Aeropyrum camini sp. nov., a strictly aerobic, hyperthermophilic archaeon from a deep-sea hydrothermal vent chimney

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A novel hyperthermophilic archaeon, designated strain SY1^T, was isolated from a deep-sea hydrothermal vent chimney sample collected from the Suiyo Seamount in the Izu-Bonin Arc, Japan, at a depth of 1385 m. The cells were irregular cocci (1.2 to 2.1 µm in diameter), occurring singly or in pairs, and stained Gram-negative. Growth was observed between 70 and 97 °C (optimum, 85 °C; 220 min doubling time), pH 6.5 and 8.8 (optimum, pH 8.0), and salinity of 2.2 and 5.3 % (optimum, 3.5 %). It was a strictly aerobic heterotroph capable of growing on complex proteinaceous substrates such as yeast extract and tryptone. The G + C content of the genomic DNA was 54.4 mol%. Phylogenetic analysis based on the 16S rDNA sequence of the isolate indicated that the isolate was closely related to *Aeropyrum pernix* strain K1^T. However, no significant genetic relatedness was observed between them by DNA–DNA hybridization. On the basis of the molecular and physiological traits of the new isolate, the name *Aeropyrum camini* sp. nov. is proposed, with the type strain SY1^T (=JCM 12091^T=ATCC BAA-758^T).

The family *Desulfurococcaceae* comprises eight genera, *Desulfurococcus*, *Aeropyrum*, *Ignicoccus*, *Staphylothermus*, *Stetteria*, *Sulfophobococcus*, *Thermosphaera* and *Thermoterrivibrio* (Huber & Stetter, 2001). Members of the family *Desulfurococcaceae*, growing optimally at 85–95 °C, have been isolated from marine and terrestrial hydrothermal systems, and are strict anaerobes with fermentative metabolism or anaerobic respiration using sulfur or thiosulfate as electron acceptors, except for *Aeropyrum pernix* (Huber & Stetter, 2001). *A. pernix* is a strictly aerobic, heterotrophic and hyperthermophilic archaeon. The type strain of the species, strain K1^T, was isolated from a coastal solfataric thermal vent in Kodakara-Jima Island, south-western Japan (Sako *et al.*, 1996a), and 11 additional strains were isolated from another coastal shallow hydrothermal vent and a coastal hot spring in south-western Japan (Nomura *et al.*, 2002). In the phylum *Crenarchaeota*, *A. pernix* strain K1^T is unique in its strictly aerobic, sulfur-independent and neutrophilic growth. The complete genome sequence of this archaeon was determined for the first time in *Crenarchaeota*

(Kawarabayasi *et al.*, 1999), and a number of molecular biological characterizations of its hyperthermophilic enzymes have been reported (e.g. Sako *et al.*, 1997; Yamano *et al.*, 1999; Tachibana *et al.*, 2000; Hansen *et al.*, 2002; Daimon *et al.*, 2002; Jeon & Ishikawa, 2002). Although *A. pernix* strain K1^T has become one of the most extensively studied archaea with respect to its molecular biology, little is known about its ecological impacts, physiological diversity and distribution in natural environments.

To our knowledge, the habitats for members of the genus *Aeropyrum* are highly restricted to coastal geothermal fields in south-western Japan, at temperatures higher than 85 °C and neutral to weakly alkaline pH (Sako *et al.*, 1996a; Nomura *et al.*, 2002). Similarly, hyperthermophilic archaea, capable of growing under an atmosphere of air such as *Acidianus* (Seeger *et al.*, 1986; Zillig *et al.*, 1986), *Sulfurisphaera* (Kurosawa *et al.*, 1998), *Sulfolobus* (Zillig *et al.*, 1980; Grogan *et al.*, 1990; Jan *et al.*, 1999; Suzuki *et al.*, 2002) and *Pyrobaculum* (Sako *et al.*, 2001; Amo *et al.*, 2002) species isolated in terrestrial hot springs, have been never isolated from deep-sea hydrothermal environments. However, we have recently isolated strictly aerobic thermophiles, *Mariniterrivibrio hydrothermalis* strain T1^T and *Rhodothermus* sp. from a deep-sea hydrothermal vent chimney (Sako *et al.*, 2003). In addition, several strictly or facultatively aerobic thermophiles have been isolated from deep-sea

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The DDBJ/EMBL/GenBank accession number for the almost complete 16S rDNA sequence (1562 bp) of strain SY1^T is AB109559.

Growth curves are available as supplementary material in IJSEM Online.

hydrothermal chimney structures (Marteinsson *et al.*, 1995, 1999; Blöchl *et al.*, 1997; Miroshnichenko *et al.*, 2003a, b). In this study, we sought to cultivate aerobic hyperthermophiles from a deep-sea hydrothermal vent chimney at the Suiyo Seamount in the Izu-Bonin Arc, Japan.

Sample collection, enrichment and purification

Sample collection and subsampling procedures were described previously (Sako *et al.*, 2003; Nakagawa *et al.*, 2003; Takai *et al.*, 2003a). A portion of the subsample (approx. 10 g) obtained from the chimney surface (1–3 mm) was suspended with 20 ml sterilized MJ synthetic sea water (Sako *et al.*, 1996b) containing 0.05 % (w/v) sodium sulfide in a 100 ml glass bottle (Schott) tightly sealed with a butyl rubber cap under a N₂ atmosphere. The suspended slurry was used to inoculate a series of media including MJYPV medium (Sako *et al.*, 2003) and the cultures were incubated at 70 and 85 °C in a dry oven on board.

Growth of aerobic thermophiles was observed in MJYPV medium after 1 days incubation at both temperatures. Tubes inoculated by other parts of the chimney provide no positive enrichment even after 5 days incubation at both temperatures. The enrichment culture at 70 °C contained rod-shaped cells, while the culture at 85 °C contained highly motile irregular cocci. Successive transfers of the irregular cocci grown at 85 °C were accomplished by using JX medium (Sako *et al.*, 1996a) at 85 °C, because those cells grew inconsistently in MJYPV medium.

To obtain a pure culture of irregular cocci grown at 85 °C, the enriched cells were streaked onto JX plates hardened with 0.5 % (w/v) Gelrite gellan gum (Sigma). The plates were incubated at 85 °C in a tightly sealed polycarbonate jar to prevent evaporation. After 3–5 days incubation, small, spherical (1–2 mm in diameter) and light-yellow colonies were formed on the surface of the plates. Well-isolated colonies were picked, and the cells were incubated in fresh liquid JX medium at 85 °C. In order to ensure purity, the streaking and isolation step was repeated at least three times. The first pure culture was designated strain SY1^T (= JCM 12091^T = ATCC BAA-758^T) and was investigated in detail. The purity was confirmed routinely by microscopic examination and by repeated partial sequencing of the 16S rDNA using several PCR primers.

Morphology

Cells were routinely observed with a differential interference microscope (UFX; Nikon). Transmission electron microscopy of negatively stained cells and thin sections of the cells was carried out as described by Zillig *et al.* (1990) and Sako *et al.* (2003). Cells grown in JX medium at 85 °C in the mid-exponential growth phase were used for electron microscopy. The cells were Gram-negative cocci, which were about 1.2–2.1 µm in diameter. Electron microscopy of thin sections showed that the cell envelope is composed of a cytoplasmic membrane, a periplasmic space and a thin

Fig. 1. Electron micrograph of a thin section of *Aeropyrum camini* strain SY1^T cells showing cytoplasmic membrane (arrowhead) and thin electron-dense layer (arrow), presumably representing the S-layer. Bar, 1 µm.

electron-dense layer, presumably representing the S-layer of protein complexes (Fig. 1). Flagella were not observed although motility was evident under the light microscopic observation. No sporulation was observed. Pili-like filaments were often observed under epifluorescence microscopic observation. These morphological properties of the new isolate were generally similar to those of *A. pernix* strain K1^T (Sako *et al.*, 1996a).

Growth characteristics

The isolate was originally enriched in MJYPV medium (Sako *et al.*, 2003), containing 1 g yeast extract (Difco) and tryptone (Difco), and 10 ml vitamin solution (Balch *et al.*, 1979) per litre of MJ synthetic sea water (Sako *et al.*, 1996b). Then, the isolate was purified and routinely cultivated with JX medium (Sako *et al.*, 1996a), which contained 1 g yeast extract (Difco) and tryptone (Difco) per litre of Jamarin S (JS) synthetic sea water (Jamarin Laboratory, Osaka, Japan). The isolate showed no growth on JXTm medium, which was suitable for the cultivation of *A. pernix* strain K1^T (Nomura *et al.*, 2002). Marine broth 2216 (Difco) could be used in place of JX medium to culture the isolate.

Growth of the new isolate under various conditions was determined by direct cell counts after staining with 4',6-diamidino-2-phenylindole (DAPI) (Porter & Feig, 1980) using a Nikon Eclipse E800 microscope equipped with a colour chilled 3 CCD camera system (C5810; Hamamatsu Hokutokusu). To determine temperature, pH and salinity ranges for growth, duplicate cultures were grown in cotton-plugged 300 ml Erlenmeyer flasks containing 100 ml medium in an air-batch rotary shaker (RGS-32.TT; Sanki Seiki) at 100 r.p.m. in all cases. At temperatures above 100 °C, screw-capped test tubes were used to prevent the evaporation of the medium. The isolate grew at 70–97 °C, showing optimum growth at 85 °C, and the generation time

at 85 °C was 220 min. No growth was observed below 65 °C or above 100 °C (see supplementary Fig. 1a in IJSEM Online). The effects of pH and salinity on the growth of the isolate were determined at 85 °C. To determine the effect of pH on growth, the pH of JX medium containing 20 mM MES (pH 5.0–6.0), PIPES (pH 6.25–7.0), HEPES (pH 7.3–8.0) or Tris (pH 8.5 and above) was adjusted to the designated values with H₂SO₄ or NaOH at room temperature. The pH value was checked after autoclaving and was aseptically readjusted with H₂SO₄ or NaOH at room temperature if necessary. The pH values of all media were stable during the cultivation period. Growth of the isolate occurred between pH 6.5 and 8.8, with optimum growth at about pH 8.0. No growth was detected below pH 6.0 or above pH 9.2 (supplementary Fig. 1b in IJSEM online). To determine the effect of salinity on the growth of the isolate, JX medium was prepared with varying dilutions of 3 × JS synthetic sea water (1 × JS synthetic sea water contains 35 g sea salts l⁻¹). The isolate grew at 2.2–5.3 % (w/v) salinity, with optimum growth at 3.5 %, which is the salinity of JX medium; no growth was observed below 1.8 % or above 6.0 % salinity (supplementary Fig. 1c in IJSEM Online). The effect of thiosulfate on the growth of the isolate was determined at 85 °C with JXT medium (Sako *et al.*, 1996a), which contains 0.1 % (w/v) sodium thiosulfate in JX medium. The maximum cell density of the isolate was increased about twofold by the addition of thiosulfate, and reached up to 1.2 × 10⁹ cells ml⁻¹, although the generation time was not varied. Other sulfur-bearing compounds such as 0.1 % (w/v) Na₂SO₃, Na₂SO₄ or cysteine hydrochloride did not affect the growth of the isolate, however 3 % of S⁰ completely inhibited growth. The effect of O₂ concentration in the gas phase on the growth of the isolate was determined at 85 °C as described before (Nakagawa *et al.*, 2003). O₂ final concentrations of 0–25 % (v/v) were tested at 350 kPa. For comparison, *A. pernix* strain K1^T was cultured under the same conditions. Both strains were found to grow optimally with 5.0 % O₂.

In an attempt to examine whether or not the new isolate was able to grow under anaerobic conditions, 80 % H₂ + 20 % CO₂ or 80 % N₂ + 20 % CO₂ (200 kPa) were tested as a gas phase instead of air with JX medium in the presence or absence of possible alternative electron acceptors such as 0.1 % (w/v) NaNO₃, Na₂SO₃, NaNO₂ or Na₂S₂O₃. In order to ensure anaerobic growth conditions, 10 % (w/v) Na₂S₉H₂O solution (pH 7.5, adjusted with H₂SO₄ and autoclaved separately) was added to the medium at a final concentration of 0.05 % (w/v). Autotrophic growth was determined with air using JS synthetic sea water containing 1 % (v/v) vitamin solution (Balch *et al.*, 1979), supplemented with a possible electron donor (0.1 % of Na₂S₂O₃, NaNO₂ or Na₂SO₃) and 0.1 % (w/v) NaHCO₃ as a carbon source. The isolate was found to be unable to grow under any of the anaerobic or autotrophic conditions tested in this study.

In an attempt to find organic substrates that could support

the growth of the isolate, various organic substrates were tested instead of both yeast extract and tryptone in JX medium. Each of the following substrates was added at concentrations of 0.02 or 0.2 % (w/v): L-arginine, L-asparagine, L-aspartate, L-glutamate, L-phenylalanine, L-proline, L-serine, L-valine, Casamino acids, gelatin, D-(–)-fructose, D-(+)-glucose, galactose, *myo*-inositol, D-sorbitol, D-(+)-xylose, D-(+)-cellobiose, lactose, maltose, D-(+)-trehalose, sucrose, chitin, starch, sodium acetate, citrate, glycerol, L-malate, sodium pyruvate, casein, yeast extract (Difco) and tryptone (Difco). These tests were performed at temperatures of 85 and 75 °C in shake flasks, and run in duplicate. The isolate was found to be able to utilize only complex proteinaceous substrates such as yeast extract and tryptone as energy and carbon sources, but not casein.

Sensitivity to antibiotics chloramphenicol, ampicillin, rifampicin and kanamycin at a final concentration of 100 µg ml⁻¹ was determined in JX medium at 70 °C. These antibiotics were confirmed to be effective at 70 °C as described previously (Sako *et al.*, 1996a). The isolate was not sensitive to ampicillin or kanamycin, but was sensitive to chloramphenicol and rifampicin. This susceptibility to antibiotics of the isolate is similar to that of *A. pernix* strain K1^T (Sako *et al.*, 1996a).

Isolation and base composition of DNA

Genomic DNA was prepared as described by Lauerer *et al.* (1986). The G + C content (mol%) of the genomic DNA was determined by direct analysis of the deoxyribonucleotides using HPLC with a DNA-GC kit (Yamasa Shouyu) after digestion of the DNA with nuclease P1 (Tamaoka & Komagata, 1984). The G + C content of the genomic DNA of strain SY1^T was found to be 54.4 mol%, which was similar to that of *A. pernix* strain K1^T (Table 1).

Phylogenetic analyses

The 16S rRNA gene (rDNA) was amplified by PCR using Arch 21F and 1492R primers (Lane, 1985; DeLong, 1992). Although a PCR product of approximately 1.5 kb was expected with this primer set, a product of 1.6 kb was obtained from the reaction. The result indicated that the 16S rDNA of the isolate contained intron-like intervening sequences as observed in other hyperthermophilic archaea such as *Pyrobaculum* (Burggraf *et al.*, 1993; Takai & Horikoshi, 1999b), *Thermoproteus* (Itoh *et al.*, 1998) and *Aeropyrum* (Nomura *et al.*, 1998, 2002). The sequence of the PCR product was directly determined in both strands using the dideoxynucleotide chain-termination method with a DNA sequencer model 3100 (Perkin Elmer/Applied Biosystems). Based on the multiple alignments with the rDNA sequences of the members of the family *Desulfurococcaceae*, the insertion sites of intron-like sequences were roughly assumed, and then the secondary structures around exon–intron junction sites were manually constructed according to the convention proposed by Thompson &

Table 1. Comparison of properties between *Aeropyrum camini* strain SY1^T and *A. pernix* strain K1^T

Data were taken from this study and Sako *et al.* (1996a).

Property	<i>A. camini</i> strain SY1 ^T	<i>A. pernix</i> strain K1 ^T
Origin	Deep-sea hydrothermal vent chimney	Coastal solfataric thermal vent
Cell diameter (µm)	1.2–2.1	0.8–1.0
Inhibition of growth by S ⁰	+	–
Temperature range (°C)	70–97	70–100
Temperature optimum (°C)	85	90–95
pH range	6.5–8.8	5.0–9.0
pH optimum	8.0	7.0
Salinity range (% w/v)	2.2–5.3	1.8–7.0
Salinity optimum (% w/v)	3.5	3.5
Genomic DNA G + C content (mol%)	54.4	56.3*

*From genome sequence.

Daniels (1988) and Lykke-Andersen *et al.* (1997). As a result, two putative introns were found in the rDNA sequence. Both of them had a secondary structure specific to archaeal rRNA introns, containing the bulge–helix–bulge core structure; 3-base loops on opposite strands separated by a 4 bp helix.

In order to determine the phylogenetic position of the isolate, the sequence of the putative exon for 16S rRNA gene was manually aligned with a subset of 16S rDNA sequences obtained from DDBJ and the Ribosomal Database Project II (RDP-II) (Maidak *et al.*, 2001) by using CLUSTAL X (Thompson *et al.*, 1997). Phylogenetic analyses were restricted to nucleotide positions that were unambiguously alignable in all sequences (Takai & Horikoshi, 1999a, Takai & Sako, 1999). Neighbour-joining analysis (Saitou & Nei, 1987) of 938 bases of sequence from each organism was accomplished using CLUSTAL X. Bootstrap analysis was used for 1000 trial replications to provide confidence estimates for the phylogenetic tree topologies. The phylogenetic tree demonstrated that the new isolate was a close relative of *A. pernix* strain K1^T (Fig. 2). The similarity of the putative exon sequence for 16S rDNA between the isolate and

A. pernix strain K1^T was 99.0%. These results indicated that the isolate was a member of the genus *Aeropyrum*.

DNA–DNA hybridization analysis between the isolate and *A. pernix* strain K1^T was performed at 37.4 °C for 3 h and was measured fluorometrically using photobiotin, according to the method of Ezaki *et al.* (1989). Although the phylogenetic analysis based on the 16S rRNA gene sequence indicated that the new isolate was closely related to *A. pernix* strain K1^T, the mean hybridization value for the isolate and *A. pernix* strain K1^T was only 5.1%. This result indicated that the new isolate could be genotypically differentiated from *A. pernix* strain K1^T.

Comparison with *A. pernix* strain K1^T

The phylogenetic analysis based on the 16S rDNA sequence indicated that strain SY1^T is closely related to *A. pernix* strain K1^T. Analyses of G + C content, metabolism, morphological properties, antibiotic susceptibility and nutrition of strain SY1^T also support the strain being a member of the genus *Aeropyrum*, except that the cell diameter of the isolate was larger than that of *A. pernix*.

Fig. 2. Phylogenetic tree of representative members of hyperthermophilic archaea, inferred from 16S rDNA sequences (accession numbers in parentheses) by the neighbour-joining method using 938 homologous sequence positions for each organism. The numbers are the bootstrap values for the branches (based on 1000 replicates). Bar, 2 substitutions per 100 nucleotides.

strain K1^T (Table 1). As is often observed in 16S rRNA genes of some strains of *A. pernix* (Nomura *et al.*, 2002), strain SY1^T possessed two potential short introns with no apparent ORF inside, which were inserted in the identical positions to the rRNA introns I δ and I ϵ of strain TB1 (Nomura *et al.*, 2002). The introns in the 16S rDNA of strain SY1^T, however, were different from those of *A. pernix* strains TB1–8 in length and sequence; 63 bp for the position of I δ (62 bp in strain TB1) and 141 bp for the position of I ϵ (122 bp in strain TB1), and the sequence similarities (91.9 and 71.4 % in I δ and I ϵ , respectively). The genetic diversity of the rRNA introns may be an important taxonomic feature within the rRNA intron-containing hyperthermophilic archaeal genus *Aeropyrum*.

The optimum temperature for the growth of strain SY1^T was 5–10 °C lower than that of *A. pernix* strain K1^T. The optimum pH for the growth of strain SY1^T was higher than that of *A. pernix* strain K1^T, and it is the highest among those of the members of the phylum *Crenarchaeota*. The pH and salinity ranges for growth of strain SY1^T was narrower than those of *A. pernix* strain K1^T (Table 1). The effect of thiosulfate on the growth of strain SY1^T was relatively weak; the maximum cell density of strain SY1^T was increased about twofold by thiosulfate, in contrast to eightfold stimulation of cell density observed in *A. pernix* strain K1^T (Sako *et al.*, 1996a). In addition, the growth of strain SY1^T was completely inhibited by S⁰, although *A. pernix* K1^T was not affected (Sako *et al.*, 1996a). These physiological properties strongly suggest that strain SY1^T can be classified as a different species from the type strain of the genus *Aeropyrum*, *A. pernix* strain K1^T. Finally, DNA–DNA hybridization analysis clearly indicated that the new isolate could be genotypically differentiated from *A. pernix* strain K1^T. On the basis of these physiological and genetic properties, we propose a new species, *Aeropyrum camini*; the type strain is strain SY1^T (=JCM 12091^T=ATCC BAA-758^T).

Strain SY1^T is the first strictly aerobic hyperthermophilic micro-organism isolated from a deep-sea hydrothermal environment, although a number of strictly anaerobic and heterotrophic hyperthermophiles such as the members of *Thermococcales* (e.g. Erauso *et al.*, 1993; González *et al.*, 1995, 1998; Godfroy *et al.*, 1997; Canganella *et al.*, 1998; Takai *et al.*, 2000), *Archaeoglobales* (Burggraf *et al.*, 1990; Kashefi *et al.*, 2002) and *Desulfurococcales* (Fiala *et al.*, 1986; Pley *et al.*, 1991) have been isolated. Successful cultivation of *A. camini* strain SY1^T only from the surface zone of the chimney structure provides an important clue into delineation of the ecological niche of the strictly aerobic hyperthermophiles at deep-sea hydrothermal systems. In very steep physical and geochemical gradients formed in the chimney structure, the highly reductive and the relatively oxidative microhabitats concomitantly occur by means of mixing between anoxic hot fluid and oxygenated sea water. We also succeeded in isolating strictly aerobic thermophiles, *Marinithermus hydrothermalis* strain T1^T and *Rhodothermus*

sp. (Sako *et al.*, 2003), and also the facultatively anaerobic thermophile *Persephonella hydrogeniphila* strain 29W^T (Nakagawa *et al.*, 2003) and the strictly anaerobic thermophile *Deferribacter desulfuricans* strain SSM1^T (Takai *et al.*, 2003a) from the same subsample used in this study. These new thermophiles from a surface layer of the chimney represent a glimpse into the potentially vast microbial diversity at the thin interface zone between hot fluid and cold sea water. The ecological potential of decomposition of organic materials by strictly aerobic, hyperthermophilic heterotrophs in the deep-sea hydrothermal vent environments is the focus of our future investigation.

Description of *Aeropyrum camini* sp. nov.

Aeropyrum camini (ca'mi.ni. L. gen. n. *camini* of a chimney, relating to its isolation from a hydrothermal vent chimney).

Cells are motile and irregular cocci, about 1.2–2.1 μ m in diameter. Gram-negative. Growth occurs at 70–97 °C (optimum, 85 °C), at pH 6.5–8.8 (optimum, 8.0) and at 2.2–5.3 % salinity (optimum, 3.5 %). Optimal doubling time is about 4 h. Strictly aerobic heterotroph. Growth is completely inhibited by S⁰. Utilizes only complex proteinaceous compounds, such as yeast extract and tryptone as sole energy and carbon sources. G + C content is 54.4 mol% (HPLC). DNA–DNA relatedness to *A. pernix* K1^T is low.

The type strain is SY1^T (=JCM 12091^T=ATCC BAA-758^T), which was isolated from a deep-sea hydrothermal vent chimney at Suiyo Seamount in the Izu-Bonin Arc, Japan (28°34'28.7' N, 140°38'66.3' E; depth 1385 m).

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