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Biochemical and phylogenetic characterization of a novel terrestrial hyperthermophilic archaeon pertaining to the genus *Pyrococcus* from an Algerian hydrothermal hot spring

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Abstract A hyperthermophilic anaerobic archeon, strain HT3, was isolated from hydrothermal hot spring in Northeast Algeria. The strain is a regular coccus, highly motile, obligatory anaerobic, heterotrophic. It utilizes proteinaceous complex media (peptone, tryptone or yeast extract). Sulfur is reduced to Hydrogen sulfide and enhances growth. It shares with other *Pyrococcus* species the heterotrophic mode of nutrition, the hyperthermophily, the ability to utilize amino acids as sole carbon and nitrogen sources and the ether lipid composition. The optimal growth occurs at 80–85°C, pH 7.5 and 1.5% NaCl. The G + C content was 43 mol%. Considering its morphology, physiological properties, nutritional features and phylogenetic analyses based on 16S rRNA gene sequencing, this strain is described as a new terrestrial isolate pertaining to the genus *Pyrococcus*.

Keywords Archaea · Hyperthermophile · *Pyrococcus* · Hydrothermal hot spring

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Introduction

The Thermococcales order constitutes a distinctive and unique group of hyperthermophilic microorganisms belonging to the Protoarchaea class, the Eurythermea Superclass, the Euryarchaeota subdivision within the Archaeabacteria division (Woese et al. 1990; Cavalier-Smith 2002). These microorganisms are obligately heterotrophic, sulfur-dependant hyperthermophilics and grow at temperatures from 50 to over 100°C. This archaeal order is currently composed of two major genera: *Thermococcus* (Zillig et al. 1983) and *Pyrococcus* (Fiala and Stetter 1986), and a third genus, *Paleococcus* (Takai et al. 2000).

Species belonging to the genus *Pyrococcus* have been isolated from various deep-sea and shallow marine hydrothermal areas. All of these strains have quite similar physiological characteristics (Godfroy et al. 1997). Only five species have been described: *Pyrococcus woesei*, *Pyrococcus furiosus, Pyrococcus horikoshii*, "*Pyrococcus abyssi*", *Pyrococcus glycovorans* (Zillig et al. 1987; Fiala and Stetter 1986; González et al. 1998; Erauso et al. 1993; Barbier et al. 1999). It has been proposed to rename the hyperthermophilic *P. woesei* as *P. furiosus* spp. *woesei* (Kanoksilapatham et al. 2004). These species are able to use proteinaceous carbon substrates in the absence or presence of elemental sulfur, which presence seems to enhance growth. *Thermococcus zilligii* (Klages and Morgan 1994; Rominus et al. 1997) and *Thermococcus waiotapuensis* (González et al. 1999) isolated from New Zealand hot springs were the unique terrestrial species described to date.

In this paper, we describe a new hyperthermophilic archeon, the strain HT3, isolated from "El Biban" hot spring. This strain is a highly motile, coccoid obligatory anaerobic, heterotrophic and sulfur reducing archaeum.

Materials and methods

Sample collection

Samples of water were collected from the hydrothermal alkaline hot spring ''El Biban'' situated in the Northeast of Algeria. The samples were collected at a depth of 13 m by submersing sterile glass bottles. The content of each bottle was transferred into N_2 filled sterile penicillin flasks, sealed with black butyl rubber stoppers, and containing a few drops of neutralized 10% Na_2S reducing solution, by using a syringe. Flasks were transported to the laboratory at ambient temperature.

Culture conditions

Strict anaerobic procedures were followed according to Balch and Wolfe (1976) enrichment cultures and cultivations were carried out in 20-ml penicillin flasks.

The strain HT3 was grown at 80°C in BHI-S liquid medium in closed glass bottles. The culture medium contained (per liter) 9.25 g brain/heart infusion (BHI), 15 g NaCl, 5 g elemental sulfur, 0.7 g KCl, 0.2 g $CaCl_2$, 10 ml of a minimal mineral solution [3 g/l $MgSO_4 \cdot 7H_2O$, 0.1 g/l $ZnSO_4$] were added in presence of 1 mg of resazurin. Inocula were routinely grown in closed glass bottles at pH 7.5 (6.05 g/l PIPES [piperazin-*N*, *N*′ bis(2-ethanesulfonic acid)] buffer). The medium was sterilized by tyndallization (40 min at 100°C three times spaced by at least 12-h intervals) and the inoculation was performed in an anaerobic chamber (Plas Labo, Lansing, MI, USA) filled with a $N_2/H_2/CO_2$ (90/5/5) gas atmosphere. The final anaerobic conditions were achieved by the addition of 20 ml/l of 2.5% (w/v) solution of $Na_2S \cdot 9H_2O$.

Isolation

Enrichments were checked by microscopic observations. Pure cultures were obtained by serial dilutions of positive enrichments (Antoine et al. 1995). The cultures were finally plated onto Gelrite™ solid medium. After incubation of the plates, each isolated colony was transferred and grown in liquid 2216 S (Belkin and Jannasch 1985) and BHI media then plated once more. This purification step was repeated three times. Estimation of the purity of the isolates was conducted on the basis of microscopic examination and the presence of a single colony type after repeated transfers in liquid and onto solid medium.

Storage

The pure isolate was transferred and stored at room temperature. Cultures in exponential growth phase were stored at 4°C after the gas phase had been briefly flushed with N_2 in order to remove the H_2S produced during growth. These cultures could remain active for at least 1 year. For long-time storage, pure cultures were stored anaerobically at − 80°C in culture medium containing 20%(v/v) glycerol.

Micrography of strain HT3

Phase contrast microphotographs of strain HT3 in mid-log phase from a BHI + S liquid culture were taken with an Olympus model BH-2 microscope.

Cell counts and determination of growth kinetics

Growth was determined by direct cell counting using a Thoma chamber hemocytometer (depth 0.02 mm) and phase contrast microscopy at a magnification of 400 (Olympus model BH-2 microscope).

Determination of growth kinetics was performed according to González et al. (1998). Growth rates were obtained from a regression line of ln N plotted against t, where N is the number cell and t is the incubation time.

Determination of growth parameters

HT3 was grown in black butyl rubber stoppers sealed penicillin flasks containing 40 ml of 2216S medium. The pH and salinity growth curves were determined at 80°C. For the determination of specific growth rates at different pH the following chemicals (Sigma) were used to buffer the medium, each at 10 mM: pH 5–6: MES; pH 6.5–7.5: PIPES; pH 8–8.5: HEPES and pH 9 Glycine–NaOH. The pH was adjusted with NaOH or HCl. Salt requirement was determined on both 2216S and BHI + S media with different concentrations of NaCl. Three replicates were simultaneously studied at each temperature, pH and salinity.

Determination of growth requirements

HT3 was grown in black butyl rubber stoppers sealed penicillin flasks containing 10 ml of 2216S medium in which yeast extract and peptone were replaced by 0.4 g ammonium chloride per liter, 10 ml/l of mineral solution, 10 ml/l of vitamin solution (Godfroy et al. 1997) in order to determine the ability of isolate to utilize various carbon sources. Tubes were inoculated with an exponentially growing culture at a final concentration of 5×10^5 cells/ml and incubated at 80°C. Individual carbon sources were added to the mineral base medium (Godfroy et al. 1997) 2216S supplemented with sulfur (0.5%). Most of the carbon sources were tested at concentration of 5 g/l, the exception being starch, which was tested at concentration of 10 g/l. In this experiment, the headspace gas was $N_2/CO_2/H_2$ (90/5/5). To check the

possible growth by fermentation or by other energy yielding reactions, these tests were run with and without sulfur added.

Some nitrogenous compounds were tested for suitability as a nitrogen source, using ammonium free mineral medium with 40 mM pyruvate as the carbon and energy source, and with 0.05% yeast extract to stimulate growth. Nitrogenous compounds were added at 20 mM final concentration except for organic complex which was added at 0.04% (w/v).

Antibiotic sensitivity

The sensitivity to antibiotics was tested at 80°C for 12 h in both BHI + S and 2216S media at concentrations of 50, 100 and 150 µg/ml. Controls were performed with an antibiotic sensitive eubacterium, *Thermotoga maritima* cultivated under the same conditions to demonstrate the efficiency of the antibiotic at the test temperature. The selected antibiotics (ampicillin, amoxicillin, cephalosporin (GIII), gentamycin, rifampicin and oxacillin) were tested according to Erauso et al. (1993).

Oxygen sensitivity

The culture conditions were the same as previously described, but with air in the penicillin flasks headspace.

The oxygen effect on growth is determined by cell counting in Thoma chamber after 12 h at 80°C (Erauso et al. 1993).

Analysis of sulfur metabolic products

Samples for H_2S determination were stabilized as ZnS by combining the samples with an excess of $ZnSO_4$ in 1 N NaOH (Ingoverson and Jörgensen 1979). Sulfide detection was carried out according to Cline (1969).

Lipid analysis

The fatty acids analysis was performed by gas chromatography of the methyl esters by DSMZ (Deutsche Sammlung von Mikororganism und Zellkulturen GmbH, Braunschweig, Germany) services on a sample from a 400 ml culture on BHI + S. Cells were collected after two centrifugations: first at 5,500 rpm for 10 min (Sigma 3 K18 Bioblock Scientific) and the second at 10,000 rpm for 10 min in an Eppendorf tube, washed twice in water and freeze-dried in a cryotube overnight at − 80°C, Freeze drying was performed in an EC apparatus Inc. Super Modulyo device.

Core ether lipids were analyzed by the whole cell acid methanolysis method (De Rosa and Gambacorta 1988). Lyophilized cells (100 mg) were mixed with 3.5 ml toluene, 3.5 ml of methanol, and 0.1 ml concentrated H_2SO_4 in a reaction tube. The tube was heated at 50°C overnight, and then core lipids were extracted with 1.5 ml hexane. The sample was evaporated and the residue was redissolved in a small amount of chloroform. The samples were analyzed by thin layer chromatography on silica gel plates (Merck Kieselgel 60; Merck, NJ, USA) developed in petroleum ether–diether (85:15, v/v). The lipids resolved on the plates were visualized with 10% (v/v) phosphomolybdic acid in ethanol.

Isolation of DNA and DNA base composition

Cells were cultured in 5 × 800 ml of 2216S medium at 80°C and harvested at the end of the exponential growth phase. After centrifugation, the cell pellet was suspended in 5 ml of lysis buffer (100 mM Tris–HCl, 100 ml NaCl, 0.5 mM EDTA, pH 8). 1% (w/v) Sarcosyl (*N*-lauroylsarcosine, sodium salt) and then 1% (w/v) sodium dodecyl sulfate and proteinase K (final concentration 0.4 mg/l) were added for cell lysis. After 3 h of incubation at 40°C, three phenol–chloroform–isoamyl alcohol (24:24:1) extractions and one chloroform extraction were performed. The DNA was precipitated by adding two volumes of ethanol 70% at − 20°C. The DNA pellet was resuspended in TE buffer (10 mM Tris–HCl, 2 mM EDTA, pH7.4) and treated with RNase (5 µg/l) for 1 h at 60°C. The DNA base composition was determined by thermal denaturation method (Marmur and Doty 1962).

16 S RNA gene sequence analysis

Cells from a 100 ml culture grown for 12 h on BHI + S medium were collected after centrifugation at 5,500 rpm for 10 min (Sigma 3 K18, Bioblock Scientific), resuspended in 0.7 ml supernatant. Isopropanol (0.7 ml) was added as a preservative. The genomic DNA extraction, PCR mediated amplification of the 16S rDNA gene and purification of the PCR product was carried out by the DSMZ services as described by Rainey et al. (1996). Purified PCR products were sequenced using the CEQTM DTCS-Quick Start Kit (Beckmann Coulter) as directed in the manufacturer's protocol. Sequence reactions were electrophoresed using the CEQ™ 8000 Genetic Analysis system.

Phylogenic analysis

A phylogenic analysis was performed on a set of selected sequences of all named species of *Pyrococcus* (five species) and *Thermococcus* (30 species). A total of 37 sequences, including those of strain HT3 and *Thermoproteus tenax* (used as an out group), were aligned using "pileup" (Accelrys GCG Version 11.1, San Diego CA, USA, http://www.accelrys.com). Nucleotide positions that could be unambiguously aligned for all 16S rRNA

genes compared were included in the analysis. The aligned sequences spanned over 1,533 positions. The distances were calculated using "distances" (Accelrys GCG) with the two-parameter correction method (Kimura 1980). A tree was built using Treecon 1.2 for Windows (Van de Peer and De Wachter 1994) by the neighbor-joining method.

Results

Morphology

Observation by phase-contrast microscopy revealed that strain HT3 cells were cocci with a diameter of 0.8–2 μm occurring singly (Fig. 1). During the exponential growth phase cocci were often seen in pairs. The aggregation increased with the culture time. The cells were highly motile.

Growth kinetics

The growth rates of strain HT3, incubated at different temperatures and cultured at various pH and salt concentration are reported in Fig. 2**.** The optimum growth temperature was around 80–85°C, which was the value registered at the hydrothermal hot spring during sampling and the temperature used for culture enrichment. Strain HT3 grew optimally between pH 7 and 8, and pH 7.5 gave the fastest growth. Studies on hyperthermophilic archaea have shown that genera belonging to the Euryarchaeota are viably growing at pH values from 6 to 9 (Veille and Zeikus 2000). The isolate grew in media containing 1–3.5% NaCl with an optimum between 1.5 and 2.0%.

Growth parameters and nutritional requirements

The biochemical characteristics of strain HT3 grown in the presence of elemental sulfur are summarized Table 1. Most of the carbon sources tested gave good growth rates, especially starch, maltose and dextrin III in presence of yeast extract at 0.1%, with the exception of xylose, arabinose, mannitol, xylan and raffinose. In these experiments, the headspace gas was $N_2/CO_2/H_2$ (90/5/5) and the assays were run with and without added sulfur.

The peptone, tryptone, yeast extract, ammonium chloride and some amino acids were suitable nitrogen source. The combination of yeast extract and peptone gave the maximal growth rate (data not shown).

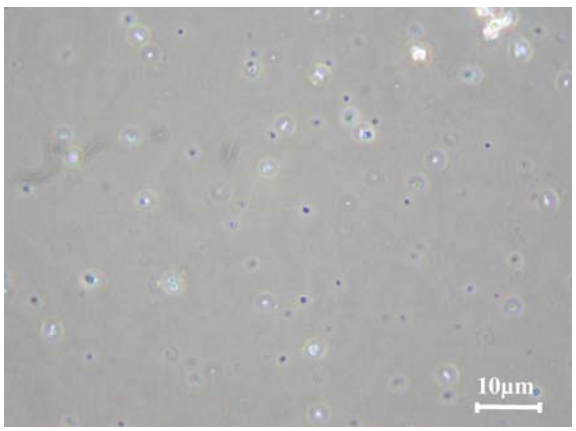

Fig. 1 Phase contrast micrograph of strain HT3. *Bar* = 10 μm

Antibiotic sensitivity

The strain was resistant to the tested antibiotics at concentrations of 50, 100 and 150 μg/ml. Hilpert et al. (1981) have already reported resistance of Archaea's strains towards antibiotics. *Thermotoga maritima* was used to establish the effectiveness of antibiotics at 80°C. It has exhibited the expected pattern of antibiotic sensitivity (Huber et al. 1986).

Oxygen sensitivity

The isolate HT3 did not grow when oxygen or air were present in the culture medium. The survival of the cells under these conditions decreased rapidly within the first 22 h.

Determination of sulfur metabolic products

No growth was observed in sulfur-free medium under either H_2/CO_2 (80/20) or $N_2/CO_2/H_2$ (90/5/5). H_2S was not produced in uninoculated control. The metabolic end product H_2 was inhibitory to growth and the sulfur reduction appeared as a hydrogen sink (Stetter 1998). The solubility of elemental sulfur in water at elevated temperatures is not known (Schauder and Kröger 1993). According to these authors, it is not excluded that the soluble sulfur level reaches values similar to the substrate concentrations under these conditions. Sulfur might penetrate through the bacterial membrane, due to its lipophilic nature, to form polythionates in the presence of O_2, or polysulfides in its absence at the more neutral pH that may prevail in the cytoplasm.

Lipid composition

As expected for members of the kingdom Archaea, the saponification/methylation procedure did not reveal fatty acids in significant quantities (DSMZ analysis). The occurrence of the typical archaeal lipids (phytanylglycerol diethers and dibiphytanyl tetraethers) in strain HT3 was further confirmed by thin layer chromatography (results not shown).

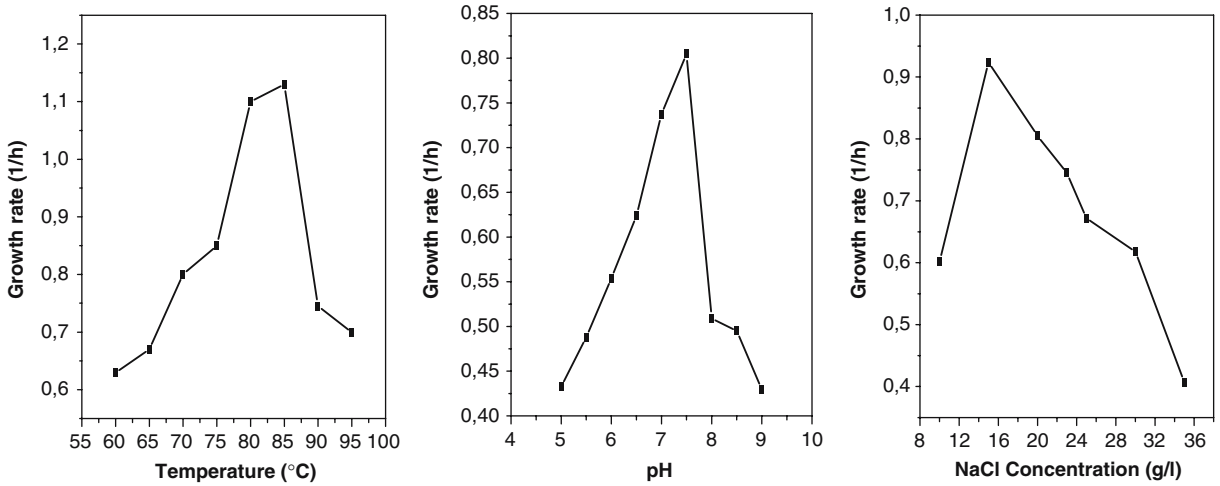

Fig. 2 Influence of temperature, pH and NaCl concentration on strain HT3 growth

Table 1 Biochemical characteristics of strain HT3 in the presence of $N_2/CO_2/H_2$ (90/5/5) and elemental sulfur

Organic substrates[a]					
Glucose	+ +	Trehalose	+	Lactate	+
Galactose	+	Raffinose	–	Sucrose	+ +
Xylose	–	Dextrine III	+ +	Beef extract	+ + +
Arabinose	–	Soluble starch	+ +	Casein hydrolysate	+ +
Sorbitol	+	Glycogen	+	BHI	+ + +
Mannose	+	Pullulan	+	Casamino acids	+
Mannitol	–	Xylan	–	Pyruvate	+ +
Fructose	+ +	Cellobiose	+ + +	Peptone	+ + +
Rhamnose	+	Pectine	+ +	Tryptone	+ + +
Lactose	+	Galacturonic acid	+ +	Amino acids	+ + +
Maltose	+ +	Citrate	–		
Melibiose	–	Acetate	–		

Minerals[b]		Nitrogen sources[c]		Nitrogen sources[c]	
Control[d]	+	Control[e]	+	Alanin	+
$Fe(SO_4)_2$	+	NH_4Cl	+	Aspartic acid	+
$(NH_4)_2SO4$	+	Peptone	+ +	Asparagin	–
$Fe(SO_4)_2 \cdot (NH_4)SO_4$	–	Tryptone	+	Threonine	–
$NaSO_3$	+	Gelatine	–	Isoleucine	+ +
$NaNO_2$	+	Yeast extract	+ + +	Phenylalanine	–
S°	+ + +			Methionine	+ +
Mn^{2+}	+			Arginine	+

Cell counts (22 h growth) per ml of culture: – = no growth; + = 1.5×10^6–1.5×10^7; + + = 1×10^7–2×10^7; + + + = 3×10^7–3×10^8 and $\geq 10^8$. In the absence of S°, no growth occurred for either substrates tested when H_2 or air was present in headspace

[a]Concentration of organic acids and proteinaceous substrates were 0.2% (w/v); sugars and polysaccharides were at 0.5% except for starch (1%); in the presence of 0.1% yeast extract

[b]Minerals were tested on 2216S medium at 20 mM as described by Blumentals et al. (1990) cited by Erauso et al. (1993)

[c]Tested with pyruvate as carbon and energy source in mineral base medium without NH_4Cl; nitrogen sources were at 20 Mm except for complex organics which were at 0.4% (w/v)

[d]Growth occurred by fermentation in controls without electron acceptors

[e]Control with no nitrogen sources added, but trace amount of yeast extract (0.05% w/v)

Phylogenetic analysis

A total of 930 nucleotides of the 16S rRNA gene sequence from strain HT3 were determined. A database search using the Sequence_Match tool version 2.7 on the RDP II server (Online access on Feb. 2006 http://www.rdp8.cme.msu.edu/Cole et al. 2003) revealed that strain HT3 belongs to *Thermococcales* and clusters within the *P. furiosus* subgroup. A phylogenetic analysis of the 16S sequences from strain HT3 and extant

named species from both genera *Pyrococcus* and *Thermococcus* further showed that strain HT3 is associated with the genus *Pyrococcus* (Fig. 3).

The partial 16S rRNA sequence of isolate *Pyrococcus* sp. HT3 has been deposited in the EMBL nucleotide sequence database under accession number AM183944.

DNA base composition

The G + C content of the strain HT3 was 43 mol%. The DNA base composition was determined by thermal denaturation method (Marmur and Doty 1962).

Discussion

The hyperthermophilic strain HT3 isolated from a terrestrial hydrothermal hot spring can be clearly assigned to the archaeal domain (Woese et al. 1990; Holt et al. 1994) on the basis of its resistance to antibiotics (Hilpert et al. 1981), the presence of glycerol diether core lipids and its 16S rDNA sequence. Moreover results clearly indicate that HT3 belongs to the *Thermococcales* order (Zillig 1992).

All known species of *Thermococcales* are anaerobes: they are round and slightly irregular cocci; they preferably use peptides, yeast extract, proteins and, rarely, carbohydrates as carbon sources (Canganella et al. 1997). Members of *Thermococcales* contain simple diether lipids in their membranes that are mainly made up of one or two phospholipids and only the trace of tetraether components (De Rosa and Gambacorta 1988).

Strain HT3 is morphologically similar to others strains of *Pyrococcus*, but it has optimal growth temperature around 85°C, which is the optimal value for growth in the genus *Thermococcus* (Goodfroy et al. 1996, 1997).

The optimal growth temperature of strain HT3 is similar to those of *Thermococcus* sp. (Tachibana et al. 1999), *Archaeoglobus profundus*, *Archaeoglobus fulgidus* and *Thermoproteus tenax* (Oliver and Thomm 2000). Some species of the genus *Pyrococcus* can live at temperatures above of 110°C at slightly alkaline pHs (Barbier 1995). The terrestrial strain *T. waiotapuensis* is unusual in that it shows relatively good growth over a

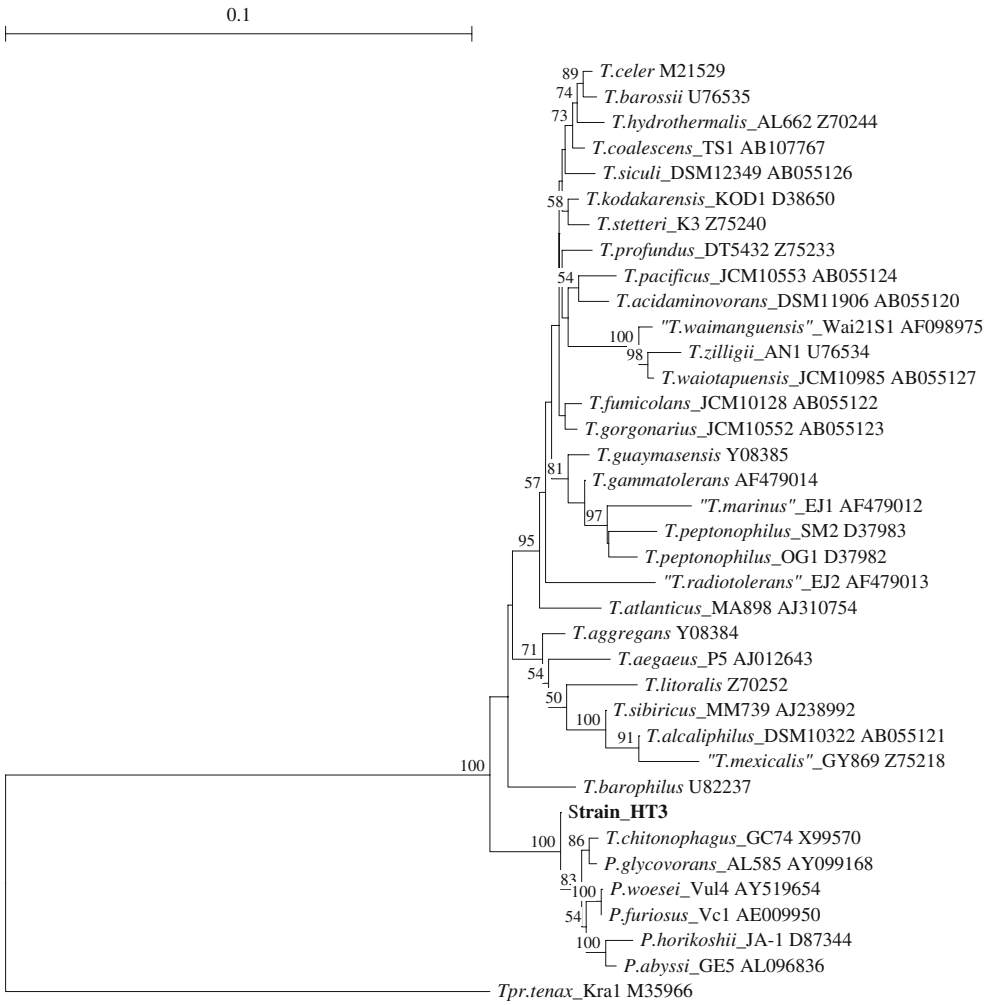

Fig. 3 Phylogenetic relatedness among members of the genera *Pyrococcus* and *Thermococcus* (phylum: Euryarchaeota) based upon 16S rRNA gene sequence comparison. *Thermoproteus tenax* (phylum: Crenarchaeota) was used as the outgroup. The dendrogram was generated by neighbor-joining analysis (Tajima and Nei 1984). *Numbers within the dendrogram* indicate the occurrence percentages of the branching order in 100 bootstrapped trees (only values of 50% and above are shown). *Bar* = 0.1 nucleotide substitution per 100 nucleotides

Table 2 Comparison of the characteristics of strain HT3 with other *Pyrococcus* and two terrestrial *Thermococcus* strains

	P. furiosus[a]	*P. abyssi*[b]	*P. glycovorans*[c]	*P. horikoshii*[d]	HT3	*T. zilligii*[e]	*T. waiotapuensis*[f]
Growth temperature (°C)							
Optimum	100	96	95	98	85	75–80	85
Range	70–103	67–102	75–104	80–102	70–95	55–85	60–90
pH							
Optimum	7	6.8	7.5	7	7.5	7.4	7
Range	5–9	4–8.5	2.5–9.5	5–8	6–9	5.4–9.2	5–8
Salt concentration (%)							
Optimum	2	3	3	2.4	2	0.29	0.54
Range	0.5–5	0.7–5	3–6	1–5	1–2.5	≤ 1.16	≤ 1.39
Sulfur requirements	S°	S°, cystine polysulfides	S°, cystine	S°	S°, cystine	S°, cystine,	S°, cystine, thioglycollate
Carbon sources	Peptone, tryptone, yeast extract, meat extract, extract of eu- and archaebacterial cells, casein, maltose, starch, casamino acids, pullulan, cellobiose	BHI, yeast extract, meat extract, peptone, tryptone, mixture of 20 amino acids	BHI, yeast extract, meat extract, peptone, starch, chitin, maltose, cellobiose, glucose, mixture of 20 amino acids	Yeast extract, peptone, tryptone, beef extract, non-hydrolysed casein, mixture of 21 amino acids	BHI, yeast extract, meat extract, peptone, tryptone, casein, pyruvate, starch, maltose, cellobiose, glucose, mixture of amino acids	Peptides, some amino acids, glucose, pyruvate	Peptides, amino acids, starch, maltose, pyruvate
Required amino acids	Peptides	Peptides	Peptides	Trp	Peptides	Peptides	Arg, His, Ile, Leu, Phe, Ser, Thr, Trp, Tyr, Val
G + C content (mol%)	38	45	47	44	43	46.2	50.4
Resistance to rifampicin	ND	ND	Resistant	Resistant	Resistant	Resistant	Sensitive
Doubling time (min)	37	33	ND	32	35	ND	54
Strain and origin	Vc 1 Porto di Levante Vulcano Island, Italy	GE5 North Fiji Basin, SW Pacific	AL585 Hydrothermal vent East Pacific Rise	JA-1 Hydrothermal vent Okinawa Trough, NE Pacific	HT3 Hydrothermal hot spring, El Biban Algeria	AN1 Freshwater geothermal pool Kuirau Park, Rotorua, New Zealand	WT1 Fresh water hot spring, (Lake Taupo area) New Zealand

Data from: [a]Fiala and Stetter (1986), [b]Erauso et al. (1993), [c]Barbier et al. (1999), [d]González et al. (1998), [e]Klages and Morgan (1994), [e]Rominus et al. (1997) and [f]González et al. (1999)
S° elemental sulfur, *ND* data not available

wide range of temperatures between 75 and 90°C (González et al. 1998).

As reported by Erauso et al. (1993), the pressure could extent the upper growth temperature of these thermophilic organisms. Miller et al. (1988), working with the deep-sea thermophilic methanogen *Methanococcus jannaschii*, have demonstrated that hyperbaric helium pressure up to 750 atm stimulated growth and extended the higher temperature limit for methanogenesis by several degrees (from 90 to 98°C).

The optimal pH for strain HT3 is neutral as with most of *Thermococcales*, exceptions being *Thermococcus alcaliphilus* (optimum 9; Keller et al. 1995) and *Thermococcus fumicolans* (optimum 8; Godfroy et al. 1996), *T. zilligii* was shown to be able to grow up to pH 9.2 (Klages and Morgan 1994; Rominus et al. 1997) whereas *T. waiotapuensis* was reported to be unable to grow above pH 8 (González et al. 1999). The generation time (35 min) of strain HT3 is lower than that of *Thermococcus* spp. with the exception of *T. peptoniphilus* (25 min, González et al. 1995) but close to those of *Pyrococcus* species. Marine strains of *Thermococcales* grow optimally at salt concentration of 20–40 g/l NaCl. It has been reported that *T. zilligii* and *T. waiotapuensis* which were isolated from two New Zealand hot springs have, respectively, a optimal growth at 2.9 and 5.4 g/l NaCl (Rominus et al. 1997; González et al. 1999).

Strain HT3 can utilize carbohydrates especially glucose, in comparison to other species of the genus *Pyrococcus*, and show very good growth on complex carbohydrates, starch, maltose, glycogen, pyruvate, peptone, tryptone, brain heart infusion, yeast extract and some of amino acids taken alone as carbon and nitrogen source.

Nutrient requirements vary considerably among described species of *Thermococcus*. *T. zilligii* is able to grow on complex proteinaceous compounds. *T. waiotapuensis* is able to utilize starch, maltose and pyruvate. *T. zilligii*, the closest *Thermococcus* species to *T. waiotapuensis* requires peptides for growth and is able to utilize glucose and pyruvate (Table 2). *T. waiotapuensis* requires the presence of at least ten amino acids for growth, *T. zilligii* requires complex proteinaceous compounds. Resistance to the antibiotic rifampicin is another difference between strain HT3 and *T. waiotapuensis*. *T. zilligii, P. glycovorans and* "*P. abyssi*" also show resistance to this antibiotic.

The G + C content of the strain HT3 is slightly lower (43%) as compared to those *Thermococcus* and *Pyrococcus*. It is between those reported for coastal strain *P. furiosus* (40.8%), and those of abyssal strains *P. horikoshii* (44%), "*P. abyssi*" (45%) and *P. glycovorans* (47%). As for other *Pyrococcus* spp. it has a short doubling time (35 min).

Characteristics of the strain HT3 and other strains of *Pyrococcus* and terrestrial *Thermococcus* are shown in Table 2.

Strain HT3 was isolated from a geographically specific area in a hydrothermal continental hot spring in Northeast Algeria, at 13 m depth. It has in common with other *Pyrococcus* the heterotrophic mode of nutrition, the hyperthermophily, the ability to use amino acids as sole carbon and nitrogen sources and their ether lipid composition.

The metabolic end products detected were H_2S, CO_2 and H_2. The presence of the latter strongly inhibits the growth of the organism. This effect is abolished in the presence of S°, which is converted into H_2S. *Pyrococcus* is a very efficient consumer of the organic material found on the geothermally heated sea floor of Vulcano Island (Fiala and Stetter 1986).

On the basis of our phylogenetic data (16S rRNA sequence comparison), mol% G + C content, chemotaxonomic analyses, and physiological traits (doubling time, sugar utilization, and morphology), isolate HT3 appears to be a new strain of *Pyrococcus*. Further analysis is required to confirm its taxonomic position.

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References

Antoine E, Guezennec J, Meunier JR, Lesongeur F, Barbier G (1995) Isolation and characterization of extremely thermophilic Archaeabacteria related to the genus *Thermococcus* from deep-sea hydrothermal guaymas basin. Curr Microbiol 31(3):186–192

Balch WE, Wolfe RS (1976) New approach to cultivation of methanogenic bacteria: 2-mercaptoethane-sulfonic acid (HS-CoM)-dependent growth of *Methanobacterium ruminantium* in a pressurized atmosphere. Appl Environ Microbiol 32:781–791

Barbier G (1995) Micro-organismes thermophiles. Bull Soc Fr Microbiol 9:13–18

Barbier G, Godfroy A, Meunier JR, Quérellou J, Cambon MA, Lesongeur F, Grimont PAD, Raguénès G (1999) *Pyrococcus glycovorans* sp. nov., a hyperthermophilic archaeon isolated from the East Pacific Rise. Int J Syst Bacteriol 49(4):1829–1837

Belkin S, Jannasch HW (1985) A new extremely thermophilic sulfur-reducing heterotrophic marine bacterium. Arch Microbiol 141(3):181–186

Blumentals II, Itoh M, Olson GJ, Kelly RM (1990) Role of polysulfides in reduction of elemental sulfur by the hyperthermophilic archaebacterium *Pyrococcus furiosus*. Appl Environ Microbiol 56(5):1255–1262

Canganella F, Jack Jones W, Gambacorta A, Antranikian G (1997) Biochemical and phylogenetic characterization of two novel deep-sea *Thermococcus* isolates with potentially biotechnological applications. Arch Microbiol 167(4):233–238

Cavalier-Smith T(2002) The neomuran origin of archaeabacteria, the negibacterial root of the universal tree and bacterial megaclassification. Int J Syst Evol Microbiol 52(1):7–76

Cline JD (1969) Spectrophotometric determination of hydrogen sulfide in natural waters. Limnol Oceanogr 14:454–458

Cole JR, Chai B, Marsh TL, Farris RJ, Wang Q, Kulam SA, Chandra S, McGarrell DM, Schmidt TM, Garrity GM, Tiedje JM (2003) The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. Nucleic Acids Res 31(1):442–443

De Rosa M, Gambacorta A (1988) The lipids of archaebacteria. Prog Lipid Res 27(3):153–175

Erauso M, Raysenbach AL, Godfroy A, Meunier JR, Crump B, Partensky F, Baross A, Marteinsson V, Barbier G, Pace NR, Prieur D (1993) *Pyrococcus abyssi* sp. nov., a new hyperthermophilic archaeon isolated from a deep-sea hydrothermal vent. Arch Microbiol 160(5):338–349

Fiala G, Stetter KO (1986) *Pyrococcus furiosus* sp. nov. represents a new genus of marine heterotrophic archaeabacteria growing optimally at 100°C. Arch Microbiol 145:56–61

Godfroy A, Meunier JR, Guezennec J, Lesongeur F, Raguénès G, Rimbault A, Barbier G (1996) *Thermococcus fumicolans* sp. nov., a new hyperthermophilic archaeon isolated from a deep-sea hydrothermal vent in the North Fiji basin. Int J Syst Bacteriol 46(4):1113–1119

Godfroy A, Lesongeur F, Raguénès G, Quérellou J, Antoine E, Meunier JR, Guezennec J, Barbier G (1997) *Thermococcus hydrothermalis* sp. nov., a new hyperthermophilic archaeon isolated from a deep-sea hydrothermal vent. Int J Syst Bacteriol 47(3):622–626

González JM, Kato C, Horikoshi K (1995) *Thermococcus peptonophilus* sp. nov., a fast growing, extremely thermophilic archeabacterium isolated from deep-sea hydrothermal vents. Arch Microbiol 164(3):159–164

González JM, Masuchi Y, Robb FT, Ammerman JW, Maeder DL, Yanagibayashi M, Tamaoka J, Kato C (1998) *Pyrococcus horikoshii*, sp. nov., a hyperthermophilic archaeon isolated from a hydrothermal vent at the Okinawa Trough. Extremophiles 2(2):123–130

González JM, Sheckells D, Viebahn M, Krupatkina D, Borges KM, Robb FT (1999) *Thermococcus waiotapuensis* sp. nov., an extremely thermophilic archaeon isolated from a freshwater hot spring. Arch Microbiol 172(2):95–101

Hilpert R, Winter J, Hammes W, Kandler O (1981) The sensivity of archaebacteria to antibiotics. Zentralbl Bakt. Hyg 1 Abt Orig C 2:11–20

Holt JG, Krieg NR, Staley JT (1994) The *Archaeabacteria*. In: Holt JG (ed) Bergey's manual of determinative bacteriology, 9th edn. Williams & Wilkins, Baltimore, pp 747–755

Huber R, Langworthy TA, König H, Thomm M, Woese CR, Sleytr UB, Stetter KO (1986) *Thermotoga maritima* sp. nov. represents a new genus of unique extremely thermophilic eubacteria growing up to 90°C. Arch Microbiol 144(4):324–333

Ingoverson K, Jörgensen BB (1979) Combined measurement of oxygen and sulfide in water samples. Limnol Oceanogr 24:390–393

Kanoksilapatham W, González JM, Maeder DL, DiRuggiero J, Robb FT (2004). A proposal to rename the hyperthermophile *Pyrococcus woesei* as *Pyrococcus furiosus* subsp. *woesei*. Archaea 1:277–283

Keller M, Braun FJ, Dirmeier R, Hafenbradl D, Burggraf S, Rachel R, Stetter KO (1995) *Thermococcus alcaliphilus* sp. nov., a new hyperthermophilic archaeum growing on polysulfide at alkaline pH. Arch Microbiol 164(6):390–395

Kimura J (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120

Klages KU, Morgan HW (1994) Characterization of an extremely thermophilic sulfur metabolizing archaebacterium belonging to the *Thermococcales*. Arch Microbiol 162:261–266

Marmur J, Doty P (1962) Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. J Mol Biol 5:109–118

Miller JF, Shah NN, Nelson CN, Ludlow JM, Clark DS (1988) Pressure and temperature effects on growth and methane production of the extreme thermophile *Methanococcus jannaschii*. Appl Environ Microbiol 54:3029–3042

Oliver O, Thomm M (2000) A simplified method for the cultivation of extreme anaerobic archaea based on the use of sodium sulfite as reducing agent. Extremophiles 4:247–252

Rainey FA, Ward-Rainey N, Kroppenstedt RM, Stackebrandt E (1996) The genus *Nocardiopsis* represents a phylogenetically coherent taxon and a distinct Actinomycete lineage: proposal of *Nocardiopsaceae* fam. nov. Int J Syst Bacteriol 46(4):1088–1092

Rominus RS, Reysenbach A-L, Musgrave DR, Morgan HW (1997) The phylogenetic position of the *Thermococcus* isolate AN1 based on 16S rRNA gene sequence analysis: a proposal that AN1 represents a new species, *Thermococcus zilligii* sp. nov. Arch microbiol 168:245–248

Stetter KO (1998) Hyperthermophiles: isolation, classification and properties. In: Horikoshi K, Grant WD (eds) Extremophiles: microbial life in extreme environments. Wiley, New York, pp 1–24

Schauder R, Kröger A (1993) Bacterial sulphur respiration. Arch Microbiol 159(6):491–497

Tachibana Y, Karumura A, Shirasaka N, Suzuki Y, Yamamoto T, Fujiwara S, Takaji M, Imanaka T (1999) Purification and characterization of an extremely thermostable cyclomaltodextrin glucanotransferase from a newly isolated hyperthermophilic archaeon, a *Thermococcus* sp. Appl Environ Microbiol 65(5):1991–1997

Tajima F, Nei M (1984) Estimation of evolutionary distances between nucleotide sequences. Mol Biol Evol 1(3):269–285

Takai K, Sugai A, Itoh T, Horikoshi K (2000) *Paleococcus ferrophilus* gen. nov., sp. nov., a barophilic, hyperthermophilic archeon from a deep-sea hydrothermal vent chimney. Int J Syst Evol Microbiol 50(2):489–500

Van de Peer Y, De Wachter R (1994) TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. Comput Appl Biosci 10:569–570

Veille C, Zeikus G (2000) Hyperthermophilic enzymes: sources, uses and molecular mechanisms for thermostability. Microbiol Mol Biol Rev 65(1):1–43

Woese CR, Kandler O, Wheelis ML (1990) Towards a natural system of organisms. Proposal for domains Archaea, Bacteria and Eucarya. Proc Nat Acad Sci USA 87(15):4576–4579

Zillig W (1992) The order Thermococcales. In: Balows A, Trüper HG, Dworkin M, Harder W, Schleifer K-H (eds) The prokaryotes. A handbook on the biology of bacteria: ecophysiology, isolation, identification, applications, vol 1, 2nd edn. Springer, Berlin Heidelberg New York, pp 702–706

Zillig W, Holz I, Janekovic D, Schäfer W, Reiter WD (1983) The archaeabacterium *Thermococcus celer* represents a novel genus within the thermophilic branch of the archaebacteria. Syst Appl Microbiol 4:88–94

Zillig W, Holz I, Klenk H-P, Trent J, Wunderl S, Janekovic D, Imsel E, Haas B (1987) *Pyrococcus woesei* sp. nov., an ultrathermophilic marine archaebacterium representing a novel order, *Thermococcales*. Syst Appl Microbiol 9:62–70