Caldisphaera lagunensis gen. nov., sp. nov., a novel thermoacidophilic crenarchaeote isolated from a hot spring at Mt Maquiling, Philippines

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Four novel, thermoacidophilic, crenarchaeotic cocci that grew anaerobically and heterotrophically were isolated from an acidic hot spring in the Philippines; two representative strains were characterized in detail. Most cells were regular cocci, 0.8–1.1 µm in width, which occurred singly or in pairs. They were non-motile and grew at 45–80 °C (optimum 70–75 °C) and pH 2.3–5.4 (optimum 3.5–4.0). They utilized starch, glycogen, gelatin, beef extract, yeast extract and peptone as carbon and energy sources. Growth was stimulated by the presence of sulfur as an electron acceptor. The lipid fraction contained cyclic and acyclic tetraether core lipids. The DNA G + C content was 31 mol%; phylogenetic analysis based on 16S rDNA sequences showed that the novel cocci represent an independent lineage in the phylum *Crenarchaeota*, distantly related to *Acidilobus aceticus* and an allied strain, NC12. *Caldisphaera lagunensis* gen. nov., sp. nov. is proposed to accommodate the four strains. The type strain is IC-154^T(=JCM 11604^T=MCC-UPLB 1331^T=ANMR 0165^T).

INTRODUCTION

In the domain *Archaea*, hyperthermophilic and extremely thermophilic archaea are distributed widely over the phyla *Crenarchaeota* and *Euryarchaeota* (Stetter, 1998). Until now, all *Crenarchaeota* species that have been isolated are either hyperthermophilic or extremely thermophilic, although the existence of mesophilic and psychrophilic *Crenarchaeota* is suggested by culture-independent molecular phylogenetic analyses (Hershberger *et al.*, 1996). The phylum *Crenarchaeota* currently comprises the orders *Sulfolobales*, *Thermoproteales* and *Desulfurococcales*, which are well-supported by 16S rDNA sequence data and by phenotypic properties, such as cell morphology and lipid composition (Burggraf *et al.*, 1997; Reysenbach, 2001). Members of the order *Desulfurococcales* are coccoid or disc-shaped, strictly anaerobic [except for *Aeropyrum pernix* (Sako *et al.*, 1996)]

and neutrophilic or weakly acidophilic, growing optimally at pH 5.5–7.5 (Huber & Stetter, 2001). Two families, *Desulfurococcaceae* and *Pyrodictiaceae*, are known in the order *Desulfurococcales*. On the 16S rDNA-based phylogenetic tree, members of the family *Pyrodictiaceae* (with optimal growth at ≥100 °C) form a coherent cluster, whereas members of the family *Desulfurococcaceae*, with optimal growth at 85–95 °C, are more diverse. At present, there are three and seven genera with validly published names, respectively, in these families [i.e. *Pyrodictium*, *Hyperthermus* and *Pyrolobus* in the family *Pyrodictiaceae*, and *Desulfurococcus*, *Aeropyrum*, *Ignicoccus*, *Staphylothermus*, *Stetteria*, *Sulfophobococcus* and *Thermosphaera* in the family *Desulfurococcaceae* (Huber & Stetter, 2001)]. In addition, the recently described species *Acidilobus aceticus* (Prokofeva *et al.*, 2000) and crenarchaeote strain NC12 ('*Caldococcus noboribetus*'; Aoshima *et al.*, 1996) seem to be distantly affiliated to the order *Desulfurococcales*.

During the course of a search for novel thermophilic archaea from a hot spring named 'Mud Spring' on the side of Mt Maquiling, Philippines, we have isolated a number of

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thermophilic *Archaea* (unpublished data). Among the four rod-shaped isolates, two strains were identified as *Caldivirga maquilingsensis*, a member of the family *Thermoproteaceae* (Itoh *et al.*, 1999). In the present study, we characterize another group of the archaeal isolates, which are cocci distantly related to the genus *Acidilobus* and strain NC12 (based on phylogenetic analysis of 16S rDNA), and propose the name *Caldisphaera lagunensis* gen. nov., sp. nov.

METHODS

Isolation and culture conditions. Samples of hot spring water, mud and soil, collected from a hot spring called 'Mud Spring' on the side of Mt Maquilung, Laguna, Philippines (Itoh *et al.*, 1999), were incubated in an enrichment medium under the following growth conditions. Gas phase: air, N₂ or H₂/CO₂ (4:1, v/v; 100 kPa); temperature, 70 or 85 °C; pH, 2.5 or 5.0 (adjusted at room temperature). The enrichment medium was composed of *Sulfolobus* medium (Brock *et al.*, 1972) supplemented with yeast extract (Difco) (1.0 g l⁻¹ for aerobes and 0.5 g l⁻¹ for anaerobes) and 10 g sulfur l⁻¹. For the isolation of anaerobic strains in N₂ or H₂/CO₂ (4:1, v/v; 100 kPa), the enrichment medium was further supplemented with 1.0 mg resazurin l⁻¹ and reduced with 0.5 g Na₂S.9H₂O l⁻¹. After 1 week of cultivation, cultures were diluted serially (1:10) and incubated under the same conditions as for the enrichment cultivation. Following the serial dilution-cultivation method (three times), the highest dilution that showed growth was again diluted serially (1:10) and each dilution was divided equally into five tubes/vials and cultivated. This step was repeated for one grown culture at the highest dilution. Finally, one grown culture at the highest dilution was assigned an IC number, as shown in Table 1. Two isolates (IC-154^T and IC-163) were cultivated routinely in modified TCD medium (Itoh *et al.*, 1999) in an atmosphere of N₂ at 75 °C.

Phenotypic and genetic studies. Morphology, growth characteristics, utilization of carbon sources, possible electron acceptors, antibiotic sensitivity, lipid composition and DNA base composition were determined as described previously (Itoh *et al.*, 1998). Unless

otherwise stated, modified TCD medium (Itoh *et al.*, 1999) was used as the basal medium for phenotypic studies, and cultures were incubated at 75 °C in an atmosphere of N₂. For electron microscopy, cells were placed on a collodion-coated grid, shadowed with platinum-palladium and examined with a transmission electron microscope (H-300; Hitachi). To determine the pH range for growth, the pH was adjusted at room temperature; it was almost the same at 75 °C in the presence of 10 mM trisodium citrate as buffer. To test tolerance of O₂, the strains were inoculated into sulfur-free, non-reduced TCD medium in an atmosphere of N₂ that contained various levels of air, and incubated on a reciprocal shaker (80 r.p.m.). Possible electron acceptors were identified by using test medium (Itoh *et al.*, 1998) reduced with 0.5 g Na₂S.9H₂O l⁻¹ in an atmosphere of N₂. Utilization of O₂ as an electron acceptor was examined in the same test medium, but not reduced, in an atmosphere of N₂ that contained 1 % O₂. Growth was estimated by fluorescence intensity after treatment with NanoOrange dye (Molecular Probes) following the supplier's protocol, or by direct counting with a Petroff-Hauser counting chamber (0.02 mm in depth). A good correlation between the two methods was obtained. Metabolic products were detected by using a gas chromatograph (GC-7A, Shimadzu) equipped with a 2 m glass column (FAL-M 25 %, 80/100 mesh) and a flame-ionization detector. Likewise, H₂ and CO₂ were detected by GLC with a thermal conductivity detector [column: H₂ with MS-5A, and CO₂ with PoraPak Q (both from Supelco)]. 16S rDNA was amplified with primers A-20F (5'-TCCGGTTGATCCTGCCG-3', corresponding to positions 8–24 in the *Escherichia coli* numbering system) and A-1530R (5'-GGAGGTGATCCAGCCG-3', positions 1540–1525). Initially, partial 16S rDNA sequences of all isolates were determined by using a sequencing primer, A-520R (5'-GTATTACCGCGGCGCTG-3', positions 536–519), and almost-whole 16S rDNA sequences of the representative strains (IC-154^T and IC-163) were determined as described previously (Itoh *et al.*, 1999). The 16S rDNA sequences were first aligned with the CLUSTAL X program (Thompson *et al.*, 1997) and edited manually with the aid of the SSU rRNA database (Van de Peer *et al.*, 2000). Evolutionary distances were calculated after gaps, ambiguous bases and unalignable regions had been eliminated. The phylogenetic tree was constructed by using the neighbour-joining method (Saitou & Nei, 1987) and was evaluated by bootstrap resampling (Felsenstein, 1985).

Table 1. Profile of isolated cocci

Gas phase was kept at atmospheric pressure (air and N₂) or 100 kPa (H₂/CO₂, 4:1) at room temperature. pH was adjusted at room temperature. Most closely related species were determined by partial 16S rDNA sequence similarity (388–439 bp).

Isolate(s)	Isolation conditions			Most closely related species	Sequence similarity (%)
	Gas phase	Temp (°C)	pH		
IC-147	Air	70	2.5	<i>Sulfolobus yangmingensis</i> *	99.1
IC-144, IC-145, IC-148	Air	70	5.0	<i>Metallosphaera sedula</i> /prunae	99.5
IC-151	Air	85	2.5	<i>Sulfolobus yangmingensis</i> *	99.1
IC-146, IC-153	Air	85	5.0	<i>Sulfolobus yangmingensis</i> *	99.1
IC-163, IC-165	N ₂	70	5.0	<i>Acidilobus aceticus</i> †	87.9
IC-156	H ₂ /CO ₂	70	5.0	<i>Sulfolobus yangmingensis</i> *	99.1
IC-154 ^T , IC-158				<i>Acidilobus aceticus</i> †	87.9
IC-155, IC-157, IC-160	H ₂ /CO ₂	85	2.5	<i>Sulfolobus yangmingensis</i> *	99.1
IC-159, IC-161	H ₂ /CO ₂	85	5.0	<i>Sulfolobus yangmingensis</i> *	99.1

*Also affiliated to *Sulfurisphaera ohwakuensis* and *Sulfolobus tokodaii* with 98.9 and 98.6 % sequence similarity, respectively.

†Also affiliated to '*Caldococcus noboribetus*' NC12 with 88.4 % sequence similarity.

RESULTS AND DISCUSSION

Isolation

Seventeen strains with coccoid cells were obtained and tentatively assigned to one of three crenarchaeotic species, based on partial 16S rDNA sequence similarities, as shown in Table 1. Three *Metallosphaera* strains were obtained under one set of growth parameters (air, 70 °C, pH 5.0) and ten strains related to *Sulfolobus yangmingensis*, *Sulfurisphaera ohwakuensis* and *Sulfolobus tokodaii* were obtained under several sets of growth conditions [i.e. air or H₂/CO₂ (4:1, v/v; 100 kPa), 70 or 85 °C, pH 2.5 or 5.0]. On the other hand, four strains (IC-154^T, IC-158, IC-163 and IC-165), isolated under N₂ or H₂/CO₂ (4:1, v/v; 100 kPa), at 70 °C, pH 5.0, showed low similarity values to *Acidilobus aceticus* (Prokofeva *et al.*, 2000) and strain NC12 [representing '*Caldococcus noboribetus*' (Aoshima *et al.*, 1996)], suggesting that they represent a novel taxon related to the genus *Acidilobus*. Of these four isolates, strains IC-154^T and IC-163, isolated under H₂/CO₂ (4:1, v/v; 100 kPa) and N₂, respectively, were selected for further characterization.

Morphology and phenotypic characteristics

Cells of strains IC-154^T and IC-163 were mostly regular cocci, 0.8–1.1 µm in diameter (Fig. 1). Cells 2.0–2.5 µm in diameter were occasionally observed. They usually occurred singly or in pairs, and sometimes in aggregates of several

Fig. 1. Transmission electron micrographs of strain IC-154^T (top) and IC-163 (bottom). Bars, 1 µm.

cells. Of the two strains characterized, only IC-163 had pili attached to its cells (up to three pili). The two strains were non-motile and grew under strictly anaerobic conditions with N₂, N₂/CO₂ (4:1, v/v; 100 kPa) or H₂/CO₂ (4:1, v/v; 100 kPa) as the gas phase. They also grew in a low-oxygen atmosphere (up to 2 % O₂), but not in 5 % O₂ or higher. They grew at 45–80 °C, and at pH 2.3–5.4 in the presence of 10 mM trisodium citrate as buffer. No growth was observed at 40 or 82 °C (at pH 4.0), or at pH 2.0 or 6.0 (at 75 °C). In buffered medium, strain IC-154^T grew optimally at 70–78 °C and at pH 3.5–4.0. The doubling time under optimal conditions (75 °C, pH 3.7) was 5 h and, at stationary phase, the cultures contained approximately 1.4–1.5 × 10⁷ cells ml⁻¹. The two strains did not grow under autotrophic conditions: medium with no yeast extract under H₂/CO₂ (4:1, v/v; 100 kPa). They utilized starch, glycogen, gelatin, beef extract, yeast extract and peptone as carbon and energy sources, but not D-arabinose, D-fructose, D-galactose, D-glucose, lactose, maltose, mannose, D-ribose, sucrose, D-xylose, acetate, butyrate, citrate, formate, fumarate, lactate, L-malate, propionate, pyruvate, succinate, methanol, formamide, methylamine or trimethylamine (final concentrations: 0.05 % for proteinaceous substances, 0.5 % for sugars and 0.2 % for other compounds). Only strain IC-154^T could utilize casamino acids (weakly). Addition of a vitamin mixture (Balch *et al.*, 1979) to the medium weakly promoted growth of the two strains. Both strains tolerated up to 1.5 % NaCl in the growth medium (no growth occurred at 1.75 % NaCl). Growth was significantly enhanced by the presence of sulfur as an electron acceptor in the medium. Likewise, growth was slightly promoted by the presence of fumarate, sulfate (only for IC-163) and O₂ (1 %, only for IC-154^T), but not by thiosulfate, cystine, oxidized glutathione, malate, nitrate or FeCl₃. Hydrogen sulfide was detected from cultures grown with sulfur. Acetate, propionate, isobutyrate, butyrate and isovalerate, as well as H₂ and CO₂, were detected as metabolic products from cultures grown with or without sulfur. Both strains were sensitive to erythromycin, novobiocin and rifampicin, but resistant to ampicillin, chloramphenicol, kanamycin, oleandomycin, streptomycin and vancomycin (100 µg ml⁻¹). The core lipid fractions contained cyclic and acyclic tetraethers.

DNA base composition and 16S rDNA analysis

The DNA G+C contents of strains IC-154^T and IC-163 were 30.7 and 30.9 mol%, respectively, as determined by the HPLC method of Tamaoka (1994). The almost-entire 16S rDNA sequences (1466 bases) determined for the two strains were identical. The G+C ratio was 61.8 %. The 16S rDNA sequence of IC-154^T was free from chimeric artefacts, according to the CHECK_CHIMERA program of the Ribosomal Database Project (Maidak *et al.*, 2001). It contained all of the small-subunit rRNA signature bases that define the crenarchaeotes (Woese *et al.*, 1993), except for two bases at positions 34 and 1335 (see Table 2). Phylogenetic analysis was conducted with other

Table 2. 16S rRNA sequence signatures that distinguish the *Acidilobus* group and/or *Desulfurococcales* in the phylum *Crenarchaeota*

Sequence position*	<i>Acidilobus</i> group	<i>Desulfurococcales</i>	<i>Thermoproteales</i> – <i>Sulfolobales</i>
Specific for <i>Acidilobus</i> group or <i>Desulfurococcales</i>			
34	T	C	C
321:332	C:G	A:G	A:G
605:633	T:G	C:G	T:G
1308:1329	T:A	C:G	C:G
1335	C	G	G (A)
1393	T	C	C
Specific for <i>Acidilobus</i> group and <i>Desulfurococcales</i>			
784:798	C:G	C:G	G (A):C (T)
1364	T	T	A

*According to the *E. coli* numbering system.

crenarchaeote species by comparing 1066 bases in order to construct a phylogenetic tree (Fig. 2). The analysis revealed that the two strains were most closely associated with the order *Desulfurococcales* within the phylum *Crenarchaeota*. However, they formed an independent lineage that was related distantly to *Acidilobus aceticus* and strain NC12, which is tentatively named '*Caldococcus noboribetus*' (sequence similarity, 91.5–92.6%). Sequence similarity to members of the orders *Desulfurococcales* (except for *Acidilobus aceticus* and strain NC12), *Sulfolobales* and

Thermoproteales was 88.8–91.4, 84.8–86.9 and 86.2–88.1%, respectively. On the phylogenetic tree, strain IC-154^T, *Acidilobus aceticus* and strain NC12 clustered together with a relatively high bootstrap value (94%), thereby this group of strains is tentatively named the *Acidilobus* group. As shown in Table 2, signature sequence analysis revealed that the *Acidilobus* group had five sequence positions (i.e. positions 34, 321:332, 1308:1329, 1335 and 1393) that are unique to this group among the phylum *Crenarchaeota*, and only two positions (i.e. positions 784:798 and 1364) that are shared with the remaining members of the order *Desulfurococcales*.

Fig. 2. 16S rDNA sequence-based phylogenetic tree, showing the position of *Caldisphaera lagunesis* IC-154^T (1066 base positions). Numbers at nodes indicate bootstrap values of 1000 trials (values >80% are shown). GenBank accession numbers are given in parentheses. Bar, 0.02% evolutionary distance.

Proposal of a novel genus and species

Strains IC-154^T and IC-163 are strictly anaerobic cocci that thrive at high temperatures (70–80 °C) and acidic pH (3–4.5) and grow organotrophically with facultative reduction of sulfur. In addition to these phenotypic properties, lipid composition and 16S rDNA sequence-based phylogenetic analysis indicated that the two strains are related to the order *Desulfurococcales* of the phylum *Crenarchaeota* (Burggraf *et al.*, 1997; Huber & Stetter, 2001). Furthermore, 16S rDNA sequence analysis revealed that the two strains are most closely related to *Acidilobus aceticus* (Prokofeva *et al.*, 2000) and strain NC12, a representative of '*Caldococcus noboribetus*' (Aoshima *et al.*, 1996), and form a cluster named the *Acidilobus* group, as described above. In addition to the phylogenetic analysis, members of the *Acidilobus* group share certain phenotypic properties: all are acidophilic cocci (optimal pH, 3.0–4.0), are strictly anaerobic and organotrophic, use sulfur as an electron acceptor and inhabit terrestrial acidic hot springs. It is noteworthy that all remaining species of the order *Desulfurococcales*, including the terrestrial genera *Desulfurococcus* (Zillig *et al.*, 1982; Bonch-Osmolovskaya *et al.*, 1988), *Sulfolobococcus* (Hensel *et al.*, 1997) and

Thermosphaera (Huber *et al.*, 1998), favour weakly acidic to neutral pH for growth (optimal pH, 5.5–7.5). Considering the outlying position of the *Acidilobus* group on the phylogenetic tree (Fig. 2) and the fact that only a few signature sequences are shared with other species of the order *Desulfurococcales* (Table 2), inclusion of the *Acidilobus* group in the order *Desulfurococcales* may be controversial. At the moment, however, sequence analysis of 16S rDNA from more related strains, or of molecules other than 16S rDNA, is required to settle the taxonomic status of the *Acidilobus* group at order or family level.

Strains IC-154^T and IC-163 can be differentiated from *Acidilobus aceticus*, and even from strain NC12, by the following characteristics. Strains IC-154^T and IC-163 are extreme thermophiles that grow optimally around 75 °C and not at 82 °C, whereas *Acidilobus aceticus* and NC12 are hyperthermophilic and grow optimally at 85 and 92 °C, respectively. Moreover, *Acidilobus aceticus* has a genomic DNA G+C content of 53.8 mol%, requires yeast extract when grown with starch as the carbon source and does not produce H₂ during growth. On the phylogenetic tree, strains IC-154^T and IC-163 occupy an independent position and show at least 7.7 % sequence dissimilarity from any other crenarchaeote strains. Therefore, these two strains represent a novel genus in the *Acidilobus* group. Except for a few phenotypic differences between strains IC-154^T and IC-163 (e.g. presence or absence of pili and spectrum of electron acceptors), the two strains are similar to each other and their 16S rDNA sequences are identical, suggesting that they belong to a single species. Thus, we propose the name *Caldisphaera lagunensis* gen. nov., sp. nov. to accommodate the four strains IC-154^T, IC-158, IC-163 and IC-165. The type strain of the novel species is IC-154^T (=JCM 11604^T=MCC-UPLB 1331^T=ANMR 0165^T).

Our attempt to isolate thermophilic organisms from an acidic hot spring in the Philippines resulted in the detection of five different crenarchaeotic species, as determined by analysis of their partial 16S rDNA sequences. Rod-shaped crenarchaeotes, *Caldivirga maquilangensis* and *Thermoproteus* sp., were isolated in an atmosphere of N₂ at pH 5.0 and 85 °C, as shown previously (Itoh *et al.*, 1999). Otherwise, coccoid crenarchaeotes, *Metallosphaera* and *Sulfolobus*–*Sulfurisphaera*-related strains and *Caldisphaera lagunensis* were obtained, as shown in this study. The growth conditions for isolation of *Metallosphaera* strains (air, pH 5.0, 70 °C) are consistent with the optimal growth conditions of known *Metallosphaera* species, except for pH (range, 1.0–4.5; Huber *et al.*, 1989; Fuchs *et al.*, 1995). The *Sulfolobus*–*Sulfurisphaera* isolates prevailed in enrichment cultures grown under various conditions, as shown in Table 1. Indeed, representative strains (IC-146, IC-147, IC-155, IC-156 and IC-157) of the *Sulfolobus*–*Sulfurisphaera* isolates grew aerobically and anaerobically in a H₂/CO₂ (4:1, v/v) gas atmosphere (data not shown). *Sulfolobus yangmingensis* is phylogenetically most closely related to our *Sulfolobus*–*Sulfurisphaera* isolates; nevertheless, *Sulfolobus*

yangmingensis, as well as *Sulfolobus tokodaii*, are described as obligate aerobes (Jan *et al.*, 1999; Suzuki *et al.*, 2002), whereas *Sulfurisphaera ohwakuensis* (Kurosawa *et al.*, 1998) is able to grow anaerobically by utilizing H₂ as an electron donor and sulfur as an electron acceptor (which is the same as our isolates). The taxonomic delineation of the genera *Sulfolobus* and *Sulfurisphaera* should be re-evaluated in the near future.

Caldisphaera lagunensis was able to grow by dissimilatory fermentative sulfur reduction in an atmosphere of N₂. Therefore, employment of N₂ as the gas phase in enrichment cultures may be advantageous for selective isolation of strains related to the genus *Caldisphaera* from sample sites where *Caldisphaera* cohabits with anaerobically growing *Sulfolobales* species, such as *Sulfurisphaera ohwakuensis*. The isolation of two genera (*Caldivirga* and *Caldisphaera*) from a single hot spring site suggests that further undescribed archaeal genera may be isolated from unexplored geothermal habitats.

Description of *Caldisphaera* gen. nov.

Caldisphaera (Cal.di.sphae'ra. L. adj. *caldus* hot; L. fem. n. *sphaera* sphere; N.L. fem. n. *Caldisphaera* a hot spherical cell).

Cells are mostly regular cocci, 0.8–1.1 µm in diameter, and occur singly or in pairs. Pili may be present. Non-motile. Extremely high temperature (70–78 °C) and acidic conditions (pH 3.5–4.5) are preferred for growth. Grows anaerobically. Resistant to chloramphenicol, kanamycin, oleandomycin, streptomycin and vancomycin. Sensitive to erythromycin, novobiocin and rifampicin. Possesses cyclic and acyclic tetraether core lipids. DNA G+C content of the type species is 31 mol%. The 16S rDNA is typical of a crenarchaeote in sequence signature analysis. Phylogenetically, the genus represents an independent lineage related to the order *Desulfurococcales*. Inhabits terrestrial hot springs. The type species is *Caldisphaera lagunensis*.

Description of *Caldisphaera lagunensis* sp. nov.

Caldisphaera lagunensis (la.gu.nen'sis. N.L. fem. adj. *lagunensis* pertaining to Laguna, the province in the Philippines where the type strain was isolated).

Grows anaerobically and tolerates low levels of oxygen (up to 2 %). Heterotrophic. Growth occurs at 45–80 °C and pH 2.3–5.4. Under optimal growth conditions, doubling time is 5 h. Growth occurs at low salinity (≤1.5 % NaCl). Chemo-organotrophic; utilizes starch, glycogen, gelatin, beef extract, yeast extract and peptone as carbon and energy sources. Forms acetate, propionate, isobutyrate, butyrate, isovalerate, H₂ and CO₂ as metabolic products. Growth is stimulated by the presence of sulfur. Hydrogen sulfide is formed. DNA G+C content is 31 mol%.

The type strain is IC-154^T (=JCM 11604^T=MCC-UPLB

1331^T=ANMR 0165^T). Reference strains are IC-158 (=ANMR 0169), IC-163 (=JCM 11605=MCC-UPLB 1332=ANMR 0174) and IC-165 (=ANMR 0176). Isolated from a hot spring on Mt Maquiling, Laguna, the Philippines.

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REFERENCES

- Aoshima, M., Nishibe, Y., Hasegawa, M., Yamagishi, A. & Oshima, T. (1996). Cloning and sequencing of a gene encoding 16S ribosomal RNA from a novel hyperthermophilic archaeobacterium NC12. *Gene* **180**, 183–187.
- Balch, W. E., Fox, G. E., Magrum, L. J., Woese, C. R. & Wolfe, R. S. (1979). Methanogens: reevaluation of a unique biological group. *Microbiol Rev* **43**, 260–296.
- Bonch-Osmolovskaya, E. A., Slesarev, A. I., Miroshnichenko, M. L., Svetlichnaya, T. P. & Alekseev, V. A. (1988). Characterization of *Desulfurococcus amylolyticus* n. sp. – a new extremely thermophilic archaeobacterium isolated from thermal springs of Kamchatka and Kunashir island. *Microbiology* (English translation of *Mikrobiologiya*) **57**, 78–85.
- Brock, T. D., Brock, K. M., Belly, R. T. & Weiss, R. L. (1972). *Sulfolobus*: a new genus of sulfur-oxidizing bacteria living at low pH and high temperature. *Arch Mikrobiol* **84**, 54–68.
- Burggraf, S., Huber, H. & Stetter, K. O. (1997). Reclassification of the crenarchaeal orders and families in accordance with 16S rRNA sequence data. *Int J Syst Bacteriol* **47**, 657–660.
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.
- Fuchs, T., Huber, H., Teiner, K., Burggraf, S. & Stetter, K. O. (1995). *Metallosphaera prunae*, sp. nov., a novel metal-mobilizing, thermoacidophilic *Archaeum*, isolated from a uranium mine in Germany. *Syst Appl Microbiol* **18**, 560–566.
- Hensel, R., Matussek, K., Michalke, K., Tacke, L., Tindall, B. J., Kohlhoff, M., Siebers, B. & Dielenschneider, J. (1997). *Sulfophobococcus zilligii* gen. nov., spec. nov. a novel hyperthermophilic archaeum isolated from hot alkaline springs of Iceland. *Syst Appl Microbiol* **20**, 102–110.
- Hershberger, K. L., Barns, S. M., Reysenbach, A.-L., Dawson, S. C. & Pace, N. R. (1996). Wide diversity of *Crenarchaeota*. *Nature* **384**, 420.
- Huber, H. & Stetter, K. O. (2001). Order II. *Desulfurococcales* ord. nov. In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol. 1, pp. 179–180. Edited by D. R. Boone & R. W. Castenholz. New York: Springer.
- Huber, G., Spinnler, C., Gambacorta, A. & Stetter, K. O. (1989). *Metallosphaera sedula* gen. nov. and sp. nov. represents a new genus of aerobic, metal-mobilizing, thermoacidophilic archaeobacteria. *Syst Appl Microbiol* **12**, 38–47.
- Huber, R., Dyba, D., Huber, H., Burggraf, S. & Rachel, R. (1998). Sulfur-inhibited *Thermosphaera aggregans* sp. nov., a new genus of hyperthermophilic archaea isolated after its prediction from environmentally derived 16S rRNA sequences. *Int J Syst Bacteriol* **48**, 31–38.
- Itoh, T., Suzuki, K. & Nakase, T. (1998). *Thermocladium modestius* gen. nov., sp. nov., a new genus of rod-shaped, extremely thermophilic crenarchaeote. *Int J Syst Bacteriol* **48**, 879–887.
- Itoh, T., Suzuki, K., Sanchez, P. C. & Nakase, T. (1999). *Caldivirga maquilingensis* gen. nov., sp. nov., a new genus of rod-shaped crenarchaeote isolated from a hot spring in the Philippines. *Int J Syst Bacteriol* **49**, 1157–1163.
- Jan, R.-L., Wu, J., Chaw, S.-M., Tsai, C.-W. & Tsen, S.-D. (1999). A novel species of thermoacidophilic archaeon, *Sulfolobus yangmingensis* sp. nov. *Int J Syst Bacteriol* **49**, 1809–1816.
- Kurosawa, N., Itoh, Y. H., Iwai, T., Sugai, A., Uda, I., Kimura, N., Horiuchi, T. & Itoh, T. (1998). *Sulfurisphaera ohwakuensis* gen. nov., sp. nov., a novel extremely thermophilic acidophile of the order *Sulfolobales*. *Int J Syst Bacteriol* **48**, 451–456.
- Maidak, B. L., Cole, J. R., Lilburn, T. G. & 7 other authors (2001). The RDP-II (Ribosomal Database Project). *Nucleic Acid Res* **29**, 173–174.
- Prokofeva, M. I., Miroshnichenko, M. L., Kostrikin, N. A., Chernykh, N. A., Kuznetsov, B. B., Tourova, T. P. & Bonch-Osmolovskaya, E. A. (2000). *Acidilobus aceticus* gen. nov., sp. nov., a novel anaerobic thermoacidophilic archaeon from continental hot vents in Kamchatka. *Int J Syst Evol Microbiol* **50**, 2001–2008.
- Reysenbach, A.-L. (2001). Class I. *Thermoprotei* class. nov. In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol. 1, p. 169. Edited by D. R. Boone & R. W. Castenholz. New York: Springer.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Sako, Y., Nomura, N., Uchida, A., Ishida, Y., Morii, H., Koga, Y., Hoaki, T. & Maruyama, T. (1996). *Aeropyrum pernix* gen. nov., sp. nov., a novel aerobic hyperthermophilic archaeon growing at temperatures up to 100 °C. *Int J Syst Bacteriol* **46**, 1070–1077.
- Stetter, K. O. (1998). Hyperthermophiles: isolation, classification, and properties. In *Extremophiles: Microbial Life in Extreme Environments*, pp. 1–24. Edited by K. Horikoshi & W. D. Grant. New York: Wiley-Liss.
- Suzuki, T., Iwasaki, T., Uzawa, T., Hara, K., Nemoto, N., Kon, T., Ueki, T., Yamagishi, A. & Oshima, T. (2002). *Sulfolobus tokodaii* sp. nov. (f. *Sulfolobus* sp. strain 7), a new member of the genus *Sulfolobus* isolated from Beppu Hot Springs, Japan. *Extremophiles* **6**, 39–44.
- Tamaoka, J. (1994). Determination of DNA base composition. In *Chemical Methods in Prokaryotic Systematics*, pp. 463–470. Edited by M. Goodfellow & A. G. O'Donnell. Chichester: Wiley.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.
- Van de Peer, Y., De Rijk, P., Wuyts, J., Winkelmans, T. & De Wachter, R. (2000). The European small subunit ribosomal RNA database. *Nucleic Acids Res* **28**, 175–176.
- Woese, C. R. (1993). The archaea: their history and significance. In *The Biochemistry of Archaea (Archaeobacteria)*, pp. vii – xxix. Edited by M. Kates, D. J. Kushner & A. T. Matheson. Amsterdam: Elsevier.
- Zillig, W., Stetter, K. O., Prangishvili, D., Schäfer, W., Wunderl, S., Janekovic, D., Holz, I. & Palm, P. (1982). *Desulfurococcaceae*, the second family of the extremely thermophilic, anaerobic, sulfur-respiring *Thermoproteales*. *Zentbl Bakteriol Parasitenkd Infektkrankh Hyg Abt I Orig C3*, 304–317.