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Palaeococcus ferrophilus gen. nov., sp. nov., a barophilic, hyperthermophilic archaeon from a deep-sea hydrothermal vent chimney

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A novel barophilic, hyperthermophilic archaeon was isolated from a deep-sea hydrothermal vent chimney at the Myojin Knoll in the Ogasawara-Bonin Arc, Japan. The cells were found to be irregular cocci and motile with multiple polar flagella. Growth was observed between 60 and 88 °C (opt. 83 °C; 30 min doubling time), pH 4·0 and 8·0 (opt. pH 6·0), 20 and 73 g sea salts l^{-1} (opt. 47 g l^{-1}) and 0·1 and 60 MPa (opt. 30 MPa). The isolate was a strictly anaerobic chemoorganotroph capable of utilizing proteinaceous substrates such as yeast extract, peptone, tryptone and casein in the presence of elemental sulfur or ferrous iron. The G+C content of the genomic DNA was 53·5 mol%. Phylogenetic analysis based on 16S rDNA sequences indicated that the isolate was a member of an ancient lineage of the *Thermococcales* that diverged prior to the formation of the two genera *Thermococcus* and *Pyrococcus*. On the basis of the physiological and molecular properties of the new isolate, the name *Palaeococcus ferrophilus* gen. nov., sp. nov. is proposed. The type strain is strain DMJ^T (= JCM 10246^T).

Keywords: hyperthermophilic archaeon, barophilic, *Thermococcales*, deep-sea hydrothermal vent

INTRODUCTION

Since the discovery of deep-sea hydrothermal vents in 1977, a number of hyperthermophiles have been isolated from various sites in deep-sea environments (Prieur *et al*., 1995). These hyperthermophiles are physiologically and phylogenetically diverse groups of micro-organisms and include members of both the bacterial and archaeal domains (Jones *et al*., 1983; Fiala *et al*., 1986; Jannasch *et al*., 1988; Huber *et al*., 1989, 1997; Pledger & Baross, 1989, 1991; Burggraf *et al*., 1990; Stetter *et al*., 1990; Pley *et al*., 1991; Gonzalez *et al*., 1995, 1998; Godfroy *et al*., 1996, 1997; Antoine *et al*., 1997; Blöchl *et al*., 1997; Canganella *et al*., 1997). Members of the *Thermococcales* are the most frequently isolated hyperthermophiles and these organisms are regarded as the major decomposers of organic matter within marine hot-water ecosystems (Zillig *et al*., 1983; Fiala & Stetter, 1986; Miroshnichenko *et al*., 1989; Pledger & Baross, 1989, 1991; Neuner *et al*., 1990; Gonzalez *et al*., 1995, 1998; Godfroy *et al*., 1996, 1997; Antoine *et al*., 1997; Canganella *et al*., 1997). They are strictly anaerobic, obligately heterotrophic micro-organisms capable of using complex substrates for growth, such as yeast extract, peptone, bacterial and archaeal cell homogenates and polymers such as casein, gelatin, starch and chitin. Their growth is also strongly associated with the reduction of elemental sulfur (S^0).

It has been generally considered that sulfur reduction played an important role in the early microbial respiration system, because most ancient lineages of micro-organisms are anaerobic, sulfur-reducing hyperthermophiles, including members of the *Thermococcales* (Achenbach-Richter *et al*., 1987). However, it seems likely that iron was more frequently used in early microbial metabolism in the light of the geochemical properties of the habitats of hyperthermophiles (Walker, 1987; Ehlich, 1990; Cairns-Smith *et al*., 1992; Seyfried & Mottl, 1995). Hafenbradl *et al*. (1996) isolated the first hyperthermophilic archaeon capable of oxidizing ferrous iron anaerobically at neutral pH, from a shallow marine hydrothermal vent

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environment. In addition, Vargas *et al*. (1998) have demonstrated that various hyperthermophilic archaea and bacteria can reduce ferric iron and conserve energy to support their growth. These findings are viewed as significant microbiological evidence of the importance of iron as the primary electron donor/acceptor element on the early Earth, and studies focusing on the isolation and cultivation of ferric-iron-reducing thermophilic bacteria have also supported the importance of iron in early microbial metabolism (Slobodkin *et al*., 1997; Slobodkin & Wiegel, 1997).

In this study, we succeeded in the isolation and cultivation of a novel hyperthermophilic archaeon from a black smoker chimney in an area with deep-sea hydrothermal vents at the Myojin Knoll in the Ogasawara-Bonin Arc, Japan. This organism displayed an absolute requirement for either elemental sulfur (S^0) or ferrous iron (Fe^{2+}) for growth. Ferrous iron seemed to be necessary for fermentative metabolism. Phylogenetic analysis based on 16S rRNA sequences indicated that the isolate is a member of a deep-branching lineage of the *Thermococcales*, placed prior to the divergence between two genera *Thermococcus* and *Pyrococcus*. We describe the characterization of this new isolate and propose the new genus name *Palaeococcus*.

METHODS

Sample collection. A sample from a black smoker chimney was obtained from the hydrothermal field at Myojin Knoll in the Ogasawara Trough, Japan (32 ° 06·208′ N, 139 ° 52·004′ E) at a depth of 1338 m by means of the manned submersible *Shinkai 2000* in a dive (dive #1007) performed in May 1998. The tip of the chimney was brought to the sea surface in a sample box and immediately frozen at −85 °C. The sample was stored at −85 °C prior to incubation.

Enrichment and purification. Portions of the thawed and fractured sample were used to inoculate a series of media including MJYP* medium (described below), which was based on MJYP medium and supplemented with 20 mM $NaNO_3$ and 5 mM $FeSO_4$ as possible energy sources or factors supporting growth, and the cultures were incubated at 75, 84 or 95 °C. All tubes of MJYP* medium inoculated with portions of the chimney sample became turbid after 2 d incubation at 75 or 84 °C and each contained highly motile, irregular cocci. In order to obtain a pure culture, the dilution-to-extinction technique was employed (Baross, 1995). After the cell density of the enrichment culture had reached approximately 10^8 cells ml^{-1}, 10 separate dilution series were prepared in which five 1:10 dilutions of the culture were made, followed by twenty 1:2 dilutions. Each dilution was made in MJYP* medium and the cultures were incubated for at least 4 d at 84 °C. The culture in the tube showing growth at the highest dilution was designated strain DMJ^T.

Sources of organisms. *Pyrococcus furiosus* (DSM 3638^T), *Thermococcus celer* (DSM 2476^T), '*Thermococcus litoralis*' (DSM 5473^T), *Thermococcus stetteri* (DSM 5262^T) and *Sulfolobus acidocaldarius* (DSM 639^T) were purchased from the Japan Collection of Microorganisms (JCM). '*Pyrococcus abyssi*' (strain GE5), *Thermococcus peptonophilus* (JCM 9653^T) and *Thermococcus profundus* (JCM 9378^T) were obtained from the culture collection of our institute. *Thermococcus aggregans* (JCM 10137^T) and *Thermococcus guaymasensis* (JCM 10136^T) were kindly provided by Francesco Canganella (University of Tusia, Vierbo, Italy). *Rhodothermus obamensis* (JCM 9785^T) was provided by Yoshihiko Sako (Kyoto University, Kyoto, Japan) (Sako *et al*., 1996) and *Thermaerobacter marianensis* (JCM 10246^T) was isolated by us (Takai *et al*., 1999).

Culture media and conditions. The new isolate was routinely cultivated in MJYP* medium, which was based on MJYP medium [containing 2 g yeast extract, 2 g Trypticase peptone, 0·5 g $Na_2S.9H_2O$ and 1 mg resazurin l^{-1} MJ (−N) synthetic sea water] and supplemented with 20 mM $NaNO_3$ and 5 mM $FeSO_4$. When 20 mM $NaNO_3$ and 3 % (w/v) elemental sulfur (S^0) were added to MJYP medium, this was defined as MJYPS medium. MJ (−N) synthetic sea water consists of (l^{-1}): NaCl, 30·0 g; K_2HPO_4, 0·14 g; $CaCl_2.2H_2O$, 0·14 g; $MgSO_4.7H_2O$, 3·4 g; $MgCl_2.6H_2O$, 4·18 g; KCl, 0·33 g; $NiCl_2.6H_2O$, 0·5 mg; $Na_2SeO_3.5H_2O$, 0·5 mg; $Fe(NH_4)_2(SO_4)_2.6H_2O$, 0·01 g; trace mineral solution, 10 ml (Balch *et al*., 1979). To prepare MJYP* medium, 2 g yeast extract, 2 g Trypticase peptone and 1 mg resazurin were dissolved in 1 litre of MJ (−N) synthetic sea water and the medium was autoclaved. Thereafter, the medium was degassed with 100 % N_2 and was completed by adding separately prepared anaerobic solutions of $NaNO_3$ (degassed with 100 % N_2 and autoclaved; pH 7·0), iron sulfate (degassed with 100 % N_2 and autoclaved; pH 7·5) and sodium sulfide (degassed with 100 % N_2 and autoclaved; pH 7·5). The pH of the medium was adjusted to 6·0 with HCl at room temperature unless noted otherwise. These procedures after autoclaving were all performed in an anaerobic chamber under a gas phase of 100 % N_2. The anaerobic cultivation technique employed was that described by Balch & Wolfe (1976). The medium was dispensed at 20 % of the total volume of a bottle or tube, which was then sealed tightly with a butyl rubber stopper and the gas phase was exchanged with N_2 at 200 kPa for routine cultivation or with other gasses at 200 kPa for testing the effect of the gas phase.

All experiments described below were conducted in duplicate. In an attempt to examine whether the oxidation and reduction of inorganic substances supported or stimulated growth, various nitrogen compounds (NH_4Cl, $NaNO_2$ and $NaNO_3$ at 20 or 2 mM), various sulfur compounds (Na_2SO_4, Na_2SO_3, $Na_2S_2O_3$ and $Na_2S_2O_5$ at 5 or 0·5 mM, S^0 at 3 %) and various iron sulfide forms (FeS, FeS_2 and Fe_2S_3 at 5 or 0·5 mM) were used alone or in various combinations, instead of the combination of $NaNO_3$ and $FeSO_4$ of MJYP* medium, in the presence or absence of sodium sulfide.

In addition, the stimulation of growth by various metal sulfates ($CuSO_4$, $NiSO_4$, $MnSO_4$, $CoSO_4$, $ZnSO_4$ and VSO_4 at 5 mM) was also examined in place of $FeSO_4$ of MJYP* medium in the presence of sodium sulfide. To test the effect of pH on growth, the pH of MJYP* medium was adjusted to various values with HCl or NaOH at room temperature. The pH was found to be stable during the cultivation period. To test the effects of sea salt concentration on growth, varying dilutions of 2 × MJ (−N) synthetic sea water supplemented with 0·2 % (w/v) yeast extract, 0·2 % (w/v) Trypticase peptone, 20 mM $NaNO_3$ and 5 mM $FeSO_4$ (pH 6·0) were used instead of MJYP* medium. Autotrophic growth was tested in MJ (−N) synthetic sea water containing 0·1 % (w/v) $NaHCO_3$, 0·1 % (v/v) trace vitamin solution (Balch *et al*., 1979), 20 mM $NaNO_3$ and either 5 mM $FeSO_4$ or 3 % (w/v) S^0 (pH 6·0) in the presence of sodium sulfide under a gas phase of H_2/CO_2 (80:20, 200 kPa) or N_2/CO_2 (80:20,

200 kPa) for anaerobic conditions and in the absence of sodium sulfide under a gas phase of $H_2/CO_2/N_2/O_2$ (60:10:25:5, 200 kPa) for microaerobic conditions.

Effect of pressure on growth. The effect of hydrostatic pressure on growth was examined by using the 'DEEP-BATH' system, a high-pressure/high-temperature bioreactor, as described previously (Moriya *et al*., 1995; Takai *et al*., 1999). Cells pre-cultured in MJYP* medium at 84 °C were transferred to a 1·5 l glass vessel containing 1 litre MJYP* medium, pre-heated to 84 °C and pre-pressurized at 0·1, 10, 20, 30, 40, 50 or 60 MPa under a gas phase of 100 % N_2, which was then weakly agitated. For tests under hydrostatic pressures of 0·1, 10, 20 or 30 MPa, a sample pre-cultured under the same hydrostatic pressure was used as the inoculum. In case of higher pressures (40, 50 or 60 MPa), cells grown at 30 MPa were used for the inocula. Samples were taken at regular intervals and growth under each pressure was determined by direct cell counting.

Organic substrates for growth. In an attempt to find organic substrates that could support growth of the isolate, experiments were conducted in which the yeast extract and Trypticase peptone in MJYP* medium were replaced with other organic materials as potential substrates. These were added at concentrations of 0·02 or 0·2 % (w/v). The cells were pre-cultured in each medium prior to inoculation of the same medium. These tests were performed in duplicate at 80 °C.

Determination of growth. Growth of the new isolate was determined by direct cell counting after staining with 4′,6-diamidino-2-phenylindole (DAPI) (Porter & Feig, 1980) using a Nikon Optishot microscope equipped with a Nikon FX-II camera system. Cultures were prepared in duplicate. The cells were grown in 100 ml glass bottles (Schott Glaswerke), each containing 20 ml medium, in a temperature-controlled dry oven (Taitec). The pH growth curve was determined at 80 °C and the growth conditions for all other cultivation tests were 80 °C and pH 6·0 adjusted at room temperature, unless noted otherwise.

Antibiotic susceptibility. Susceptibility to the antibiotics vancomycin, streptomycin, chloramphenicol and rifampicin at final concentrations of 50, 100 and 150 μg ml^{-1} was determined in MJYP* medium. *Rhodothermus obamensis* and *Thermaerobacter marianensis* were used as controls to check the effectiveness of the antibiotics at 80 °C.

Light and electron microscopy. Cells were routinely observed under a phase-contrast microscope (Optishot 2; Nikon). For microscopy at 80 °C, a drop of culture at 80 °C was placed on a slide preheated to 85 °C and observed immediately. Micrographs were obtained by using a Nikon Optishot microscope equipped with a Nikon FX-II camera system. Transmission electron microscopy of negatively stained cells was carried out as described by Zillig *et al*. (1990). Cells grown in MJYP* medium at 80 °C in the mid-exponential phase of growth were negatively stained with 2 % (w/v) uranyl acetate and observed under a JEOL JEM-1210 electron microscope at an accelerating voltage of 80 kV. For ultrathin sectioning, cells grown in MJYP* medium at 80 °C in the mid-exponential phase of growth were fixed with 4 % (w/v) paraformaldehyde for 1 h at room temperature. Thin sections were prepared and stained with uranyl acetate and lead citrate and observed with a JEOL JEM-1210 electron microscope at an accelerating voltage of 80 kV.

Techniques for analysis of inorganic metabolites. The concentrations of inorganic substances were monitored during growth of the isolate in MJYP medium supplemented with 5 mM $FeSO_4$ ($NaNO_3$-free) or in MJYP* medium (20 mM $NaNO_3$) in the absence of sodium sulfide at 80 °C. The concentration of soluble ferrous iron was measured by using ferrozine (Lovley & Phillips, 1986) and the concentration of soluble ferric iron was determined by the same method after reduction with 0·28 M hydroxyl-ammonium chloride in a sample acidified with 1 M HCl and heated at 100 °C for 10 min; the ferrous iron concentration was subtracted. For cultivation on ferric iron-dependent medium, 10 mM iron(III) citrate and poorly crystalline iron(III) oxide (10 mM) (Lovley & Phillips, 1986) were added to MJYP medium without sodium sulfide. To monitor nitrate reduction, the diazotization method was employed to determine the concentration of nitrate/nitrite (Matsunaga & Nishimura, 1969) and Nessler's reagent was employed to measure the ammonia concentration in the medium (Allen *et al*., 1974). The production of sulfite was checked by using a semi-quantitative test paper, Quantofix Sulphite (Macherey-Nagel). H_2S was analysed quantitatively by the methylene blue method (Fonselius, 1983) and H_2 production was tested by using a H_2 detection tube (GASTEC).

Lipid analysis. Core ether lipids were analysed by the modified whole-cell acid methanolysis method (De Rosa & Gambacorta, 1988). Lyophilized cells (300 mg) were mixed with 1 ml methanol containing 5 % (w/v) HCl and heated at 100 °C for 2 h. Next, core lipids were extracted with 1 ml chloroform and the sample was evaporated. The residue was dissolved in a small amount of chloroform and analysed by TLC on silica gel plates (Merck Kieselgel 60; Merck) developed in hexane/ethyl acetate (7:3, v/v) or chloroform/ethanol (9:1, v/v). All compounds were visualized by spraying with 50 % (w/v) sulfuric acid, followed by heating at 160 °C for 5 min. The intensity of coloured spots corresponding to each compound was measured by using a spectrophotoscanner (Shimadzu) and expressed as the integral optical density (IOD). Authentic core ether lipids extracted from *Sulfolobus acidocaldarius* were used as a reference.

Isolation and base composition of DNA. DNA was extracted and purified as described by Marmur & Doty (1962) and Lauerer *et al*. (1986). The G+C content of DNA was determined by direct analysis of deoxyribonucleotides by HPLC (Tamaoka & Komagata, 1984). Nonmethylated DNA from bacteriophage λ (G+C content 49·8 mol %; TaKaRa) (Sanger *et al*., 1982) was used as reference material.

Amplification of 16S rRNA gene and sequence determination. The 16S rRNA gene (rDNA) was amplified by the PCR using Arch 21F (Fuhrman *et al*., 1992) and 1492R (DeLong, 1992) primers. The 1·5 kb PCR product was sequenced directly by the dideoxynucleotide chain termination method using a DNA sequencer (model 373As; Perkin Elmer ABI). The rDNA sequence was analysed by using SIMILARITY_RANK and ALIGN_SEQUENCE from the Ribosomal Database Project (RDP) (Larsen *et al*., 1993) and the gapped BLAST search algorithm (Altschul *et al*., 1997; Benson *et al*., 1998) to estimate the degree of similarity to other archaeal 16S rDNA sequences.

Data analysis. The almost complete sequence (1409 bp) of 16S rDNA of strain DMJ^T was aligned manually to 16S rDNA sequences from the RDP based on primary and secondary structure considerations by using the Genetic Data Environment (GDE) multiple-sequence editor. Phylogenetic analyses were restricted to nucleotide positions that were unambiguously alignable in all sequences. Least-

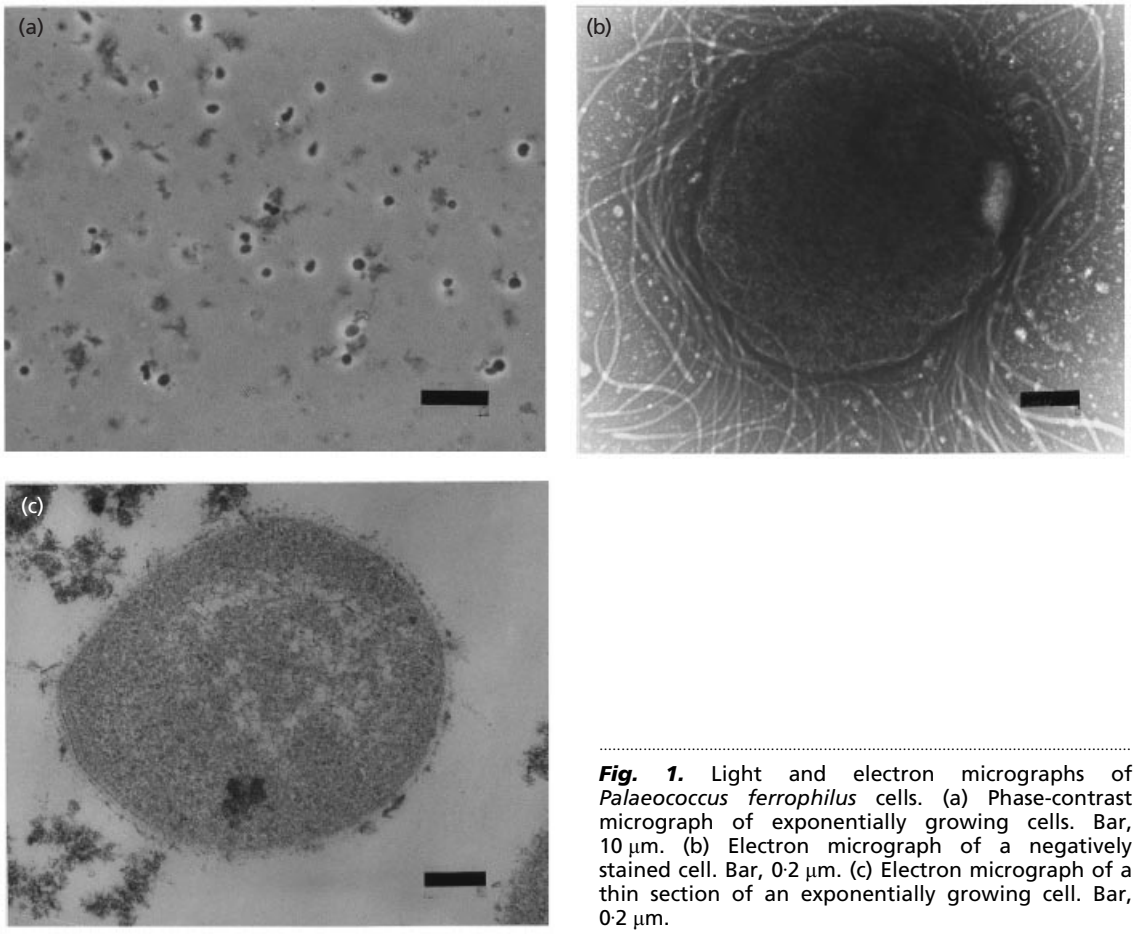

Fig. 1. Light and electron micrographs of *Palaeococcus ferrophilus* cells. (a) Phase-contrast micrograph of exponentially growing cells. Bar, 10 μm. (b) Electron micrograph of a negatively stained cell. Bar, 0·2 μm. (c) Electron micrograph of a thin section of an exponentially growing cell. Bar, 0·2 μm.

squares distance matrix analysis (Olsen, 1988), based on evolutionary distances, was performed by using the correction of Kimura (1980). Neighbour-joining analysis was accomplished by using the ODEN software package (version 1.1, National Institute of Genetics, Mishima, Japan). Maximum-likelihood analysis was performed by using the PHYLIP package (version 3.5; obtained from J. Felsenstein, University of Washington, Seattle). Bootstrap analysis was used to provide confidence estimates for phylogenetic tree topologies.

Cultivation test. Representative members of the *Thermococcales* were cultivated in media containing various inorganic substances to examine the effect of iron on their growth. *Pyrococcus* species were cultivated at 95 °C and *Thermococcus* species were cultivated at 80 °C under a gas phase of N_2 (100 %, 200 kPa). The pH of all media was adjusted to 6·0 at room temperature.

RESULTS

Enrichment and purification

Enrichment from a black smoker chimney sample in MJYP* medium occurred at 75 and 84 °C, and only highly motile, irregular coccoid cells were observed after incubation for 2 d. Although the cells could not form colonies in the tubes of MJYP* medium containing 2 or 3 % (w/v) agar (melting temperature 80 °C; Nacarai Tesque) at 75 °C, they were successfully purified by the dilution-to-extinction technique in 10 separate series at 75 °C. The culture in the tube showing growth at the highest dilution was designated strain DMJ^T (= JCM 10246^T). The purity of this culture was confirmed routinely by microscopic examination and by repeated partial sequencing of the 16S rRNA gene using several PCR primers. All further experiments were performed with isolate DMJ^T.

Morphology

The cells were irregular cocci, 0·5–2·0 μm in diameter, often appearing singly or in pairs (Fig. 1a). As observed by light microscopy at 80 °C, the cells were highly motile, and transmission electron microscopic examination of negatively stained cells indicated that the cells had multiple flagella (Fig. 1b). Electron micrographs of thin sections indicated the presence of an *S*-layer-like structure (Fig. 1c). As described by Blöchl *et al*. (1997), the periplasmic space was little stained by the conventional method of chemical fixation and dehydration at room temperature (Fig. 1c). The dense layer in the outer part presumably revealed the heads of the *S*-layer-anchoring protein complexes (Baumeister & Lembcke, 1992).

Table 1. The effect of inorganic substances on growth of *Palaeococcus ferrophilus*

Cells were grown at 80 °C under a gas phase of N_2 (100 %, 200 kPa) and the pH of all media was 6·0 at room temperature. MJYP, MJYP* and MJYPS media were described in Methods. Maximum cell yield is given as: ++, 1×10^8–5×10^8 cells ml^{-1} culture; +, 5×10^7–1×10^8 cells ml^{-1} culture; −, no growth.

Medium	Maximum cell yield
MJYP*	++
MJYP with 20 mM $NaNO_3$ and 0·5 mM $FeSO_4$	+
MJYP with 2 mM $NaNO_3$ and 5 mM $FeSO_4$	+
MJYP with 2 mM $NaNO_3$ and 0·5 mM $FeSO_4$	+
MJYP with 20 mM $NaNO_3$ and 5 mM $FeSO_4$ without Na_2S	++
MJYP	−
MJYP with 0·5 mM $FeSO_4$	+
MJYP with 5 mM $FeSO_4$	++
MJYP with 2 mM $NaNO_3$	−
MJYP with 20 mM $NaNO_3$	−
MJYP with 20 mM NH_4Cl and 5 mM $FeSO_4$	++
MJYP with 2 mM NH_4Cl and 5 mM $FeSO_4$	++
MJYP with 20 mM $NaNO_3$ and 5 mM FeS	−
MJYP with 20 mM $NaNO_3$ and 5 mM FeS_2	++
MJYP with 20 mM $NaNO_3$ and 0·5 mM FeS_2	++
MJYP with 20 mM $NaNO_3$ and 5 mM FeS_2 without Na_2S	++
MJYP with 20 mM $NaNO_3$ and 5 mM Fe_2S_3	++
MJYP with 20 mM $NaNO_3$ and 0·5 mM Fe_2S_3	+
MJYP with 20 mM $NaNO_3$ and 5 mM Fe_2S_3 without Na_2S	++
MJYPS	++ (H_2S formed)
MJYP with 20 mM $NaNO_3$ and 5 mM $Na_2S_2O_3$	−
MJYP with 20 mM $NaNO_3$ and 5 mM $Na_2S_2O_5$	−
MJYP with 20 mM $NaNO_3$ and 5 mM Na_2SO_3	−
MJYP* with 3 % (w/v) S^0	+ (H_2S formed)
MJYP* with 5 mM $Na_2S_2O_3$	++

Antibiotic susceptibility

The control bacteria *Rhodothermus obamensis* and *Thermaerobacter marianensis* exhibited the expected pattern of antibiotic susceptibility at 80 °C, indicating that the antibiotics used in this study were effective at this cultivation temperature. The new isolate was found to be resistant to vancomycin, streptomycin and chloramphenicol at 150 μg ml^{-1} and to rifampicin at 100 μg ml^{-1}.

Growth parameters

The new isolate grew only under strictly anaerobic culture conditions (Table 1). The isolate was found to be a heterotroph; it did not grow under any of the autotrophic culture conditions tested. Under a N_2 gas phase, growth on MJYP* medium, which contains $FeSO_4$, was almost the same as growth on MJYPS, which contains S^0. However, in the presence of H_2, no growth was observed in MJYP* medium, whereas growth was unaffected on MJYPS medium. H_2 was produced during growth in MJYP* medium, while H_2S was detected as a major gas product during growth in MJYPS medium.

The isolate grew over the temperature range of about 60 to 88 °C, showing optimum growth at 83 °C, and the generation time at 83 °C was about 30 min at pH 6·0 (Fig. 2a). No growth was observed at 90 °C. Growth of the new isolate at 80 °C occurred between pH 4·0 and 8·0, with optimum growth at about pH 6·0 (Fig. 2b). No growth was detected below pH 4·0 or above pH 8·0.

The new isolate required sea salts for growth. It grew over the concentration range of about 20 to 73 g sea salts l^{-1}, with optimum growth at around 43 g sea salts l^{-1} at 80 °C and pH 6·0 (Fig. 2c). Below 20 or above 73 g sea salts l^{-1}, no growth was observed and the cells lysed rapidly.

The growth rate and cell yield of the isolate were enhanced at hydrostatic pressures of 30 and 10 MPa, respectively (Fig. 2d). These results indicated that the new isolate was a barophilic micro-organism with an optimum pressure for growth (30 MPa) higher than

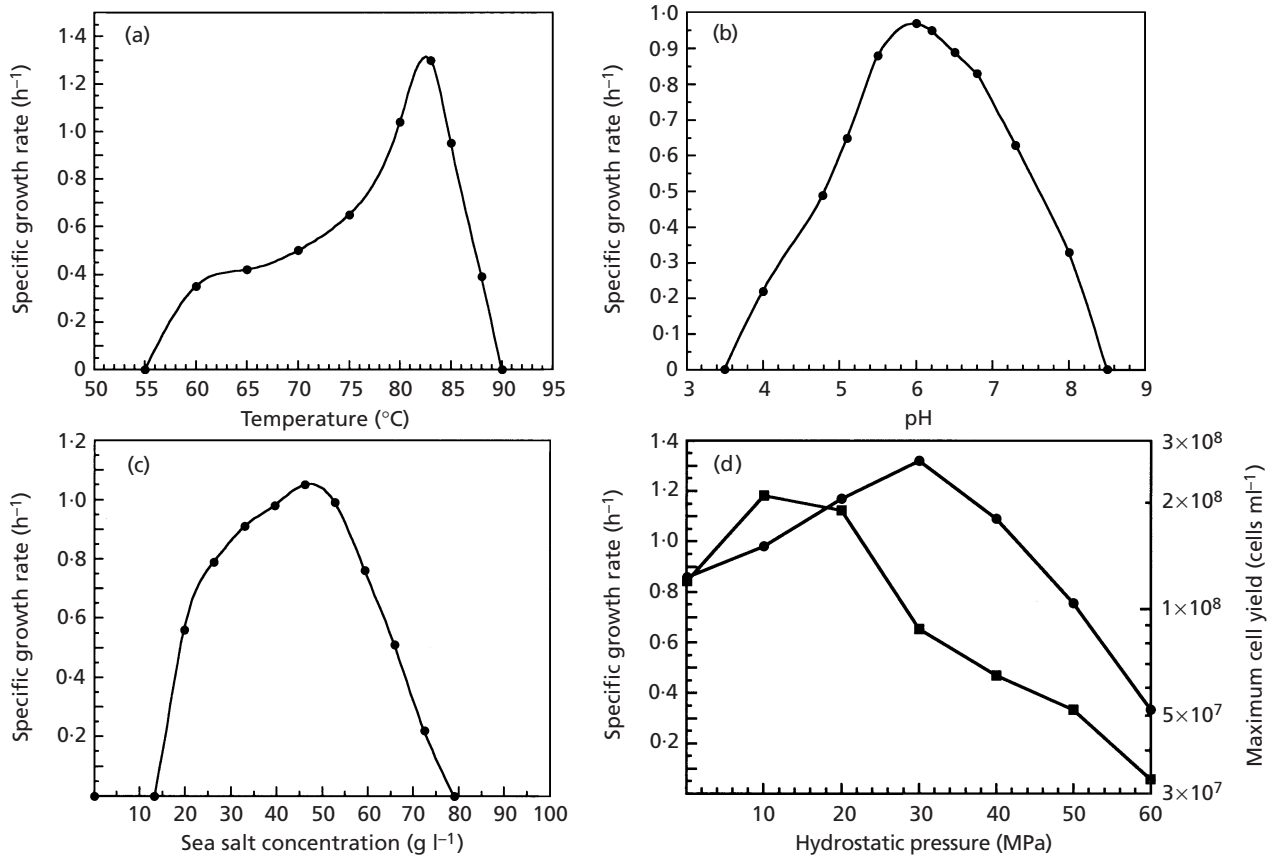

Fig. 2. Effects of temperature (a), pH (b), sea salt concentration (c) and hydrostatic pressure (d) on growth of *Palaeococcus ferrophilus*. (a) Growth was determined in MJYP* medium at pH 6·0. No growth occurred below 60 °C or above 88 °C. (b) Growth was determined in MJYP* medium at 80 °C. The pH of the medium was adjusted with HCl or NaOH at room temperature. No growth occurred below pH 4·0 or above pH 8·0. (c) Growth was determined in varying dilutions of 2× MJ (−N) synthetic sea water containing 0·2 % (w/v) yeast extract and 0·2 % (w/v) Trypticase peptone supplemented with 20 mM $NaNO_3$ and 5 mM $FeSO_4$ (pH 6·0), instead of MJYP* medium. No growth was observed at sea salt concentrations below 20 or above 73 g l^{-1}. (d) Growth rate (●) and maximum cell yield (■) were determined by using the 'DEEP-BATH' system in MJYP* medium at 84 °C and pH 6·0.

the *in situ* hydrostatic pressure (13 MPa) at the isolation site. When the isolate was cultivated in MJYPS medium instead of MJYP* medium, the highest growth rate and cell yield were obtained at 30 MPa (data not shown). In the 'DEEP-BATH' system (Moriya *et al.*, 1995; Takai *et al.*, 1999), the dissolution of gas components increases with increasing hydrostatic pressure. H_2 was probably produced in the course of growth in the presence of $FeSO_4$, and accumulation of H_2 may have inhibited the subsequent growth of the isolate and reduced the final cell yield. Hence, the lower optimum pressure in terms of cell yield from the culture in the presence of $FeSO_4$ may have been due to H_2 accumulation.

Inorganic and organic substrates

Since the isolate did not grow in a medium consisting only of synthetic sea water and organic substrates such as yeast extract and Trypticase peptone, various inorganic substances were added to the medium to determine which served to support growth of the isolate as energy sources or growth factors (Table 1). The isolate was capable of growth using yeast extract and Trypticase peptone when $FeSO_4$, FeS_2, Fe_2S_3 or S^0 were added to the medium (Table 1). The presence of $NaNO_3$ or NH_4Cl was not required absolutely for growth of the isolate and no requirement for other inorganic substances was observed. In the presence of S^0, H_2S was produced during growth, as determined by the methylene blue method, indicating that sulfur reduction was associated strongly with growth of the isolate. In contrast, H_2 was produced during growth in the presence of $FeSO_4$, FeS_2 or Fe_2S_3, while little H_2S was detected. The presence of both S^0 and $FeSO_4$ resulted in a somewhat lower cell yield (Table 1). When the new isolate was grown at varying concentrations of $FeSO_4$, FeS_2 or Fe_2S_3, the maximum cell yield was increased with increasing concentration of each substrate (Fig. 3). This result also supported the conclusion that the new isolate required iron during growth in the absence of sulfur.

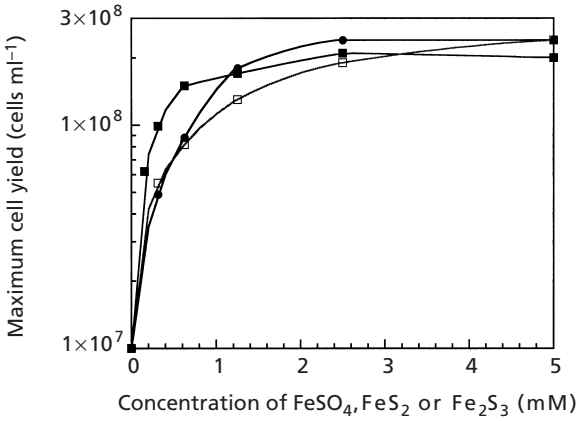

Fig. 3. Effect of concentration of $FeSO_4$ (●), FeS_2 (■) or Fe_2S_3 (□) on growth of *Palaeococcus ferrophilus*. The maximum cell yields were determined in MJYP medium containing 20 mM $NaNO_3$ and various concentrations of iron compounds at 80 °C and pH 6·0.

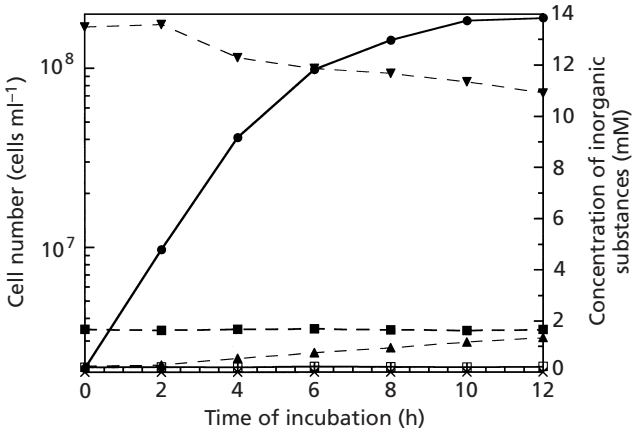

Fig. 4. Change in inorganic metabolites during growth of *Palaeococcus ferrophilus*. The cell number (●) and concentrations of Fe^{2+} (■), Fe^{3+} (□) NO_3^- (▼), NO_2^- (×) and NH_4^+ (▲) were determined during the period of growth in MJYP* medium in the absence of sodium sulfide at 80 °C and pH 6·0.

In order to determine which form of iron, ferrous or ferric, was required for growth and how the iron functioned for growth, the concentrations of soluble ferrous and ferric iron in the medium were monitored during growth in the presence or absence of nitrate. When the cells were cultivated in MJYP medium supplemented with 5 mM $FeSO_4$ ($NaNO_3$-free) in the absence of sodium sulfide, the concentrations of soluble ferrous and ferric iron were 1·4 and 0·2 mM immediately after culture inoculation and were stable during the period of growth. In the case of the growth in MJYP* medium, the concentrations of soluble ferrous and ferric iron were maintained at their initial levels (1·6 mM and 0·2 mM) (Fig. 4). This result implied that iron was not associated with either ferrous iron oxidation or ferric iron reduction during growth. Although little production of ammonium ions was observed during growth on $NaNO_3$-free medium, the ammonium ion concentration increased with decreasing nitrate ion concentration during growth on MJYP* medium (Fig. 4). The reduced nitrate ion concentration and the elevated ammonium ion concentration were not observed during incubation of uninoculated MJYP* medium. These results suggested that the new isolate was able to use nitrate as an electron acceptor and to form ammonium in the presence of iron, whereas nitrate reduction was not absolutely required for growth. In either medium in the absence or presence of $NaNO_3$, H_2 was produced during growth and no H_2S was detected.

To determine the effect of ferric iron on growth, the new isolate was cultivated in MJYP medium supplemented with 10 mM iron(III) citrate or poorly crystalline iron(III) oxide (10 mM) without sodium sulfide (Lovley & Phillips, 1986). However, no growth occurred on the medium containing ferric iron alone. The growth characteristics with various inorganic substances suggested strongly that the new isolate required either elemental sulfur or ferrous iron absolutely for growth. Because ferrous iron oxidation did not occur during growth, nitrate reduction was not associated with iron oxidation.

Organic substrates that served to support heterotrophic growth were determined in the presence of ferrous iron or elemental sulfur as cofactors for growth. In either case, the isolate grew only in media containing proteinaceous substrates such as yeast extract, Trypticase peptone, tryptone or casein. In addition, the maximum cell yield was enhanced by increased concentrations of these substrates. No growth occurred with starch, Casamino acids, any of various sugars (maltotriose, cellobiose, maltose, lactose, trehalose, sucrose, glucose, galactose, fructose or xylose), organic acids (tartrate, succinate, propionate, 2-aminobutyric acid, malate, lactate, pyruvate or acetate) or amino acids.

Lipid analysis

In common with other members of the *Archaea*, the isolate was found to have ether lipid membranes. The major core lipids were C_{40} tetraether lipids (>80%) and a small proportion of C_{20},C_{20} diether lipids was also detected (<20%).

DNA base composition

The G+C content of the genomic DNA of strain DMJ^T was found to be 53·5 mol%.

Phylogenetic analyses

Almost the complete sequence (1409 bp) of the 16S rRNA gene was determined from strain DMJ^T. The 16S rDNA sequence of the new isolate was determined

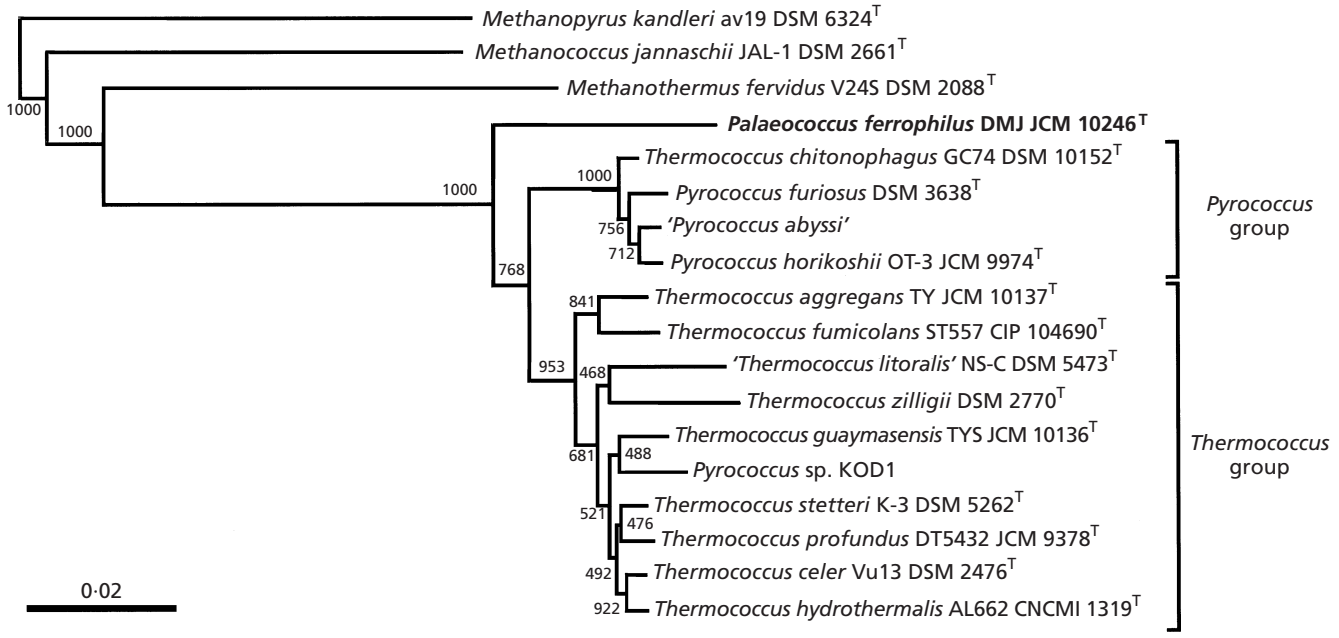

Fig. 5. Phylogenetic tree of representative members of the *Thermococcales* within the Euryarchaeota inferred by the neighbour-joining method from 16S rDNA sequences using 1229 homologous sequence positions for each organism. Each number represents the bootstrap value for branching (1000 replicates). The scale bar indicates two substitutions per 100 nucleotides. The 16S rDNA sequences in this figure are from GenBank with the accession numbers: *Methanopyrus kandleri*, M59932; *Methanococcus jannaschii*, M59126; *Methanothermus fervidus*, M32222; *Pyrococcus furiosus*, U20163; *Pyrococcus horikoshii*, D87344; '*Pyrococcus abyssi*', L19921; *Pyrococcus* sp. KOD1, D38650; *Thermococcus chitonophagus*, X99570; *Thermococcus aggregans*, Y08384; *Thermococcus fumicolans*, Z70250; '*Thermococcus litoralis*', Z70252; *Thermococcus zilligii*, U76534; *Thermococcus guaymasensis*, Y08384; *Thermococcus stetteri*, Z75240; *Thermococcus profundus*, Z75233; *Thermococcus celer*, M21529; *Thermococcus hydrothermalis*, Z70244; *Palaeococcus ferrophilus*, AB019239.

repeatedly to check culture purity and the reproducibility of PCR amplification of the rDNA sequence was confirmed. Furthermore, inspection of the predicted secondary structure and evaluation by the CHECK_CHIMERA program of the RDP (Larsen *et al.*, 1993) indicated that the rDNA sequence of DMJ^T was free from chimeric artifacts (data not shown). The rDNA sequence of the isolate was most closely related to those of *Thermococcus* and *Pyrococcus* species, but showed less than 93% similarity to either of these genera (92·9% to *Thermococcus guaymasensis* and *Thermococcus profundus*; 92·8% to *Thermococcus celer*; 92·6% to *Thermococcus stetteri*, *Thermococcus aggregans* and *Thermococcus hydrothermalis*; 92·3% to *Thermococcus zilligii*; 91·9% to '*Thermococcus litoralis*'; 91·6% to *Thermococcus fumicolans*; 91·4% to *Thermococcus chitonophagus*; 92·5% to *Pyrococcus* sp. KOD1; 92·2% to *Pyrococcus furiosus*; 91·8% to *Pyrococcus horikoshii* and '*Pyrococcus abyssi*'; 84·4% to *Methanothermus fervidus*; 83·8% to *Methanococcus jannaschii*; 83·4% to *Archaeoglobus fulgidus*; 78·6% to *Methanopyrus kandleri*). These findings imply that the new isolate is a member of the order *Thermococcales* within the Euryarchaeota, even though the isolate appears to be phylogenetically distant from the two genera within the *Thermococcales*. For phylogenetic placement of the isolate, phylogenetic trees were constructed by the neighbour-joining and maximum-likelihood methods.

Phylogenetic analyses by the neighbour-joining and maximum-likelihood methods indicated exactly identical topologies in the affiliation of the new isolate, placing this organism prior to the divergence between *Pyrococcus* and *Thermococcus* (Fig. 5). On the basis of its phylogenetic placement, the new isolate is deemed to be a member of an ancient lineage of *Thermococcales*, representing a genus distinct from *Pyrococcus* and *Thermococcus*. The bootstrap confidence estimate also revealed high significance in the placement of strain DMJ^T (Fig. 5).

Cultivation test

Representative members of the *Thermococcales* were cultivated in media containing various inorganic substances to examine the effect of iron on their growth (Table 2). Although all members of the *Thermococcales* including the new isolate grew in MJYPS medium, only *Thermococcus stetteri* and *Thermococcus aggregans* in addition to the new isolate grew on MJYP* medium (Table 2). These two species also grew on MJYP medium containing 20 mM $NaNO_3$ and on other media without elemental sulfur or ferrous iron. The results indicated that these *Thermococcus* species

Table 2. Comparison of the growth of members of the *Thermococcales* in various media

Pyrococcus species were cultivated at 95 °C and *Palaeococcus* and *Thermococcus* species were cultivated at 80 °C under a gas phase of N_2 (100%, 200 kPa). All media were based on MJYP medium containing 20 mM $NaNO_3$. The various media were then supplemented with: A, no addition; B, 3% S^0; C, 5 mM $FeSO_4$; D, 5 mM FeS_2; E, 5 mM Fe_2S_3; F, 5 mM FeS; G, 5 mM $CuSO_4$, $NiSO_4$, $MnSO_4$, $CoSO_4$ or VSO_4. The pH of all media was adjusted to 6·0 at room temperature. Maximum cell yield is given as: ++, 1×10^8–5×10^8 cells ml^{-1} culture; +, 5×10^7–1×10^8 cells ml^{-1} culture; −, no growth.

Taxon	Maximum cell yield						
	A	B	C	D	E	F	G
Palaeococcus ferrophilus	−	++	++	++	++	−	−
Pyrococcus horikoshii	−	++	−	−	−	−	−
Pyrococcus furiosus	−	++	−	−	−	−	−
'*Pyrococcus abyssi*'	−	++	−	−	−	−	−
Thermococcus aggregans	++	++	++	++	++	++	++
'*Thermococcus litoralis*'	−	++	−	−	−	−	−
Thermococcus guaymasensis	−	++	−	−	−	−	−
Thermococcus stetteri	++	++	++	++	++	++	++
Thermococcus peptonophilus	++	++	−	−	−	−	−
Thermococcus profundus	−	++	−	−	−	−	−
Thermococcus celer	−	++	−	−	−	−	−

were able to grow on media without elemental sulfur or ferrous iron and that the inorganic substances had little effect on their growth. An absolute requirement for ferrous iron was observed only in the new isolate among the members of the *Thermococcales* tested.

DISCUSSION

A novel barophilic, hyperthermophilic archaeon was isolated from a black smoker chimney at the Myojin Knoll in the Ogasawara-Bonin Arc, Japan. The isolate was found to be a strictly anaerobic, obligately heterotrophic micro-organism capable of growth using proteinaceous substrates such as yeast extract, peptone, tryptone and casein in the presence of either elemental sulfur (S^0) or ferrous iron (Fe^{2+}). The isolate produced H_2S when cultivated in the presence of S^0 or in the presence of both S^0 and Fe^{2+}, while it produced H_2 during growth in the presence of Fe^{2+}. These features of fermentative-type metabolism are similar to those displayed by members of the *Thermococcales*, most of which are capable of growth using peptides in the presence or absence of S^0 (Zillig *et al.*, 1983; Fiala & Stetter, 1986; Miroshnichenko *et al.*, 1989; Pledger & Baross, 1989, 1991; Neuner *et al.*, 1990; Gonzalez *et al.*, 1995, 1998; Godfroy *et al.*, 1996, 1997; Antoine *et al.*, 1997; Canganella *et al.*, 1997). The major core lipids of the new isolate were tetraether and diether lipids, which are the most predominant core lipids in the *Thermococcales* (Zillig *et al.*, 1983; Pledger & Baross, 1991; Godfroy *et al.*, 1996, 1997; Gonzalez *et al.*, 1998), and the 16S rDNA sequence was most closely related to those of members of the *Thermococcales* (Fig. 5). However, the representative species of the *Thermococcales* tested did not exhibit an absolute dependence on ferrous iron for growth in the absence of sulfur (Table 2). This is the first report of a member of the *Thermococcales* displaying an absolute requirement for ferrous iron for fermentative growth in the absence of sulfur. Phylogenetic analysis revealed that the new isolate is most closely related to members of the *Thermococcales*, representing an ancient lineage distinct from *Pyrococcus* and *Thermococcus*. Although most of the morphological and physiological properties of the new isolate were similar to those of other members of the *Thermococcales*, it seemed likely that the absolute requirement of the new isolate for ferrous iron for fermentative growth in the absence of sulfur was one of the discriminative properties. Furthermore, the phylogenetic position of the new isolate should be considered as important evolutionary evidence that discriminates the new isolate clearly from other members of the *Thermococcales* at the genus level. Therefore, we propose a new genus, *Palaeococcus* gen. nov. The type species is *Palaeococcus ferrophilus* sp. nov., of which the type strain is strain DMJ^T (= JCM 10246^T).

Recently, there has been increasing interest in the role of iron in early microbial metabolism in view of geochemical and microbiological findings (Walker, 1987; Ehlich, 1990; Cairns-Smith *et al.*, 1992; Seyfried & Mottl, 1995; Hafenbradl *et al.*, 1996; Slobodkin *et al.*, 1997; Slobodkin & Wiegel, 1997; Vargas *et al.*, 1998). Vargas *et al.* (1998) have demonstrated that many hyperthermophilic archaea and bacteria can reduce ferric iron and conserve energy to support growth. They have also suggested that not only sulfur-

reducing hyperthermophiles but also those that grow by fermentation are capable of using ferric iron as a terminal electron acceptor (Vargas *et al*., 1998). Hafenbradl *et al*. (1996) isolated the first hyperthermophilic archaeon found to be capable of oxidizing ferrous iron anaerobically at neutral pH, from a shallow marine hydrothermal vent environment, an organism named *Ferroglobus placidus*. On the basis of its metabolic properties, it has been speculated that the formation of ferric iron by such unique microorganisms under anaerobic conditions might have contributed to banded iron formation in the early history of the Earth (Hafenbradl *et al*., 1996). Compared with these iron-reducing or iron-oxidizing hyperthermophiles, *Palaeococcus ferrophilus* requires ferrous iron for fermentative growth but does not utilize iron as an electron acceptor or donor. In the fermentative-type metabolism of *Thermococcus* and *Pyrococcus* species, S^0 reduction is thought to be a mechanism for detoxifying inhibitory H_2 (Fiala & Stetter, 1986), or it may play a role in energy conservation (Schicho *et al*., 1993). Ma *et al*. (1995) have reported that the production of H_2 and some alcohols is increased because of the enhanced activities of iron hydrogenase and iron-alcohol dehydrogenase when *Thermococcus* sp. ES1 is cultivated under S^0-limited conditions. These enzymes are thought to function in the disposal of excess reductant and may play an important role in S^0-limited growth (Ma *et al*., 1995). Likewise, *Palaeococcus ferrophilus* produces H_2S when cultivated in the presence of S^0 but H_2 is produced instead during growth in the presence of Fe^{2+}. It seems likely, therefore, that there are several enzymes, such as iron hydrogenase and iron-alcohol dehydrogenase in *Thermococcus* sp. ES1, that are regulated by ferrous iron, and relatively high concentrations of ferrous iron might be required for proper operation of the iron(II)-regulated fermentation pathway in *Palaeococcus ferrophilus*. Considering that iron is one of the most abundant chemical materials in the black smoker fluids and chimneys (Seyfried & Mottl, 1995), iron could play a key role in the fermentative metabolism of organisms inhabiting black smoker environments, such as *Palaeococcus ferrophilus*. Further investigation of the function of ferrous iron in *Palaeococcus ferrophilus* may shed light on an as yet undiscovered role of iron in early microbial metabolism besides its function as an electron donor/acceptor.

Description of *Palaeococcus* gen. nov.

Palaeococcus (Pa.la.eo.coc′cus. Gr. adj. *palaios* ancient; Gr. n. *kokkos* grain or kernel; M.L. masc. n. *Palaeococcus* ancient spherical cell, denoting the ancient lineage of this organism).

Irregular coccoid, highly motile with multiple flagella. Anaerobic, barophilic and hyperthermophilic. Neutrophilic heterotroph. Requirement for sea salts for growth. Able to utilize proteinaceous substrates such as yeast extract, peptone, tryptone and casein in the presence of elemental sulfur (S^0) or ferrous iron (Fe^{2+}) as a cofactor for growth. G+C content of genomic DNA is about 54 mol%. Major core lipids are C_{40} tetraether and C_{20} diether lipids. On the basis of 16S rRNA gene analysis, the genus *Palaeococcus* is most closely related to the genera *Pyrococcus* and *Thermococcus* and is a member of the order *Thermococcales*. Members of the genus *Palaeococcus* occur in deep-sea hydrothermal vent environments, so-called 'black smoker' environments. The type species is *Palaeococcus ferrophilus*.

Description of *Palaeococcus ferrophilus* sp. nov.

Palaeococcus ferrophilus (fer.ro′phi.lus. L. n. *ferrum* iron; Gr. adj. *philos* loving; M.L. adj. *ferrophilus* iron-loving, indicating that it requires iron for growth in the absence of sulfur).

Irregular cocci, 0·5–2·0 μm in diameter. Cells occur singly or in pairs. Exhibits high motility with multiple flagella. Strictly anaerobic. The temperature range for growth is 60–88 °C with the optimum being 83 °C. The pH range for growth is from 4·0 to 8·0, with optimum growth occurring at pH 6·0. Sea salts in the concentration range of 20–73 g l^{-1} are required for growth, with optimum growth occurring at 43 g l^{-1}. The optimum hydrostatic pressure for growth is 30 MPa. Growth using yeast extract, peptone, tryptone or casein occurs in the presence of elemental sulfur (S^0) or ferrous iron (Fe^{2+}) as a required cofactor. Major core lipids are C_{40} tetraether (>80%) and C_{20} diether lipids (<20%). The G+C content of genomic DNA is about 53·5 mol% (HPLC). The 16S rDNA sequence exhibits 93% similarity to those of members of the genera *Pyrococcus* and *Thermococcus*. The organism was isolated from a black smoker sample obtained from the Myojin Knoll in the Ogasawara-Bonin Arc, Japan, at a depth of 1338 m.

The type strain of *Palaeococcus ferrophilus* is strain DMJ^T, which has been deposited in the Japan Collection of Microorganisms, Institute of Physical and Chemical Research (RIKEN), Wako-shi, Saitama, Japan, under accession number JCM 10246^T.

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