***Pyrococcus glycovorans* sp. nov., a hyperthermophilic archaeon isolated from the East Pacific Rise**

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A hyperthermophilic archaeon, strain AL585^T, was isolated from a deep-sea hydrothermal vent located on the East Pacific Rise at latitude 13 ° N and a depth of 2650 m. The isolate was a strictly anaerobic coccus with a mean cell diameter of 1 µm. The optimum temperature, pH and concentration of sea salt for growth were 95 °C, 7.5 and 30 g l⁻¹. Under these conditions, the doubling time and cell yield were 0.5 h and 5 × 10⁸ cells ml⁻¹. Strain AL585^T grew preferentially in media containing complex proteinaceous carbon sources, glucose and elemental sulfur. The G+C content of the DNA was 47 mol %. Sequencing of the 16S rDNA gene showed that strain AL585^T belonged to the genus *Pyrococcus* and was probably a new species. This was confirmed by total DNA hybridization. Consequently, this strain is described as a new species, *Pyrococcus glycovorans* sp. nov.

Keywords: archaea, *Pyrococcus*, hyperthermophile, deep-sea, hydrothermal vent

INTRODUCTION

From the beginning of the last decade, the scientific community has been increasingly interested in hyperthermophiles, i.e. strains with temperature optima for growth above 80 °C (Brock, 1992). Such organisms are attractive objects for basic research into the molecular basis of thermophily, thermostability and the origin of life as well as for applied research (Blöchl *et al.*, 1995). At present, the industrial applications of hyperthermophiles are related to their ability to produce thermostable enzymes (Leuschner & Antranikian, 1995). In this regard, the *Thermococcales* is the most frequently studied archaeal order and *Pyrococcus* is the most frequently studied genus within the hyperthermophiles (Leuschner & Antranikian, 1995). At present, the genus *Pyrococcus* contains only four species: *Pyrococcus furiosus* (Fiala & Stetter, 1986), *Pyrococcus woesei* (Zillig *et al.*, 1987), '*Pyrococcus abyssi*' (Erauso *et al.*, 1993) and '*Pyrococcus horikoshii*' (Gonzalez *et al.*, 1998). *P. furiosus* and *P.*

woesei are subjective synonyms (Erauso *et al.*, 1993) and their 16S rDNA and DNA polymerases sequences are identical (J. Quéréllou, unpublished data). Both were isolated from samples collected from marine solfataras of Vulcano island, Italy. '*P. abyssi*' and '*P. horikoshii*' were isolated from deep-sea vents of the North Fiji Basin (Erauso *et al.*, 1993) and Okinawa Trough (Gonzalez *et al.*, 1998), respectively. *Pyrococcus* species are coccoid marine archaea, strictly anaerobic, heterotrophic and sulfur-reducing with optimal temperatures for growth in the range 95–100 °C. Substrates for growth include complex organic substrates such as yeast extract, peptone, tryptone, meat extract and peptides (Fiala & Stetter, 1986). *P. furiosus* can also grow on starch, maltose (Fiala & Stetter, 1986; Raven & Sharp, 1997), glycogen, pullulan (Brown *et al.*, 1990), pyruvate (Schäfer & Schönheit, 1991) and cellobiose (Kengen *et al.*, 1993). *P. woesei* can grow on glycogen, starch and gellan gum (Zillig *et al.*, 1987; Koch *et al.*, 1991; Rüdiger *et al.*, 1992, 1995). '*P. abyssi*' and '*P. horikoshii*' are unable to grow on carbohydrates (Erauso *et al.*, 1993; Gonzalez *et al.*, 1998). This report describes a novel hyperthermophilic species of the genus *Pyrococcus* isolated from samples collected on the East Pacific Rise.

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The GenBank/EMBL accession number for the 16S rDNA sequence of strain AL585^T is Z70247.

METHODS

Reference strains. *P. furiosus* DSM 3638^T and *Thermotoga maritima* DSM 3109^T were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig-Stöckheim, Germany. '*P. abyssi*' was provided by Gaël Erauso (Centre National de la Recherche Scientifique, Station Biologique, Roscoff, France).

Culture conditions. BHI-S medium contained (l⁻¹): 9 g brain heart infusion (Difco), 23 g NaCl (instead of sea salt, to prevent formation of mineral precipitate), 6.05 g PIPES buffer (Sigma), 10 g sulfur (Prolabo) and 1 mg resazurin (Sigma). 2216-S medium (Belkin & Jannasch, 1985) contained (l⁻¹): 2 g peptone (Difco), 0.5 g yeast extract (Difco), 30 g sea salt (Sigma, ref. S-9883), 6.05 g PIPES buffer, 10 g sulfur and 1 mg resazurin. 20AA-S medium contained (l⁻¹): 30 g sea salt, 6.05 g PIPES buffer, 10 g sulfur, 1 mg resazurin, 10 ml of a mineral solution (Balch *et al.*, 1979), 10 ml of a vitamin solution (Balch *et al.*, 1979) and each of the 20 classical amino acids at a concentration of 2 mM. A stock solution of the 20 amino acids each at a concentration of 2 mM was sterilized separately by 0.22 µm filtration (Nalgene). The pH was adjusted with 5 M NaOH and 5 M HCl (measured at room temperature and atmosphere). The media were steam-sterilized at 100 °C (in order to avoid melting of sulfur and solidification in blocks at higher temperatures) for 30 min on two successive days, transferred into an anaerobic chamber (La Calhène, France) containing a gas mixture of N₂, H₂ and CO₂ (90:5:5), reduced by addition of sodium sulfide (Sigma) at a final concentration of 0.5 g l⁻¹ and then distributed into Hungate tubes or into 50 ml serum vials that were closed with a butyl rubber stopper and an aluminium cap. Unless indicated otherwise, cultures were grown on BHI-S or 2216-S medium at pH 7.5 (measured at room temperature) and always incubated at atmospheric pressure (100 kPa) under the anaerobic gas mixture mentioned above at 95 °C. Cultures were inoculated with previous cultures at 5% (v/v).

Enrichment cultures and purification. After collection, samples were stored on board for 2–3 weeks at room temperature and then in the laboratory at 4 °C in serum vials filled with sterile sea water (sea salt, 30 g l⁻¹) under anaerobic conditions (N₂ gas phase and 0.5 g sodium sulfide l⁻¹). Samples from the MVT90 cruise were ground and used for inoculation (10% v/v) of 2216-S and BHI-S media prepared at different pHs (3.5, 5.5 and 7.5) and incubated at 60, 80, 95, 100 and 110 °C. Positive cultures were identified by the appearance of turbidity and the presence of cells as revealed by microscopic observation. Cultures were then purified by streaking enrichment samples onto the same media solidified with Gelrite (Scott Laboratories) as gelling agent and incubated at 95 °C in glass Petri dishes set down in jars prepared under the gas mixture (Godfroy *et al.*, 1997). A collection of 125 thermophilic strains was obtained from those samples (Raguénès *et al.*, 1995).

Storage. Purified isolates were grown in BHI-S liquid culture media. Cultures in exponential growth phase were stored anaerobically in cryotubes (useful volume of 1.8 ml) for long-term storage, at –20 °C and –70 °C, after addition of dimethyl sulfoxide (5% v/v) (Sigma) as cryoprotectant. Cultures in exponential growth phase were also stored at 4 °C and were usable for at least 1 year.

Optical microscopy and determination of cell densities. Cell number was determined with an Olympus (Japan) microscope, model BH-2, and a Thoma chamber (depth of 0.02 mm). Specific coloration using the Spot Test Flagella

(Difco) was done for microscopic observation of flagella. When many non-microbial particles were observed (for instance with starch or at temperatures higher than 100 °C), cells were counted by epifluorescent microscopy (Hobbie *et al.*, 1977). Samples were diluted in sterile water containing sea salt (30 g l⁻¹), formalin (2.5%) and acridine orange (0.01%) and filtered onto black nuclepore polycarbonate membrane filters (pore size 0.2 µm; Costar). The acridine orange counts were confirmed by staining additional cultures with the DNA-specific stain 4',6-diamidino-2-phenylindole (Porter & Feig, 1980).

For determination of the optimum pH and salinity for growth, the turbidity of cultures in 2216-S medium was also quantified with a spectrophotometer (Spectronic 301; Milton Roy). Before taking measurements, Hungate tubes were gently mixed by inversion. After 6 min, sulfur particles had settled and OD₆₀₀ were measured. Absorbance varied linearly with cell numbers from 2.5 × 10⁷ to 1.5 × 10⁸ cells ml⁻¹ or OD₆₀₀ of 0.05–0.25. A regression through the origin gave the following formula: cells ml⁻¹ = 4.5 × 10⁸ × OD₆₀₀ (*n* = 7, *r*² = 0.98).

For flow-cytometric analysis, cells were fixed for 15 min at room temperature with 1% paraformaldehyde (final concentration). Filtered artificial sea water (Sigma, ref. S-1649) was used to dilute samples if necessary. Nucleic acid staining was achieved by addition of SYBR Green-I (Molecular Probes) at a final dilution of 10⁻⁴ of the commercial solution and 0.1% Triton X-100. Samples were incubated for 15–30 min at room temperature in the dark. Enumeration of cells were performed with a FACSsort flow cytometer (Becton Dickinson) equipped with an air-cooled laser providing 15 mW at 488 nm and with the standard filter set-up. Forward and right-angle light scatters (FSC and SSC) as well as the green fluorescence of the complex DNA-SYBR Green were collected on logarithmic signals. Samples were run at a calibrated rate of 50 µl min⁻¹ and dilutions of the samples in filtered artificial sea water were adapted such that the event rate was below 800 cells s⁻¹ to avoid coincidence. Data were recorded as list-mode files and processed with custom-designed software CYTOWIN (D. Vaulot, unpublished; available online at <http://www.sb-roscoff.fr>), which discriminates cell populations by using a combination of all the parameters recorded.

Electron microscopy. For transmission electron microscopy, cells were harvested at the end of the exponential phase of growth and centrifuged. The pellet was resuspended and fixed for 1 h at 4 °C in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2, 1100 mosmol) and then post-fixed for 1 h at 4 °C in 1% OsO₄ in 0.1 M sodium cacodylate buffer (pH 7.2, 1100 mosmol). After dehydration with increasing ethanol concentrations (70, 95 and 100%, v/v), cells were embedded in EMBed 812 resin, thin-sectioned, contrasted with 7% (w/v) uranyl acetate in methanol and a 2.5% (w/v) aqueous solution of lead citrate and examined with a model 100CX transmission microscope (JEOL).

Determination of growth parameters. In order to determine the optimum temperature, pH and salinity for growth, cells were grown in Hungate tubes containing 6 ml 2216-S medium. The headspace gas was N₂/H₂/CO₂ (90:5:5) at 100 kPa below and 200 kPa above 100 °C. Temperatures were maintained with aluminium heating blocks (Barnstead) and were monitored with temperature probes placed in control tubes. pH and salinity effects on growth were studied at 95 °C. Optimum pH was determined with 2216-S medium modified as follows: pH 2.5, 3.5 and 4.5, no buffer; pH 5.5 and 6.5, MES buffer (Sigma), 1 g l⁻¹; pH 7.5, HEPES buffer

(Sigma), 1 g l⁻¹; and pH 8.5 and 9.5, AMPSO buffer (Sigma), 1 g l⁻¹. pH was adjusted with 5 M NaOH and 5 M HCl (measured at room temperature and pressure). Salt requirement was determined with 2216-S medium modified with different dilutions of sea salt. Three replicates were simultaneously studied at each temperature, pH or salinity.

Determination of growth requirements. Utilization of different individual carbon sources for growth was tested. Individual carbon sources were added to a defined medium that contained (l⁻¹): 30 g sea salt, 0.4 g ammonium chloride, 6.05 g PIPES buffer, 10 ml mineral solution (Balch *et al.*, 1979), 10 ml vitamin solution (Balch *et al.*, 1979), 10 g sulfur and 1 mg resazurin. Carbon sources were added at concentrations of 5 g l⁻¹ except for starch, chitin, cellulose and lactose, which were added at 10 g l⁻¹, and ethanol, which was added at 5 ml l⁻¹. Growth on 20AA-S was also tested. Negative controls were performed with tubes without any addition of nitrogen and carbon substrate. Such tubes, prepared with H₂/CO₂ (80:20) headspace gas mixture as sole carbon source, were used to examine the possibility of autotrophic growth. To examine growth without elemental sulfur, cells were cultured simultaneously on BHI-S and BHI (i.e. BHI-S medium without sulfur) media. For this experiment, the headspace gases on BHI medium were N₂/H₂/CO₂ (90:5:5) and H₂/CO₂ (80:20) at a pressure of 100 kPa and the reductant was titanium nitritotriacetate (Moensch & Zeikus, 1983) at a concentration of 200 µM. Growth on cystine (10 g l⁻¹) and on polysulfide (10 mM) (Blumentals *et al.*, 1990) were also investigated. For all these experiments, the pH was adjusted to 7.5 (measured at room temperature and atmosphere).

Growth rates and confidence intervals. Growth rates were determined by linear regression of semi-logarithmic plots of cell concentration against time. The doubling time (*t*_d) was calculated from the slope (μ): $t_d = \log_{10}(2)/\mu$. Confidence intervals were calculated using standard errors of the slope (*se* _{μ}) as: $\mu \pm 2 se_{\mu}$. When replicates (*n*) were available, the mean growth rate and related standard error were calculated as: $\mu = (\Sigma \mu_i)/n$ and $se_{\mu} = [\Sigma (se_{\mu_i})^2/n^2]^{0.5}$.

Antibiotic sensitivity. Sensitivity to antibiotics (chloramphenicol, kanamycin, penicillin, rifampicin, streptomycin and vancomycin; all from Sigma) was tested at 80 °C in BHI-S medium at concentrations of 50, 100 and 150 µg ml⁻¹. *Thermotoga maritima* was used as a bacterial control to test the efficiency of antibiotics at 80 °C. '*P. abyssi*' (strain ST549) was used as an archaeal control to test the growth of another archaeon in the presence of antibiotics.

DNA extraction and base composition. DNA was prepared from cells harvested at the end of the exponential phase of growth. After centrifugation, the cell pellet was suspended in 5 ml lysis buffer (100 mM Tris/HCl, 100 mM NaCl, 0.5 mM EDTA; pH 8) and then 1% (w/v) Sarkosyl, 1% (w/v) SDS and 0.4 mg proteinase K l⁻¹ were added to achieve cell lysis. After 3 h incubation at 40 °C, three extractions with cold phenol/chloroform/isoamyl alcohol (24:24:1) and one extraction with chloroform were performed (1:1 with lysate). Between the first and second phenol/chloroform/isoamyl alcohol extractions, DNA was treated with RNase (5 µg ml⁻¹) for 1 h at 60 °C. DNA was then precipitated by addition of 2 vols ethanol at -20 °C. The dried DNA pellet was resuspended in TE (10 mM Tris/HCl, 2 mM EDTA; pH 7.4). DNA concentration and purity was tested spectrophotometrically at 230, 260, 280 and 320 nm with a GenQuant II spectrophotometer (Pharmacia). DNA base composition was determined by thermal denaturation

(Marmur & Doty, 1962) under the conditions reported by Raguénès *et al.* (1997) using DNA from *Escherichia coli*, *Clostridium perfringens* and *Micrococcus luteus* (Sigma) as standards.

16S rDNA sequence analysis. The 16S rDNA was amplified by PCR (Saiki *et al.*, 1988) as described previously (Dauga & Grimont, 1991), except that an archaea-specific primer was used. The primer sequences were 5'-TCCGGTTGATCCT-GCCGGAC-3' (*E. coli* 16S rRNA gene, positions 2-21) and 5'-CTTTCGGTTCGCCCCCTACT-3' (*Thermococcus celer* 23S rRNA, positions 257-234). The PCR product was precipitated and dissolved in 30 µl distilled water. The PCR product was cloned into pUC18 by using a SureClone ligation kit (Pharmacia) and also subcloned as *Hind*III-*Eco*RI insertions into M13mp18 and M13mp19 phage vectors. Both strands of one clone were sequenced by the dideoxy chain-termination method (Sanger *et al.*, 1977), except that we used a HotTub DNA sequencing kit (Amersham) to resolve numerous compressions observed during sequencing of genes of hyperthermophilic microorganisms. PCR products were also sequenced directly by using a cycle sequencing kit (type CSDS; Gibco-BRL) and ³²P-labelled primers (Amersham). As a control, the DNA sequence was also obtained with an automatic DNA analysis system (Applied Biosystems); this work was done at Euro Sequence Gene Service (ESGS, France). A total of 1925 bp was sequenced. The determined sequence was then aligned with available reference 16S rRNA sequences. Reference sequences used were: *Thermococcus hydrothermalis* (Z70244), *Thermococcus fumicolans* (Z70250), *P. furiosus* (U20163), '*P. abyssi*' (L19921) and '*P. horikoshii*' (D87344). The alignment was performed with 1271 bp. A multiple sequence file was obtained by using the MEGALIGN program of the DNASTAR package (Promega), similarities and alignment were obtained by the CLUSTAL W method with weighted residues (Thompson *et al.*, 1994) and phylogenetic reconstruction was produced using PHYLO-WIN (Galtier *et al.*, 1996) with the following conditions: distance, Jukes-Cantor, and successively the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Lake, 1987) methods. Bootstrap values were determined according to Felsenstein (1985).

Quantitative DNA-DNA hybridization. DNA from the reference strains *P. furiosus* and '*P. abyssi*' was used as labelled probes in a single hybridization experiment with strain AL585^T. Four to five micrograms of each reference-strain DNA was labelled by incorporation of both [³H]dATP and [³H]dGTP by using a Megaprime kit (Amersham). The nuclease S1 method for quantitative DNA-DNA hybridization was carried out as described by Popoff & Coynault (1980) by using DE81 filters (Whatman) and a β matic IV scintillation counter (Kontron Instruments).

RESULTS

Collection of samples

Chimney walls of active, deep-sea vents, frequently covered with alvinellid communities, were sampled from different sites on the East Pacific Rise at latitudes 11, 13 and 21° N with the deep-submergence vehicle *Alvin* during the American oceanographical cruise MVT'90 in 1990. These samples, chimney wall pieces and alvinellid tubes were stored in serum vials filled with sterile sea water under anaerobic conditions obtained by injection of sodium sulfide to a final

Fig. 1. Transmission electron micrographs of strain AL585^T. (a) Single cells and division by constriction (arrow). Bar, 1 µm. (b) View of the cell wall. Arrows indicate surface layer (SL), periplasmic space (PS) and cytoplasmic membrane (CM). Bar, 0.1 µm.

concentration of 0.5 g l⁻¹. Samples were brought back to the laboratory at ambient temperature immediately after the cruise. Strain AL585^T was isolated from a sample containing pieces of one smoker and its covering of alvinellid tubes collected at a site called Totem, latitude 13° N, depth 2650 m.

Enrichment and purification

Strain AL585^T was obtained at 95 °C in BHI-S medium, pH 7.5. Purification of strain AL585^T was performed by successive plating of culture samples onto solid BHI-S medium prepared with Gelrite. Small, translucent, colourless colonies appeared within a few days, indicating that growth was slow in such conditions compared with liquid medium. One isolated colony was transferred to liquid medium and stored.

Morphology

Observations by phase-contrast microscopy revealed that strain AL585^T cells were cocci occurring singly, in pairs or in small aggregates. The cells appeared to be motile and polar flagella were observed by phase-contrast microscopy after specific coloration. Numerous diplococci were observed during the exponential growth phase, indicating that cells probably divided by constriction. Under electron microscopy, more or less spherical cocci were observed (Fig. 1a). These cells were delimited by a cytoplasmic membrane (5 nm) covered by a bilayered cell envelope with an inner periplasmic space (15 nm) and an external, densely

stained layer (5 nm), probably corresponding to a surface-layer protein as already described for other thermococcales (Huber *et al.*, 1995) (Fig. 1b). Cells divided by constriction, as visible in Fig. 1(a), but budding cells were not observed. Clumps of dense, spherical particles were frequently present inside the cytoplasm and fibres and dense particles were sometimes attached externally to the cell envelope. Electron microscopy permitted the study of the diameter distribution. The diameters of single cells ranged from 0.5 to 1.5 µm, depending on their size and the axis of measurement. The diameters measured were normally distributed and the mean diameter was 1 µm.

Growth parameters

Near atmospheric pressure, strain AL585^T grew slowly at 75 and 104 °C, but growth was not observed at 107 °C. The maximum growth rate was observed at 95 °C (Fig. 2a). The highest cell yield was also obtained at this temperature, which can be considered as optimal. Growth was slow at pH 2.5 and 9.5 and the optimum pH was between 6.5 and 8.5 (Fig. 2b). The highest cell yield was obtained at pH 7.5, which can be considered as the optimum pH. Growth was observed at sea-salt concentrations ranging from 20 to 60 g l⁻¹. Calculated growth rates were not significantly different between 30, 40 and 60 g l⁻¹. Strain AL585^T was unable to grow without sea salt or at a concentration of 80 g l⁻¹ (Fig. 2c). The highest cell concentrations at the end of the exponential phase were obtained for 30 g l⁻¹ sea salt, which can be considered as optimal. Under the

Fig. 2. Temperature, pH and sea-salt concentration optima for growth of strain AL585^T on 2216-S medium. (a) Growth rate as a function of temperature (30 g sea salt l⁻¹, pH 7.5). (b) Growth rate as a function of pH (30 g sea salt l⁻¹, 95 °C). (c) Growth rate as a function of sea-salt concentration (95 °C, pH 7.5). Bars indicate confidence intervals.

supposed optimal conditions (95 °C, pH 7.5, 30 g l⁻¹ sea salt), the mean doubling time from three replicates was 93 min and the highest cell concentration observed was around 5×10^8 cells ml⁻¹. This doubling time was higher than the lowest previously described for other *Pyrococcus* species: 37 min for *P. furiosus* (Fiala & Stetter, 1986), 35 min for *P. woesei* (Zillig *et al.*, 1987), 33 min for '*P. abyssi*' (Erauso *et al.*, 1993) and 32 min for '*P. horikoshii*' (Gonzalez *et al.*, 1998).

Considering this mean doubling time under optimal conditions, we observed that the duration of cultures made with highly diluted inocula (dilution of 10^{-4} v/v, for instance) until the end of the exponential growth

Fig. 3. Growth curve as determined by flow-cytometry counts.

phase was abnormally short. In order to observe the growth kinetics at relatively low concentrations directly, we determined cell numbers against time using flow cytometry. A volume of 0.1 ml of a culture containing 2×10^8 cells ml⁻¹ was inoculated in 30 ml BHI-S medium and cultivated at 95 °C. This culture was sampled over 8.7 h. Flow cytometry counts are shown in Fig. 3. Three observations were made during the exponential phase of growth and the three corresponding points, from 6×10^5 to 6.2×10^7 cells ml⁻¹, were perfectly aligned ($r^2 = 0.9999$). From these points, a doubling time of 27 min was calculated. These results suggested that growth rates for strain AL585^T calculated at microscopically or densitometrically countable concentrations (from 10^7 to 5×10^8 cells ml⁻¹) were suboptimal. This signifies that 'optimal growth' (i.e. maximum growth rate) in serum vials or Hungate tubes was only observable at relatively low cell concentrations, not easily compatible with the techniques commonly used for cell counting. When using these techniques, Thoma chamber counts or OD measurements, we observed a transition phase between the exponential growth phase and the stationary phase, during which the growth rate decreased.

Nutritional requirements

Elemental sulfur clearly stimulated growth of strain AL585^T in complex medium (maximum concentrations greater than or equal to 2×10^8 cells ml⁻¹) (Fig. 4). We repeatedly observed that cystine had less effect (maximum concentrations between 5×10^7 and 2×10^8 cells ml⁻¹) and that polysulfide failed to stimulate growth (maximum concentrations less than or equal to 5×10^7 cells ml⁻¹). Elemental sulfur appeared to prolong growth, which was inhibited by metabolic products in its absence. A high concentration (80%) of H₂ in the gas phase also strongly inhibited growth. In the presence of elemental sulfur, cultures produced large amounts of hydrogen sulfide during growth (data not shown). These results suggest that addition of elemental sulfur prevents H₂ inhibition of growth, as proposed by Fiala & Stetter (1986).

Fig. 4. Influence of the presence and absence of sulfur compounds and of the gas phase on growth of strain AL585^T in BHI medium. Gas phases tested were N₂/H₂/CO₂ (90:5:5), unsupplemented (Δ) or supplemented with sulfur ($\blacktriangle$), cystine ($\circ$) or polysulfide ($\blacksquare$), and H₂/CO₂ (80:20) ($\square$) (without added sulfur compounds).

Strain AL585^T appeared to be an obligate heterotroph. No growth was observed in mineral medium, in the absence of a carbon source, with an H₂/CO₂ gas phase (80:20), with or without elemental sulfur (data not shown). Yeast extract plus peptone (2216-S medium) and brain heart infusion (BHI-S medium) supported rapid and efficient growth of strain AL585^T (maximum concentrations greater than or equal to 2×10^8 cells ml⁻¹). This strain was also able to grow equally efficiently with peptone or glucose as carbon and energy sources. Less efficient growth was observed with starch and maltose as carbon sources (maximum concentrations between 5×10^7 and 2×10^8 cells ml⁻¹). A small amount of growth was obtained with meat extract, yeast extract, chitin, cellobiose and a mixture of 20 amino acids (maximum concentrations between 1.5×10^7 and 5×10^7 cells ml⁻¹). None of the other substrates tested as carbon sources permitted growth.

Antibiotic sensitivity

Strain AL585^T was resistant to rifampicin, vancomycin, penicillin, kanamycin, streptomycin and chloramphenicol at concentrations of 50, 100 and 150 µg ml⁻¹. *Thermotoga maritima* exhibited the expected pattern of antibiotic sensitivity (Huber *et al.*, 1986) at 80 °C, demonstrating that the antibiotics were active at high temperature.

DNA composition

The G+C content of the DNA of strain AL585^T was 47 ± 1 mol %. The G+C contents of *P. furiosus* and '*P. abyssi*', determined under the same conditions, were 38 ± 1 and 44 ± 1 mol %. These two values are identical to the values published previously (Fiala & Stetter, 1986; Erauso *et al.*, 1993).

Fig. 5. Unrooted phylogenetic tree of strain AL585^T and other reference species from the *Thermococcales*. The tree was based on an alignment done with CLUSTAL W and established using PHYLO-WIN with the following parameters: distance, Jukes-Cantor, and successively neighbour-joining, maximum-likelihood and maximum-parsimony methods. Bootstrap values (encircled numbers) are those of the neighbour-joining method with 500 replicates. ***, All three methods give the same branching. Numbers on the branches give the evolutionary distances.

16S rRNA sequence analysis

The sequence of the 16S rDNA of strain AL585^T was determined and revealed that this isolate belonged to the genus *Pyrococcus*. Consequently, the sequence was aligned with other available 16S rDNA sequences for *Pyrococcus* species; sequence similarities were found to be 98% to *P. furiosus*, '*P. abyssi*' and '*P. horikoshii*', 96% to *Thermococcus hydrothermalis* and 94% to *Thermococcus fumicolans*. Fig. 5 gives the corresponding phylogenetic tree. In this tree, strain AL585^T appeared closer to *P. furiosus* than to '*P. abyssi*' and '*P. horikoshii*'.

DNA-DNA hybridization

Labelled probes prepared from total DNA from *P. furiosus* and '*P. abyssi*', the two *Pyrococcus* species previously described, closely related to strain AL585^T, were hybridized with total DNA from strain AL585^T. The levels of DNA reassociation measured were 20 and 18%, respectively. We can observe that the values are far below 70%, which is the percentage to consider for definition of a new species (Wayne *et al.*, 1987). Those results confirm that strain AL585^T is a new species of *Pyrococcus*.

DISCUSSION

The novel marine hyperthermophilic strain AL585^T can be assigned to the archaeal domain (Woese *et al.*, 1990) because of its resistance to antibiotics and its 16S rDNA sequence. This latter result (Fig. 5) indicates

Table 1. Comparison of characteristics of strain AL585^T and other *Pyrococcus* species

Data were taken from Fiala & Stetter (1986), Brown *et al.* (1990), Schäfer & Schönheit (1991), Kengen *et al.* (1993) and Raven & Sharp (1997) (*P. furiosus*); Zillig *et al.* (1987), Koch *et al.* (1991) and Rüdiger *et al.* (1992, 1995) (*P. woesei*); Erauso *et al.* (1993) (*P. abyssi*); and Gonzalez *et al.* (1998) (*P. horikoshii*). ND, Not determined.

Characteristic	<i>P. furiosus</i> Vc1 ^T (= DSM 3638 ^T)	<i>P. woesei</i> DSM 3773 ^T	<i>P. abyssi</i> GE5 (= CNCM I-1302)	<i>P. horikoshii</i> JCM 9974	Strain AL585 ^T
G + C content (mol %)	37–38	37.5	44–45	44	47
Growth temperature (°C):					
Optimum	100	100–103	96	98	95
Range	70–103	Up to 105*	67–102	80–102	75–104
pH:					
Optimum	7	ND	6.8	7.0	7.5
Range	5–9	ND	4.0–8.5	5–8	2.5–9.5
NaCl concentration (g l ⁻¹):					
Optimum	20	30	30	24	26
Range	5–50	ND	7–50	10–50	17–52
Carbon sources used	Yeast extract, peptone, tryptone, meat extract, casein, starch, maltose, cellobiose, glycogen, pullulan, pyruvate	Yeast extract, tryptone, glycogen, starch, gellan	Brain heart infusion, yeast extract, meat extract, peptone, tryptone, 20 amino acids	Peptone, yeast extract, beef extract, tryptone, casein, 20 amino acids	Brain heart infusion, yeast extract, peptone, meat extract, starch, chitin, maltose, cellobiose, glucose, 20 amino acids

* No minimum temperature for growth was given.

that this strain belongs to the order *Thermococcales* (Zillig *et al.*, 1987), which is so far represented by two genera, *Pyrococcus* (Fiala & Stetter, 1986) and *Thermococcus* (Zillig *et al.*, 1983), and the strain is included in the genus *Pyrococcus*. The new strain has a morphology similar to the previously described species of *Pyrococcus* and, similar to other strains of *Pyrococcus*, strain AL585^T has an optimal growth temperature above 90 °C, while *Thermococcus* species grow optimally below 90 °C (Godfroy *et al.*, 1996, 1997; Miroshnichenko *et al.*, 1998; Canganella *et al.*, 1998; Duffaud *et al.*, 1998). Characteristics of strain AL585^T and other species of the genus *Pyrococcus* are shown in Table 1. Strain AL585^T has a G + C content higher than other strains of *Pyrococcus*, and this criterion distinguishes the abyssal strains (*P. abyssi*, *P. horikoshii* and strain AL585^T) very clearly from the coastal strains (*P. furiosus* and *P. woesei*). However, the growth responses to temperature, pH and salinity do not differentiate strain AL585^T from the other species of *Pyrococcus*. The maximum growth rate observed for strain AL585^T corresponds to a doubling time of 0.5 h, which is similar to that observed for other *Pyrococcus* species. However, in the case of strain AL585^T, the maximum growth rate was much lower (doubling time of 1.5 h) at the end of the exponential phase of growth, in the range of cell concentrations usually studied. We can hypothesize that this transition phase could reveal the existence of a limiting factor for growth or an inhibiting effect of metabolic products when growth occurs in closed vessels. This last hypothesis will have to be tested further by studying the behaviour of strain AL585^T when grown in a fermenter.

As with other *Pyrococcus* species, strain AL585^T grows efficiently in the presence of proteinaceous substrates. Unlike *P. abyssi* and *P. horikoshii*, strain AL585^T is also able to use different carbohydrates, an ability

already described for *P. furiosus* and *P. woesei*. This is in line with 16S rDNA sequence comparisons (Fig. 5), which separated *Pyrococcus* species in two groups corresponding with these phenotypic differences. However, strain AL585^T is able to use glucose as a carbon source for its growth, a feature that appears to be unique in the genus *Pyrococcus*. This ability to ferment proteins, amino acids and carbohydrates in the presence of sulfur appears to correspond to the characteristics of the original ecosystem surrounding this isolate. Strain AL585^T was isolated from pieces of an active smoker covered with an alvinellid colony, made mainly of *Alvinella pompejana* individuals. Current knowledge of the ecology of this species (Desbruyères *et al.*, 1998) clearly indicates that all the major elements we used to prepare culture media (sea salt, sulfur, proteins and carbohydrates) are present in the immediate vicinity of this animal.

On the basis of its physiological characteristics, its 16S rDNA sequence and DNA–DNA hybridization data, strain AL585^T represents a new species of *Pyrococcus*, and we propose to name it *Pyrococcus glycovorans* sp. nov.

Description of *Pyrococcus glycovorans* Barbier, Godfroy, Meunier and Raguénès sp. nov.

Pyrococcus glycovorans (gly.co.vo'rans. Gr. adj. *glykos* sweet, referring to glucose; L. part. pres. *vorans* eating, devouring; M.L. part. adj. *glycovorans* eating glucose).

Cells are cocci with a mean diameter of 1 µm (range 0.5–1.5 µm), motile with polar flagella. Cell division occurs by constriction. Obligately anaerobic. Grows optimally at 30 g l⁻¹ sea salt and pH 7.5. At atmospheric pressure, growth occurs between 75 and 104 °C. Obligately chemo-organotrophic. Ferments protein

and proteolytic products and carbohydrates, especially glucose. Sulfur is not necessary for growth but significantly enhances final cell concentrations when grown in closed culture vessels. Sulfur is reduced to H_2S . At low cell concentrations, under optimal conditions and at atmospheric pressure, doubling time is 0.5 h and maximal concentrations at the beginning of the stationary phase can reach 5×10^8 cells ml^{-1} . G + C content is 47 mol %. 16S rDNA sequence comparisons locate *Pyrococcus glycovorans* within the *Thermococcales*, in the archeal domain. The GenBank/EMBL accession number for the 16S rDNA sequence is Z70247.

The type strain, AL585^T (= CNCM I-2120^T), was isolated from pieces of a deep-sea smoker and its covering of alvinellid tubes collected at 13° N at a depth of 2650 m on the East Pacific Rise.

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REFERENCES

- Balch, W. E., Fox, G. E., Magrum, L. J., Woese, C. R. & Wolfe, R. S. (1979). Methanogens: reevaluation of a unique biological group. *Microbiol Rev* **43**, 260–296.
- Belkin, S. & Jannasch, H. W. (1985). A new extremely thermophilic, sulfur-reducing heterotrophic, marine bacterium. *Arch Microbiol* **141**, 181–186.
- Blöchl, E., Burggraf, S., Fiala, G. & 7 other authors (1995). Isolation, taxonomy and phylogeny of hyperthermophilic microorganisms. *World J Microbiol & Biotechnol* **11**, 9–16.
- Blumentals, I. I., Itoh, M., Olson, G. J. & Kelly, R. M. (1990). Role of polysulfides in reduction of elemental sulfur by the hyperthermophilic archaeobacterium *Pyrococcus furiosus*. *Appl Environ Microbiol* **56**, 1255–1262.
- Brock, T. D. (1992). Research on thermophiles. In *Thermophiles: Science and Technology*, pp. A3–A12. Reykjavik: Ictec.
- Brown, S. H., Costantino, H. R. & Kelly, R. M. (1990). Characterization of amylolytic enzyme activities associated with the hyperthermophilic archaeobacterium *Pyrococcus furiosus*. *Appl Environ Microbiol* **56**, 1985–1991.
- Canganella, F., Jones, W. J., Gambacorta, A. & Antranikian, G. (1998). *Thermococcus guaymasensis* sp. nov. and *Thermococcus aggregans* sp. nov., two novel thermophilic archaea isolated from the Guaymas Basin hydrothermal vent site. *Int J Syst Bacteriol* **48**, 1181–1185.
- Dauga, P. A. D. & Grimont, P. A. D. (1991). Nucleotide sequence of 16S rRNA from ten *Serratia* species. *Res Microbiol* **141**, 1139–1149.
- Desbruyères, D., Chevaldonné, P., Alayse, A.-M. & 15 other authors (1998). Biology and ecology of the 'Pompeii worm' (*Alvinella pompejana* Desbruyères and Laubier), a normal dweller of an extreme deep-sea environment: a synthesis of current knowledge and recent developments. *Deep-Sea Res Part II Top Stud Oceanogr* **45**, 383–422.
- Duffaud, G. D., d'Hennezel, O. B., Peek, A. S., Reysenbach, A.-L. & Kelly, R. M. (1998). Isolation and characterization of *Thermococcus barossii*, sp. nov., a hyperthermophilic archaeon isolated from a hydrothermal vent flange formation. *Syst Appl Microbiol* **21**, 40–49.
- Erauso, G., Reysenbach, A. L., Godfroy, A. & 8 other authors (1993). *Pyrococcus abyssi* sp. nov., a new hyperthermophilic archaeon isolated from a deep-sea hydrothermal vent. *Arch Microbiol* **160**, 338–349.
- Felsenstein, J. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. *J Mol Evol* **17**, 368–376.
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **30**, 783–791.
- Fiala, G. & Stetter, K. O. (1986). *Pyrococcus furiosus* sp. nov. represents a novel genus of marine heterotrophic archaeobacteria growing optimally at 100 °C. *Arch Microbiol* **145**, 56–61.
- Galtier, N., Gouy, M. & Gautier, C. (1996). SEAVIEW and PHYLO-WIN: two graphic tools for sequence alignment and molecular phylogeny. *Comput Appl Biosci* **12**, 543–548.
- Godfroy, A., Meunier, J.-R., 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**, 1113–1119.
- Godfroy, A., Lesongeur, F., Raguénès, G., Quérellou, J., Antoine, E., Meunier, J.-R., 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**, 622–626.
- Gonzalez, J. M., Masuchi, Y., Robb, F. T., Ammerman, J. W., Maeder, D. L., 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**, 123–130.
- Hobbie, J. E., Daley, R. J. & Jasper, S. (1977). Use of nucleopore filters for counting bacteria by fluorescence microscopy. *Appl Environ Microbiol* **33**, 1225–1228.
- Huber, R., Langworthy, T. A., König, H., Thomm, M., Woese, C. R., Sleytr, U. B. & Stetter, K. O. (1986). *Thermotoga maritima* sp. nov. represents a new genus of unique extremely thermophilic eubacteria growing up to 90 °C. *Arch Microbiol* **144**, 324–333.
- Huber, R., Stöhr, J., Hohenhaus, S., Rachel, R., Burggraf, S., Jannasch, H. W. & Stetter, K. O. (1995). *Thermococcus chitonophagus* sp. nov., a novel, chitin-degrading, hyperthermophilic archaeum from a deep-sea hydrothermal vent environment. *Arch Microbiol* **164**, 255–264.
- Kengen, S. W. M., Luesink, E. J., Stams, A. J. M. & Zehnder, A. J. B. (1993). Purification and characterization of an extremely thermostable β -glucosidase from the hyperthermophilic archaeon *Pyrococcus furiosus*. *Eur J Biochem* **213**, 305–312.

- Koch, R., Spreinat, A., Lemke, K. & Antranikian, G. (1991). Purification and properties of a hyperthermoactive α -amylase from the archaeobacterium *Pyrococcus woesei*. *Arch Microbiol* **155**, 572–578.
- Lake, J. A. (1987). A rate-independent technique for analysis of nucleic acid sequences: evolutionary parsimony. *Mol Biol Evol* **4**, 167–191.
- Leuschner, C. & Antranikian, G. (1995). Heat-stable enzymes from extremely thermophilic and hyperthermophilic microorganisms. *World J Microbiol & Biotechnol* **11**, 95–114.
- Marmur, J. & Doty, P. (1962). Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *J Mol Biol* **5**, 109–118.
- Miroshnichenko, M. L., Gongadze, G. M., Rainey, F. A., Kostyukova, A. S., Lysenko, A. M., Chernyh, N. A. & Bonch-Osmolovskaya, E. A. (1998). *Thermococcus gorgonarius* sp. nov. and *Thermococcus pacificus* sp. nov.: heterotrophic extremely thermophilic archaea from New Zealand submarine hot vents. *Int J Syst Bacteriol* **48**, 23–29.
- Moensch, T. T. & Zeikus, J. G. (1983). An improved preparation method for a titanium(III) media reductant. *J Microbiol Methods* **1**, 199–202.
- Popoff, M. Y. & Coynault, C. (1980). Use of DEAE-cellulose filters in the S1 nuclease method for bacterial deoxyribonucleic acid hybridization. *Ann Microbiol (Paris)* **131A**, 151–155.
- Porter, K. G. & Feig, Y. S. (1980). The use of DAPI for identifying and counting microflora. *Limnol Oceanogr* **25**, 943–948.
- Raguénès, G., Meunier, J.-R., Antoine, E., Godfroy, A., Caprais, J.-C., Lesongeur, F., Guézennec, J. & Barbier, G. (1995). Biodiversité d'archaea hyperthermophiles des sites hydrothermaux du Pacifique oriental. *C R Acad Sci Ser III Sci Vie* **318**, 395–402.
- Raguénès, G., Christen, R., Guézennec, J., Pignet, P. & Barbier, G. (1997). *Vibrio diabolicus* sp. nov., a new polysaccharide-secreting organism isolated from a deep-sea hydrothermal vent polychaete annelid, *Alvinella pompejana*. *Int J Syst Bacteriol* **47**, 989–995.
- Raven, N. D. H. & Sharp, R. J. (1996). Development of defined and minimal media for the growth of the hyperthermophilic archaeon *Pyrococcus furiosus* Vc1. *FEMS Microbiol Lett* **146**, 135–141.
- Rüdiger, A., Ogbonna, J. C., Märkl, H. & Antranikian, G. (1992). Effect of gassing, agitation, substrate supplementation and dialysis on the growth of an extremely thermophilic archaeon *Pyrococcus woesei*. *Appl Microbiol Biotechnol* **37**, 501–504.
- Rüdiger, A., Jorgensen, P. L. & Antranikian, G. (1995). Isolation and characterization of a heat-stable pullulanase from the hyperthermophilic archaeon *Pyrococcus woesei* after cloning and expression of its gene in *Escherichia coli*. *Appl Environ Microbiol* **61**, 567–575.
- Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. & Erlich, H. A. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**, 487–491.
- Saitou, M. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**, 5463–5467.
- Schäfer, T. & Schönheit, P. (1991). Pyruvate metabolism of the hyperthermophilic archaeobacterium *Pyrococcus furiosus*. *Arch Microbiol* **155**, 366–377.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.
- Wayne, L. G., Brenner, D. J., Colwell, R. R. & 9 other authors (1987). Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.
- Woese, C. R., Kandler, O. & Wheelis, M. L. (1990). Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. *Proc Natl Acad Sci USA* **87**, 4576–4579.
- Zillig, W., Holz, I., Janekovic, D., Schäfer, W. & Reiter, W. D. (1983). The archaeobacterium *Thermococcus celer* represents a novel genus within the thermophilic branch of the archaeobacteria. *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 ultra-thermophilic marine Archaeobacterium, representing a novel order, *Thermococcales*. *Syst Appl Microbiol* **9**, 62–70.