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

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A novel hyperthermophilic, anaerobic archaeon, strain Bio-pl-0405IT2\(^T\), was isolated from a hydrothermal chimney sample collected from the East Pacific Rise at 2700 m depth in the ‘Sarah Spring’ area (7\(^\circ\) 25’ 24” S 107\(^\circ\) 47’ 66” W). Cells were irregular, motile cocci (0.8–1.5 \(\mu\)m in diameter) and divided by constriction. Growth was observed at temperatures between 60 \(^\circ\)C and 95 \(^\circ\)C with an optimum at 80 \(^\circ\)C. The pH range for growth was between pH 4.0 and pH 8.0 with an optimum around pH 7.0. Strain Bio-pl-0405IT2\(^T\) grew at salt concentrations of 1–5 % (w/v) \(\text{NaCl}\) with an optimum at 2 %. The novel isolate grew by fermentation or sulphur respiration on a variety of organic compounds. It was a chemoorganoheterotrophic archaeon growing preferentially with yeast extract, peptone and tryptone as carbon and energy sources and sulphur and organic compounds as electron acceptors; it also grew on maltose and starch. Sulphur or \(L\)-cystine were required for growth and were reduced to hydrogen sulfide. The strain was resistant to rifampicin, chloramphenicol, vancomycin and kanamycin (all at 100 \(\mu\)g ml\(^{-1}\)) but was sensitive to tetracycline. The G+C content of its genomic DNA was 53.6 mol\%. Phylogenetic analysis of the almost complete 16S rRNA gene sequence (1450 bp) of strain Bio-pl-0405IT2\(^T\) showed that the novel isolate belonged to the genus Thermococcus. DNA–DNA hybridization values with the two closest relatives Thermococcus hydrothermalis AL662\(^T\) and Thermococcus celer JCM 8558\(^T\) were below the threshold value of 70 %. On the basis of the physiological and genotypic distinctness, we propose a novel species, Thermococcus prieurii sp. nov. The type strain is Bio-pl-0405IT2\(^T\) (=CSUR P577\(^T\)=JCM 16307\(^T\)).

Hydrothermal deep-sea vents represent one of the most extreme environments on Earth. These ecosystems are characterized by steep physicochemical gradients, high hydrostatic pressures, high temperatures, obscurity and prevalence of chemosynthesis. The hydrothermal fluids are acidic, reduced and enriched with heavy metals, methane and hydrogen sulfide. These ecosystems, inhospitable from an anthropocentric point of view, host diverse communities of animals and micro-organisms. Thermococcales are among the most abundant prokaryotes isolated from these singular habitats (Prieur, 2002).

The order Thermococcales is the branch between the order Methanopyrales and the order Methanococcales and belongs to the phylum Euryarchaeota, domain Archaea (Zillig, 1992). At the time of writing, the order Thermococcales includes three genera: Thermococcus, Pyrococcus and Palaeococcus (Achenbach-Richter et al., 1988; Stetter, 1996; Takai & Sako, 1999). Representatives of these genera differ in their 16S rRNA gene sequences, their DNA G+C contents and their optimal growth temperatures. At the time of writing, the order Thermococcales encompasses two species of the genus Palaeococcus, six species of the genus Pyrococcus and 33 species of the genus Thermococcus. Members of the genus Thermococcus are organotrophic, strictly anaerobic hyperthermophiles growing on proteinaceous compounds in the absence or presence of sulphur. Commonly, they exhibit better growth when they are grown with sulphur compounds and produce hydrogen sulphide.

In this study, a new member of the genus Thermococcus originating from a deep-sea hydrothermal vent is described. The novel micro-organism, strain Bio-pl-0405IT2\(^T\), was isolated from a hydrothermal chimney sample collected in 2004, by the submersible Nautilus from the ‘Sarah Spring’ area (at 2700 m depth) (7\(^\circ\) 25’ 24” S 107\(^\circ\) 47’ 66” W) on the East Pacific Rise, during the cruise BioSpeedo.
Onboard, samples were immediately transferred into an anaerobic chamber and placed into sterile flasks filled with reduced sterile seawater under anaerobic conditions (N₂ headspace gas). Enrichment and isolation cultures were performed anaerobically, as described by Balch & Wolfe (1976), in Ravot medium and incubated at 85 °C for 2 or 3 days. The medium contained, per litre of distilled water: 1 g NH₄Cl, 0.2 g MgCl₂·6H₂O, 0.1 g CaCl₂·2H₂O, 0.1 g KCl, 0.83 g CH₃COONa·2H₂O, 20 g NaCl, 5 g yeast extract, 5 g tryptone, 3.45 g piperazine-N,N′-bis-(2-ethanesulfonic acid) (PIPS buffer) and 0.001 g resazurin. The pH was adjusted to 7 before autoclaving. After autoclaving, the following sterile solutions were added aseptically: 5 ml of 6% (w/v) K₂HPO₄ solution and 5 ml of 6% (w/v) KH₂PO₄ solution.

The medium was dispensed (50 ml) into 100 ml sterile flasks and supplemented with 10 g (w/v) elemental sulphur l⁻¹ previously tyndallized. Anaerobiosis was obtained by applying a vacuum to the medium and saturating it with dinitrogen. Finally, a sterile solution of reduced sterile seawater under anaerobic conditions (N₂ headspace gas). Enrichment and isolation cultures were performed anaerobically, as described by Balch & Wolfe (1976), in Ravot medium and incubated at 85 °C for 2 or 3 days. The medium contained, per litre of distilled water: 1 g NH₄Cl, 0.2 g MgCl₂·6H₂O, 0.1 g CaCl₂·2H₂O, 0.1 g KCl, 0.83 g CH₃COONa·2H₂O, 20 g NaCl, 5 g yeast extract, 5 g tryptone, 3.45 g piperazine-N,N′-bis-(2-ethanesulfonic acid) (PIPS buffer) and 0.001 g resazurin. The pH was adjusted to 7 before autoclaving. After autoclaving, the following sterile solutions were added aseptically: 5 ml of 6% (w/v) K₂HPO₄ solution and 5 ml of 6% (w/v) KH₂PO₄ solution.

Thermococcus celer JCM 8558ᵀ, Thermococcus hydrothermalis AL662ᵀ, Thermococcus siliaci DSM 12349ᵀ, Thermococcus gammatolerans JCM 11827ᵀ, Thermococcus barophilus DSM 11836ᵀ, Thermococcus guaymasensis JCM 10136ᵀ were used as reference strains and were provided by the Brittany Culture Collection (UBOCC).

Transmission electron microscopy (TEM) of negatively stained cells was carried out using a 100 CXII electron microscope (JEOL) at an accelerating voltage of 60 kV. To prepare samples for TEM, 5 µl of culture was placed onto a Formvar-carbon grid and the cells were negatively stained with 2% (w/v) uranyl acetate. Scanning electron microscopy (SEM) was performed with a S-3200N scanning electron microscope (Hitachi). To observe thin sections, cells were fixed with 2% glutaraldehyde in the Ravot medium, and post-fixed with 1% osmium tetroxide at 4 °C overnight. The samples were then dehydrated with ethanol, embedded in Spurr’s resin and thin-sectioned as described previously (Honda & Inouye, 2002).

Cells of the novel isolate were irregular coccii and occurred singly or in pairs (Fig. 1). Cells were generally 0.8–1.5 µm in diameter (Fig. 1a). They were motile with a polar tuft of flagella (Fig. 1b) and divided by constriction (Fig. 1c). Thin sections revealed that the cytoplasm was homogeneous after a growth of 12 h (Fig. 1d). Cytoplasmic membranes were typically thick and separated from a 5 nm in thickness cell wall by 10 nm of periplasm (Fig. 1e).

To determine the optimal growth conditions of the novel isolate, temperatures, NaCl concentrations and pH range of the culture medium were varied over a wide range of conditions. These experiments were all carried out under anaerobic conditions, under an atmosphere of N₂ (100%, 100 kPa). Growth was routinely monitored by direct cell counting using a phase-contrast microscope (BX60; Olympus) and a modified Thoma chamber (depth 10 µm). Growth rates were calculated using linear regression analysis from three to seven points along the logarithmic portion of the resulting growth curves. To determine the optimum temperature for growth, cells were grown in Hungate tubes containing 6 ml Ravot medium. Growth temperatures were tested over the range 50 °C–100 °C in thermostated ovens. Strain Bio-pl-0405IT2ᵀ grew at temperatures between 60 °C and 95 °C. No growth was detected at 55 °C or 100 °C and the optimal temperature was 80 °C (Fig. S1a, available in IJSEM Online). The effects of pH and salinity were determined at 80 °C. To determine growth rates at different pH, Ravot medium was modified by using the following buffers (Sigma), each at a concentration of 10 mM: for pH 3.0–4.0, no buffer; for pH 5.0–6.0, MES buffer [2-(N-morpholino)ethanesulfonic acid]; for pH 6.5–7.0, PIPES buffer (piperazine-N,N′-bis-2-ethanesulfonic acid); for pH 7.5–8.5, HEPES buffer [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]; and for pH 9.0, no buffer. The optimal pH for growth was close to pH 7.0. No growth was observed at pH 3.0 or pH 8.5 (Fig. S1b). To determine the optimal NaCl concentration for growth, increasing concentrations of NaCl were added to Ravot medium, over the range 0–10% (w/v) in 1% increments. The optimal NaCl concentration for growth was 2% (w/v) and no growth was observed at 0 or 6% (w/v) NaCl (Fig. S1c). Three replicates were studied simultaneously at each temperature, pH or salinity. Under optimal growth conditions (temperature, pH and NaCl concentration), strain Bio-pl-0405IT2ᵀ grew fast with a generation time of approximately 23 min.

Over a period of 30 h in batch culture, no sudden decrease in cell numbers was observed and the final cell concentration reached a maximum of 9 × 10⁹ cells ml⁻¹. Pressure assays (0.1, 15, 25, 35 and 50 MPa) were also carried out at 80 °C. Syringes containing 10 ml Ravot medium were filled in an anaerobic chamber with 0.2 ml culture in late exponential phase. Samples were processed in duplicate and transferred into a high pressure and temperature incubation system, custom built by Top Industrie (Vaux le Pénil, France). This experiment demonstrated that the novel isolate was able to grow under high pressures; the classical growth curve was maintained at 15, 25 and 35 MPa. At 50 MPa, the cellular concentration was lower (data not shown). According to these results, the novel isolate seems piezotolerant but not piezophilic.
As for all the species of the genus *Thermococcus*, the novel isolate grew under strictly anaerobic conditions. Elemental sulphur or L-cystine was required for growth. Cord-Ruwisch tests demonstrated that sulphur compounds were reduced to hydrogen sulphide (Cord-Ruwisch, 1985). Strain Bio-pl-0405IT2 had no capacity to grow in the presence of thiosulphate, sulphate, sulphite, fumarate, nitrate and nitrite (all at 10 mM). To investigate the capability of the strain to grow with single carbon sources, incubations were performed on Ravot medium prepared with 0.02% yeast extract and without tryptone (minimal medium). The following carbon sources were tested in the presence of elemental sulphur: yeast extract, tryptone, peptone, casein and Casamino acids (all at 12 g l$^{-1}$), starch, cellobiose, glucose, maltose, galactose, glycogen, acetate, pyruvate (all at 20 mM), and methanol and ethanol (both at 0.2% (w/v)). The minimal medium was used as a negative control.

The novel isolate was an obligate organotroph, utilizing complex proteinaceous substrates such as peptone, tryptone and yeast extract as carbon and energy sources. Poor growth was observed on starch and maltose and no growth was observed for other substrates.

Growth of strain Bio-pl-0405IT2 was not inhibited by rifampicin, chloramphenicol, vancomycin and kanamycin at 10 μg ml$^{-1}$, 25 μg ml$^{-1}$, 50 μg ml$^{-1}$, 75 μg ml$^{-1}$ or 100 μg ml$^{-1}$. Growth was inhibited by tetracycline at 25 μg ml$^{-1}$. The susceptibility to antibiotics was determined under the standard growth conditions. *Thermosiphon japonicus* IHB1T (=JCM 10495T) (Takai & Horikoshi, 2000) was used as a bacterial control to check the effectiveness of the antibiotics at 80 °C.

The DNA G+C content of Bio-pl-0405IT2 was determined by HPLC by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). It was 53.6 mol%.

The 16S rRNA gene sequence of the novel isolate was amplified by PCR using the forward and reverse primers 4F specific for Archaea (5′-TCCGATTATCCTGCGGGG-3′) and 1492R universal (5′-GGTACTCCTGTTACGACTT-3′). The reaction was performed in a volume of 25 μl containing 50 ng template, 10 mM of each primer, 10 mM dNTPs, 25 mM MgCl$_2$, 1× buffer (Promega), and 1 U polymerase. The resulting PCR products were purified by

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**Fig. 1.** Micrographs of cells of strain Bio-pl-0405IT2. (a) Scanning electron micrograph of cells; (b) electron micrograph of negatively stained cells; (c–e) thin sections of cells of strain Bio-pl-0405IT2 grown for 12 h in Ravot medium. CM, Cytoplasmic membrane; CW, cell wall. Bars, 1 μm (a), 200 nm (b), 100 nm (c–d), 30 nm (e).
PCR QIAquick (Qiagen) kit and double-strand sequenced (Cogenics Grenoble). The alignment was performed with 1450 bp. The sequence was compared with those available in the public databases by using the BLAST program. A BLAST search (Altschul et al., 1997) against the GenBank database revealed a high level of similarity (>$97\%$) with sequences from other species of the genus *Thermococcus*. Among the order *Thermococcales*, 16S rRNA gene sequence analyses are valuable to separate the three genera, but they do not allow discrimination of species within a genus. Consequently, the novel isolate was concluded to belong to the order *Thermococcales* but further investigations were necessary to determine the precise affiliation within this order. The sequence was then aligned with sequences of closest

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**Fig. 2.** Dendrogram showing the relationship between strain Bio-pl-0405IT2$^\mathrm{T}$ and other members of the order *Thermococcales*. The topology shown corresponds to an unrooted tree calculated by the neighbour-joining algorithm with the modifications of Jukes and Cantor. Bootstrap values are indicated at the branch nodes. Bar, 2 nt substitution per 1000 nt.
relatives obtained from the GenBank database using the BioEdit software (Version 7.0.9.0). A phylogenetic tree showing the relationship of strain Bio-pl-0405IT2T with other species was constructed by the neighbour-joining (NJ) method (Saitou & Nei, 1987) using CLUSTAL W (Thompson et al., 1994) for the alignment and PHYLO_WIN to run the NJ algorithm. Bootstrapping was used to determine the reliability of phylogenetic trees (Fig. 2). Bootstrap resampling was performed 1000 times. Pairwise distance between Bio-pl-0405IT2T sequence and its closest neighbours were respectively 0.006 and 0.008 for Thermococcus celer (JCM 8558T) and Thermococcus hydrothermalis AL 662T.

DNA–DNA hybridizations were carried out by the DSMZ as described by De Ley et al. (1970) with the modifications described by Huss et al. (1983) using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostated 6 × 6 multiecell changer and a temperature controller with in-situ temperature probe (Varian). The DNA–DNA hybridizations with labelled DNA from strain Bio-pl-0405IT2T gave the following values: 33 % with Thermococcus celer JCM 8558T and 34 % with Thermococcus hydrothermalis AL 662T (Table 1). These low values, clearly inferior to the species threshold value of 70 %, strongly suggested that strain Bio-pl-0405IT2T represented a novel species (Wayne et al., 1987).

In comparison with phylogenetically related species of the genus Thermococcus (Table 2), the novel isolate Bio-pl-0405IT2T was distinguishable from T. celer (JCM 8558T) by its ability to grow on starch and maltose, by its optimal temperature for growth and by its requirement for sulphur. It was distinguishable from T. hydrothermalis (AL 662T) by its short doubling time. Strain Bio-pl-0405IT2T can also be distinguishable from the other species by the presence of three extra-chromosomal elements inside the cells. These elements, one virus TP1 (Thermococcus prieurii virus 1; Gorlas et al., 2012) and two plasmids, are double stranded circular DNA (21.5 kb, 3 kb and 2 kb respectively) and were discovered by using the modified alkaline lysis procedure (Birnboim & Doly, 1979) (Fig. S2). So far, very few plasmids and viruses have been described from the order Thermococcales. Only one other virus, PAV1 isolated from ‘Pyrococcus abyssi’ strain GE23, has been described from hyperthermophilic euryarchaeotes (Geslin et al., 2003; 2007). At the time of writing, only eight plasmids have been well described and sequenced. Four plasmids (pGT5, pGE2, PRT1 and pP12-1) were isolated from strains of ‘Pyrococcus abyssi’ and species of the genus Pyrococcus (Erauso et al., 1996; Benbouzid-Rollet et al., 1997; Ward et al., 2002; Soler et al., 2010) and four plasmids (pTN1, pTN2, pT26-2 and pAMT11) were isolated from strains of species of the genus Thermococcus (Soler et al., 2007; Soler et al., 2010; Gonnert et al., 2011). For Thermococcales which are good models for physiological and molecular studies on hyperthermophilic micro-organisms, such elements could provide interesting properties to their hosts by the unusual proteins they encode. Indeed, they are a reservoir of additional genes.

On the basis of the 16S rRNA gene sequence and the low DNA–DNA hybridization values with its closest relatives, strain Bio-pl-0405IT2T represents a novel species of the genus Thermococcus, for which we propose the name Thermococcus prieurii sp. nov.

**Description of Thermococcus prieurii sp. nov.**

Thermococcus prieurii (pri.eu’ri.i. N.L. gen. masc. n. prieurii of Prieur, in honour of Professor Daniel Prieur, in recognition of his important contribution to the microbiology of extreme environments).

Cells are irregular, motile cocci (diameter 0.8–1.5 µm). Cell division occurs by constriction. The doubling time is 23 min. Sulphur or l-cystine is necessary for growth. Does not use thiosulphate, sulphate, sulphite, fumarate, nitrate or nitrite for growth. Growth occurs between 60 and 95 °C (optimum, 80 °C), from pH 4.0 to pH 8.0 (optimum, pH 7.0) and with 1–5.0 % (w/v) NaCl (optimum, 2 %). Strictly anaerobic, obligately chemo-organotrophic and grows preferentially on proteolysis products such as yeast extract, peptone and tryptone compounds. Poor growth is observed on starch and maltose and no growth is observed on cellobiose, glucose, galactose, glycogen, acetate, pyruvate, methanol, ethanol, casein and Casamino acids. Resistant to chloramphenicol, rifampicin, vancomycin and kanamycin (all at 100 µg ml⁻¹), but sensitive to tetracycline (25 µg ml⁻¹).

The type strain, Bio-pl-0405IT2T (≡CSUR P577T=JCM 16307T), was isolated from an active chimney recovered from the ‘Sarah Spring’ hydrothermal site on the East-Pacific Rise. The DNA G+C content of the type strain is 53.6 mol%.

| Table 1: DNA–DNA hybridizations among members of the genus Thermococcus |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Strain                     | DNA G+C content (mol%)      | DNA–DNA hybridization (%) with: |
|                            |                            | Bio-pl-0405IT2T | T. celer JCM 8558T | T. hydrothermalis AL 662T |
| Bio-pl-0405IT2T            | 53.6                       | 100           | 33             | 34             |
| T. celer JCM 8558T         | 56.6                       | 34            | 100            | 26             |
| T. hydrothermalis AL 662T  | 58                         | 29            | 26             | 100            |
Table 2. Characteristics distinguishing strain Bio-pl-0405IT2\(^T\) from phylogenetically related species of the genus Thermococcus

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*Data for reference strains from this study.

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References


