**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 DMJ¹ (≡ JCM 10246T).

**Keywords:** hyperthermophilic archaea, 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.*, 1988, 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 (S⁰).

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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The GenBank/EMBL/DDBJ accession number for the 16S rDNA sequence of strain DMJ is AB019239.
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 archaean 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 (S⁰) or ferrous iron (Fe⁴⁺) 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 NaNO₃ and 5 mM FeSO₄ 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 10⁶ 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 DMJ².

**Sources of organisms.** Pyrococcus furiosus (DSM 3638⁹), Thermococcus celer (DSM 2476⁵), Thermococcus litoralis (DSM 5473⁵), Thermococcus setteri (DSM 5262⁵) and Sulfolobus acidocaldarius (DSM 639⁹) were purchased from the Japan Collection of Microorganisms (JCM). Pyrococcus abyssi (strain GE5), Thermococcus peptonophilus (JCM 9653⁹) and Thermococcus profundus (JCM 9378⁹) were obtained from the culture collection of our institute.

Thermococcus aggregans (JCM 10137⁹) and Thermococcus guaymasensis (JCM 10136⁹) were kindly provided by Francesco Canganella (University of Tusia, Vierbo, Italy). Rhodothermus oahumansis (JCM 9785⁵) was provided by Yoshihiko Sako (Kyoto University, Kyoto, Japan) (Sako et al., 1996) and Thermoaerobacter marianensis (JCM 10246⁹) 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 Na₂S•9H₂O and 1 mg resazurin 1⁻¹ MJ (−N) synthetic sea water] and supplemented with 20 mM NaNO₃ and 5 mM FeSO₄. When 20 mM NaNO₃ and 3% (w/v) elemental sulfur (S⁰) were added to MJYP medium, this was defined as MJYPS medium. MJ (−N) synthetic sea water consists of (l⁻¹): NaCl, 30.0 g; K₂HPO₄, 0.14 g; CaCl₂•2H₂O, 0.14 g; MgSO₄•7H₂O, 3.4 g; MgCl₂•6H₂O, 4.18 g; KCl, 0.33 g; NiCl₂•6H₂O, 0.5 mg; Na₂SeO₃•5H₂O, 0.5 mg; Fe(NH₄)₂(SO₄)•6H₂O, 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% N₂ and was completed by adding separately prepared anaerobic solutions of NaNO₃ (degassed with 100% N₂ and autoclaved; pH 7.0), iron sulfate (degassed with 100% N₂ and autoclaved; pH 7.5) and sodium sulfide (degassed with 100% N₂ 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% N₂. 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 N₂ 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 (NH₄Cl, NaNO₃, and NaNO₂ at 20 or 2 mM), various sulfur compounds (Na₂S₂O₃, Na₂S₃O₄ and Na₂S₄O₆ at 5 or 0.5 mM, S²⁻ at 3%) and various iron sulfide forms (FeS, FeS₂ and FeS₃ at 5 or 0.5 mM) were used alone or in various combinations, instead of the combination of NaNO₃ and FeSO₄ of MJYP* medium, in the presence or absence of sodium sulfide.

In addition, the stimulation of growth by various metal sulfates (CuSO₄•5H₂O, NiSO₄•6H₂O, MnSO₄•4H₂O, CoSO₄, ZnSO₄ and VSO₄ at 5 mM) was also examined in place of FeSO₄ 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 x MJ (−N) synthetic sea water supplemented with 0-2% (w/v) yeast extract, 0-2% (w/v) Trypticase peptone, 20 mM NaNO₃ and 10 mM FeSO₄ (pH 6.0) were used instead of MJYP* medium. Autotrophic growth was tested in MJ (−N) synthetic sea water containing 0-1% (w/v) NaHCO₃, 0-1% (w/v) trace vitamin solution (Balch et al., 1979), 20 mM NaNO₃ and either 5 mM FeSO₄ or 3% (w/v) S²⁻ (pH 6.0) in the presence of sodium sulfide under a gas phase of H₂/CO₂ (80:20, 200 kPa) or N₂/CO₂ (80:20, 200 kPa).
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 1:51 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% N₂, 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 Optiphot microscope equipped with a Nikon FX-II camera system. Cultures were prepared in duplicate. The cells were grown in 100 ml glass bottles (Schott Glasswerke), 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 Thermoaerobacter 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 (Optiphot 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 Optiphot 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 FeSO₄ (NaNO₃-free) or in MJYP* medium (20 mM NaNO₃) 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). H₂S was analysed quantitatively by the methylene blue method (Fonselius, 1983) and H₂ production was tested by using a H₂ 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/ethanol (9:1, 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 spectrophotocaner (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 using the dideoxyribonucleotide 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* 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-
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 DNADIST 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 N₂ (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 DMJT (= JCM 10246T). 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 DMJT.

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 (Bau-meister & Lembcke, 1992).
Table 1. The effect of inorganic substances on growth of *Palaeococcus ferrophilus*

<table>
<thead>
<tr>
<th>Medium</th>
<th>Maximum cell yield</th>
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<tr>
<td>MJYP* with 20 mM NaNO₃ and 0.5 mM FeSO₄</td>
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<td>MJYP with 2 mM NaNO₃ and 5 mM FeSO₄</td>
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<td>MJYP with 2 mM NaNO₃ and 0.5 mM FeSO₄</td>
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<td>MJYP with 20 mM NaNO₃ and 5 mM FeSO₄ without Na₂S</td>
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<td>MJYP</td>
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<tr>
<td>MJYP with 0.5 mM FeSO₄</td>
<td>+</td>
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<tr>
<td>MJYP with 5 mM FeSO₄</td>
<td>+ +</td>
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<tr>
<td>MJYP with 20 mM NaNO₃</td>
<td>-</td>
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<tr>
<td>MJYP with 20 mM NH₄Cl and 5 mM FeSO₄</td>
<td>+ +</td>
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<tr>
<td>MJYP with 20 mM NaNO₃ and 5 mM FeS</td>
<td>+ +</td>
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<td>MJYP with 20 mM NaNO₃ and 0.5 mM FeS</td>
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<td>MJYP with 20 mM NaNO₃ and 5 mM FeS without Na₂S</td>
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**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 N₂ gas phase, growth on MJYP* medium, which contains FeSO₄, was almost the same as growth on MJYPS, which contains S². However, in the presence of H₂S, no growth was observed in MJYP* medium, whereas growth was unaffected on MJYPS medium. H₂S was produced during growth in MJYP* medium, while H₂S 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 NaNO₃ and 5 mM FeSO₄ (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. H₂ was probably produced in the course of growth in the presence of FeSO₄, and accumulation of H₂ 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 FeSO₄ may have been due to H₂ 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 FeSO₄, FeS₄, Fe₂S₃ or S⁰ were added to the medium (Table 1). The presence of NaNO₃ or NH₄Cl was not required absolutely for growth of the isolate and no requirement for other inorganic substances was observed. In the presence of S⁰, H₂S 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, H₂ was produced during growth in the presence of FeSO₄, FeS₄ or Fe₂S₃, while little H₂S was detected. The presence of both S⁰ and FeSO₄ resulted in a somewhat lower cell yield (Table 1). When the new isolate was grown at varying concentrations of FeSO₄, FeS₄ or Fe₂S₃, 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.
implied that iron was not associated with either ferrous levels (1). Ferrous and ferric iron were maintained at their initial concentrations during the period of growth. In MJYP* medium, the concentrations of soluble iron functioned for growth, the concentrations of soluble iron were monitored during growth in the presence or absence of nitrate. The cell number (●) and concentrations of Fe²⁺ (■), Fe³⁺ (□) NO₃⁻ (◇), NO₂⁻ (×) and NH₄⁺ (▲) 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 oxidation or ferric iron reduction during growth. Although little production of ammonium ions was observed during growth on NaNO₃-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 NaNO₃, H₂S was produced during growth and no H₂S 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 (Lovely & 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-aminoctanoic 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 C₅₅ tetraether lipids (>80%) and a small proportion of C₂₀,C₂₀ diether lipids was also detected (<20%).

**DNA base composition**

The G+C content of the genomic DNA of strain DMJ 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. 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, MS9932; Methanococcus jannaschii, MS9126; Methanothermus fervidus, M32222; Pyrococcus furiosus, U20163; Pyrococcus horikoshii, D87344; *Pyrococcus abyssi*, L19921; Pyrococcus sp. KOD1, D38650; Thermococcus chitonophagus, X99570; Thermococcus aggregans, Y08384; Thermococcus fumiculosus, 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 predict secondary structure and evaluation by the predict structure program of the RDP (Larsen et al., 1993) indicated that the rDNA sequence of DMJ 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 fumiculosus; 91.4% to Thermococcus chitonophagus; 92.5% to Pyrococcus sp. KOD1; 92.2% to Pyrococcus furiosus; 91.8% to *Pyrococcus abyssi*; 84.4% to Methanothermus fervidus; 83.8% to Methanothermus 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 (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 MJYPS* medium (Table 2). These two species also grew on MJYP medium containing 20 mM NaNO₃ and on other media without elemental sulfur or ferrous iron. The results indicated that these *Thermococcus* species
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 (S⁰) or ferrous iron (Fe²⁺). The isolate produced H₂S when cultivated in the presence of S⁰ or in the presence of both S⁰ and Fe²⁺, while it produced H₂ during growth in the presence of Fe²⁺. 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 S⁰ (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 DMJ¹ (= JCM 10246T).

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-

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

<table>
<thead>
<tr>
<th>Taxon</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palaeococcus ferrophilus</td>
<td>–</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Pyrococcus horikoshii</td>
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<td>–</td>
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<tr>
<td>Pyrococcus furiosus</td>
<td>–</td>
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<td>–</td>
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<tr>
<td>‘Pyrococcus abyssi’</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Thermococcus aggregans</td>
<td>+</td>
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<tr>
<td>‘Thermococcus litoralis’</td>
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<td>+</td>
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<tr>
<td>Thermococcus guaymasensis</td>
<td>–</td>
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<tr>
<td>Thermococcus stetteri</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Thermococcus peptonophilus</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Thermococcus profundus</td>
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<tr>
<td>Thermococcus celer</td>
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</tr>
</tbody>
</table>

Pyrococcus species were cultivated at 95 °C and Palaeococcus and Thermococcales species were cultivated at 80 °C under a gas phase of N₂ (100%, 200 kPa). All media were based on MJYP medium containing 20 mM NaNO₃ addition; B, 3% S°; C, 5 mM FeSO₄; D, 5 mM FeS₂; E, 5 mM FeS₃; F, 5 mM FeS; G, 5 mM CuSO₄, NiSO₄, MnSO₄, CoSO₄ or VSO₄. The pH of all media was adjusted to 6.0 at room temperature. Maximum cell yield is given as: + +, 1 × 10⁸–5 × 10⁹ cells ml⁻¹ culture; +, 5 × 10⁷–1 × 10⁸ cells ml⁻¹ culture; –, no growth.
Fe mechanism for detoxifying inhibitory H
organism named shallow marine hydrothermal vent environment, an
ing ferrous iron anaerobically at neutral pH, from a
thermophilic archaeon found to be capable of oxidiz-
et al
H
Palaeococcus ferrophilus
1995). Likewise,
when
alcohols is increased because of the enhanced activities
have reported that the production of H
conservation (Schicho
Pyrococcus
species, S
498
International Journal of Systematic and Evolutionary Microbiology
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Palaeococcus
Palaeococcus
utilize iron as an electron acceptor or donor. In the
fermentative-type metabolism of Thermococcus and Pyrococcus species, S
is reduction thought to be a
mechanism for detoxifying inhibitory H
(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
and some
alcohols is increased because of the enhanced activities
of iron hydrogenase and iron-alcohol dehydrogenase
when
sp. ES1 is cultivated under S
limited conditions. These enzymes are thought to
function in the disposal of excess reductant and may
play an important role in S
limited growth (Ma et al., 1995). Likewise,
Palaeococcus ferrophilus produces
when cultivated in the presence of S
but
is produced instead during growth in the presence of
Fe
. 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 concen-
trations of ferrous iron might be required for proper
operation of the iron(II)-regulated fermentation path-
way 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 metab-
ilism 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. Neutro-
philic 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
) or ferrous iron (Fe
) as a cofactor for growth. G+C content of genomic
DNA is about 54 mol%. Major core lipids are C
50
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 Thermo-
coccus 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 Palaeo-
coccus 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
 are required for growth,
with optimum growth occurring at 43 g l
. The

optimydraulic pressure for growth is 30 MPa.
Growth using yeast extract, peptone, tryptone or
casein occurs in the presence of elemental sulfur (S
) or
ferrous iron (Fe
) as a required co-factor. Major core
lipids are C
50
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
, 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
.

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