***Thermocladium modestius* gen. nov., sp. nov., a new genus of rod-shaped, extremely thermophilic crenarchaeote**

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Three strains of novel, extremely thermophilic, rod-shaped crenarchaeotes were isolated from acidic hot spring areas in Japan. Cells of the three strains were straight or slightly curved rods and occasionally branched out singly or extensively, or had spherical bodies protruding at the ends of the cells. They were heterotrophs that grew anaerobically or microaerobically. The presence of CO₂ in the gas phase, archaeal cell-extracts and a vitamin mixture stimulated growth of the strains. Growth occurred at 45–82 °C and pH 2.6–5.9 and was optimal around 75 °C and pH 4.0. The strains utilized glycogen, starch, gelatin and various proteinaceous complex compounds as carbon sources. They required sulfur, thiosulfate or L-cystine as possible electron acceptors. The lipids mainly consisted of various cyclic glycerol-bisdiphytanyl-glycerol tetraethers. The G+C contents of the genomic DNAs were 52 mol%. Comparison of the 16S rDNA sequences indicated that they belonged to a separate lineage in the family *Thermoproteaceae*. The three strains were included in a single species due to high levels of DNA–DNA hybridization values. Based upon these results, the new isolates were assigned to a new genus and species in the family *Thermoproteaceae*, *Thermocladium modestius* gen. nov., sp. nov. The type strain is *Thermocladium modestius* IC-125^T (= JCM 10088^T).

Keywords: *Thermocladium modestius*, extreme thermophile, crenarchaeotes, *Thermoproteaceae*

INTRODUCTION

Accumulation of 16S rRNA/rDNA sequence data has revealed that the domain *Archaea* is composed of two major branches, the kingdoms *Euryarchaeota* (euryarchaeotes) and *Crenarchaeota* (crenarchaeotes) (Woese *et al.*, 1990). Currently isolated species of the crenarchaeotes are all extreme thermophiles or hyperthermophiles (Stetter, 1996), although crenarchaeal signatures have been discovered in nucleic acids from mesophilic or psychrophilic habitats (Hershberger *et al.*, 1996). The thermophilic crenarchaeotes have been mainly isolated from geothermally heated terrestrial or submarine environments (Huber & Stetter, 1992; Segerer & Stetter, 1992). Such biotopes allow diverse microbial communities, including a variety of cren-

archaeote genera and species to occur, as exemplified by a hot spring sample collected from Yellowstone National Park, USA (Barns *et al.*, 1994).

In this paper, the isolation and the taxonomic and phylogenetic characteristics of three rod-shaped crenarchaeote strains thriving around 75 °C and pH 4.0 are described; a new genus and species in the family *Thermoproteaceae* is proposed for these three strains, *Thermocladium modestius* gen. nov., sp. nov.

METHODS

Isolation procedure. Samples of hot spring water, muds and soils were collected from several sites associated with volcanic activity in Japan. The samples were transported in sterile plastic tubes at ambient temperature. The basal salts medium for *Sulfolobus* (Brock *et al.*, 1972), supplemented with 0.2 g yeast extract l⁻¹ (Difco), 1.0 mg resazurin l⁻¹, 5.0 g sulfur powder l⁻¹ and adjusted to pH 5.0 at room tem-

The DDBJ accession number for the 16S rDNA sequence of strain IC-125^T reported in this paper is AB005296.

perature, was used as an enrichment medium. The basal salts medium and yeast extract [a 10.0% (w/v) solution] were separately autoclaved at 121 °C for 15 min. Sulfur was sterilized by autoclaving at 105 °C for 1 h. Ten millilitres of the medium, contained in a serum bottle, was flushed with a H_2/CO_2 gas mixture (4:1, v/v) and was reduced with sterile $Na_2S \cdot 9H_2O$ solution (final concn 0.5 g l⁻¹). After readjusting the pH to 5.0 with 0.5 M H_2SO_4 , the bottle was sealed with a butyl rubber stopper. After inoculation, the culture was pressured to 100 kPa with H_2/CO_2 (4:1, v/v) and incubated at 70 °C for 1 week.

Culture conditions. TCD medium was developed to compare the effect of medium composition on growth. It consisted of 1.0 l basal salts medium of the *Sulfolobus* medium (Brock *et al.*, 1972), 0.5 g yeast extract, 10 ml archaeal cell-extract solution, 1.0 mg resazurin and 10.0 g sulfur powder. The pH was adjusted to 4.0–4.5 at room temperature. The gas phase was either N_2 or a H_2/CO_2 (4:1, v/v; 100 kPa) gas mixture; the medium was reduced by the addition of 0.5 g l⁻¹ (final concn) $Na_2S \cdot 9H_2O$ just before use. The archaeal cell-extract solution was prepared by the modified method of Fiala *et al.* (1986). Namely, 1 g packed cells of *Sulfolobus acidocaldarius*, *Methanosarcina barkeri*, *Halobacterium salinarum* or *Thermoplasma acidophilum* was suspended in 100 ml distilled water and disrupted by an ultrasonic oscillator (Sonifier model 250; Branson) for 30–45 min (50% duty cycle; i.e. 0.5 s ultrasonic exposure and 0.5 s non-exposure per cycle) on ice. The homogenate was centrifuged at 12000 g for 10 min and the supernatant was filter-sterilized (pore size 0.2 µm; Minisart, Sartorius). This solution (1%, w/v) was stored at 4 °C until use. To obtain sufficient biomass for isolation of DNAs and lipids, yeast extract concentration in the medium was increased to 1.0 g l⁻¹.

Morphology. The cells were observed directly under a phase-contrast light-microscope (Optiphot-2; Nikon), or shadowed with platinum/palladium and examined with a transmission electron microscope (model H-300; Hitachi).

Growth characteristics. Unless otherwise indicated, isolates were cultivated in TCD medium under an N_2 atmosphere at 75 °C. The cell-extract solution was prepared from *Sulfolobus acidocaldarius* JCM 8929^T. To test for aerobic and microaerobic growth, $Na_2S \cdot 9H_2O$ was omitted from the TCD medium and the gas phase was either air or N_2 containing 5% (v/v) air, respectively. Trisodium citrate (10 mM, pH 2.0–5.5) or MES (10 mM, pH 5.0–7.0) was added to the TCD medium to examine the effect of pH. The pH of the medium was adjusted at room temperature by the addition of H_2SO_4 or NaOH. Sulfide production was determined qualitatively as described by Huber *et al.* (1986).

Protein assay. Due to the highly variable size of cells, it was difficult to estimate the total growth yield of the culture by counting cell numbers. Therefore, cellular protein content was determined as an estimate of the growth yield. The cultures were centrifuged at 12000 g for 10 min and the pelleted cells were suspended in 5% (w/v) trichloroacetic acid solution followed by incubation at 90 °C for 15 min. The suspension was centrifuged again and the precipitated cellular protein was solubilized in 0.5 M NaOH solution. The protein concentration was measured by the BCA (bicinchoninic acid) protein assay kit (Pierce) as described by the supplier's protocol. BSA was used as a standard protein.

Utilization of carbon sources. Each of the test compounds was added to the TCD medium prepared without yeast extract. D-Arabinose, D-fructose, D-galactose, D-glucose,

lactose, maltose, D-mannose, D-ribose, sucrose, D-xylose, glycogen and soluble starch were added at final concentrations of 0.5% (w/v). Acetate, butyrate, citrate, formate, fumarate, lactate, L-malate, propionate, pyruvate, succinate, L-alanine, L-arginine, L-asparagine, L-aspartic acid, L-cysteine, L-glutamic acid, L-glutamine, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-valine, methanol, ethanol, formamide, monomethylamine, trimethylamine and gelatin (Difco) were added at 0.2%. Beef extract, casamino acids, malt extract, peptone, tryptone and yeast extract (all from Difco) were added at 0.05%.

Possible electron acceptors. The following basal medium was designed to test possible electron acceptors (made up in 1.0 l distilled water): 0.53 g NH_4Cl ; 0.28 g KH_2PO_4 ; 0.2 g $MgCl_2 \cdot 6H_2O$; 0.07 g $CaCl_2 \cdot 2H_2O$; 0.5 g yeast extract; 10 ml archaeal cell-extract solution; 9.0 ml trace mineral solution of Zeikus (1977); and 1.0 mg resazurin. The medium was adjusted to pH 4.0 at room temperature with HCl. Sulfur, sodium thiosulfate, sodium sulfate, sodium sulfite, L-cystine, oxidized glutathione, fumarate, L-malate, sodium nitrate or $FeCl_3$ was added to the basal medium at a final concentration of 20 mM or 6.4 g l⁻¹ (sulfur only). Each of the media was reduced by the addition of titanium (III) citrate (final concn 1.3 mM) as described by Zehnder & Wuhrmann (1976).

Sensitivity to antibiotics. Ampicillin, chloramphenicol, erythromycin, kanamycin, oleandomycin, rifampicin or vancomycin was added to the TCD medium at a concentration of 100 µg ml⁻¹. Degradation of the antibiotics during the cultivation was estimated using *Bacillus sphaericus* JCM 2502 as a test organism.

Lipid analysis. Whole-cell acid methanolysis was performed as described by Ross *et al.* (1985). The degraded products were analysed by thin-layer chromatography on Kieselgel 60 (Merck). Diether- and tetraether core lipids were detected on the thin-layer chromatogram developed with hexane/diether (80:20, v/v) (Ross *et al.*, 1985) and hexane/ethyl acetate (70:30, v/v) (Trincon *et al.*, 1988), respectively.

Isolation of DNA, G+C content analysis and DNA–DNA hybridization. Packed cells were suspended in standard saline/citrate solution and lysed with 1% (w/v) SDS at 60 °C for 5 min. Genomic DNAs were isolated from the lysates and purified; their G+C content was determined as described by Tamaoka (1994). DNA–DNA hybridization studies were undertaken according to the fluorescent method of Ezaki *et al.* (1989). *Thermoproteus tenax* JCM 9277^T (= DSM 2078^T) was used as a reference strain.

16S rDNA analysis. Nearly complete 16S rDNA of the strain IC-125^T was amplified by PCR, cloned into pDIRECT vectors, and transformed into *Escherichia coli* DH5α competent cells using the PCR-Direct Cloning System (Clontech). The PCR primers, which include PCR-Direct sequences (underlined) at the 5' ends, were designed as follows: forward primer, 5' CTCGCTCGCCACCGGTTGATCC-TGCC (positions 9–23 in *E. coli* numbering system); reverse primer, 5' CTGGTTCGGCCAAAGGAGGTGATCCARCCG (positions 1540–1525). The ALFred DNA sequencer and the AutoCycle Sequencing kit of Pharmacia Biotech were used for sequencing. For sequencing both strands of DNA, the M13-universal primer, the M13-reverse primer and the following primers were used: 5' GGCACGTGAGCAAGGGCC (positions 318–335), 5' GGATTCRCCCCTAC (positions 699–685), 5' GGGTAGGGCGAAAT-

CC (positions 683–699), 5' GAGAGGAGGTGCATGGC-CG (positions 1043–1061) and 5' GGYGGGGGTCTCG-CTCG (positions 1119–1102). The sequence obtained was aligned with those of some crenarchaeotes and *Thermococcus celer* as the root organism with the CLUSTAL W program (Thompson *et al.*, 1994). Nucleotide substitution rates were calculated after gaps and unknown bases were eliminated. The phylogenetic tree was constructed according to the neighbour-joining method (Saitou & Nei, 1987), and was evaluated by bootstrap sampling (Felsenstein, 1985). The following 16S rRNA/rDNA sequences were used for the phylogenetic analysis: *Acidianus brierleyi* DSM 1651 (X90477), *Acidianus infernus* DSM 3191 (X89852), *Aeropyrum pernix* K1 (D83259), *Desulfurococcus mobilis* (M36474), *Metallosphaera sedula* DSM 5348 (X90481), *Pyrobaculum aerophilum* im2 (L07510), *Pyrobaculum islandicum* geo3 (L07511), *Pyrodicticum occultum* PL19 (M21087), '*Pyrolobus fumarii*' 1A (X99555), *Stetteria hydrogenophila* 4ABC (Y07784), *Sulfolobus acidocaldarius* ATCC 33909 (D14876), *Sulfolobus metallicus* DSM 6482 (X90479), *Sulfolobus shibatae* DSM 5389 (M32504), *Thermococcus celer* DSM 2476 (M21529), *Thermophilum pendens* DSM 2475 (X14835) and *Thermoproteus tenax* (M35966).

RESULTS

Isolation

Seventeen samples of hot spring water, solfataric soils and muds were collected from nine different solfataric areas in Japan. The samples were inoculated in the enrichment medium at 70 °C and pH 5.0 under H₂/CO₂ (4:1, v/v; 100 kPa) gas mixture. After 1 week cultivation, regular or irregular coccoid cells prevailed in most of the cultures. However, rod-shaped cells were seen, accompanied by lesser numbers of coccoid cells, in enrichment cultures of the following three samples: mud from a spring pool, Noji-onsen, Fuku-

shima; water from a flowing spring, Kawayu-onsen, Hokkaido; and water from a spring pool, Tamagawa-onsen, Akita. The original temperatures of the first and last samples were 80 and 65 °C, respectively. The pH values of the three samples measured at room temperature were 2.4, 2.0 and 1.8, respectively. Initially, the serial dilution method (1:10 dilution) with the medium used for the enrichment failed to separate the rod-shaped cells from the coccoid cells. However, when the serial dilutions were repeated with the enrichment medium containing an archaeal cell-extract solution, the coccoid cells were eliminated in the growth culture at the highest dilution. Therefore, the enrichment medium containing the archaeal cell-extract solution was used for the following isolation procedures, and each of the diluted cultures was incubated for 2 weeks. The serial dilutions (1:10 dilution) were repeated twice; then, the growth culture at the highest dilution was again serially diluted (1:10), and each of the dilutions was further divided into five bottles and incubated. One of the cultures at the highest dilution was again serially diluted (1:10 dilution). The final growth cultures were assigned IC-125^T (from Noji-onsen), IC-139 (from Kawayu-onsen) and IC-142 (from Tamagawa-onsen). The purity of the strains was confirmed by microscopic observations for the cultures grown under various growth conditions (e.g. temperature range of 37–85 °C, pH range of 2.0–7.5).

In addition to the three rod-shaped strains, coccoid strains were obtained from these three samples following enrichment under aerobic and/or acidic (pH 2.5) conditions, or under an N₂ atmosphere (70 °C, pH 5.0). Moreover, other rod-shaped strains were isolated from the samples of Noji-onsen and

Fig. 1. Phase-contrast micrographs of the isolate IC-125^T. Cells were rods (a) and occasionally branched out singly (b) or extensively (c). Some cells bore spherical bodies at the ends of the cells (b, c). Bars, 5 µm.

Fig. 2. Transmission electron micrographs of IC-125^T isolates. Cells seemed to multiply by constriction (a) or branching (b). Bars, 2 μ m.

Table 1. Effect of the cell-extract and vitamin mixture on the growth of the isolate IC-125^T

The relative growth yield was determined by means of the protein content after 1 week of cultivation under a H_2/CO_2 (4:1, v/v; 100 kPa) atmosphere. The yield of the culture with the cell-extract from *Sulfolobus acidocaldarius* JCM 9062 is expressed as 100% (corresponded to 171 μ g protein ml^{-1}). The solutions of the cell-extracts and the vitamin mixture were added to the media at final concentrations of 1.0% (v/v), respectively.

Additive	Relative growth yield (%)
None	43.0
Cell-extract from:	
<i>Sulfolobus acidocaldarius</i> JCM 9062	100.0
<i>Methanosarcina barkeri</i> JCM 10043 ^T	86.7
<i>Halobacterium salinarum</i> JCM 8978 ^T	86.3
<i>Thermoplasma acidophilum</i> JCM 9062 ^T	82.1
<i>Escherichia coli</i> JCM 1649 ^T	39.3
Vitamin mixture*	84.0

* According to Balch *et al.* (1979).

Tamagawa-onsen with enrichment at 85 °C and pH 5.0 under an N_2 atmosphere (T. Itoh, K. Suzuki & T. Nakase, unpublished results).

Morphology

Cells of the three strains were mostly regular rods, straight or slightly curved with various length (Figs 1a and 2a). Most cells were within the range 0.4–0.5 $\times$ 5–20 μ m. Frequently, long rods of up to 50 μ m were observed. In addition, bent cells, branched cells forming T-shapes or Y-shapes, and extensively branched cells existed (Fig. 1b, c; Fig. 2b). The proportion of the branching cells exceeded 20% of the total cells at the early stationary growth phase. The ends of the cells were rectangular to round. They sometimes had protruding spherical bodies, which measured 1–3 μ m in diameter, at the ends of the cells. Cells showing constriction were observed, as shown in Fig. 2(a), by transmission electron microscopy. This

would imply that the cells divide by the constriction mode. Motility and flagella were not observed.

Growth characteristics

The three isolates proliferated under strictly anaerobic conditions with N_2 , H_2/CO_2 (4:1, v/v; 100 kPa) or N_2/CO_2 (4:1, v/v; 100 kPa) in the gas phase. To estimate the effect of the gas phase on the growth of the isolates, growth yields were determined by means of the protein contents after 1 week cultivation. When growth yields of the cultures under a H_2/CO_2 (4:1, v/v; 100 kPa) gas phase were regarded as 100% (corresponding to 163–204 μ g protein ml^{-1}), growth yields of the cultures under N_2/CO_2 (4:1, v/v; 100 kPa) and N_2 gas phases were 88–109% and 60–66%, respectively. Thus, the presence of CO_2 in the gas phase enhanced growth to some extent. Sulfide was detected following growth. Under a gas phase of 5% air, growth occurred while the medium was oxidized

Fig. 3. Effect of temperature on the growth of isolate IC-125^T. Strain IC-125^T was grown in TCD medium at pH 4.2 under a H₂/CO₂ (4:1, v/v; 100 kPa) atmosphere. Trisodium citrate (10 mM) was added as a buffer agent. Growth rates were calculated from 4–5 points on the logarithmic part of the growth curve.

Fig. 4. Effect of pH on the growth of isolate IC-125^T. Strain IC-125^T was grown in TCD medium at 75 °C under a H₂/CO₂ (4:1, v/v; 100 kPa) atmosphere. The buffer was 10 mM trisodium citrate. Growth rates were calculated from 3–6 points on the logarithmic part of the growth curves.

(as judged by the colour of resazurin). The growth yields of the microaerobic cultures were the same or less than those of the cultures with an N₂ gas phase. No growth was observed if the gas phase was air. All of the archaeal cell-extract solutions examined, as well as a vitamin mixture solution of Balch *et al.* (1979) (final concn 1.0%, v/v), enhanced growth of strain IC-125^T; however, the extract of *E. coli* JCM 1649^T did not enhance growth, as shown in Table 1. The archaeal cell-extract solution or the vitamin mixture solution did not replace yeast extract as the carbon source (data not shown). None of the isolates grew autotrophically, in the absence of a carbon source, under a H₂/CO₂ (4:1, v/v; 100 kPa) or a N₂/CO₂ (4:1, v/v; 100 kPa) atmosphere. They grew at 45–80 °C under an N₂ atmosphere. Under a H₂/CO₂ atmosphere, they grew up to 82 °C, but failed to grow at 84 °C. When strain IC-125^T was cultivated at various temperatures (60, 65, 70, 75 and 80 °C), optimal growth was observed at 75 °C as shown in Fig. 3. The effect of pH on the growth was examined with either 10 mM trisodium citrate (pH 2.0–5.5) or 10 mM MES (pH 5.0–7.0) as buffer agents. In the absence of these buffer agents, the pH of the cultures, which was initially adjusted to pH 4.0, increased up to 5.2–5.3 following growth. In the presence of 10 mM trisodium citrate, strains IC-125^T and IC-142 grew in a pH range of 3.0–5.1, and strain IC-139 grew in a pH range of 2.6–5.1. No strains grew at pH 1.9 or 5.6. In the media buffered with 10 mM MES, all of the isolates grew at pH up to 5.9, but not at pH 6.1. When strain IC-125^T was cultivated at various pH (3.0, 3.7, 4.2, 4.7 and 5.0) with 10 mM trisodium citrate, optimal growth was observed at

pH 4.2, as shown in Fig. 4. Under optimal growth conditions [75 °C, pH 4.2 under H₂/CO₂ (4:1, v/v; 100 kPa)], strain IC-125^T grew with a doubling time of 15–18 h. The stationary cultures contained 200–230 µg protein ml⁻¹ and 3–4 × 10⁷ cells ml⁻¹. The three isolates grew in the medium containing 1.0% (w/v) NaCl, but not in 1.5% (w/v) NaCl.

Utilization of carbon sources

Of the carbon sources tested, the three strains grew well in the presence of yeast extract and gelatin. Glycogen, starch, beef extract, casamino acids, malt extract, peptone and tryptone supported moderate growth. No or negligible growth was obtained on any of the mono- and disaccharides, organic acids, alcohols, amide and amines studied so far. When strain IC-125^T was grown under a H₂/CO₂ (4:1, v/v; 100 kPa) atmosphere, a similar utilization pattern was obtained. Yeast extract was added as a carbon source at various concentrations (0.02, 0.05, 0.1, 0.2, 0.5 and 1.0%, w/v); the three strains grew at concentrations up to 0.5%, but not at 1.0%. The highest growth yields were obtained with 0.2% yeast extract, but the growth was slower than in cultures grown with 0.1% yeast extract (data not shown).

Possible electron acceptors

The three stains showed no or negligible growth on the basal medium without an electron acceptor. Addition of sulfur, thiosulfate or L-cystine supported growth, and sulfate supported scanty growth. When strain IC-125^T was grown under a H₂/CO₂ (4:1, v/v; 100 kPa)

Table 2. Levels of DNA relatedness between isolates and *Thermoproteus tenax* JCM 9277^T

Strain	Relatedness (%) with biotinylated DNA from:			
	IC-125 ^T	IC-139	IC-142	JCM 9277 ^T
Isolate IC-125 ^T	100	79	81	2
Isolate IC-139	79	100	79	2
Isolate IC-142	90	87	100	1
<i>Thermoproteus tenax</i> JCM 9277 ^T	2	2	2	100

atmosphere, slight growth occurred in the presence of nitrate or FeCl₃.

Antibiotic sensitivity

The isolates were insensitive to chloramphenicol, oleandomycin and kanamycin. Even though the antimicrobial activity of rifampicin was drastically reduced after 3 d in the medium, the isolates did not grow even after 2 weeks. No antimicrobial activity of vancomycin, ampicillin and erythromycin remained after 3 d; these antibiotics had no effect on the growth of the isolates.

Lipid analysis

The three isolates contained large amounts of tetraether core lipids and small amounts of diether core lipids. No fatty acid was found. On the thin-layer chromatogram developed with hexane/diethylether (70:30, v/v), there were at least five spots which corresponded to glycerol-bisdiphytanyl-glycerol tetraethers. All of these lipids are considered to be different cyclized variations in the isoprenoid chains.

G + C contents of DNAs

The G + C contents of DNAs from the strains IC-125^T, IC-139 and IC-142 were 52.4, 51.9 and 52.4 mol %, respectively.

DNA-DNA hybridization

Values for DNA-DNA hybridization between the three isolates and *Thermoproteus tenax* JCM 9277^T are shown in Table 2. The three isolates IC-125^T, IC-139 and IC-142 showed high levels of hybridization values (> 79 %) to each other.

16S rDNA analysis

For the 16S rDNA of strain IC-125^T, the sequence of 1469 positions was determined. The G + C content of the rDNA was 63.9 mol %. The sequence was aligned with those of other crenarchaeotes and *Thermococcus celer*. All of the small subunit rRNA sequence signatures defining the crenarchaeotes (Winker & Woese, 1991), except for that at positions 1244:1293, were

Fig. 5. Phylogenetic tree showing the position of *Thermocladium modestius* strain IC-125^T. The tree is constructed by the neighbour-joining method derived from the 16S rRNA/rDNA sequences. The numbers indicate the bootstrap scores of 1000 trials. The bar represents 2% nucleotide substitution. Accession numbers of nucleotide sequences cited are: *Pyrobaculum aerophilum* im2 (L07510); *Pyrobaculum islandicum* geo3 (L07511); *Thermococcus celer* DSM 2476 (M21529); *Thermofilum pendens* DSM 2475 (X14835); and *Thermoproteus tenax* (M35966). The clade of the order Sulfolobales comprises: *Acidianus brierleyi* DSM 1651 (X90477); *Acidianus infernus* DSM 3191 (X89852); *Metallosphaera sedula* DSM 5348 (X90481); *Sulfolobus acidocaldarius* ATCC 33909 (D14876); *Sulfolobus metallicus* DSM 6482 (X90479); and *Sulfolobus shibatae* DSM 5389 (M32504). The clade of the families Desulfurococcaceae and Pyrodictiaceae comprises: *Aeropyrum pernix* K1 (D83259); *Desulfurococcus mobilis* (M36474); *Pyrodictium occultum* PL19 (M21087); '*Pyrolobus fumarii*' 1A (X99555); and *Stetteria hydrogenophila* 4ABC (Y07784).

found in the sequence from strain IC-125^T. The base pair at positions 1244:1293 in IC-125^T were T:A, whereas those of the other crenarchaeotes are R (A/G):Y (T/C). To calculate the similarities between strain IC-125^T and the other crenarchaeotes, 1213 bases of each sequence were compared. The G + C contents of the sequences compared were 59.2–67.5 mol %. The phylogenetic tree derived from the 16S rRNA/rDNA sequences is shown in Fig. 5. Strain IC-125^T clustered with members of the family *Thermoproteaceae*. This cluster is supported by a high bootstrap value (100 %). Strain IC-125^T formed a separate lineage in this cluster, and the similarity values between strain IC-125^T and the species of *Thermoproteus* and

Pyrobaculum were in the range 90.6–90.9%. The representative species of another family *Thermofilaceae*, *Thermofilum pendens*, was branched outside the family *Thermoproteaceae*; however, bootstrap sampling gave only 67% confidence in this position. The transversion analysis (Woese *et al.*, 1991) did not affect the branching patterns, except for a small change in the position of *Sulfolobus metallicus* (tree not shown). The partial sequences of strains IC-139 (positions 401–657 in the *E. coli* system) and IC-142 (positions 363–657) were identical with that of strain IC-125^T.

DISCUSSION

The three extremely thermophilic, rod-shaped isolates, IC-125^T, IC-139 and IC-142, were obtained from samples taken from the acidic hot spring areas in Japan. These samples were considered to house a variety of thermophilic micro-organisms, and enrichment under several growth conditions in regard to temperature, pH and gas phase enabled isolation of different types of strains. The three rod-shaped strains were enriched in medium at 70 °C and pH 5.0 under a H₂/CO₂ (4:1, v/v; 100 kPa) atmosphere, and they grew optimally around 75 °C and pH 4.0. Although the three strains grew under an N₂ atmosphere, the enrichment of the samples under an N₂ atmosphere (70 °C, pH 5.0) resulted in coccoid isolates. The temperatures of the original samples were adequate for the strains to grow (i.e. 65–80 °C). In contrast, the pH values of these sources were lower than their growth pH ranges. They may extend their growth pH ranges in the sampling sites, or they may be allochthonous organisms.

The presence of ether lipids and absence of fatty acids support the conclusion that the three isolates belong to the domain *Archaea*. In addition, the 16S rDNA sequences of the isolates indicated that they were crenarchaeotes. Rod-shaped crenarchaeotes are currently included in the order *Thermoproteales*, which has been revised recently on the grounds of 16S rRNA/rDNA sequence similarities (Brugggraf *et al.*, 1997). Two families, *Thermofilaceae* and *Thermoproteaceae*, are known in this order. The former family contains only the genus *Thermofilum* (Zillig *et al.*, 1983), whereas the latter family comprises two genera, *Pyrobaculum* (Huber *et al.*, 1987; Völkl *et al.*, 1993) and *Thermoproteus* (Bonch-Osmolovskaya *et al.*, 1990; Zillig, 1989; Zillig *et al.*, 1981). Both genera are commonly encountered in continental solfataric areas, although *Pyrobaculum aerophilum* was isolated from a boiling marine waterhole (Völkl *et al.*, 1993). The two families are also differentiated by cell thickness (i.e. the cell widths of members of the family *Thermofilaceae* are less than 0.35 µm, while those of members of the family *Thermoproteaceae* are at least 0.4 µm). The distinctive rod shape and the cell widths (0.4–0.5 µm) of our isolates are also consistent with their placement within the family *Thermoproteaceae*.

The morphology of the three isolates is similar to that

found within the genera *Thermoproteus* and *Pyrobaculum*. The isolates formed regular rods measuring 0.4–0.5 µm in width; cells were bent, branched, or had terminal spheres which sometimes looked like 'golf clubs'. These morphological features are common to the two genera *Thermoproteus* and *Pyrobaculum* (Huber *et al.*, 1987; Völkl *et al.*, 1993; Zillig, 1989; Zillig *et al.*, 1981). The new isolates, however, tended to form extensively branched cells more frequently than the strains of these genera. Unlike *Pyrobaculum* species, the isolates had neither flagella nor motility. Branching, budding and cell constriction would be common cell-multiplication modes for the order *Thermoproteales*.

Although the three isolates favoured strictly anaerobic growth, they grew under low amounts of air. Except for *Pyrobaculum aerophilum*, all of the *Thermoproteaceae* species are strict anaerobes and do not grow microaerobically. *Pyrobaculum aerophilum* grows under a low concentration of O₂ (< 5%), and uses O₂ as an electron acceptor (Völkl *et al.*, 1993). Although our isolates tolerated 5% (v/v) air in the gas phase, it is unlikely that O₂ was an electron acceptor because it did not increase growth yields. The archaeal cell-extract solution stimulates growth of the new isolates. This preparation seems to be related to the growth factor required for *Thermofilum pendens* (Zillig *et al.*, 1983). Unlike *Thermofilum pendens*, growth of the isolates is enhanced by the cell-extract solution from *Thermoplasma* or by a vitamin mixture (Balch *et al.*, 1979). At the moment, therefore, it is not clear whether or not the actual elements contributing to the growth enhancement of our isolates and *Thermofilum pendens* are identical. The growth temperature ranges of the isolates were significantly lower than those of other members of the order *Thermoproteales*, which grow optimally at hyperthermophilic ranges (i.e. 85–100 °C). The three isolates grew optimally at 75 °C; thus, they are the first examples of non-hyperthermophilic strains among the rod-shaped crenarchaeotes. Furthermore, the optimal growth pH was lower than that of the three described genera. The three isolates were insensitive to chloramphenicol, kanamycin and oleandomycin; however, they were sensitive to rifampicin, like strains of the genus *Pyrobaculum* (Huber *et al.*, 1987; Völkl *et al.*, 1993).

The G + C contents of DNAs of the three isolates are 52 mol% and clearly different from those of *Thermoproteus* species (Bonch-Osmolovskaya *et al.*, 1990; Zillig *et al.*, 1981; T. Itoh, K. Suzuki & T. Nakase, unpublished results) and *Thermofilum pendens* (Zillig *et al.*, 1983) which are in the range 56–60 mol%. Although the G + C contents of *Pyrobaculum* species are reported to be 44–52 mol% (Huber *et al.*, 1987; Völkl *et al.*, 1993), the values for the type strains of the three species were estimated in our laboratory to be about 50 mol% (T. Itoh, K. Suzuki & T. Nakase, unpublished results). The 16S rDNA analysis showed that strain IC-125^T clustered with the members of the family *Thermoproteaceae*. This cluster is supported by

a high bootstrap value (100%), and strain IC-125^T apparently belongs in an independent lineage within the cluster. Accordingly, in line with the phenotypic differences, particularly those of the growth temperature, growth pH and the G + C contents of genomic DNAs, the three strains should be recognized as a new genus in the family *Thermoproteaceae*. The three isolates belong to one species because of the phenotypic similarities described above and high levels of DNA–DNA relatedness.

On the basis of our results, the name *Thermocladium modestius* gen. nov., sp. nov. is proposed for the three isolates. The type strain of the new species is IC-125^T. The three strains IC-125^T, IC-139 and IC-142 have been deposited in the Japan Collection of Microorganisms, The Institute of Physical and Chemical Research (RIKEN), Saitama, Japan, with accession numbers JCM 10088^T, JCM 10089 and JCM 10090, respectively.

Description of *Thermocladium* gen. nov.

Thermocladium (Ther.mo.cla'di.um. Gr. adj. *thermus* hot; Gr. dim. n. *cladion* small branch; M.L. neut. n. *Thermocladium* a hot small twig, indicating branching cells in a hot environment).

Cells are straight or slightly curved rods, and sometimes bend and branch out singly or extensively. Most cells are 0.4–0.5 × 5–20 µm. Spherical bodies may protrude at the ends of the cells. Cells multiply by branching and budding and divide by the constriction mode. No flagella or motility are observed. Grows anaerobically or microaerobically. Prefers extremely thermophilic and weakly acidic conditions to grow (e.g. 70–75 °C, pH 4.0–4.5). Insensitive to chloramphenicol, kanamycin and oleandomycin, but sensitive to rifampicin.

Possesses several (at least five) tetraether core lipids containing cyclopentane rings in the isopranyl chains. The G + C content of genomic DNA is 52 mol %. The 16S rDNA is typical of a crenarchaeote in the sequence signature analysis, except for base pair positions 1244:1293. Phylogenetically, falls under an independent lineage in the family *Thermoproteaceae*. Inhabits acidic, extremely thermophilic (i.e. 65–80 °C), terrestrial hot spring area. Monospecific, at present. The type species is *Thermocladium modestius*.

Description of *Thermocladium modestius* sp. nov.

Thermocladium modestius [mo.des'ti.us. L. comp. adj. *modestior* (neut. *modestius*) modester, referring to the growth temperature range].

Cells are rod-shaped. Grows anaerobically or microaerobically. Heterotrophic. Growth is stimulated by CO₂ in the gas phase and by archaeal cell-extract or a vitamin mixture. Growth occurs at 45–82 °C and is optimal around 75 °C. Growth occurs at pH 2.6–5.1 and is optimal at pH 4.0 when buffered with 10 mM

trisodium citrate. Growth pH is extended to pH 5.9 when buffered with 10 mM MES. Under optimal conditions [75 °C, pH 4.2, under H₂/CO₂ (4:1, v/v; 100 kPa) gas mixture], strain IC-125^T grows with a doubling time of 15–18 h. Grows at low salinity (0–1.0% NaCl). Utilizes glycogen, starch, beef extract, casamino acids, peptone, malt extract, tryptone and yeast extract as carbon sources. Grows best with 0.1–0.2% yeast extract, but does not grow with 1.0% yeast extract. Requires sulfur, thiosulfate, L-cystine or sulfate as electron acceptors. With H₂/CO₂ (4:1, v/v; 100 kPa) as gas phase, may utilize nitrate or FeCl₃ as electron acceptors. Produces sulfide during growth. The G + C content of genomic DNA is 52 mol %. The type strain, IC-125^T (= JCM 10088^T), was isolated from solfataric mud at Noji-onsen, Fukushima, Japan. Two additional strains, IC-139 (= JCM 10089) and IC-142 (= JCM 10090), are also included in this species.

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