Thermogymnomonas acidicola gen. nov., sp. nov., a novel thermoacidophilic, cell wall-less archaeon in the order *Thermoplasmatales*, isolated from a solfataric soil in Hakone, Japan

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A novel thermoacidophilic, cell wall-less archaeon, strain IC-189^T, was isolated from a solfataric field in Ohwaku-dani, Hakone, Japan. The cells were irregular cocci, sometimes lobed, club-shaped or catenated, and were highly variable in size, ranging from 0.8 to 8.0 µm in diameter. The strain grew at temperatures in the range 38–68 °C (optimally at 60 °C) and at pH 1.8–4.0 (optimally at around pH 3.0). Strain IC-189^T was obligately aerobic and heterotrophic, requiring yeast extract for growth. Yeast extract, glucose and mannose served as carbon and energy sources. The polar lipids consisted mainly of cyclic or acyclic glycerol-bisdiphytanyl-glycerol tetraethers, and the predominant quinone was a menaquinone with seven isoprenoid units (MK-7). The G + C content of total DNA was 56.1 mol%. 16S rRNA gene sequence analysis revealed that strain IC-189^T was a member of the order *Thermoplasmatales*, but diverged from the hitherto known species of the genera *Thermoplasma*, *Picrophilus* and *Ferroplasma* (86.2–91.0 % sequence similarity). These phenotypic and phylogenetic properties clearly support a separate taxonomic status for this strain. Therefore, strain IC-189^T represents a novel genus (order *Thermoplasmatales*) and species, for which the name *Thermogymnomonas acidicola* gen. nov., sp. nov. is proposed, with type strain IC-189^T (=JCM 13583^T=DSM 18835^T).

The order *Thermoplasmatales* was created in 2001 to accommodate the moderately thermophilic, aerobic or facultatively anaerobic, acidophilic, euryarchaeotic genera *Thermoplasma* and *Picrophilus*, growing optimally at around 60 °C and at pH values below 2 (Darland *et al.*, 1970; Schleper *et al.*, 1996; Reysenbach, 2001). The non-thermophilic genus *Ferroplasma* was subsequently included in this order (Golyshina *et al.*, 2000). Because of the marked divergence of the 16S rRNA gene sequences and biochemical properties among these three genera, each genus represents only the respective family, i.e. *Thermoplasmataceae*, *Picrophilaceae* or *Ferroplasmaceae*. The genus *Thermoplasma* comprises two species, *Thermoplasma acidophilum* and *Thermoplasma volcanium*, which were isolated from self-heated coal-refuse piles and solfataras, respectively (Darland *et al.*, 1970; Segerer *et al.*, 1988; Segerer & Stetter, 1992a). *Thermoplasma* species lack cell walls and show various cell morphologies, from coccoid shapes to

filamentous or plate-like ones. They are facultative anaerobes and obligate heterotrophs. The genus *Picrophilus* is represented by two species, *Picrophilus oshimae* and *Picrophilus torridus* (Schleper *et al.*, 1996), which were found in solfataras in Hokkaido, Japan. *Picrophilus* species have S-layer cell walls and are hyperacidophilic, thriving at pH 0–1. These species are also obligately heterotrophic, but do not grow anaerobically. The genus *Ferroplasma* was created for a non-thermophilic acidophile that was isolated from bioleaching pilot plants (Golyshina *et al.*, 2000). The genus is currently monospecific, being represented by *Ferroplasma acidiphilum*; however, two species without validly published names, '*Ferroplasma acidarmanus*' and '*Ferroplasma cupricumulans*', have also been described (Edwards *et al.*, 2000; Dopson *et al.*, 2004; Hawkes *et al.*, 2006). The latter species is moderately thermophilic, like members of the genera *Thermoplasma* and *Picrophilus*. *F. acidiphilum* lacks cell walls and shows pleomorphic cell morphology (like *Thermoplasma* species) and grows chemo-organotrophically or chemomixotrophically on ferrous iron and yeast extract or sugars.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain IC-189^T is AB269873.

Here, we describe the isolation and phenotypic and phylogenetic characterization of a novel moderately thermophilic and acidophilic archaeon, IC-189^T, isolated from a solfataric field in Hakone, Kanagawa, Japan. Its physiological and morphological features resemble those of the aforementioned *Thermoplasmatales* species; however, its 16S rRNA gene sequence, its DNA G+C content and some of its physiological features warrant a taxonomic status within a novel genus in this order.

A soil sample (60 °C, pH 1.4) was collected from a solfataric field in Ohwaku-dani (Hakone, Kanagawa Prefecture, Japan) on 19 July 2002 and brought back to our laboratory in a sterile, hermetically sealed, plastic tube on the same day. In an attempt to isolate various micro-organisms by using a single culture medium, a soil suspension was diluted serially and each dilution was distributed in multiple wells and cultivated (as in the most-probable number method). Approximately 1 g (wet weight) soil sample was suspended in 10 ml *Sulfolobus* medium (pH 2.5; Brock *et al.*, 1972) and 10²-, 10³-, and 10⁴-fold dilutions in the same medium were distributed in wells of PCR plates (96 wells for each dilution, 100 µl per well), sealed with caps and incubated at 55 °C for 2 weeks. Microbial growth was observed in 70 wells at the 10⁴-fold dilution. Growth in the wells could be divided roughly into four cultural types: 47 wells showed a predominance of relatively large coccoid cells (approx. 3–5 µm in diameter); four wells contained numerous small cocci (<1 µm in diameter); 18 wells contained coexisting large and small cocci; and one well contained only rod-shaped cells. One of the cultures dominated by small cocci was further diluted serially (1:10) and each dilution was divided equally into 10 tubes and cultivated. This step was repeated for one growth-positive culture at the highest dilution. Then, the serial dilution (1:10)/cultivation method was repeated twice to obtain a pure culture, which was designated IC-189^T and studied further (as described below). On the other hand, the strain that comprised large cocci and the strain that comprised rod-shaped cells were identified as a *Picrophilus* species and an actinobacterium, respectively, on the basis of 16S rRNA gene sequences (data not shown). After strain IC-189^T had been purified, it was cultivated routinely at 60 °C in a modified *Thermoplasma* medium (JCM medium no. 180, but with the pH adjusted to 3.0). The purity of the strain was confirmed routinely by microscopic examination and repeated partial sequencing of the 16S rRNA gene using several primers. In addition, no 16S rRNA gene fragments were PCR-amplified with bacteria-specific primers (Namwong *et al.*, 2005), *Nanoarchaeota*-specific primers (Hohn *et al.*, 2002) or *Sulfolobales*-specific primers (T. Itoh, unpublished data). Unless otherwise indicated, the modified *Thermoplasma* medium was used for the phenotypic characterization of strain IC-189^T.

Cells of strain IC-189^T were mostly irregular cocci and sometimes showed lobe- and club-shaped forms (Fig. 1). Despite the cultural type observed in the PCR plate wells,

cells grown in the modified *Thermoplasma* medium (and even in the *Sulfolobus* medium) were dominated by cocci 1–3 µm in diameter; larger (up to 8 µm) and smaller (around 0.8 µm) cocci were frequently observed (Fig. 1). Most cells occurred singly, but some were accompanied by budding daughter cells or were catenated like beads on a string (Fig. 1c). No motile cells were observed. Under a light microscope, some large cells seemed to have inclusion bodies (Fig. 1b). The cells showed moderate natural fluorescence when examined under a fluorescence microscope (BX51; Olympus) (excitation, 330–385 nm; absorption, 420 nm). Platinum–palladium-shadowed cells, prepared and observed under a transmission electron microscope as described previously (Itoh *et al.*, 1998), revealed pili (one or a few per cell) (Fig. 1d). To prepare thin sections, cells were fixed with 2.5 % glutaraldehyde at 4 °C for 2 h; this was followed by post-fixation with 2 % OsO₄ in 0.1 M cacodylate buffer at 4 °C for 2 h. After dehydration with increasing ethanol concentrations, the cells were embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead acetate and then examined under a transmission electron microscope (JEM-1230; JEOL). The cells apparently lacked cell walls and were each surrounded by a single, thin layer approximately 10 nm thick (Fig. 1e). This cell-envelope width is comparable to those of the cell wall-less genera *Thermoplasma* and *Ferroplasma*. On a plate medium solidified with 0.8 % (w/v) Phytagel (Sigma), strain IC-189^T produced colourless, small (<1.5 mm in diameter), flat or slightly umbonate colonies with a 'fried-egg' appearance (Fig. 1f).

Strain IC-189^T was strictly aerobic and did not grow anaerobically, even in the presence of sulfur (0.1 M), nitrate (10 mM) or ferric iron [as 1 mM Fe(III)citrate or 1 mM Fe(III)–EDTA]. It was obligately heterotrophic and required 0.001–0.2 % (w/v) yeast extract for growth in medium containing 1.0 % glucose. No growth was observed without yeast extract or at a 0.5 % concentration. The yeast extract could not be replaced by either a vitamin mixture (Balch *et al.*, 1979) or a trace-element solution (Seeger & Stetter, 1992b). The strain grew at temperatures in the range 38–68 °C (optimally at 60 °C) and at pH 1.8–4.0 (optimally at pH 3.0). Under optimal growth conditions, the strain multiplied with a doubling time of 4.8 h.

To evaluate utilization of carbon and energy sources, the modified *Thermoplasma* medium (pH 3.0) was altered further as follows: yeast extract concentration was reduced to 0.005 %, and glucose was replaced by each of the test substrates. Strain IC-189^T utilized glucose and mannose, but not arabinose, fructose, galactose, lactose, maltose, D-ribose, sucrose, xylose, starch, glycogen (at 0.5 %), acetate, butyrate, citrate, formate, fumarate, L-lactate, malate, propionate, pyruvate, succinate, methanol, ethanol, formamide, methylamine, trimethylamine, gelatin (at 0.2 %), beef extract, peptone, malt extract or tryptone (at 0.05 %). Glutamate supported weak growth, but none of the other 19 amino acids supported growth (at 0.2 %). The strain

Fig. 1. Light micrographs (a–c), transmission electron micrograph of cells shadowed with platinum–palladium (d), ultrathin sections stained with uranyl acetate and lead acetate (e) and colonies (f) of strain IC-189^T. Bars, 5.0 µm (a, b); 10.0 µm (c); 2.0 µm (d); 40.0 nm (e); 0.6 mm (f).

also grew on yeast extract as the sole source of carbon and energy. The addition of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.5 %) to the medium was inhibitory to growth. The strain tolerated the presence of NaCl at concentrations up to 2.5 %, but not at 3.0 %. Growth was inhibited by the addition of erythromycin, novobiocin and rifampicin in the early exponential phase, but not by the addition of ampicillin, chloramphenicol, kanamycin, oleandomycin, penicillin, streptomycin or vancomycin (all at $100 \mu\text{g ml}^{-1}$).

TLC analysis of the lipid fraction, extracted as described previously (Itoh *et al.*, 1998), detected a large amount of tetraether core lipids, including at least three acyclic or cyclic moieties. Diether core lipid was not detected. On a thin-layer chromatogram of the quinone fraction (Itoh *et al.*, 1985), a single band that corresponded to a menaquinone moiety was detected. HPLC analysis revealed that it contained MK-7 as a major component.

The G + C content of the genomic DNA was 56.1 mol%, as determined by the HPLC method of Tamaoka (1994). The almost-complete 16S rRNA gene was PCR-amplified with primers A-20F and A-1530R and then sequenced as described previously (Itoh *et al.*, 2002). The G + C content of the 1434 positions determined was 57.9 mol%. In a Southern hybridization study performed with a labelled 16S rRNA gene of strain IC-189^T, a single band was detected from total DNA sample digested with *Bgl*II, *Eco*RV or *Bst*XII, indicating that there was a single copy of the 16S rRNA gene in the genomic DNA (data not shown). A BLAST search of the 16S rRNA gene sequence of strain IC-189^T revealed that, of the archaeal orders, it was related most closely to the order *Thermoplasmatales* (Reysenbach, 2001). Therefore the sequence was aligned with those of the

previously described *Thermoplasmatales* species by using the CLUSTAL_X program (Thompson *et al.*, 1997). However, strain IC-189^T was found to be related only distantly to those *Thermoplasmatales* species: the levels of sequence similarity with respect to the two *Picrophilus* species were 91.0 %, this being followed by 90.9 % for *T. acidophilum*, 90.8 % for *T. volcanium* and 86.2 % for *F. acidiphilum*. Therefore, the sequence was compared again with a dataset that included several uncultured clones belonging to the order *Thermoplasmatales* in GenBank. After the removal of ambiguous bases and unreliable alignment sections, 1349 base positions were compared and a phylogenetic tree (Fig. 2) was reconstructed by using the neighbour-joining method (Saitou & Nei, 1987). Strain IC-189^T was also shown to be related distantly to these clones, with 90.2–92.8 % sequence similarity, but it showed some affinity (73 % bootstrap support) with clones isolated from acid mine drainage and related habitats; these clones are known as the ‘alphabet (A to E) plasma’ groups (Baker & Banfield, 2003).

Strain IC-189^T is an aerobic, moderately thermophilic, acidophilic, heterotrophic archaeon devoid of a cell wall. Furthermore, the strain predominantly contained tetraether core lipids and possessed MK-7 as a major respiratory quinone. These morphological, physiological and biochemical properties correspond well with those of the order *Thermoplasmatales*. In addition, 16S rRNA gene sequence analysis placed strain IC-189^T in the clade of the order *Thermoplasmatales*. However, strain IC-189^T can be separated from the known genera in this order, namely *Thermoplasma*, *Picrophilus* and *Ferroplasma* (Darland *et al.*, 1970; Segerer *et al.*, 1988; Segerer & Stetter, 1992a; Schleper

Fig. 2. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of strain IC-189^T within the order *Thermoplasmatales*. '*Aciduliprofundum boonei*' was used as an outgroup. Numbers at nodes indicate bootstrap percentages (based on 1000 resamplings). Bar, 0.01 substitutions per site.

et al., 1996; Golyshina *et al.*, 2000), on the basis of its higher pH for optimal growth (around pH 3.0) and its higher DNA G+C content (56.1 mol%). Further phenotypic differences between strain IC-189^T and these three genera are shown in Table 1. On the basis of the phylogenetic analysis using 16S rRNA gene sequences, strain IC-189^T was related distantly to the known species of the three genera. Thus, it may represent a different family in this order; however, such a conclusion should await the isolation of other strains related to IC-189^T and the performance of more detailed comparative studies of all genera of the order *Thermoplasmatales*. On the other hand, it is clear that strain IC-189^T represents a novel genus and species in the order *Thermoplasmatales*, for which the name *Thermogymnomonas acidicola* gen. nov., sp. nov. is proposed.

Description of *Thermogymnomonas* gen. nov.

Thermogymnomonas (Ther'mo.gym.no.mo'nas. Gr. adj. *thermos* hot; Gr. adj. *gymnos* nude; Gr. f. n. *monas* unit; N.L. fem. n. *thermogymnomonas* hot, cell wall-less unit).

Cells are pleomorphic, predominantly comprising irregular cocci that range from 1.0 to 3.0 µm in diameter. Cells lack a cell wall and are surrounded by a thin membrane (approx. 10 nm thick). Buds (daughter cells) are sometimes associated with cells. Pili are present. Non-motile. On solid medium, small colourless colonies with a 'fried-egg' appearance are formed. Strictly aerobic. Acidophilic and moderately thermophilic. Heterotrophic, requiring yeast extract for growth. Growth is inhibited by erythromycin, novobiocin and rifampicin (at 100 µg ml⁻¹). Lipids predominantly comprise tetraethers; MK-7 is the major

Table 1. Differential characteristics among the genera of the order *Thermoplasmatales*

Data for *Thermoplasma* are from Darland *et al.* (1970), Segerer *et al.* (1988) and Segerer & Stetter (1992a), data for *Picrophilus* are from Schleper *et al.* (1995) and data for *Ferroplasma* are from Golyshina *et al.* (2000).

Characteristic	<i>Thermoplasma</i>	<i>Picrophilus</i>	<i>Ferroplasma</i>	IC-189 ^T
Cell shape	Pleomorphic	Irregular coccoid	Pleomorphic	Pleomorphic
S-layer cell wall	—	+	—	—
Motility	+	—	—	—
Anaerobic growth	+	—	—	—
Autotrophic growth	—	—	+	—
Fe ²⁺ oxidation	—	—	+	—
Growth temperature				
Range	33–67	47–65	15–45	38–68
Optimum	59	60	35	60
Growth pH				
Range	0.5–4.0	0–3.5	1.3–2.2	1.8–4.0
Optimum	1–2	0.7	1.7	3.0
DNA G+C content (mol%)	38–46	36	37	56

respiratory quinone. The DNA G+C content is 56 mol%. Phylogenetically, the genus represents an independent lineage within the order *Thermoplasmatales*. Inhabits geo-thermal terrestrial habitats. The genus is monospecific, at present, and the type species is *Thermogymnomonas acidicola*.

Description of *Thermogymnomonas acidicola* sp. nov.

Thermogymnomonas acidicola (a.ci.di'co.la. N.L. neut. n. *acidum* an acid; L. suff. *-cola* from L. n. *incola* an inhabitant; N.L. n. *acidicola* an inhabitant of an acidic environment).

Morphology and growth properties are as described for the genus. Growth occurs at temperatures in the range 38–68 °C (optimum, 60 °C) and at pH 1.8–4.0 (optimum, pH 3.0). Yeast extract, as well as glucose and mannose (in the presence of yeast extract), serve as carbon and energy sources. NaCl is tolerated at concentrations up to 2.5 %. The DNA G+C content DNA is 56.1 mol%.

The type strain, IC-189^T (=JCM 13583^T=DSM 18835^T), was isolated from a sample of solfataric soil collected from Ohwaku-dani, Hakone, Japan.

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