***Pyrobaculum oguniense* sp. nov., a novel facultatively aerobic and hyperthermophilic archaeon growing at up to 97 °C**

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A novel hyperthermophilic, heterotrophic, rod-shaped archaeon was isolated from a terrestrial hot spring at Oguni-cho, Kumamoto Prefecture, Japan. The new isolate, strain TE7^T, grew under aerobic, microaerobic and anaerobic conditions. Isolate TE7^T grew optimally at 90–94 °C and pH 7.0–7.5 (adjusted at 25 °C) under atmospheric air with vigorous shaking. Strain TE7^T cells were motile rods 2–10 µm in length and covered with a surface-layer lattice. Cell yields at 90 °C under aerobic conditions were twice that under anaerobic conditions. Under aerobic conditions, growth was inhibited by elemental sulfur, but thiosulfate stimulated growth. Under anaerobic conditions, no growth was observed in the presence of nitrate and nitrite, but elemental sulfur, thiosulfate, L-cystine and oxidized glutathione stimulated growth. The 16S rDNA sequence of TE7^T exhibited a close relationship to the sequences of *Pyrobaculum aerophilum* and *Thermoproteus neutrophilus*, which belong to the cluster of the genus *Pyrobaculum*. DNA–DNA hybridization analysis showed a low level of DNA similarity between TE7^T and previously described *Pyrobaculum* species. As TE7^T is phenotypically and phylogenetically different from the other members of this genus, it is described as a new species named *Pyrobaculum oguniense* (type strain TE7^T = JCM 10595^T = DSM 13380^T).

Keywords: *Pyrobaculum oguniense* sp. nov., facultative aerobe, hyperthermophile, archaeon

INTRODUCTION

A number of hyperthermophiles, growing above 90 °C, have been isolated from geothermal environments such as hydrothermal vents, terrestrial hot springs and solfataric fields (Stetter, 1996; Huber & Stetter, 1998). Because oxygen has a low solubility at high temperature and reducing gases are present, many of the organisms in these environments are anaerobes (Stetter, 1996; Huber & Stetter, 1998; Adams & Kelly, 1998). In fact, characterized hyperthermophiles mostly consist of obligate anaerobes, a few facultative microaerophiles (e.g. *Pyrobaculum aerophilum*, *Pyrolobus fumarii* and *Aquifex pyrophilus*) (Adams & Kelly, 1998;

Blöchl *et al.*, 1997) and one facultative aerophile (*Acidianus infernus*) (Seeger *et al.*, 1986). In 1996, the first obligately aerobic and neutrophilic hyperthermophile was reported, namely *Aeropyrum pernix*, which can grow only under atmospheric air (Sako *et al.*, 1996); recent studies have shown that this species possesses biotechnological features of commercial interest (Sako *et al.*, 1997; Nomura *et al.*, 1998; Morii *et al.*, 1999; Chavez Crocker *et al.*, 1999). Moreover, the full genome sequence of *A. pernix* was fully examined (Kawarabayashi *et al.*, 1999). The isolation of *A. pernix* suggested that there are other unidentified hyperthermophiles highly adapted to aerobic conditions above 100 °C, even in reductive high-temperature environments. However, there have been no reports of the isolation of other novel aerobic hyperthermophiles.

In this study, we tried to isolate a second aerobic and neutrophilic hyperthermophile that can grow under atmospheric air: we succeeded in the cultivation and

Abbreviations: DAPI, 4',6'-diamidino-2-phenylindole dihydrochloride; ITS, internal transcriptional spacer; RDP, Ribosomal Database Project; S-layer, surface layer.

The DDBJ accession number for the 16S rDNA sequence of strain TE7^T is AB029339.

isolation of a novel facultatively aerobic, hyperthermophilic archaeon (growing at temperatures up to 97 °C) from a slightly alkaliphilic terrestrial hot spring called the Tuetate hot spring in Oguni-cho, Kumamoto Prefecture, Japan. Here, we describe the unique characterization of the new isolate, the first report of a facultatively aerobic and hyperthermophilic archaeon that grows at temperatures above 90 °C under neutrophilic conditions.

METHODS

Strains. *P. aerophilum* IM2^T (JCM 9630^T, DSM 7523^T), *Pyrobaculum organotrophum* H10^T (JCM 9190^T, DSM 4185^T), *Pyrobaculum islandicum* geo3^T (JCM 9189^T, DSM 4184^T) and *Thermoproteus neutrophilus* V24Sta^T (JCM 9278^T, DSM 2338^T) were obtained from Japan Collection of Microorganisms (JCM; Wako, Japan).

Collection of samples. Effluent water samples (97 °C, pH 8 *in situ*) were collected from a terrestrial hot spring, namely the Tuetate hot spring, Oguni-cho, Kumamoto Prefecture, Japan. The samples were stored under aerobic conditions at 5 °C.

Isolation and culture conditions. For enrichment, isolation and cultivation, modified Allen's basal medium (Allen, 1959; Brock *et al.*, 1972) supplemented with 0.1% (w/v) yeast extract (Difco) and 0.1% (w/v) Na₂S₂O₃·5H₂O was used as the standard medium (MS medium). The pH of this medium was adjusted to 7.0–7.5 with NaOH at 25 °C before autoclaving. The enrichment was incubated in screw-capped test tubes (Pyrex; 180 mm × 18 mm) containing 7 ml of the medium at 88 °C under aerobic conditions for 2 d. A serial dilution method was employed and repeated several times to obtain pure cultures (Sako *et al.*, 1996).

Aerobic batch cultures were grown in cotton-plugged 300 ml Erlenmeyer flasks containing 100 ml medium in an air-bath rotary shaker (RGS-32.TT; Sanki Seiki) with vigorous rotary shaking (130 r.p.m.). Anaerobic growth conditions were achieved using a technique of Balch *et al.* (1979). Autoclaved MS medium (200 ml) in a 500 ml glass bottle (Schott Glaswerke) with a butyl rubber plug was reduced by 0.05% (w/v) Na₂S·9H₂O and pressurized with N₂ (100%, 200 kPa). Resazurin (0.0001%, w/v) was used as a redox indicator. For microaerobic growth, the gas phase was replaced with N₂ (100%, 200 kPa) and the Na₂S·9H₂O solution was omitted. In this medium, resazurin turns red, indicating the presence of O₂.

For the determination of the autotrophic growth under anaerobic conditions, modified Allen's basal medium was supplemented with 0.1% (w/v) Na₂S₂O₃·5H₂O or 1% (w/v) S⁰, and the gas phase was H₂:CO₂ (80:20, 200 kPa) or N₂:CO₂ (80:20, 200 kPa). For autotrophic growth under aerobic conditions, only 0.1% (w/v) Na₂S₂O₃·5H₂O was added.

P. aerophilum IM2^T (= JCM 9630^T = DSM 7523^T) was grown in BSY medium (Völkl *et al.*, 1993). *P. organotrophum* H10^T (= JCM 9190^T = DSM 4185^T) and *P. islandicum* geo3^T (= JCM 9189^T = DSM 4184^T) were grown in JCM 184 medium (JCM Catalogue of Strains, 7th edition, 1999). *T. neutrophilus* V24Sta^T (= JCM 9278^T = DSM 2338^T) was grown in JCM 195 medium. Anaerobic culture conditions for *P. aerophilum*, *P. organotrophum* and *P. islandicum* were achieved by using the same methods as those described

above. For *T. neutrophilus*, the gas phase was replaced with H₂:CO₂ (80:20, 200 kPa). The cultivation temperature of these organisms was 90 °C.

Light- and electron microscopy. Cells were observed with a differential interference microscope (UFX; Nikon). For observations with transmission electron microscopy, cells were fixed with 2% (v/v) glutaraldehyde in 0.1 M Na₂HPO₄/KH₂PO₄ (pH 7.2) buffer and post-fixed with 1% (v/v) OsO₄. They were then dehydrated with ethanol and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead acetate and examined under an H-700H electron microscope (Hitachi) at an accelerating voltage of 100 kV. Negative staining of cells for electron microscopy was achieved with 2% (v/v) phosphotungstic acid.

Determination of growth. Cell densities were determined by direct counting of cells stained with 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI), using an epifluorescence microscope (UFX-IIA; Nikon).

pH and temperature ranges for growth. The pH range for growth was determined in MS medium under aerobic conditions with rotary shaking (130 r.p.m.). For the determination of the pH-dependency of growth, the pH value of the medium was adjusted at 25 °C (pH_{25 °C}) with NaOH and H₂SO₄; then the pH values were also determined at 80 °C (pH_{80 °C}). The pH_{80 °C} values of 5.4, 6.1, 6.3, 6.6, 6.8 and 7.4 corresponded to pH_{25 °C} values of 5.5, 6.5, 7.0, 7.5, 8.0 and 9.0, respectively. For the determination of the temperature range for growth, the cultures were incubated at different temperatures under aerobic conditions at pH_{25 °C} 7.0 (pH_{80 °C} = 6.3).

Substrate utilization. To determine organic-substrate utilization, each of following substrates (at 0.1% w/v unless specified otherwise) was added to modified Allen's basal medium, supplemented with Na₂S₂O₃·5H₂O (0.1%) (pH_{25 °C} adjusted to 7.0): yeast extract, trypticase peptone (BBL), yeast extract (0.05%) and trypticase peptone (0.05%), gelatin (Sigma), casein (Sigma), casamino acids (Difco), starch (Wako), D-(+)-glucose, sucrose, lactose, maltose, sodium acetate, and sodium pyruvate. To determine sulfur-compound utilization, the MS medium without Na₂S₂O₃·5H₂O, but with the following sulfur compounds, was used: Na₂SO₃ (0.1%), S⁰ (1%), glutathione (the oxidized form) (0.1%), L-cysteine HCl (0.1%), L-cysteine (0.1%). For the determination of nitrate respiration, NaNO₂ (0.1%) and NaNO₃ (0.1%) were added to the MS medium prepared under anaerobic conditions.

Antibiotic resistance. The antibiotic resistance of the isolate was tested using chloramphenicol (Sigma), penicillin G (Sigma), rifampicin (Sigma), streptomycin (Sigma) and vancomycin (Nacalai Tesque). All of the antibiotics were tested individually at a concentration of 100 µg/ml in the MS medium. Cells were grown overnight at 80 °C in either the presence or the absence of the antibiotic under aerobic conditions. The resistance of *Pyrobaculum* species to these antibiotics at 80 °C was determined previously by Huber *et al.* (1987) and Völkl *et al.* (1993).

Detection of H₂S as a metabolic product. H₂S was detected qualitatively using the technique described by Huber *et al.* (1998).

DNA isolation and base composition. DNA was isolated as described by Lauerer *et al.* (1986). The G+C content (mol%) of the genomic DNA was determined using HPLC

(Tamaoka & Komagata, 1984) with a DNA-GC kit (Yamasa Shouyu).

16S rDNA analysis. The 16S rDNA was amplified by the PCR. The primers employed in the amplification had the sequences 5'-TTC CGG TTG ATC CYG CCG GA (21F) and 5'-ACG GNT ACC TTG TTA CGA CTT (1492R), which correspond to positions 7–26 and 1512–1492, respectively, in the 16S rDNA (*Escherichia coli* numbering; DeLong, 1992). The PCR product was directly cloned into the pCRII vector (Invitrogen) and sequenced by the deoxynucleotide chain-termination method, using an ABI 373A automated DNA sequencer (Applied Biosystems). The 16S rDNA sequence was aligned with a subset of 16S rDNA sequences obtained from the DNA Database of Japan (DDBJ) and the Ribosomal Database Project (RDP) by using the ODEN software package (version 1.1, National Institute of Genetics, Mishima, Japan). Neighbour-joining analysis (Saitou & Nei, 1987) of 1371 bases of sequence from each organism was accomplished using the ODEN software package.

DNA–DNA hybridization. DNA from strain TE7^T was compared with DNA from *P. aerophilum* IM2^T (= JCM 9630^T = DSM 7523^T), *P. organotrophum* H10^T (= JCM 9190^T = DSM 4185^T), *P. islandicum* geo3^T (= JCM 9189^T = DSM 4184^T) and *T. neutrophilus* V24Sta^T (= JCM 9278^T = DSM 2338^T) by using the fluorometric DNA–DNA hybridization method, with photobiotin-labelled DNA as described by Ezaki *et al.* (1989).

Confirmation of strain purity. DNA was isolated from both aerobic and anaerobic culture. The 16S rDNA and the internal transcriptional spacer (ITS) region between the 16S rDNA and the 23S rDNA were amplified by the PCR. The primers employed in the amplification had the sequences 5'-CGGTTGGATCACCTC (1528F) and 5'-GTTGGTTTC-TTTTCCT (0213aR) in the 16S rDNA and 23S rDNA, respectively (Achenbach & Woese, 1995). The PCR product was directly cloned into the pCRII vector. All 10 16S rDNA clones from both aerobic and anaerobic cultures of TE7^T were analysed using RFLP. All 10 ITS region clones from both aerobic and anaerobic cultures of TE7^T were sequenced by using the deoxynucleotide chain-termination method, using an ABI 373A automated DNA sequencer (Applied Biosystems).

RESULTS

Enrichment and isolation

Enrichment of effluent water samples was carried out in MS medium at 88 °C. A single population of rod-shaped cells was observed after 2 d incubation. Only rod cells were observed, i.e. no coccoid, branched or variably rod-shaped cells were seen. A culture of rod-shaped cells was purified by serial dilution, and strain TE7^T (= JCM 10595^T) was isolated. The purity of this strain was routinely confirmed by microscopic examination. Furthermore, RFLP analysis of the 16S rDNA gene fragment of 10 PCR-amplified clones (data not shown) as well as sequencing analysis of the ITS region between the 16S rDNA and 23S rDNA gene of 10 additional PCR-amplified clones from both aerobic and anaerobic cultures (data not shown) were examined to confirm the purity of the strain.

Fig. 1. Transmission electron micrographs of cells of *Pyrobaculum oguniense* TE7^T. (a) Negatively stained cells at exponential growth phase. An arrow indicates the flagellum. Bar, 1 µm. (b) Negatively stained cell at stationary phase. An arrow indicates the pilus-like structure. Bar, 1 µm. (c) Ultrathin section of a whole cell of strain TE7^T. Arrow indicates the surface layer (S-layer). Bar, 0.5 µm.

Morphology

Cells of isolate TE7^T were rod-shaped, with a width of 0.6–1.0 µm and a length of 2–10 µm (Fig. 1a–c). Golf-club-like cells were observed only in the stationary growth phase of an aerobic culture grown in a test tube. These cells were found at the bottom of the test tube in a zone that was reduced by the growth of cells. However, branched cells were never observed. Motility was observed, using a differential interference microscope, even at stationary phase. Whilst monopolar flagellation is present in the exponential phase (Fig. 1a), flagella are absent in the stationary phase, and a thick pilus-like structure (Zillig *et al.*, 1983) is observed (Fig. 1b). Negatively stained and thin-section micrographs showed that the cell wall consisted of typical

Fig. 2. Effect of temperature (a) and pH at 80 °C (b) on the growth of *P. oguniense* TE7^T.

surface-layer (S-layer) protein (Fig. 1a–c). Cells appeared singly or formed X- or V-shaped aggregates of a few cells after growth in the MS medium. Aggregates with diameters of up to 1 mm were found after growth with trypticase peptone as the substrate.

Effects of temperature, pH and salt concentration on growth

TE7^T grew over a temperature range of 70–97 °C, with an optimum at 90–94 °C (Fig. 2a). Growth was not observed at ≥ 100 °C or at ≤ 60 °C. Growth of isolate TE7^T occurred between pH_{80 °C} 5.4 and 7.4 with an optimum of pH_{80 °C} 6.3–7.0. No growth was observed at pH_{80 °C} 5.0 and pH_{80 °C} 7.6 (Fig. 2b). TE7^T did not require NaCl for growth and grew in the presence of 0–1.5% (w/v) NaCl. The optimal concentration for growth was 0% NaCl. No growth was observed above 2% (w/v) NaCl (data not shown). There was no difference between aerobic and anaerobic culture in terms of these growth requirements.

Physiological characterization of growth

Under optimal conditions, the doubling time was 3–4 h in aerobic culture and 6 h in microaerobic and anaerobic culture. New isolate TE7^T could grow

heterotrophically on proteinaceous complex substrates such as 0.1% (w/v) yeast extract or 0.05% (w/v) yeast extract and 0.05% (w/v) trypticase peptone in the absence of sulfur compounds under aerobic conditions. However, in the presence of Na₂S₂O₃·5H₂O, the growth yield of TE7^T increased fourfold and the maximum cell density was about 2.3×10^8 cells ml⁻¹. With trypticase peptone as the sole substrate, a cell density of 1.0×10^8 cells ml⁻¹ was obtained. No growth was observed with casein, gelatin, casamino acids, starch, D-(+)-glucose, sucrose, lactose, maltose, sodium acetate or sodium pyruvate as the sole carbon source. Na₂SO₃ (0.1%) and S⁰ (1%) inhibited aerobic growth. H₂S was not formed under aerobic conditions.

Under microaerobic and anaerobic conditions, TE7^T grew heterotrophically and required sulfur compounds. The maximum cell density under both conditions reached from 5.6×10^7 to 6.0×10^7 cells ml⁻¹ with 0.1% (w/v) yeast extract in the presence of Na₂S₂O₃·5H₂O. Furthermore, growth was observed on 0.05% (w/v) yeast extract and 0.05% (w/v) trypticase peptone, with a cell density of about 3.5×10^7 cells ml⁻¹. Very weak growth was observed on trypticase peptone, gelatin, casamino acids and acetate, with cell densities of about 5×10^6 cells ml⁻¹. S⁰, glutathione (the oxidized form), L-cystine and Na₂S could replace Na₂S₂O₃·5H₂O, supporting a cell yield of 3.5×10^7 to 4.5×10^7 cells ml⁻¹. L-Cysteine could also support growth. However, the cell yield was only 6.5×10^6 cells ml⁻¹. H₂S was detected following growth with these sulfur compounds (data not shown). These results suggested that these sulfur compounds may be used as electron acceptors. No growth was observed in MS medium under anaerobic conditions in the presence of NaNO₂ or NaNO₃ (data not shown). Autotrophic growth was not observed under either aerobic or anaerobic conditions.

Antibiotic resistance

Isolate TE7^T was resistant to streptomycin and vancomycin and sensitive to rifampicin, like other *Pyrobaculum* spp. However, growth was slightly inhibited by chloramphenicol and penicillin G. In contrast, other *Pyrobaculum* spp. are insensitive to these antibiotics (Huber *et al.*, 1987; Völkl *et al.*, 1993).

DNA base composition

The G + C content of isolate TE7^T was 48 mol%, as determined by HPLC analysis.

16S rDNA gene sequence analysis

The size of the PCR products of 16S rDNA was 2269 nucleotides, which consists of 1472 nucleotides of exons and two introns. One intron (INT Pog-IV) is 32 bp long (1173–1204; numbers are relative to the beginning of this PCR product) and the other intron (INT Pog-V) is 665 bp long (1213–1877). The 16S rDNA sequence of isolate TE7^T was very similar to

Fig. 3. Phylogenetic tree of crenarchaeotes within the *Thermoproteales* inferred from 16S rDNA sequences by using the neighbour-joining method on 1371 bases of sequence from each organism. Each number indicates the bootstrap value from 100 trials. Bar, 1 substitution per 100 nucleotides. The GenBank accession numbers are in square brackets.

Table 1 DNA–DNA hybridization between *Pyrobaculum oguniense* strain TE7^T, other *Pyrobaculum* species and *Thermoproteus neutrophilus*

—, Not determined.

Source of microplate-bound DNA	Source of photobiotin-labelled DNA				
	Strain TE7 ^T	<i>P. aerophilum</i>	<i>P. islandicum</i>	<i>P. organotrophum</i>	<i>T. neutrophilus</i>
<i>P. oguniense</i> TE7 ^T	100	5	2	2	5
<i>P. aerophilum</i> IM2 ^T	15	100	7	1	—
<i>P. islandicum</i> geo3 ^T	3	3	100	77	—
<i>P. organotrophum</i> H10 ^T	4	4	72	100	—
<i>T. neutrophilus</i> V24Sta ^T	4	—	—	—	100

those of members of the *Thermoproteaceae*. The closest sequences were from *P. aerophilum* (99.1% similarity), *T. neutrophilus* (98.9% similarity) and *P. islandicum* (98.5% similarity). Lower similarity (96.9%) was found with *Thermoproteus tenax*. Fig. 3 shows the phylogenetic relationship between isolate TE7^T and other members of the *Thermoproteales*.

DNA–DNA hybridization

The DNA of isolate TE7^T yielded a very low hybridization signal with the isolated DNA of *P. aerophilum*, *P. islandicum*, *P. organotrophum* and *T. neutrophilus*. Even the strongest hybridization signals with *P. aerophilum* were under 20% (Table 1).

DISCUSSION

New isolate TE7^T was a rod-shaped, facultative aerobic, heterotrophic and hyperthermophilic archaeon growing optimally at a temperature above 90 °C. The phylogenetic analysis of 16S rDNA sequences showed that for isolate TE7^T the closest known species were *P. aerophilum* and *T. neutrophilus*. The type species of the genus *Pyrobaculum* is *P. islandicum*, which was included in the same cluster of *P. aerophilum* and *T. neutrophilus*. On the other hand, the type species of the genus *Thermoproteus* is *T. tenax* (Zillig *et al.*, 1981), which was less closely related. Moreover, bootstrap analysis consistently placed *T. tenax* outside the cluster of *Pyrobaculum* spp. The classification of *T. neutrophilus* and recently isolated *Pyrobaculum* spp. was not dependent on the phylogeny of 16S rDNA analysis (Fischer *et al.*, 1983; Huber

et al., 1987). In terms of physiology, *T. tenax* is an acidophile (Zillig *et al.*, 1981), but *Pyrobaculum* spp. and *T. neutrophilus* are neutrophiles (Fischer *et al.*, 1983; Huber *et al.*, 1987; Völkl *et al.*, 1993). In view of these facts, isolate TE7^T should be a member of the genus *Pyrobaculum* (Fig. 3) and *T. neutrophilus* should be reclassified within the genus *Pyrobaculum*. Whilst the isolate TE7^T, *P. aerophilum* and *T. neutrophilus* were closely related in 16S rDNA sequence analyses, the DNA–DNA hybridization analysis showed very low similarities in genomic DNA (Table 1), and the G + C content of TE7^T (48 mol%) is lower than that of *P. aerophilum* (52 mol%) (Völkl *et al.*, 1993) and *T. neutrophilus* (60 mol%) (Itoh *et al.*, 1998). Thus, TE7^T, *P. aerophilum* and *T. neutrophilus* clearly represent distinct species in the genetic analysis.

Isolate TE7^T has unique features of respiration. Under aerobic conditions, growth of TE7^T appeared to be particularly dependent on aerobic respiration, which was inhibited by S⁰ and Na₂SO₃. This phenomenon has been observed in *P. aerophilum* (Völkl *et al.*, 1993). However, *P. aerophilum* grows in microaerobic conditions (0.5–1.0% oxygen) but not in aerobic conditions with vigorous shaking under atmospheric air. Under anaerobic conditions, growth of TE7^T depends on sulfur compounds such as Na₂S₂O₃, S⁰, glutathione (the oxidized form) and L-cystine, and it produces H₂S. These growth characteristics are similar to those of *P. islandicum* and *P. organotrophum* (Huber *et al.*, 1987) under anaerobic conditions. Alternatively, nitrate reduction, which in *P. aerophilum* depends on anaerobic conditions (Völkl *et al.*, 1993), was not observed. Thus, it can be said that TE7^T has a chimeric-

type respiration, i.e. *P. aerophilum*-type respiration under aerobic conditions and *P. islandicum*-type respiration under anaerobic conditions. Furthermore, *T. neutrophilus* and *P. islandicum* grow autotrophically in the presence of H₂ and S⁰ (Fischer *et al.*, 1983; Schäfer *et al.*, 1986; Huber *et al.*, 1987), and *P. aerophilum* grows autotrophically by the oxidation of H₂ or thiosulfate (Völkl *et al.*, 1993), whereas autotrophic growth of TE7^T was not observed.

In addition, the growth of TE7^T was slightly inhibited by chloramphenicol and penicillin G, which could not inhibit the growth of other *Pyrobaculum* species (Huber *et al.*, 1987; Völkl *et al.*, 1993). Also, TE7^T was not capable of growing above 0.5% NaCl, whereas the optimum NaCl concentration for growth of *P. aerophilum* is 1.5% NaCl (Völkl *et al.*, 1993). Furthermore, the maximum growth temperature of other *Pyrobaculum* species is over 100 °C (Fischer *et al.*, 1983; Huber *et al.*, 1987; Völkl *et al.*, 1993). In contrast, TE7^T could not grow above 97 °C. The optimum pH for TE7^T is pH 7, but that for *P. islandicum* and *P. organotrophum* is pH 6 (Huber *et al.*, 1987). Therefore, on the basis of the genomic DNA comparison and the physiological properties, we propose that TE7^T represents a new species of *Pyrobaculum*, which we have named *Pyrobaculum oguniense* (named after the isolation locale, Oguni-cho). The type strain is isolate TE7^T.

The close phylogenetic relationship between *P. aerophilum* and *P. oguniense* is the first example of a relationship between a microaerophile and an aerophile in the deepest and short branches within the phylogenetic tree. Aerobic metabolic pathways have been suggested to be a modern adaptation in the history of respiration (Dickerson *et al.*, 1976; Broda & Peschek, 1979). The phylogenetic analysis of cytochrome oxidase indicates that the aerobic metabolism had a single origin and was present in the last common ancestor of all organisms (Castresana *et al.*, 1994, 1995; Castresana & Saraste, 1995; Castresana & Moreira, 1999). In addition, geochemical analysis indicates that traces of oxygen could already have been formed photochemically early in the history of the Earth (Towe, 1990). Therefore, molecular and biochemical comparison of *P. aerophilum* and *P. oguniense* may show the nature of the development of aerobic respiration and the systems for oxidative stress in ancient types of organisms. On the other hand, the differences in anaerobic respiration between *P. aerophilum* and *P. oguniense* are also interesting. Recent molecular analysis has suggested that the last common ancestor of living organisms might have also possessed nitrate, sulfate and sulfur respiration (Castresana & Moreira, 1999). In addition, *P. aerophilum* contains the gene for proteins of the enzymic machinery of sulfate and sulfur respiration (Fitz-Gibbon *et al.*, 1997), even though these physiological activities have not been detected. These facts suggest that the genus *Pyrobaculum* still possesses the respiratory ability possessed by the common ancestor of living organisms

for billion of years and that they may choose the most appropriate respiration system for their habitat. Therefore, the molecular and biochemical investigation of respiration of *P. oguniense* may provide insights into survival strategies in such extreme environments.

Description of *Pyrobaculum oguniense* TE7^T sp. nov.

Pyrobaculum oguniense (o.gu.ni.en'se. M. L. neut. adj. *oguniense* pertaining to Oguni-cho, which is the town of the Tuetate hot spring).

The cells are rod-shaped and usually between 2 and 10 µm long and between 0.6 and 1.0 µm wide. Motile by monopolar flagellation. Cell wall composed of protein surface layer. Growth between 70 and 97 °C (optimum, 90–94 °C), at pH_{80 °C} 5.4 and 7.4 (optimum pH_{80 °C} 6.3–7.0) and up to 1.5% NaCl (w/v) (optimal at 0%). Optimal doubling time of 180 min under aerobic conditions and 360 min under anaerobic and microaerobic conditions. Heterotrophic growth with yeast extract and trypticase peptone. Sulfur inhibits aerobic growth but stimulates anaerobic growth. Oxygen, thiosulfate, sulfur, L-cystine and glutathione (oxidized form) are possible electron acceptors. The G+C content of genomic DNA is about 48 mol%. 16S rDNA sequence exhibits 99.1–98.5% similarity to those of *Pyrobaculum aerophilum*, *Thermoproteus neutrophilus* and *Pyrobaculum islandicum*. Isolate obtained from water samples of a terrestrial hot spring (Tuetate hot spring), Oguni-cho, Kumamoto Prefecture, Japan. The type strain is *Pyrobaculum oguniense* TE7^T (= JCM 10595^T = DSM 13380^T).

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REFERENCES

- Adams, M. W. W. & Kelly, R. M. (1998). Finding and using hyperthermophilic enzymes. *Trends Biotechnol* **16**, 329–332.
- Allen, M. B. (1959). Studies with *Cyanidium caldarium*, an anomalously pigmented chlorophyte. *Arch Microbiol* **32**, 270–277.
- Archenbach, L. & Woese, C. (1995). 16S and 23S rRNA-like primers. In *Archaea: a Laboratory Manual*, vol. 3, *Thermophiles*, pp. 201–203. Edited by F. T. Robb & A. R. Place. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Balch, W. E., Fox, G. E., Magrum, L. J., Woese, R. C. & Wolfe, R. S. (1979). Methanogens: re-evaluation of a unique biological group. *Microbiol Rev* **43**, 260–296.
- Blöchl, E., Rachel, R., Burggraf, S., Jannasch, H. W. & Stetter, K. (1997). *Pyrolobus fumarii*, gen. and sp. nov., represents a novel group of archaea, extending the upper temperature limit for life to 113 °C. *Extremophiles* **1**, 14–21.
- Brock, T. D., Brock, M. B., Belly, R. T. & Weiss, R. L. (1972). *Sulfolobus*: a new genus of sulfur-oxidizing bacteria living at low pH and high temperature. *Arch Microbiol* **84**, 54–68.

- Broda, E. & Peschek, G. A. (1979). Did respiration or photosynthesis come first? *J Theor Biol* **81**, 201–212.
- Castresana, J. & Moreira, D. (1999). Respiratory chains in the last common ancestor of living organisms. *J Mol Evol* **49**, 453–460.
- Castresana, J. & Saraste, M. (1995). Evolution of energetic metabolism: the respiration-early hypothesis. *Trends Biochem Sci* **20**, 443–448.
- Castresana, J., Lübben, M., Saraste, M. & Higgins, D. G. (1994). Evolution of cytochrome oxidase, an enzyme older than atmospheric oxygen. *EMBO J* **13**, 2516–2525.
- Castresana, J., Lübben, M. & Saraste, M. (1995). New archaeobacterial genes coding for redox proteins: implications for the evolution of aerobic metabolism. *J Mol Biol* **250**, 202–210.
- Chavez Crocker, P., Sako, Y. & Uchida, A. (1999). Purification and characterization of intracellular heat-stable proteinase (pernilase) from the marine hyperthermophilic archaeon *Aeropyrum pernix* K1. *Extremophiles* **3**, 3–9.
- DeLong, E. F. (1992). Archaea in coastal marine environments. *Proc Natl Acad Sci USA* **89**, 5685–5689.
- Dickerson, R. E., Tomkovich, R. & Almasy, R. J. (1976). The cytochrome fold and the evolution of bacterial energy metabolism. *J Mol Biol* **100**, 473–491.
- Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid–deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.
- Fischer, F., Zillig, W., Stetter, K. O. & Schreiber, G. (1983). Chemolithoautotrophic metabolism of anaerobic extremely thermophilic archaeobacteria. *Nature* **301**, 511–513.
- Fitz-Gibbon, S., Choi, A. J., Miller, J. H., Stetter, K. O., Simon, M. I., Swanson, R. & Kim, U.-J. (1997). A fosmid-based genomic map and identification of 474 genes of the hyperthermophilic archaeon *Pyrobaculum aerophilum*. *Extremophiles* **1**, 36–51.
- Huber, H. & Stetter, K. O. (1998). Hyperthermophiles and their potential in biotechnology. *J Biotechnol* **64**, 39–52.
- Huber, R., Kristjansson, J. K. & Stetter, K. O. (1987). *Pyrobaculum* gen. nov., a new genus of neutrophilic, rod-shaped archaeobacteria from continental solfataras growing optimally at 100 °C. *Arch Microbiol* **149**, 95–101.
- Huber, R., Dyba, D., Huber, H., Burggraf, S. & Rachel, R. (1998). Sulfur-inhibited *Thermosphaera aggregans* sp. nov., a new genus of hyperthermophilic archaea isolated after its prediction from environmentally derived 16S rRNA sequences. *Int J Syst Bacteriol* **48**, 31–38.
- Itoh, T., Suzuki, K. & Nakase, T. (1998). Occurrence of introns in the 16S rRNA genes of members of the genus *Thermoproteus*. *Arch Microbiol* **170**, 155–161.
- Kawarabayashi, Y., Hino, Y., Horikawa, H. & 27 other authors (1999). Complete genome sequence of an aerobic hyperthermophilic crenarchaeon, *Aeropyrum pernix* K1. *DNA Res* **6**, 83–101.
- Lauerer, G., Kristjansson, J. K., Langworthy, T. A., König, H. & Stetter, K. O. (1986). *Methanothermus sociabilis* sp. nov., a second species within the *Methanothermaceae* growing at 97 °C. *Syst Appl Microbiol* **8**, 100–105.
- Morii, H., Yagi, H., Akutsu, H., Nomura, N., Sako, Y. & Koga, Y. (1999). A novel phosphoglycolipid archaetidyl (glucosyl) inositol with two sesterterpanyl chains from the aerobic hyperthermophilic archaeon *Aeropyrum pernix* K1. *Biochem Biophys Acta* **1436**, 426–436.
- Nomura, N., Sako, Y. & Uchida, A. (1998). Molecular characterization and postsplicing fate of three introns within the single rRNA operon of the hyperthermophilic archaeon *Aeropyrum pernix* K1. *J Bacteriol* **180**, 3635–3643.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Sako, Y., Nomura, N., Uchida, A., Ishida, Y., Morii, H., Koga, Y., Hoaki, T. & Maruyama, T. (1996). *Aeropyrum pernix* gen. nov., sp. nov., a novel aerobic hyperthermophilic archaeon growing at temperatures up to 100 °C. *Int J Syst Bacteriol* **46**, 1070–1077.
- Sako, Y., Crocker, P. C. & Ishida, Y. (1997). An extremely heat-stable extracellular proteinase (aeropyrolysin) from the hyperthermophilic archaeon *Aeropyrum pernix* K1. *FEBS Lett* **415**, 329–334.
- Schäfer, S., Barkowski, C. & Fuchs, G. (1986). Carbon assimilation by the autotrophic thermophilic archaeobacterium *Thermoproteus neutrophilus*. *Arch Microbiol* **146**, 301–308.
- Segerer, A., Neuner, A., Kristjansson, J. K. & Stetter, K. O. (1986). *Acidianus infernus* gen. nov., sp. nov., and *Acidianus brierleyi* comb. nov.: facultatively aerobic, extremely acidophilic thermophilic sulfur-metabolizing archaeobacteria. *Int J Syst Bacteriol* **36**, 559–564.
- Stetter, K. O. (1996). Hyperthermophilic prokaryotes. *FEMS Microbiol Rev* **18**, 149–158.
- Tamaoka, J. & Komagata, K. (1984). Determination of DNA base composition by reversed-phase high-performance liquid chromatography. *FEMS Microbiol Lett* **25**, 125–128.
- Towe, K. M. (1990). Aerobic respiration in the Archaeon? *Nature* **348**, 54–56.
- Völkl, P., Huber, R., Drobner, E., Rachel, R., Burggraf, S., Trincone, A. & Stetter, K. O. (1993). *Pyrobaculum aerophilum* sp. nov., a novel nitrate-reducing hyperthermophilic archaeum. *Appl Environ Microbiol* **59**, 2918–2926.
- Zillig, W., Stetter, K. O., Schäfer, W., Janekovic, D., Wunderl, S., Holz, I. & Palm, P. (1981). Thermoproteales: a novel type of extremely thermoacidophilic anaerobic archaeobacteria isolated from Icelandic solfataras. *Zentbl Bakteriol Hyg 1 Abt Orig C* **2**, 205–227.
- Zillig, W., Gierl, A., Schreiber, G., Wunderl, S., Janekovic, D., Stetter, K. O. & Klenk, H. P. (1983). The archaeobacterium *Thermofilum pendens* represents a novel genus of the thermophilic, anaerobic sulfur respiring *Thermoproteales*. *Syst Appl Microbiol* **4**, 79–87.