Vulcanisaeta thermophila sp. nov., a hyperthermophilic and acidophilic crenarchaeon isolated from solfataric soil

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An anaerobic, rod-shaped, hyperthermophilic and acidophilic crenarchaeon, designated strain CBA1501^T, was isolated from solfataric soil of the Mayon volcano in the Republic of the Philippines. Phylogenetic analysis showed that strain CBA1501^T is affiliated with the genus *Vulcanisaeta* in the phylum *Crenarchaeota*. DNA sequence similarities between the 16S rRNA gene of strain CBA1501^T and those of *Vulcanisaeta distributa* IC-017^T and *Vulcanisaeta souniana* IC-059^T were 98.5 and 97.4 %, respectively. Strain CBA1501^T grew between 75–90 °C, over a pH range of 4.0–6.0 and in the presence of 0–1.0 % (w/v) NaCl, with optimal growth occurring at 85 °C, pH 5.0, and with 0 % (w/v) NaCl. Fumarate, malate, oxidized glutathione, sulfur and thiosulfate were used as final electron acceptors, but $FeCl_3$, nitrate and sulfate were not. The DNA G+C content of strain CBA1501^T was 43.1 mol%. On the basis of polyphasic taxonomic analysis, strain CBA1501^T represents a novel species of the genus *Vulcanisaeta* in the phylum *Crenarchaeota*, for which we propose the name *Vulcanisaeta thermophila* sp. nov. The type strain is CBA1501^T (=ATCC BAA-2415^T=JCM 17228^T).

Since their discovery in 1981, hyperthermophiles growing optimally above 80 °C have been isolated from hot terrestrial, subterranean and submarine environments (Stetter, 1999, 2006, 2013). The crenarchaeal genus *Vulcanisaeta* is classified within the family *Thermoproteaceae* of the order *Thermoproteales* and currently includes two species with validly published names: *Vulcanisaeta distributa* and *Vulcanisaeta souniana* (Itoh *et al.*, 2002), based on the List of Prokaryotic Names with Standing in Nomenclature database (Euzéby, 1997; Parte, 2014). This genus was proposed for crenarchaeal strains isolated from hot springs in Japan, these strains are anaerobic, heterotrophic, rod-shaped, hyperthermophilic and acidophilic (Itoh *et al.*, 2002). Members of the genus *Vulcanisaeta* grow over pH ranges of 3.1–5.6 and temperature ranges of 65–99 °C (Itoh *et al.*, 2002). Strain CBA1501^T is proposed as a novel species in the genus *Vulcanisaeta* of the phylum *Crenarchaeota*, based on phylogenetic, phenotypic and genomic analyses conducted in this study.

A soil sample (94 °C and pH 5.9) was collected in May 2010 from a solfataric thermal field from the Mayon volcano in the province of Albay of the Bicol Region, the Republic of the Philippines (GPS position; 13° 15′ 00̸ N 123 °41′ 00̸ E) and transported to the laboratory in a sealed plastic bag under ambient conditions. Japan Collection of Microorganisms (JCM) medium number 236 (M236) was prepared anaerobically for the cultivation and isolation of crenarchaeal strains, according to the JCM culture medium guideline. Briefly, M236 medium contained the following (per litre salt base solution): 2.94 g trisodium citrate.$2H_2O$, 0.5 g yeast extract (BD), 10.0 ml trace vitamins, 1.0 mg resazurin, 0.5 g $Na_2S.9H_2O$ and 10.0 g sulfur. A 5 g soil sample was suspended in M236 medium using a serum bottle (Wheaton), sealed with butyl rubber stopper (Bellco) and aluminium cap (Wheaton), and was enriched at 80 °C for 1 month. The serial dilution

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Abbreviations: DDH, DNA–DNA hybridization; ML, maximum-likelihood; MP, maximum-parsimony; NJ, neighbour-joining.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CBA1501^T is HM594677.

method was applied three times with growth culture at the highest dilution to obtain pure cultures. One of the resulting isolated strains was designated CBA1501^T, and its purity was confirmed by the observation of uniform rod-shaped using a light microscope (BA210; Motic) and by sequencing its 16S rRNA gene, as described below. *V. distributa* DSM 14429^T and *V. souniana* DSM 14430^T were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) and used as reference strains for the DNA–DNA hybridization (DDH) analyses.

Genomic DNAs from strain CBA1501^T and the reference strains were extracted and purified as described by Sambrook *et al.* (1989). The 16S rRNA gene was amplified using a PCR PreMix kit (iNtRON Biotechnology) and the archaeal-targeting primer set Arch21F and 1492R (DeLong, 1992), using previously described PCR conditions (Roh *et al.*, 2008). The PCR product was sequenced using a PRISM 3730XL DNA Analyser (Applied Biosystems), as described (Roh *et al.*, 2008). Partial 16S rRNA gene sequences were assembled and a nearly full-length 16S rRNA gene sequence (1441 bp) was obtained. Identification of closely related taxa and calculation of pairwise 16S rRNA gene sequence similarities were performed using the EzTaxon-e server (Kim *et al.*, 2012). Multiple sequence alignments were performed using the SILVA Incremental Aligner (Pruesse *et al.*, 2012). Phylogenetic trees were reconstructed using the MEGA5 software package (Tamura *et al.*, 2011) with the neighbour-joining (NJ) (Saitou & Nei, 1987), maximum-parsimony (MP) (Kluge & Farris, 1969) and maximum-likelihood (ML) (Felsenstein, 1981) methods, based on 1000 randomly generated trees.

The 16S rRNA sequence from strain CBA1501^T was most similar to those from *V. distributa* IC-017^T (98.5 %), *V. souniana* IC-059^T (97.4 %), '*Vulcanisaeta moutnovskia*' 768-28 (96.8 %), *Caldivirga maquilingensis* IC-167^T (94.6 %), *Pyrobaculum islandicum* GEO3^T (93.5 %), and *Pyrobaculum organotrophum* H10^T (93.5 %). The 16S rRNA gene sequence similarity between strain CBA1501^T and other type species of the crenarchaeotes was less than 93.4 %. Topologies of the phylogenetic trees indicated that strain CBA1501^T formed a monophyletic clade in the genus *Vulcanisaeta* of the family *Thermoproteaceae* (Fig. 1).

Cell morphology and size were determined using an electron microscopy (SUPPA 55VP; Zeiss) as described previously (Lee *et al.*, 2013). Cells of strain CBA1501^T were rod-shaped (mainly 0.6–0.7 μm wide and 4.7–9.3 μm long). To determine optimal culture conditions, strain CBA1501^T was anaerobically cultivated at various temperatures (65–100 °C at intervals of 5 °C) and different pH ranges (pH 3.0–8.0 at intervals of 1.0 pH unit) in M236 medium for 1 month. The pH was adjusted with the following buffers: 1 M acetic acid/sodium acetate (pH 3.0); 10 mM MES (pH 4.0–6.0); 10 mM TAPS (pH 7.0 and 8.0). To test the NaCl tolerance of strain CBA1501^T, JCM medium number 297 (M297) containing the following (per litre salt base solution): 0.5 g yeast extract, 10.0 g sulfur, 0.5 g $Na_2S{\cdot}9H_2O$ and 1.0 mg resazurin was prepared anaerobically following the JCM guidelines, with the modification of adding 0–3 % (w/v) NaCl in 0.5 % increments. Growth was estimated by direct counting using a haemocytometer and light microscope (BA210; Motic). Strain CBA1501^T grew at 75–90 °C, at pH 4.0–6.0 and in the presence of 0–1.0 % (w/v) NaCl. Optimal growth occurred at 85 °C, pH 5.0 and with 0 % (w/v) NaCl. The strain grew with doubling time of 11 h (under the optimal growth conditions with sulfur and shaking at 100 r.p.m.), which is approximately two times slower than those of other members of the genus *Vulcanisaeta* (Itoh *et al.*, 2002); the culture at stationary phase contained approximately 1.0×10^6 cells ml^{-1}.

To characterize its ability to utilize available electron acceptors, CBA1501^T was cultured in M297 medium wherein sulfur was replaced with $FeCl_3$ (10 mM), fumarate (20 mM), malate (20 mM), nitrate (20 mM), oxidized glutathione (2.5 mM), sulfate (20 mM), or thiosulfate (20 mM). Yeast extract was used as a carbon source, and the pH was adjusted to 5.0 with 10 mM MES. Fumarate, malate, oxidized glutathione and thiosulfate were utilized as electron acceptors; whereas $FeCl_3$, nitrate and sulfate were not. When sulfur compounds were used as the electron acceptors, hydrogen sulfide formation was detected by the method of Cui *et al.* (2007). To analyse the utilization of different carbon sources, yeast extract was replaced by 0.5 % (w/v) each of the following substrates using M297 medium (adjusted to pH 5.0 with 10 mM MES) and thiosulfate instead of sulfur as an electron acceptor: acetate, D-arabinose, beef extract, butyrate, Casamino acids, citrate, formate, D-fructose, fumarate, D-galactose, gelatin, D-glucose, lactose, L-malate, D-maltose, D-mannose, methanol, methylamine, peptone, pyruvate, starch, succinate, sucrose, trimethylamine or D-xylose. Beef extract, Casamino acids, fumarate, D-galactose, gelatin, lactose and D-maltose were utilized as carbon sources; whereas acetate, D-arabinose, butyrate, citrate, formate, D-fructose, D-glucose, L-malate, D-mannose, methanol, methylamine, peptone, pyruvate, starch, succinate, sucrose, trimethylamine and D-xylose were not utilized. Strain CBA1501^T showed weak growth in a low-oxygen atmosphere [5.0 % (v/v) air in N_2] with sodium thiosulfate (20 mM) as an electron acceptor, but no growth in 5.5 % (v/v) air in N_2. The strain showed no growth under autotrophic conditions of a H_2/CO_2 (4 : 1, v/v) gas mixture in M297 medium without yeast extract. To test antibiotic sensitivity, strain CBA1501^T was inoculated in M297 medium with 100 μg antibiotic ml^{-1}: erythromycin, novobiocin, rifampicin, ampicillin, chloramphenicol, kanamycin, streptomycin and vancomycin. The strain was susceptible to novobiocin, rifampicin, chloramphenicol, kanamycin, streptomycin and vancomycin, but resistant to erythromycin and ampicillin. The differential characteristics between strain CBA1501^T and type strains of species of the genus *Vulcanisaeta* are shown in Table 1. The minimum temperature supporting growth of strain CBA1501^T (75 °C) was higher than that of the type strains of *V. distributa* (70 °C)

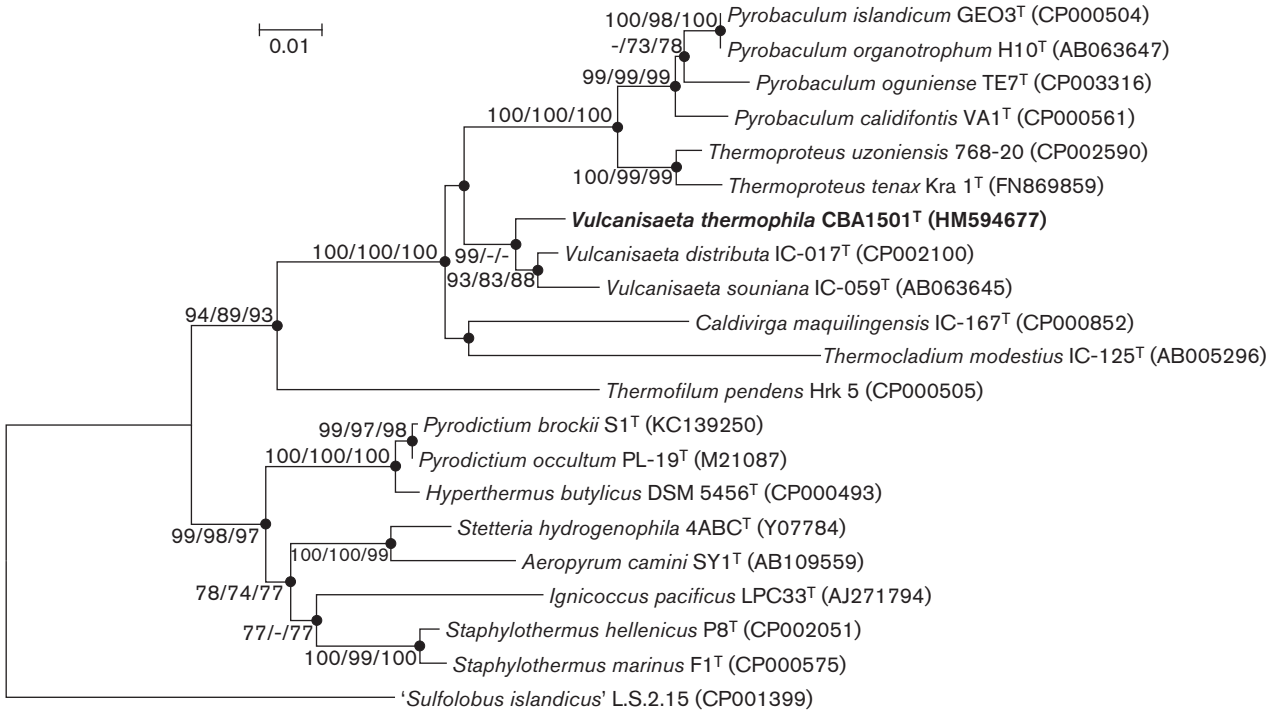

Fig. 1. Phylogenetic tree based on the NJ algorithm for the 16S rRNA gene sequences of strain CBA1501^T and the type strains of closely related species. Numbers at nodes indicate bootstrap values (>70 %) calculated using the NJ, MP and ML algorithm probabilities.Filled circles represent nodes recovered with both the MP and ML algorithms. '*Sulfolobus islandicus*' L.S.2.15 was used as an outgroup. Bar, 0.01 changes per nucleotide position.

and *V. souniana* (65 °C). Only strain CBA1501^T utilized fumarate as an electron acceptor, fumarate and lactose as a carbon source; but it did not utilize L-malate, peptone or starch as a carbon source.

DDH was performed using photobiotin-labelled DNA probes and microwell plates as described by Ezaki *et al.* (1989). The DNA–DNA hybridization values of strain CBA1501^T with *V. distributa* DSM 14429^T and *V. souniana* DSM 14430^T were 42.6 % and 20.3 %, respectively. DDH values less than 70 % indicated that strain CBA1501^T represents a distinct genospecies (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994). The DNA G+C content of strain CBA1501^T was determined to be 43.1 mol% by a fluorimetric method (González & Saiz-Jimenez, 2002), using SYBR Green I and a real-time PCR thermocycler. This value is slightly lower than the reported ranges of 44–46 mol% for other members of the genus *Vulcanisaeta* (Itoh *et al.*, 2002).

Members of the genus *Vulcanisaeta* were originally isolated from terrestrial geothermal habitats in Japan (Itoh *et al.*, 2002). Subsequently, the 16S rRNA gene sequences of *Vulcanisaeta*-like phylotypes were detected in hot springs of Yellowstone National Park, USA (Meyer-Dombard *et al.*, 2005) and '*V. moutnovskia*' strain 768-28 was isolated from a hot spring in Kamchatka, Russia (Gumerov *et al.*, 2011). These findings raise the possibility of a wider distribution of hyperthermophilic crenarchaea belonging to the genus *Vulcanisaeta* that inhabit geothermal habitats in other countries along with Japan, USA, Russia and the Philippines. In this study, strain CBA1501^T was clearly distinguished from previously described taxa of the genus *Vulcanisaeta* based on the phylogenetic, phenotypic and genomic comparisons. On the basis of polyphasic taxonomic analyses, strain CBA1501^T represents a novel species of the genus *Vulcanisaeta* in the family *Thermoproteaceae*, for which we propose the name *Vulcanisaeta thermophila* sp. nov.

Description of *Vulcanisaeta thermophila* sp. nov.

Vulcanisaeta thermophila [ther.mo′phi.la. Gr. fem. n. *thermê* heat; N.L. adj. *philus -a -um* (from Gr. adj. *philos -ê -on*), friend, loving; N.L. fem. adj. *thermophila* heat-loving].

Cells are anaerobic, hyperthermophilic, acidophilic, obligately chemo-organotrophic and rod-shaped (0.6–0.7 μm wide and 4.7–9.3 μm long). Growth occurs between 75–90 °C (optimum 85 °C), at pH 4.0–6.0 (optimum pH 5.0) and in the presence of 0–1.0 % (w/v) NaCl (optimum 0 %, w/v). Under optimal growth conditions, doubling time is 11 h. Fumarate, malate, oxidized glutathione, sulfur and thiosulfate are utilized as electron acceptors; whereas $FeCl_3$, nitrate, and sulfate are not. Beef extract, Casamino acids, fumarate, D-galactose, gelatin, lactose, D-maltose and yeast

Table 1. Differential characteristics of strain CBA1501^T and type strains of closely related species of the genus *Vulcanisaeta*

Taxa: 1, CBA1501^T; 2, *V. distributa* IC-017^T (data from Itoh *et al.*, 2002); 3, *V. souniana* IC-059^T (Itoh *et al.*, 2002). +, Positive; −, negative.

Characteristic	1	2	3
Temperature for growth (°C)			
Range	75–90	70–99	65–89
Optimum	85	90	85
pH for growth			
Range	4.0–6.0	3.1–5.6	3.5–5.0
Optimum	5.0	4.5	4.5
NaCl range for growth (%, w/v)	⩽1.0	⩽1.0	⩽1.25
Electron acceptor utilization			
Fumarate	+	−	−
Oxidized glutathione	+	+*	−*
Carbon source utilization			
Fumarate	+	−	−
D-Galactose	+	+	−
Lactose	+	−	−
L-Malate	−	+	+
Peptone	−	+	+
Starch	−	+	+
DNA G+C content (mol%)	43.1	45.4	44.9

*Data with media reduced with $Na_2S.9H_2O$.

extract are utilized as carbon sources; whereas acetate, D-arabinose, butyrate, citrate, formate, D-fructose, D-glucose, L-malate, D-mannose, methanol, methylamine, peptone, pyruvate, starch, succinate, sucrose, trimethylamine and D-xylose are not. Cells tolerate a low level of oxygen [5.0 % (v/v) air in N_2] with sodium thiosulfate as an electron acceptor.

The type strain is CBA1501^T (=ATCC BAA-2415^T=JCM 17228^T), isolated from solfataric soil from the Mayon volcano on the island of Luzon in the Republic of the Philippines. The DNA G+C content of the type strain is 43.1 mol%.

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