the stress response under low hydrostatic pressure is a characteristic of piezophiles [4,16].

These results show that strain $CDGS^T$ exhibits the broad pressure range for growth, whether among psychrophiles, mesophiles or hyperthermophiles [12], growing effectively from atmospheric pressure to at least 120 MPa, and with difficulty up to 130 MPa. These extreme pressures correspond to the pressure of a water column of 12 to 13 km depth, greater than the pressure measured at the bottom of the deepest parts of the ocean in the Mariana

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Trench. The archaea *Pyrococcus yayanosii* and *Methanocococcus thermolithotrophicus* hold the previous known record for a pressure range for growth of 100 MPa [12]. In conclusion, *Thermococcus piezophilus* represents an excellent model of archaea to study microbial adaptation to hydrostatic pressure at high temperatures.

There are many piezophilic strains within the order *Thermococcales* [12]. This feature might be helpful for their oceanic dispersal over long distances, including in the abysses and in the hadal zones.

Description of *Thermococcus piezophilus* sp. nov.

Thermococcus piezophilus (pi.e.zo.phi'lus. Gr. v. *piezo,* to press; N.L. adj. *philus -a -um* (from Gr. adj. *philos -ê -on*), loving; N.L. masc. adj. *piezophilus*, loving pressure).

Cells are irregular motile cocci. Obligately anaerobic. Obligate chemoorganoheterotroph; Grows preferentially on complex peptide organics such as yeast extract, peptone, tryptone, casamino acids and beef extract. Poor growth on a few organic acids, mono- and polysaccharides. Elemental sulphur, L-cystine or polysulphur are not required but definitely stimulate growth.

Optimal growth occurs at 75 °C, pH 6.0 and 3 % NaCl. Piezophilic, growing optimally under 50 MPa. The doubling time is 92 min under optimal growth conditions.

The type strain, CDGS^T (=ATCC TSD-33^T = UBOCC 3296^T) was isolated from a chimney wall material of the ~ 5 km water depth Beebe vent field, at the Mid-Cayman Rise.

The DNA G+C content of this strain is 51.11 mol%.

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Table 1. Characteristics differentiating strain CDGS^T from closest species of the genus *Thermococcus.*

1, Strain CDGS^T (this study); 2, '*T. onnurineus*' [21] ; 3, *T. barossii* [6]; 4, *T. profundus* [14]; 5, *T. coalescens* [15]. Legend: S, stimulatory ; R, required ; §, information deduced from the genome sequence ; ND, not determined.

Characteristic	1	2	3	4	5
Geographical origin	Beebe Vent Field, Mid-Cayman Rise	Manus basin, Papua New Guinea	Juan de Fuca Ridge	Middle Okinawa Trough	Suiyo Seamount, Izu-Bonin Arc
Sample Type	Hydrothermal chimney rock	Hydrothermal chimney rock	Hydrothermal chimney rock	Hydrothermal vent	Hydrothermal fluids
Depth (m)	4969	1650	ND	1395	1380
Size (µm)	0.8 - 2.0	0.5 - 1.0	0.7 - 3.7	1.0 - 2.0	0.7 - 3.7
Motility	+	+	-	+	+
Temperature (°C;Optimum)	60-95 °C (75 °C)	63-90 °C (80 °C)	60-92 °C (82.5 °C)	50-90 °C (80 °C)	57-90 °C (87 °C)
pH (Optimum)	5.5-9.0 (pH 6.0)	5.0-9.0 (pH 8.5)	4.0- 9.0 (pH 6.5-7.5)	4.5-8.5 (Opt. ND)	5.2-8.7 (pH 6.5)
NaCl (optimum) (%)	2.0-6.0 (3.0)	1.0-5.0 (3.5)	ND	1.0-6.0 (Opt. ND)	1.5-4.5 (2.5)
Doubling time (min)	92	92	35	50	150
Sulphur requirement	S	R	R	R	S
Growth on:					
Pyruvate	-	-	-	+	-
Maltose	-	-	+	+	-
Starch	-	+	ND	+	-
Yeast Extract	+	+	+	+	+
Peptone	+	+	+	+	ND
CO	-§	+	ND	ND	ND
DNA G+C content (mol%)	51.1	52	54.7	50	53.9

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Fig. 1. Growth rates of strain CDGS^T at different salinities (A), temperatures (B) and pHs (C). Growth yields of the novel isolate after 38h incubations at different pressures (D).

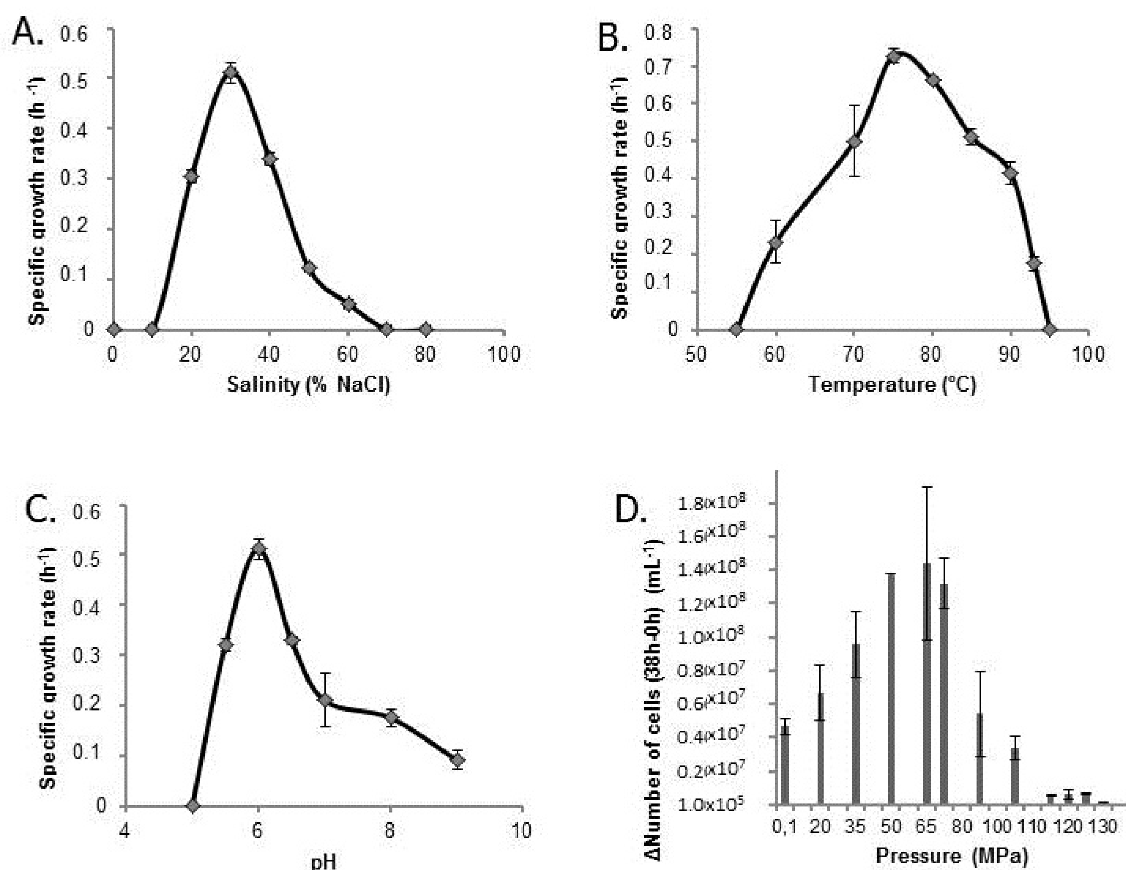

Fig. 2. Left side: Phylogenetic relationships of strain CDGS^T and other *Thermococcus* species based on ribosomal protein sequences. The topology shown was calculated with PHYML. Accession numbers are given in brackets. Bootstrap values (%) are based on 1000 replicates. Bar, 2 substitutions per 100 nt. **Right side.** Venn diagram of orthologous genes showing the pan/core genomes of strain CDGS^T, '*T. onnurineus'* str. NA1 and the five type species of the *Thermococcus* genus with publicly available genomes (*T. barophilus* MP^T, *T. gammatolerans* EJ3^T, *T. kodakarensis* KOD1^T, *T. sibiricus* MM379^T and *T. litoralis* Ns-C^T). The Venn diagram is based on a threshold of 80% amino acid identity and 80% amino acid alignment coverage. Numbers shown in the Venn diagram correspond to the orthologous gene groups given in the respective patterns. Histograms

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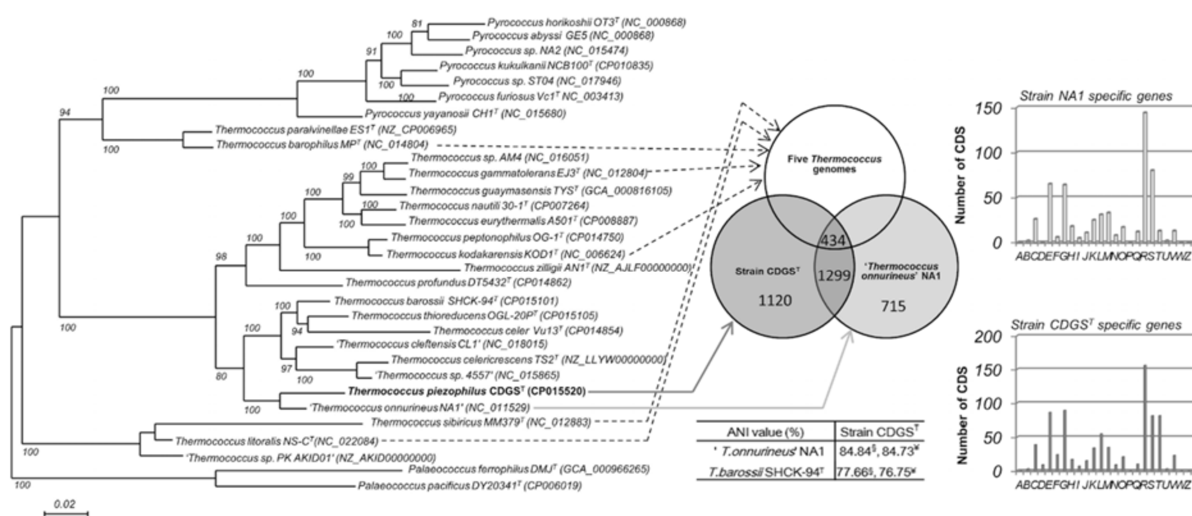
show the distribution of COG groups within the strain-specific portions of the genomes. COG categories: A, RNA modification and processing; B, Chromatin structure and dynamics; C, Energy production and conversion; D, Cell cycle control, cell division, chromosome partitioning; E, Amino acid transport and metabolism; F, Nucleotide transport and metabolism; G, Carbohydrate transport and metabolism; H, Coenzyme transport and metabolism; I, Lipid transport and metabolism; J, Translation, ribosomal structure and biogenesis; K, Transcription; L, Replication, recombination and repair; M, Cell wall/membrane/envelope biogenesis; N, Cell motility; O, Post-translational modification, protein turnover, chaperones; P, Inorganic ion transport and metabolism; Q, Secondary metabolites biosynthesis, transport and catabolism; R, General function prediction only; S, Function unknown; T, Signal transduction mechanisms; U, Intracellular trafficking, secretion and vesicular transport; V, Defense mechanisms; W, Extracellular structures; Z, Cytoskeleton. **Middle:** Average Nucleotide Identity scores between the genomes of the novel strain and its two closest relatives calculated with EzGenome (§) and JSpeciesWS (¥) programs.



Supplementary Information

Materials and Methods

Enrichment and isolation

Chimney samples were incubated anaerobically for enrichment cultures in DSM 141 modified medium (per litre of distilled water: 18 g NaCl, 4 g $MgCl_2·6H_2O$, 3.45 g $MgSO_4·6H_2O$, 2 g yeast extract, 1.95 g MES buffer, 0.34 g KCl, 0.25 g NH_4Cl, 0.14 g $CaCl_2·2H_2O$, 0.002 g $Fe(NH_4)_2(SO_4)_2·6H_2O$, 0.001 g resazurin) with a pH adjusted to 6.5. Medium was boiled for 3-5 minutes, cooled under flow of high purity nitrogen gas, then reduced with 0.5 g cysteine hydrochloride, and autoclaved (for 60 min 1 atm and 121°C). Sterile solutions of vitamins and trace elements were added 10 mL each (see recipe of DSM medium 141, below). Initial enrichment cultures were incubated in vials reduced with 0.5 g L^{-1} sodium sulphide ($Na_2S.9H_2O$) and incubated at 60°C under an atmosphere of H_2/CO_2 (80/20, 0.2 MPa). After two days of incubation, low cellular abundances were obtained in the enrichment cultures, suggesting that we did not enrich autotrophs but rather heterotrophs. The medium was then changed to stimulate heterotrophic growth. Subcultures and isolation were done in Ravot modified medium prepared without maltose, with a pH adjusted to 6.0 (per litre of distilled water: 30 g NaCl, 3.3 g PIPES buffer, 2 g tryptone, 2 g yeast extract, 0.83 g sodium acetate trihydrate, 0.5 g KCl, 0.5 g $MgCl_2·6H_2O$, 0.3 g NH_4Cl, 0.1 g $CaCl_2·2H_2O$, 0.001 g resazurin). Medium was autoclaved and supplemented with 0.26 mM KH_2PO_4, 0.20 mM K_2HPO_4 and 1 mL vitamin mixture (described in DSM medium 141). After degassing, medium was aliquoted anaerobically in vials, supplemented with 12 g/L L-cystine, and reduced with 0.5 g/L sodium sulphide ($Na_2S·9H_2O$) just before inoculation. After two days of incubation, motile cocci were observed and subcultured under the same conditions. They were subsequently purified by three repeated streaking on plates, on the same medium solidified

with gellan gum (Phytagel™, Sigma-Aldrich). Plates were incubated into anaerobic jars at 70 °C for six days. After the third transfer on plate, a single circular white colony was picked and referenced as strain CDGS^T. Purity of this isolate was confirmed by microscopy and sequencing of the 16S rRNA gene using five different primers.

DSM medium 141, with modifications

Modified mineral basis

For 1 L	g
KCl	0.34
$MgCl_2 \cdot 6H_2O$	4.00
$MgSO_4 \cdot 6H_2O$	3.45
NH_4Cl	0.25
$CaCl_2 \cdot 2H_2O$	0.14
K_2HPO_4	0.14
NaCl	18.00
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	2.00*
MES buffer	1.95
Resazurin	1 drop
Distilled water	1L

Dissolve the ingredients and then adjust the pH to 6.0.
Boil the medium for 3-5 minutes, and cool it under a flow of high purity nitrogen gas.
Add 0.5 g cysteine hydrochloride.
Autoclave for 60 min at 121°C, 1 atm.
After autoclaving, flush the medium with a mixture of high purity nitrogen gas.
Sterile solutions of vitamins and trace elements (see below) are added 10 mL each to the cold mineral basis.

Medium is then aliquoted anaerobically into sterile vials. Gas phase is then replaced by a high purity H_2/CO_2 (80/20, 2 atm, 2/3rd of gas phase in the vial) gas mixture.

Vitamin solution

For 1 L	mg
Biotin	2.00
Folic acid	2.00
Pyridoxine-HCl	10.00
Thiamine-HCl·$2H_2O$	5.00
Riboflavin	5.00
Nicotinic acid	5.00
D-Ca-pantothenate	5.00
B12 vitamin (cobalamin)	0.10
p-amino-benzoic acid	5.00
Lipoic aciod	5.00
Distilled water	1L

Sterilize by filtration (pores: 0.2 µm).

Trace element solution

For 1L	g
Nitriloacetic acid	1.50
$MgSO_4 \cdot 7H_2O$	3.00
$MnSO_4 \cdot H_2O$	0.50
NaCl	1.00
$FeSO_4 \cdot 7H_2O$	0.10
$CoSO_4 \cdot 7H_2O$	0.18
$CaCl_2 \cdot 2H_2O$	0.10
$ZnSO_4 \cdot 7H_2O$	0.18
$CuSO_4 \cdot 5H_2O$	0.01
$KAl(SO_4)_2 \cdot 12H_2O$	0.02
H_3BO_3	0.01
$Na_2MoO_4 \cdot 2H_2O$	0.01
$NiCl_2 \cdot 6H_2O$	0.03
Na_2SeO_3	0.2*
Distilled water	1 L

Dissolve first the nitriloacetic acid, adjust the pH to 6.5 and then add the other components. The final pH is adjusted to 7.0.
Autoclave at 121°C and 1 atm, for 1h.

Physiological analyses

Unless stated otherwise, the experiments were carried out anaerobically in triplicate in the presence of L-cystine and incubation was carried out in the dark, at 70 °C, pH 6.0 and with 30 g/L NaCl. Growth was monitored either by flow cytometry enumeration (CyFlow Space, Partec) or by direct cell counting onto a modified Thoma chamber (depth 10 µm). In parallel, the production of hydrogen sulphide was measured by a colorimetric method [5]. Growth rates were calculated using linear regression analysis of 4 to 6 points along the linear portions of the growth curves.

The pH range for growth of the novel isolate was determined in Ravot modified medium prepared with various buffers (each at 20 mM, Sigma-Aldrich): for pH 4.0 and 5.0, HOMOPIPES buffer; for pH 5.5-6.5, MES buffer; for pH 7.0, PIPES buffer; for pH 7.5-8.0, HEPES buffer; for pH 8.5, TAPS buffer; for pH 9.0, CAPSO buffer.

The pressure range for growth of the strain was tested in 5 mL syringes placed either into a high-pressure/high-temperature incubation system custom-built by Top-Industrie (Dammarie-les-Lys, France) or into home-made high pressure reactors (Oger laboratory, INSA Lyon). At the optimal temperature for growth (75 °C), growth yield after 14, 18, 24 and 38 hours of incubation was monitored at the following hydrostatic pressures: 0.1, 20, 35, 50, 65, 70, 90, 105, 120, 125, 130 and 135 MPa. Cell counts were done by flow cytometry and the level of cellular activity was monitored by ATPmetry using the ATPmetry HS kit (Biothema®) and the luminester C-110 (Kikkoman®). At the end of the incubations performed at the highest pressure conditions (120, 125, 130 and 135 MPa), the presence of viable cells was established by cultivation at atmospheric pressure (75°C). Cell aspect was observed by SEM imaging. Growth rates around the optimal pressure (40, 50, 60 MPa) were derived from a set of experiments performed with sampling at 2 to 3 hours intervals.

The following carbon sources were tested, in triplicate, at 75°C, in Ravot mineral medium supplemented with one of the following compound at a final concentration of 20 mM: D(-)-fructose, D(+)-galactose, D(+)-glucose, D(+)-mannose, D(-)-ribose, D(+)-xylose, dextrose (D(+)-glucose, corn sugar), D(+)-maltose, starch, xylan, methanol (5 mM), fumarate, succinate, pyruvate, acetate, formate, *L*-cystein, *L*-glutamic acid, glycine, *L*-lysin, *L*-methionin, *L*-serine, *L*-valine, peptone, tryptone, yeast extract, beef extract, casamino acids and casein (casein acid hydrolysate, vitamins free). This experiment was performed, on the one hand, with these substrates tested as sole carbon sources and, on the other hand, with 0.05 % (w/v) yeast extract for culture initiation. Unsupplemented medium was used as a negative control.

The ability of strain CDGS^T to use electron acceptors was tested by adding elemental sulphur (12 g/L), L-cystine (12 g/L), sodium thiosulphate (20 mM), sodium sulphate (20 mM), sodium sulphite (5 mM), polysulphur (0.5 mM) [11], sodium nitrate (20 mM), sodium nitrite (5 mM) and oxygen (0.5, 5 and 20% v/v) to a medium prepared without terminal electron acceptors and fermentable compounds.

Carbon source or terminal electron acceptors utilization were confirmed by three subsequent transfers.

Metabolic end products

Respiration and fermentation end products (2-methylbutyrate, 3-hydroxybutyrate, acetate, acrylate, formate, fumarate, gluconate, glycolate, isobutyrate, isovalerate, lactate, oxalate, propionate, succinate) were detected by chromatographic separation from cells grown for 20 h on Ravot medium in presence or absence of an electron acceptor (polysulphur), respectively. Cells were centrifuged and stored in an acid-rinsed glass vial at -20°C. Anions concentrations were determined using a Dionex ICS-2000 Reagent-Free Ion Chromatography

System equipped with an AS50 autosampler (Dionex Camberley UK). Chromatographic separation was conducted using two Ionpac AS15 columns (4 x 250 mm) in series at 30 °C and the determination of species was carried out using an Anion Self-Regenerating Suppressor (ASRS 300 4-mm) unit in combination with a DS6 heated conductivity cell (35 °C). The gradient program was as followed: 8 mM KOH (29.9 min), increase 28.5 mM KOH min-1 to 65 mM (30.1 min), decrease 57 mM KOH min-1 to 8 mM (9 min).

Results

Metabolic end products

Metabolic end products of the fermentation of complex peptides sources were isovalerate (124 µM), acetate (54 µM), propionate (21 µM), formate (13 µM), isobutyrate (9 µM) and 2-methylbutyrate (5 µM), when no terminal electron acceptor was provided. In the presence of polysulphur as an external terminal electron acceptor, isovalerate (820 µM), acetate (698 µM), succinate (611 µM), 2-methylbutyrate (546 µM), formate (157 µM), isobutyrate (93 µM), propionate (92 µM) and fumarate (13 µM) were the major catabolic end products of proteinaceous substrates.

Supplementary Figures and Tables

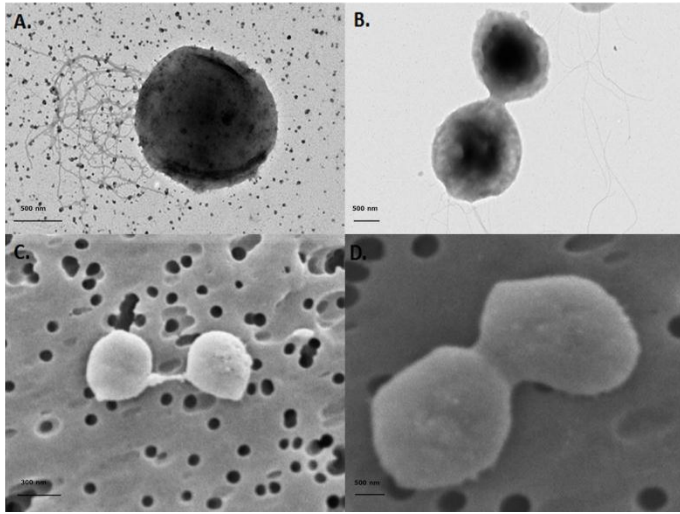


Fig. S1. Transmission (A, B) and scanning (C, D) electron micrographs of cells of strain CDGS^T in exponential growth phase (75 °C) showing division by constriction (B, D).

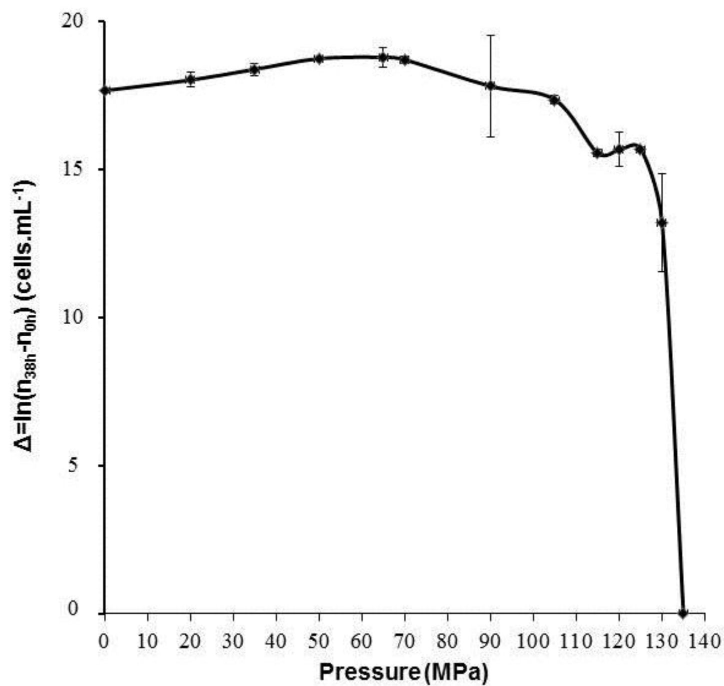


Fig. S2. Cell number evolution over 38 h of incubation at different pressures.

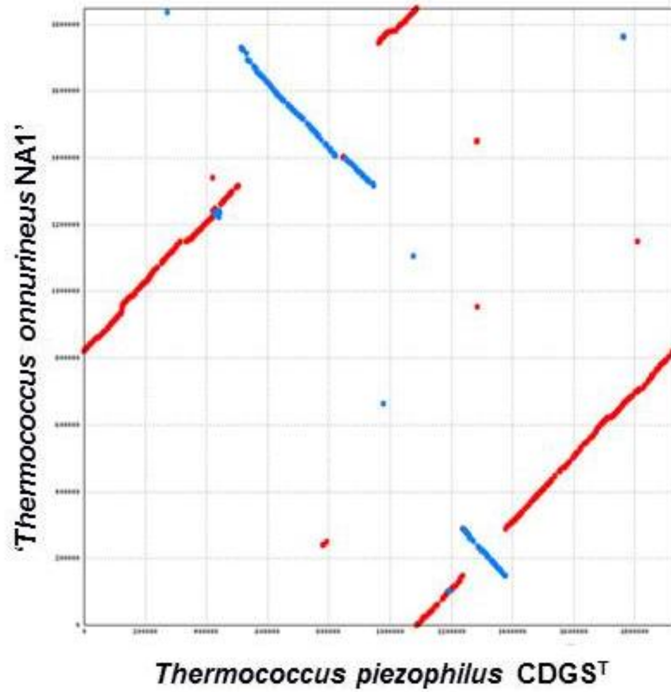


Fig. S3. Graphical representation (dot plot) of the regional self-similarity between the genomes of '*T. onnurineus*' strain NA1 and the novel isolate CDGS^T.

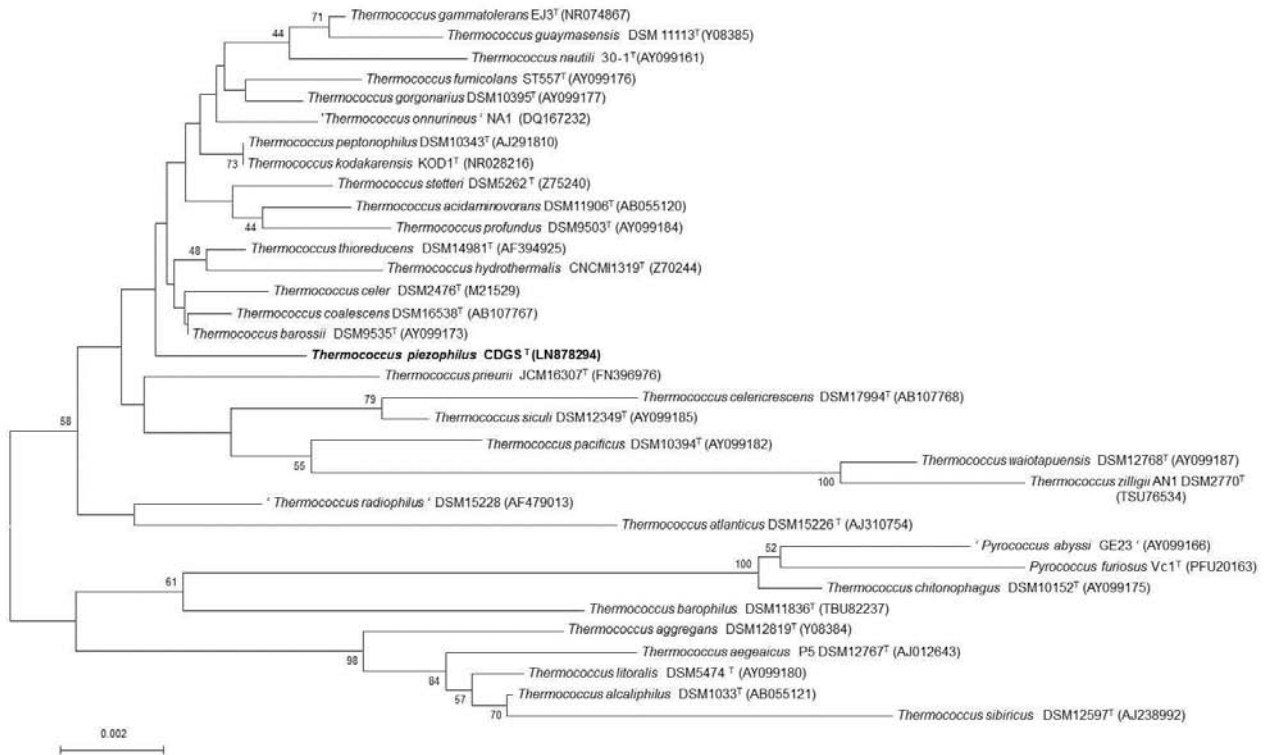


Fig. S4. Phylogenetic tree showing the relationships between strain CDGS^T and other *Thermococcus* species based on a neighbor-joining method with Jukes and Cantor corrections

for the analysis of 16S rRNA gene sequences (1300 nt). The novel isolate is indicated in bold. Accession numbers are given in brackets. Bootstrap values (%) are based on 1000 replicates. Bar, 2 substitutions per 1000 nt.

Table S1. Growth yields after 14 hours of incubation, growth rates (μ) and latency phase (Lag) duration were determined as a function of hydrostatic pressure, for pressures around the optimal pressure for growth.

	40 MPa	**50 MPa**	**55 MPa**	**60 MPa**	**65 MPa**
Growth yield (cell.mL^{-1})	$7.2·10^7$	$4.9·10^8$	$2.9·10^8$	$3.0·10^8$	$3.2·10^8$
	± 0.03	± 0.18	± 0.11	± 0.01	± 0.02
μ (h^{-1})	0.30	0.45	0.38	0.35	0.32
Lag (h)	2	0	1	1	1

Table S2. General features of strain CDGS^T and *'Thermococcus onnurineus'* NA1 genomes

	Strain CDGS^T	**'*T. onnurineus*' NA1**
Isolation source	Deep-sea hydrothermal vent – Cayman Trough	Deep-sea hydrothermal vent – East Manus Basin
Genome size (bp)	1,928,919	1,847,607
GC %	51.11	51.27
Number of CDS	2,418	2,029
Genes classified into Cluster of Orthologous Groups (COG) of functions	1,199	1,100
Average CDS length (bp)	709	831
Protein coding density (%)	87.71	90.65
23S rRNA	1	1
16S rRNA	1	1
5S rRNA	2	2
tRNA	45	46
Extrachromosomal elements	0	0

Table S3. Intracellular ATP content (i[ATP]) per cell at different pressures.

	0.1 MPa	50 MPa	120 MPa	125 MPa	130 MPa	135 MPa
Average cell number (cells/ mL)	$1.2\ 10^{8} \pm 3.3\ 10^{7}$	$1.7\ 10^{8} \pm 1.1\ 10^{7}$	$7.0\ 10^{6} \pm 9.5\ 10^{5}$	$1.8\ 10^{6} \pm 1.5\ 10^{5}$	$1.4\ 10^{6} \pm 1.0\ 10^{5}$	$2.2\ 10^{6} \pm 2.1\ 10^{5}$
[iATP]/cell (pmol)	$4.8\ 10^{-6} \pm 1.8\ 10^{-7}$	$2.1\ 10^{-6} \pm 3.3\ 10^{-7}$	$2.1\ 10^{-6} \pm 2.5\ 10^{-8}$	$1.3\ 10^{-5} \pm 1.3\ 10^{-6}$	$1.1\ 10^{-5} \pm 7.95\ 10^{-11}$	Close to 0