Pyrococcus yayanosii sp. nov., an obligate piezophilic hyperthermophilic archaeon isolated from a deep-sea hydrothermal vent

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An obligate piezophilic anaerobic hyperthermophilic archaeon, designated strain CH1^T, was isolated from a hydrothermal vent site named 'Ashadze', which is located on the Mid-Atlantic Ridge at a depth of 4100 m. Enrichment and isolation of the strain were carried out at 95 °C under a hydrostatic pressure of 42 MPa. Cells of strain CH1^T were highly motile irregular cocci with a diameter of ~1–1.5 µm. Growth was recorded at 80–108 °C (optimum 98 °C) and at pressures of 20–120 MPa (optimum 52 MPa). No growth was observed under atmospheric pressures at 60–110 °C. Growth was observed at pH 6.0–9.5 (optimum 7.5–8.0) and in 2.5–5.5 % (w/v) NaCl (optimum 3.5 %). Strain CH1^T was strictly anaerobic and grew on complex proteinaceous substrates, such as yeast extract, Peptone, and casein, as well as on sucrose, starch, chitin, pyruvate, acetate and glycerol without electron acceptors. The G + C content of the genomic DNA was 49.0 ± 0.5 mol%. Analysis of 16S rRNA gene sequences revealed that strain CH1^T belongs to the genus *Pyrococcus*. Based on its physiological properties and similarity levels between ribosomal proteins, strain CH1^T represents a novel species, for which the name *Pyrococcus yayanosii* sp. nov. is proposed. The type strain is CH1^T (=JCM 16557). This strain is also available by request from the Souchothèque de Bretagne (catalogue LMBE) culture collection (collection no. 3310).

Since the discovery of deep-sea hydrothermal vents and so-called black smokers, many mesophilic, thermophilic and

hyperthermophilic micro-organisms of the superkingdoms *Bacteria* and *Archaea* have been described. Although vent fields have been explored at depths ranging from 800 to 3600 m, rather few attempts have been made to enrich isolates under *in situ* pressures. Almost all thermophilic and hyperthermophilic vent-associated prokaryotes have been isolated under atmospheric pressure and few of them have been exposed to elevated hydrostatic pressures during culture. To our knowledge, only a few piezo-thermophilic

Abbreviation: CRP, concatenated ribosomal protein.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CH1^T is EU682399.

Three supplementary figures and two supplementary tables are available with the online version of this paper.

organisms have been described so far: *Thermococcus barophilus* (Marteinsson *et al.*, 1999a), *Palaeococcus ferrophilus* (Takai *et al.*, 2000), *Marinitoga piezophila* (Alain *et al.*, 2002), *Methanopyrus kandleri* (Takai *et al.*, 2008) and two chemolithoautotrophic strains (Takai *et al.*, 2009). *T. barophilus* and *M. piezophila* were isolated after enrichment cultures were grown at high temperatures and under elevated hydrostatic pressures, and showed highest growth rates when grown under hydrostatic pressures. Even if these organisms were able to grow under atmospheric pressure, their growth rates were much slower. Moreover, *T. barophilus* expressed a stress protein when grown under atmospheric pressure (Marteinsson *et al.*, 1999b) and *M. piezophila* formed long filaments.

In March 2007, black smoker samples were collected during the Serpentine cruise at a depth of 4100 m at the Ashadze site (12° 58.4' N 44° 51.8' W) located on the Mid-Atlantic Ridge (MAR) (Fouquet *et al.*, 2007). Samples were collected using the ROV Victor 6000, which was operated from the *Pourquoi Pas?* research vessel. The samples were immediately placed in a sterile sampling box, previously filled on-board with sterile freshwater, which was opened as close as possible to the sampling site and then tightly closed with a lid. Once on board, chimney samples were immediately crushed under sterile conditions in an anaerobic chamber and then immediately distributed into syringes containing artificial seawater, which was reduced with Na₂S. The samples were then pressurized at 40 MPa and placed at 4 °C for storage prior to analysis. The whole procedure was carried out anaerobically. In the laboratory, samples were depressurized on ice at room temperature and 0.5 to 1 ml of the rock suspensions were rapidly transferred for initial enrichment into 10 ml syringes loaded with modified MJYP medium (Takai *et al.*, 2000), which was adjusted to pH 7 and supplemented with 20 mM NaNO₃, 5 mM FeSO₄ and 1 mg rezasurin l⁻¹ for monitoring anaerobiosis. The syringes were then placed into the high-pressure/high-temperature incubation system, which was custom-built by Top Industrie (Dammarié-les-Lys, France), pressurized to 42 MPa (corresponding to the *in situ* pressure) and heated at 85, 95 or 105 °C (Marteinsson *et al.*, 1997). All experiments were conducted in duplicate.

After 2 days of incubation under strictly anaerobic conditions, a growing culture was observed and an isolate, designated strain CH1^T, was successfully purified using the dilution-to-extinction technique under 42 MPa pressure at 95 °C. The purity of the isolate was confirmed by microscopic observation and by cloning and sequencing the 16S rRNA gene. Cells of strain CH1^T were obtained during exponential growth phase and stored anaerobically in 1.8 ml cryotubes for long-term storage, at -80 °C, after addition of dimethyl sulfoxide (5%, v/v) (Sigma) as cryoprotectant.

Cells of strain CH1^T were observed using a light microscope equipped with a phase-contrast oil-immersion objective (model BH2; Olympus) and appeared as slightly irregular

cocci, 1–1.5 µm in diameter, that divided by constriction and occurred singly or in pairs but sometimes in line. The cells were highly motile and specific coloration, using the Spot Test Flagella kit (Difco), revealed a polar tuft of flagella (Supplementary Fig. S1, available in IJSEM Online). The Gram staining reaction was negative.

Since better growth occurred in *Thermococcales* Rich Medium (TRM; Zeng *et al.*, 2009), all further investigations for determination of growth parameters under high hydrostatic pressures were conducted in 5 ml syringes containing TRM inoculated with 1% late-exponential phase culture (~1.10⁸ cells ml⁻¹) grown under 42 MPa pressure at 95 °C. Cells grown under specific conditions were collected at regular intervals and growth was estimated by direct cell counting using a Thomas chamber (depth 0.02 mm). All samples were tested in duplicate.

Strain CH1^T grew only under strictly anaerobic conditions and within a temperature range of 80 to 108 °C with optimum growth at 98 °C. It grew under high-pressure conditions within the range of 20 to 120 MPa with an optimum of 52 MPa when tested at optimal growth temperature of 98 °C. No growth was observed at pressures below 20 MPa or above 120 MPa (Supplementary Fig. S2). Strain CH1^T required salt and grew at concentrations ranging from 2.5 to 5.5% (w/v) NaCl, with an optimum of ~3.5%. No growth was observed below 2.5% or above 5.5% (w/v) NaCl. The isolate grew at pH 6.0–9.5, with an optimum of ~pH 7.5–8. No growth was detected at pH 5.5 or 10.

Growth requirement and substrate utilization tests were performed in syringes using exponential-phase cells at a final concentration of 5 × 10⁵ cells ml⁻¹. Incubations were carried out at optimal pressure (52 MPa) and temperature (98 °C) for growth. Various single carbon sources were added to a basal TRM (pH 7.5), supplemented with a small amount of yeast extract (0.002%, w/v) for culture initiation. The following carbon sources, all at a final concentration of 0.2% (w/v), were tested: yeast extract, Peptone, Tryptone, casein, glycogen, starch, chitin, acetate, formate, propionate, pyruvate, succinate, cellobiose, galactose, glucose, maltose, sucrose, ethanol, glycerol, Casamino acids and tryptophan.

Nitrogen sources were tested on TRM mineral medium prepared without NH₄Cl and Tryptone but with sucrose (0.2%, w/v) as the carbon and energy source and 5 g sulfur l⁻¹ as the electron acceptor. Hydrolysis of urea, glutamate and gelatin was determined by testing each substrate at a concentration of 0.2% (w/v), while utilization of NH₄Cl, KNO₃ and KNO₂ was determined by using 20 mM of each substrate added to the basal medium. To examine the ability of the isolate to grow in the absence of elemental sulfur, cells were cultured in TRM medium without sulfur; L-cystine (5 g l⁻¹) and thiosulfate (20 mM) were also tested. Nitrate, sulfate and thiosulfate were tested as potential electron acceptors with succinate as a carbon source.

Growth was recorded after 2 and 4 days of incubation. Negative (uninoculated TRM) and positive (inoculated

TRM containing yeast extract and Peptone) controls were performed for each substrate. All tests were performed in duplicate. For all tests, positive cultures were transferred (10% inoculum) into the test media for confirmation of growth. The results of these experiments are presented in Table 1. No growth was observed on the control medium containing yeast extract (0.002%) only.

Strain CH1^T used a variety of proteinaceous substrates and carbohydrates as carbon and energy sources since sulfate, nitrate and thiosulfate were not used as electron acceptors. Growth was greatly enhanced by the addition of sulfur and

cystine but was only slightly enhanced by thiosulfate. In all cases, hydrogen sulfide was produced.

For genomic analyses, DNA was prepared from cells harvested at the end of the exponential phase using chemical extraction (Barbier *et al.*, 1999).

The G + C content of the DNA was determined by thermal denaturation (Marmur & Doty, 1962) using the conditions reported by Raguénès *et al.* (1997). A calibration curve was obtained by using ultrapure DNA (Sigma) from *Escherichia coli* Strain B (50 mol%), *Clostridium perfringens* (26.5 mol%)

Table 1. Characteristics of strain CH1^T and related members of the genus *Pyrococcus*

Taxa: 1, strain CH1^T; 2, *P. furiosus* Vc1^T (=DSM 3638^T); 3, '*P. abyssii*' GE5 (=CNCM I 1302); 4, *P. horikoshii* JCM 9974^T; 5, *P. glycovorans* AL585^T (=CNCM I-2120^T). All strains grew with complex substrates as sole carbon and energy sources including yeast extract, peptone and tryptone. +, Positive; –, negative; (+), weakly positive. ND, not determined.

Characteristic	1	2	3	4	5
Geographical origin	Ashadze, Mid-Atlantic Ridge	Vulcano Island, Italy	North Fiji Basin, SW Pacific Ocean	Okinawa Trough, Pacific Ocean	13° N, East Pacific Rise
Depth (m)	4100	0	2000	1395	2650
Motility/morphology	Highly motile cocci	Slightly irregular cocci	Highly motile cocci	Irregular cocci	Cocci
Arrangement of flagella	Polar tuft of flagella	Monopolar polytrichous flagella	Polar tuft of flagella	Polar tuft of flagella	Polar flagella
Metabolism	Strictly anaerobic	Strictly anaerobic	Anaerobic	Obligate anaerobe	Strictly anaerobic
Size (mm)	0.6–1.4	0.8–2.5	0.8–2	0.8–2	0.5–1.5
Growth conditions:					
Temperature range (°C)	80–108	70–103	67–102	80–102	75–104
Optimum temperature (°C)	98	100	96	98	95
pH range	6–9.5	5–9	4–8.5	5–8	2.5–9.5
Optimum pH	7.5–8	7	6.8	7	7.5
NaCl range (% w/v)	2.5–5.5	0.5–5	0.7–5	1–5	1.7–5.2
Optimum NaCl (% w/v)	3.5	2	3	2.4	2.6
Pressure range (MPa)	20–120	0.1–30	0.1–50	0.1–40	0.1–45
Optimum pressure	52	10	20	0.1	10
Doubling time (min)	50	37	33	32	30
DNA G + C content (mol%)	49	38	45	44	47
Carbon and energy sources					
Casein	+	+	+	+	–
Cellobiose	+	+	–	–	+
Sucrose	+	+	+	–	+
Glucose	(+)	–	–	–	+
Maltose	–	+	+	–	+
Starch	+	+	+	–	+
Chitin	+	–	–	ND	+
Pyruvate	+	+	+	–	–
Glycerol	+	–	ND	–	ND
Acetate	+	–	ND	ND	ND
Casamino acids	–	(+)	+	–	+
Tryptophan	–	–	–	+	(+)
Growth with elemental sulfur	Enhanced	Enhanced	Enhanced	Greatly enhanced	Significantly enhanced

and *Micrococcus luteus* (72 mol%) as standards. The G+C content of the genomic DNA of strain CH1^T was 49.0 ± 0.5 mol%.

16S rRNA genes were amplified using the archaeal 16S rRNA gene universal primers A8f (5'-CGGTGGATCCT-GCCGGA-3') and U1492r (5'-GTTACCTTGTTACGAC-TT-3'). Analysis of the 16S rRNA gene sequence of strain CH1^T (1424 bp) indicated that it belonged to the genus *Pyrococcus* and was most closely related to *Pyrococcus furiosus*, sharing 99.4 % sequence similarity (Supplementary Fig. S3). High 16S rRNA gene sequence similarity levels between two species of the genus *Pyrococcus* is common and was also observed between '*P. abyssi*' and *P. horikoshii* (Supplementary Table S1). With such high 16S rRNA gene sequence similarities, DNA–DNA hybridization should normally be performed (Stackebrandt & Goebel, 1994; Rosselló-Mora & Amann, 2001); however, in the present study, DNA could not be obtained in the amounts needed to perform DNA–DNA hybridization as strain CH1^T could only grow under pressure and, as such, cultures only reached low cell densities. Consequently, an alternative method was performed. It has been demonstrated that ribosomal proteins are good phylogenetic markers (Matte-Tailliez *et al.*, 2002; Brochier-Armanet *et al.*, 2008) and, in analyses, give high resolution at the species level. A complete genome sequencing project of strain CH1^T has been completed (Jun *et al.*, 2011). Ribosomal protein sequences were retrieved from available contigs of the raw genome sequence using the tblastn option in the BLAST program (Altschul *et al.*, 1990). Ribosomal protein sequences (59 in total) common to strain CH1^T, '*P. abyss*', *P. horikoshii*, *P. furiosus* and *Thermococcus kodakaraensis* were concatenated to produce a single protein sequence of ~8100 residues. Concatenated ribosomal protein (CRP) sequences (Supplementary Table S2) were aligned using CLUSTAL_X (Thompson *et al.*, 1994). CRP sequence similarities were obtained by pairwise alignment using different protein matrices: identity, Blosum 50, PAM 250 and Gonnet. CRP sequence similarities were compared with similarity levels between 16S rRNA gene sequences and with published results of DNA–DNA reassociation levels between already characterized members of the genus *Pyrococcus*. A phylogenetic tree based on CRP sequences was reconstructed using the program PHYLO-WIN (Galtier *et al.*, 1996) with *T. kodakaraensis* as an outgroup.

The type of substitution matrix used did not change the results significantly (Supplementary Table S2). When each couple of reference species was compared for both DNA–DNA reassociation and CRP sequence similarity levels, it appeared that the highest CRP similarity level (96.2 %) was observed between '*P. abyss*' and *P. horikoshii*. All other CRP sequence similarity levels between strain CH1^T and other reference species were lower (92.2–92.8 %) indicating that strain CH1^T was a distinct species. In addition, the phylogenetic tree based on CRP sequences clearly separated strain CH1^T from reference strains of the genus *Pyrococcus*, having a very strong tree topology with 100 % bootstrap

support for the node separating CH1^T from other members of the genus *Pyrococcus* (Fig. 1).

In conclusion, *P. yayanosii* appears to be the most thermophilic species among members of the order *Thermococcales* but is less thermophilic than the record holder, the piezophilic methanogen *Methanopyrus kandleri* (Takai *et al.*, 2008).

Following discussions held at the meetings of the International Committee on Systematics of Prokaryotes (ICSP) and its Judicial Commission (JC) in San Francisco in 2005, a committee, consisting of the Chairman of the ICSP, the Chairman of the JC of the ICSP and the Editor in Chief of the *International Journal of Systematic and Evolutionary Microbiology*, have granted an exception, in this instance, to Rule 27(3) of the Bacteriological Code governing the deposit of a type strain in two recognized collections in two different countries. *Pyrococcus yayanosii* CH1^T is available on request from the 'Souchthèque de Bretagne' culture collection. Attempts are still under way to complete the deposition of the type strain in a second recognized culture collection.

Description of *Pyrococcus yayanosii* sp. nov.

Pyrococcus yayanosii (ya.ya.nos'i.i. N.L. gen. n. *yayanosii* of Yayanos, in honour of Aristides Yayanos, a pioneer in the study of piezophilic bacteria).

Cells are motile cocci (1–1.5 µm in diameter) with a polar tuft of flagella. Growth occurs at 80–108 °C (optimum 95 °C) and under 20–120 MPa hydrostatic pressure (optimum 52 MPa). No growth occurs at any temperature at pressures below 20 MPa. Growth occurs at pH 6.0–9.5 (optimum pH 7.5–8.0) and in 2.5–5.5 % (w/v) NaCl (optimum 3.5 %). Growth occurs under strictly anaerobic conditions by fermentation of simple or complex substrates. Nitrate, sulfate and thiosulfate are not used as electron acceptors. Yeast extract, Peptone, Tryptone, cellobiose, sucrose, starch, chitin, pyruvate, acetate and glycerol are used as substrates for fermentation. Maltose, Casamino acids and tryptophan are not utilized. Hydrogen sulfide is produced when elemental sulfur, thiosulfate or cystine is added to the media. Growth is enhanced by elemental sulfur. Urea, glutamate, gelatin, NH₄Cl, KNO₃ and KNO₂ were not used as nitrogen sources.

Fig. 1. Neighbour-joining phylogenetic tree based on concatenated ribosomal protein sequences (8016 sites) of members of the genus *Pyrococcus*. *Thermococcus kodakaraensis* was used as an outgroup. Bootstrap values, based on 500 replicates, are given at branch points. Bar, 3.1 % sequence divergence.

The type strain, CH1^T (=JCM 16557^T), was isolated from an active deep-sea hydrothermal chimney at a depth of 4100 m at the Ashadze site on the Mid-Atlantic Ridge (12° 58.4' N 44° 51.8' W). The type strain is also available on request from the 'Souchothèque de Bretagne' (catalogue LMBE) culture collection (<http://www.ifremer.fr/souchotheque/>). The DNA G + C content of the type strain is 49 ± 0.5 mol%.

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