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\textsuperscript{T}, grew under aerobic, microaerobic and anaerobic conditions. Isolate TE7\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{T} and previously described Pyrobaculum species. As TE7\textsuperscript{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\textsuperscript{T} = JCM 10595\textsuperscript{T} = DSM 13380\textsuperscript{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 micro-aerophiles (e.g. Pyrobaculum aerophilum, Pyrolobus fumarii and Aquifex pyrophilus) (Adams & Kelly, 1998; Blöchl et al., 1997) and one facultative aerophile (Acidiamus infernus) (Segerer 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 Croocker et al., 1999). Moreover, the full genome sequence of \textit{A. pernix} was fully examined (Kawarabayashi et al., 1999). The isolation of \textit{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
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 characteristic 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® (JCM 9630®, DSM 7523®), *Pyrobaculum organotrophum* H10® (JCM 9190®, DSM 4185®), *Pyrobaculum islandicum* geo3® (JCM 9189®, DSM 4184®) and *Thermoproteus neutrophilus* V24Sta® (JCM 9278®, DSM 23383®) 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 in 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) Na$_2$SO$_4$. 5H$_2$O 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 0.5% (w/v) Na$_2$S. 9H$_2$O and pressurized with N$_2$ (100%, 200 kPa). Resazurin (0.0001%, w/v) was used as a redox indicator. For microaerobic growth, the gas phase was replaced with N$_2$ (100%, 200 kPa) and the Na$_2$S. 9H$_2$O solution was omitted. In this medium, resazurin turns red, indicating the presence of O$_2$.

For the determination of the autotrophic growth under anaerobic conditions, modified Allen’s basal medium was supplemented with 0.1% (w/v) Na$_2$SO$_4$. 5H$_2$O or 1% (w/v) S$_2$O$_3$ and the gas phase was H$_2$.CO$_2$ (80:20, 200 kPa) or N$_2$.CO$_2$ (80:20, 200 kPa). For autotrophic growth under aerobic conditions, only 0.1% (w/v) Na$_2$S. 5H$_2$O was added.

*P. aerophilum* IM2® (= JCM 9630® = DSM 7523®) was grown in BSY medium (Völkl et al., 1993). *P. organotrophum* H10® (= JCM 9190® = DSM 4185®) and *P. islandicum* geo3® (= JCM 9189® = DSM 4184®) were grown in JCM 184 medium (JCM Catalogue of Strains, 7th edition, 1999). *T. neutrophilus* V24Sta® (= JCM 9278® = DSM 23383®) 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 H$_2$.CO$_2$ (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 (UXI; Nikon). For observations with transmission electron microscopy, cells were fixed with 2% (v/v) glutaraldehyde in 0.1 M Na$_2$HPO$_4$.K$_2$HPO$_4$. (pH 7.2) buffer and post-fixed with 1% (v/v) OsO$_4$. 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 (UXF-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-dependence of growth, the pH value of the medium was adjusted to 25 °C (pH$_{25}$ °C) with NaOH and H$_2$SO$_4$; then the pH values were also determined at 80 °C (pH$_{80}$ °C). The pH$_{25}$ °C values of 5.4, 6.1, 6.3, 6.6, 6.8 and 7.4 corresponded to pH$_{80}$ °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 Na$_2$SO$_4$. 5H$_2$O (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 Na$_2$SO$_4$. 5H$_2$O, but with the following sulfur compounds, was used: Na$_2$SO$_3$ (0.1%), S$^{2-}$ (1%), glutathione (the oxidized form) (0.1%), l-cysteine HCl (0.1%), l-cystine (0.1%). For the determination of nitrate respiration, NaNO$_3$ (0.1%) and NaNO$_2$ (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 H$_2$S as a metabolic product.** H$_2$S 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.
package.

each organism was accomplished using the analysis (Saitou & Nei, 1987) of 1371 bases of sequence from Institute of Genetics, Mishima, Japan). Neighbour-joining using the 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 software package.

**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 deoxy-nucleotide 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 qden 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 qden software package.

**DNA–DNA hybridization.** DNA from strain TE7 was compared with DNA from P. aerophilum IM2 (= JCM 9630 = DSM 7523), P. organotrophum H10 (= JCM 9190 = DSM 4185), P. islandicum geo3 (= JCM 9189 = DSM 4184) and T. neutrophilus V24Sta (= JCM 9278 = DSM 2338) 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 23S rDNA were amplified by the PCR. The primers employed in the amplification had the sequences 5’-CGGTTGGATCACCTC-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 were analysed using RFLP. All 10 ITS region clones from both aerobic and anaerobic cultures of TE7 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 variantly rod-shaped cells were seen. A culture of rod-shaped cells was purified by serial dilution, and strain TE7 (= JCM 10595) 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.

**Morphology**

Cells of isolate TE7 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...
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

TE7T 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 TE7T occurred between pH80°C 5-4 and 7-4 with an optimum of pH80°C 6-3–7-0. No growth was observed at pH80°C 5-0 and pH80°C 7-6 (Fig. 2b). TE7T 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 TE7T could grow heterotrophically on proteinaceous complex substrates such as 0.1% (w/v) yeast extract or 0.05% (w/v) trypticase peptone in the absence of sulfur compounds under aerobic conditions. However, in the presence of Na2S3O3·5H2O, the growth yield of TE7T increased fourfold and the maximum cell density was about 2·3 x 10⁸ cells ml⁻¹. With trypticase peptone as the sole substrate, a cell density of 1·0 x 10⁶ 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, TE7T grew heterotrophically and required sulfur compounds. The maximum cell density under both conditions reached from 5·6 x 10⁷ to 6·0 x 10⁷ cells ml⁻¹ with 0.1% (w/v) yeast extract in the presence of Na2S3O3·5H2O. 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 x 10⁷ cells ml⁻¹. Very weak growth was observed on trypticase peptone, gelatin, casamino acids and acetate, with cell densities of about 5 x 10⁶ cells ml⁻¹. S⁰, glutathione (the oxidized form), l-cysteine and Na2S could replace Na2S3O3·5H2O, supporting a cell yield of 3·5 x 10⁷ to 4·5 x 10⁷ cells ml⁻¹. l-Cysteine could also support growth. However, the cell yield was only 6·5 x 10⁶ 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 NaNO3 or NaN3 (data not shown). Autotrophic growth was not observed under either aerobic or anaerobic conditions.

Antibiotic resistance

Isolate TE7T 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; Vökl et al., 1993).

DNA base composition

The G + C content of isolate TE7T 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 TE7T was very similar to
phylogenetic relationship between isolate TE7 found with Thermoproteus tenax and other members of the

**DISCUSSION**

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

**DNA–DNA hybridization**

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

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

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<th>Source of microplate-bound DNA</th>
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<td>Strain TE7T</td>
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Isolate TE7 has unique features of respiration. Under aerobic conditions, growth of TE7 appeared to be particularly dependent on aerobic respiration, which was inhibited by \( \text{S}^0 \) and \( \text{Na}_2\text{SO}_4 \). This phenomenon has been observed in \( \text{P. aerophilum} \) (Volkl et al., 1993). However, \( \text{P. aerophilum} \) 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 depends on sulfur compounds such as \( \text{Na}_2\text{S}_2\text{O}_3 \) and \( \text{S}^0 \), glutathione (the oxidized form) and \( \text{L-cystine} \), and it produces \( \text{H}_2\text{S} \). These growth characteristics are similar to those of \( \text{P. islandicum} \) and \( \text{P. organotrophum} \) (Huber et al., 1987) under anaerobic conditions. Alternatively, nitrate reduction, which in \( \text{P. aerophilum} \) depends on anaerobic conditions (Volkl et al., 1993), was not observed. Thus, it can be said that TE7 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 _H_2 and _S_0 (Fischer et al., 1983; Schäfer et al., 1986; Huber et al., 1987), and _P. aerophilum_ grows autotrophically by the oxidation of _H_2 or thiosulfate (Völkl et al., 1993), whereas autotrophic growth of _TE7_ was not observed.

In addition, the growth of _TE7_ 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_ 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_ could not grow above 97°C. The optimum pH for _TE7_ 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_ 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_.

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 billions 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 sp. nov.**

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