**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 \(\mu\)m 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*, *Pyrobaculum fumarii* and *Aquifex pyrophilus*) (Adams & Kelly, 1998; Blöchl et al., 1997) and one facultative aerophile (*Acidianus 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 *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...
isolation of a novel facultatively aerobic, hyper-
thermophilic 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 charac-
terization of the new isolate, the first report of a
facultatively aerobic and hyperthermophilic archaeon
that grows at temperatures above 90 °C under neutro-
philic 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 neutrophilum* V24Sta° (JCM
9278°, DSM 2338°) 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) Na2SO4, 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 auto-
claving. The enrichment was incubated in screw-capped test
vials (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
test the ability of the isolate to form colonies on agar
plates (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 (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
autoclaving. The enrichment was incubated in screw-capped test
vials (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
test the ability of the isolate to form colonies on agar
plates (Sako et al., 1996).

**Determination of growth.** Cell densities were determined by
direct counting of cells stained with 4’,6-diamidino-2-
phenylindole dihydrochloride (DAPI), using an epifo-
scopes were stained

**Light- and electron microscopy.** Cells were observed with
a differential interference microscope (UXF; 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

**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 Na2SO4, 5H2O (0.1%)
(pH 7.5 ± 0.2) at 70 °C: 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 Na2SO4, 5H2O but with the following sulfur
compounds, was used: Na2S2O3 (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, NaNO3
(0.1%) and NaNO2 (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 Volkl 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
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
differential interference microscope (UXF; 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.
(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 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 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 TE7T was compared with DNA from P. aerophilum IM2T (= JCM 9630T = DSM 7523T), P. organotrophum H10T (= JCM 9190T = DSM 4185T), P. islandicum geo3T (= JCM 9189T = DSM 4184T) and T. neutrophilus V24StaT (= JCM 9278T = DSM 2338T) by using the fluorometric DNA–DNA hybridization method, with photobiotion-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′-CGGTTGGAATCGCTC (1528F) and 5′-GTTGTGTTTCTTTTCCT (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 TE7T 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 TE7T (= JCM 10595T) 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 TE7T 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. 1. Transmission electron micrographs of cells of Pyrobaculum oguniense sp. nov.](image-url)
Optimum of pH growth was 0% NaCl. No growth was observed above
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) yeast extract and 0·05% (w/v) tryptase peptone in the absence of sulfur compounds under aerobic conditions. However, in the presence of Na2S2O3·5H2O, the growth yield of TE7T increased fourfold and the maximum cell density was about 2·3 × 10⁶ cells ml⁻¹. With tryptase peptone as the sole substrate, a cell density of 1·0 × 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 5·6×10⁷ to 6·0×10⁷ 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) tryptase peptone, with a cell density of about 3·5×10² cells ml⁻¹. Very weak growth was observed on tryptase peptone, gelatin, casamino acids and acetate, with cell densities of about 5×10⁶ cells ml⁻¹. S⁰, glutathione (the oxidized form), L-cystine and Na2S could replace Na2S2O3·5H2O, supporting a cell yield of 3·5×10⁶ to 4·5×10⁷ cells ml⁻¹. L-Cysteine could also support growth. However, the cell yield was only 6·5×10⁶ 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 NaNO₂ (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; Völkl *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...
Thermoproteus tenax found with other members of the Thermoproteales. DNA–DNA hybridization between Thermoproteus neutrophilus. Table 1 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 a acidophile (Zillig et al., 1981), but Pyrobaculum spp. and T. neutrophilus are neutrophiles (Fischer et al., 1983; Huber et al., 1987; Völk 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%) (Völk 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 S\(^\circ\) and Na\(\text{SO}_4\). This phenomenon has been observed in P. aerophilum (Völk 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 Na\(\text{S}_2\text{O}_3\), S\(^\circ\), glutathione (the oxidized form) and L-cystine, and it produces H\(\text{S}\). 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 (Völk et al., 1993), was not observed. Thus, it can be said that TE7\(^T\) has a chimeric-

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

<table>
<thead>
<tr>
<th>Source of microplate-bound DNA</th>
<th>Source of photobiotin-labelled DNA</th>
<th>Strain TE7(^T)</th>
<th>P. aerophilum</th>
<th>P. islandicum</th>
<th>P. organotrophum</th>
<th>T. neutrophilus</th>
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<tr>
<td>P. oguniense TE7(^T)</td>
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<td>100</td>
<td>5</td>
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<td>P. aerophilum IM2(^T)</td>
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<td>P. islandicum geo3(^T)</td>
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<td>3</td>
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<td>P. organotrophum H10(^T)</td>
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<td>4</td>
<td>72</td>
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<td>T. neutrophilus V24Sta(^T)</td>
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...
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 (Völk et al., 1993), whereas autotrophic growth of TE$^7$ was not observed.

In addition, the growth of TE$^7$ was slightly inhibited by chloramphenicol and penicillin G, which could not inhibit the growth of other *Pyrobaculum* species (Huber et al., 1987; Völk et al., 1993). Also, TE$^7$ was not capable of growing above 0.5% NaCl, whereas the optimum NaCl concentration for growth of *P. aerophilum* is 1.5% NaCl (Völk et al., 1993). Furthermore, the maximum growth temperature of other *Pyrobaculum* species is over 100 °C (Fischer et al., 1983; Huber et al., 1987; Völk et al., 1993). In contrast, TE$^7$ could not grow above 97 °C. The optimum pH for TE$^7$ 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 TE$^7$ represents a new species of *Pyrobaculum*, which we have named *Pyrobaculum oguniense* (named after the isolation locale, Oguni-cho). The type strain is isolate TE$^7$.

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 living 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 TE$^7$ 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$^{0.0}$ 5.4 and 7.4 (optimum pH$^{0.0}$ 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* TE$^7$ (= JCM 10595$^T$ = DSM 13380$^T$).

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