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Polyphasic Taxonomic Analysis of Novel Hyperthermophilic Crenarchaea

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February, 2016

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Polyphasic Taxonomic Analysis of Novel Hyperthermophilic Crenarchaea

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(Supervised by Professor **Soo-Je Park**)

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ABSTRACT

An anaerobic, rod-shaped, hyperthermophilic and acidophilic crenarchaeon, designated strain CBA1501^T and CBA1502^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. Sequence similarities between the 16S rRNA gene of strain CBA1501^T and those of *V. distributa* IC-017^T and *V. 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. The G+C content of strain CBA1501^T was 43.1 mol%. The 16S rRNA gene sequence of strain CBA1502^T was most closely related to that of *Thermoproteus uzoniensis* DSM 5263^T (99.2% similarity) and *T. tenax* Kra 1^T (99.0%). Strain CBA1502^T grew at 75–90 °C and pH 4.0–6.0 and in the presence of 0–0.5% (w/v) NaCl, with optimal growth at 85 °C and pH 5.0. The DNA G+C content of strain CBA1502^T was 62.0 mol%. On the basis of polyphasic taxonomic analysis, strain CBA1501^T and CBA1502^T are a novel species of the genus *Vulcanisaeta* and *Thermoproteus* in the phylum *Crenarchaeota*, respectively. I propose the names *Vulcanisaeta thermophila* sp. nov. and *Thermoproteus thermophilus* sp. nov. The type strains are CBA1501^T (= ATCC BAA-2415^T = JCM 17228^T) and CBA1502^T (= ATCC BAA-2416^T = JCM 17229^T). In addition, I present a draft genome sequence of *V. thermophila* CBA1501^T containing 2,022,594 bp, with 49.1% G+C content. The sequence will provide opportunities for biotechnological applications of hyperthermophilic enzymes.

KeyWords: Archaea, hyperthermoacidophilic crenarchaeon, solfataric soil, polyphasic taxonomy

CONTENTS

LIST OF FIGURES

LIST OF TABLES

1. Research Background

Hyperthermophilic organisms include Archaea and Bacteria that thrive in hot terrestrial, subterranean, and submarine ecosystems (Stetter, 1999; Stetter, 2006; Stetter, 2013). The optimal growth temperature for hyperthermophiles is more than 80 °C (Stetter, 1998).

Phylogenetically, the hyperthermophiles represent one of the most primitive life forms on Earth. Approximately 75 species of hyperthermophilic Archaea and Bacteria, representing 32 genera and 10 orders have been characterized (Stetter, 2000). Members of hyperthermophilic Archaea within the phylum Crenarchaeota and Euryarchaeota are reported as unusual cell morphologies such as disks, networks, golf clubs, irregular spheres, and branched or irregular rods (Huber *et al.*, 2002).

The enzymes produced by hyperthermophiles are stable at high temperature, making them industrially applicable. Most hyperthermophilic enzymes have been observed from *Pyrococcus furiosus*, *Thermotoga maritima*, or closely related hyperthermophilic members. These enzymes include extracellular and intracellular amylases, proteases, xylanases, pullulanases, dehydrogenases, oxidoreductases, and DNA polymerases (Huber & Stetter, 1998).

The Crenarchaeota has been classified as belonging to the domain Archaea. Initially, Crenarchaeota were thought to be sulfur-dependent extremophiles. The Crenarchaeota strains had been classified to the order Acidilobales, Desulfurococcales, Fervidicoccales, Sulfolobales, and Thermoproteales based on phylogenetic analysis using 16S rDNA sequences (Itoh *et al.*, 2002; Perevalova *et al.*, 2010). To date, all cultured Crenarchaeota strains are Gram negative, able to grow at temperatures as high as 121 °C (Kashefi & Lovley, 2003), and are morphologically diverse with rod, cocci, filamentous, and oddly shaped cells.

In this paper, I report novel species of the hyperthermophilic Crenarchaeota strains genera *Vulcanisaeta* and *Thermoproteus* and provide sequence information for hyperthermophilic enzymes with high biotechnological value.

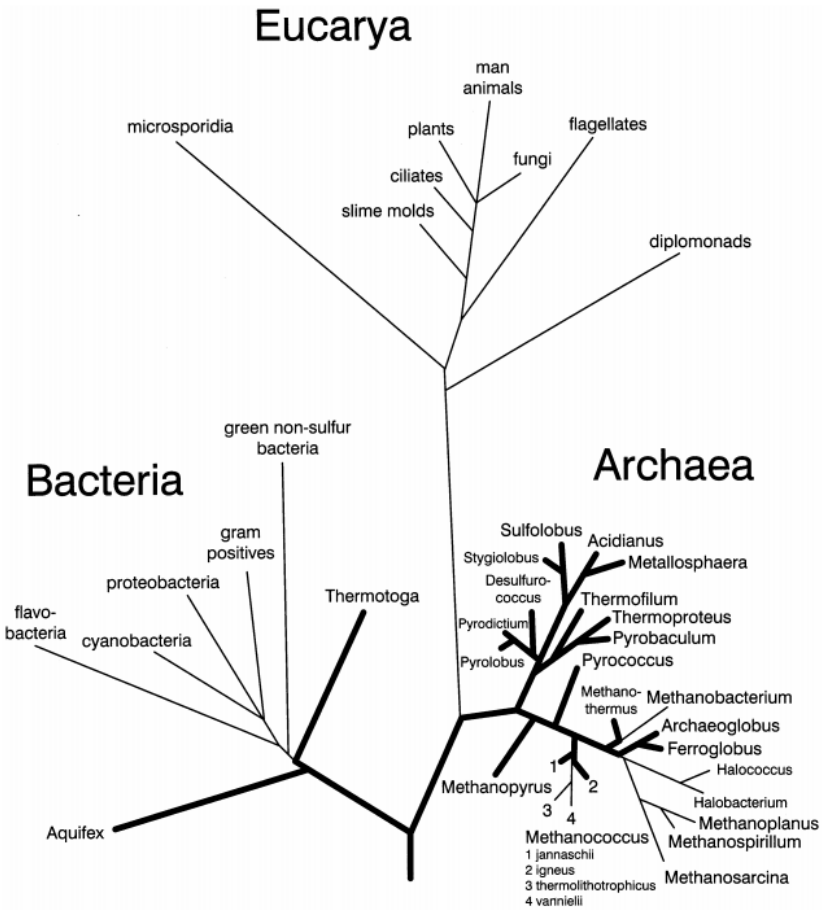

Fig. 1 Hyperthermophiles within the phylogenetic tree

(Adapted from Huber & Stetter, 1998)

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

2.1 Introduction

Since their discovery in 1981, hyperthermophiles growing optimally above 80 °C have been isolated from hot terrestrial, subterranean, and submarine environments (Stetter, 1999; Stetter, 2006; Stetter, 2013). The crenarchaeal genus *Vulcanisaeta* is classified within the family *Thermoproteaceae* of the order *Thermoproteales* and currently includes two species with validly published names: *V. distributa* and *V. 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 that are anaerobic, heterotrophic, rod-shaped, hyperthermophilic and acidophilic (Itoh *et al.*, 2002). The *Vulcanisaeta* strains 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.

2.2 Materials and Methods

2.2.1 Archaeal strains and culture conditions

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 (13°15′N, 123°41′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: 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 in 1 l salt base solution. 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 method was applied three times with growth culture at the highest dilution to obtain pure cultures.

2.2.2 Morphological, physiological and biochemical characterization

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 and sodium acetate for pH 3.0; 10 mM 2-(*N*-morpholino) ethanesulfonic acid (MES) for pH 4.0–6.0; 10 mM *N*-Tris (hydroxymethyl) methyl-3-aminopropanesulfonic acid for pH 7.0 and 8.0. To test the NaCl tolerance of strain CBA1501^T, JCM medium number 297 (M297) containing 0.5 g yeast extract, 10.0 g sulfur, 0.5 g $Na_2S·9H_2O$, and 1.0 mg resazurin in 1 l salt base solution was prepared anaerobically following the JCM guidelines, with the modification of adding 0–3% (w/v)

NaCl in 0.5% increments. Each growth was estimated by direct counting using a hemocytometer a light microscope (BA210; Motic). Cell morphology and size were determined using an electron microscopy (SUPPA 55VP; Carl Zeiss) as described previously (Lee *et al.*, 2013). 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. When sulfur compounds were used as the electron acceptors, hydrogen sulfide formation was detected by a method according to Cui *et al*. (2007a). To analyze 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) with 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. To test antibiotic sensitivity, strain CBA1501^T was inoculated in M297 medium with the following amounts (100 μg ml^{-1}): erythromycin, novobiocin, rifampicin, ampicillin, chloramphenicol, kanamycin, streptomycin and vancomycin.

2.2.3 16S rRNA gene sequence determination and phylogenetic analysis

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 Archaea-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 Analyzer

(Applied Biosystems), as described (Roh *et al.*, 2008). 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 constructed using MEGA5 (Tamura *et al.*, 2011) with the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Kluge & Farris, 1969), and maximum-likelihood (Felsenstein, 1981) methods, based on 1000 randomly generated trees.

2.2.4 DNA G+C content determination and DNA-DNA hybridization

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. DDH was performed using photobiotin-labelled DNA probe and microwell plate as described by Ezaki *et al*. (1989).

2.3 Results and Discussion

2.3.1 Nomenclature

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).

2.3.2 Morphological, physiological and biochemical characteristics

Cells of strain CBA1501^T were rod-shaped (mainly 0.6–0.7 μm wide and 4.7–9.3 μm long) (Fig. 2). The strain grew with doubling time of 11 h (under the optimal growth conditions with sulfur and shaking at 100 rpm) that is approximately 2 times slower than those of other *Vulcanisaeta* strains (Itoh *et al.*, 2002) and the culture at stationary phase contained approximately 1.0×10^6 cells ml^{-1}. Strain CBA1501^T grew at 75–90 °C, at pH 4.0–6.0 (w/v), and in presence of 0–1.0% (w/v) NaCl, with optimal growth occurring at 85 °C, at pH 5.0, and with 0% (w/v) NaCl. Fumarate, malate, oxidized glutathione, and thiosulfate were utilized as electron acceptors, whereas $FeCl_3$, nitrate, and sulfate were not. 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. 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. The strain was susceptible to novobiocin, rifampicin, chloramphenicol, kanamycin, streptomycin and vancomycin, but resistant to erythromycin and ampicillin. Differential characteristics between strain CBA1501^T and the type strains in the genus *Vulcanisaeta* are shown in Table 1. The minimum temperature supporting growth of strain CBA1501^T (75 °C) was higher than that of *V. distributa* (70 °C) and *V. souniana* (65 °C). Only strain CBA1501^T utilized fumarate as

an electron acceptor, and fumarate and lactose as a carbon source, but it did not utilize L-malate, peptone, or starch as a carbon source.

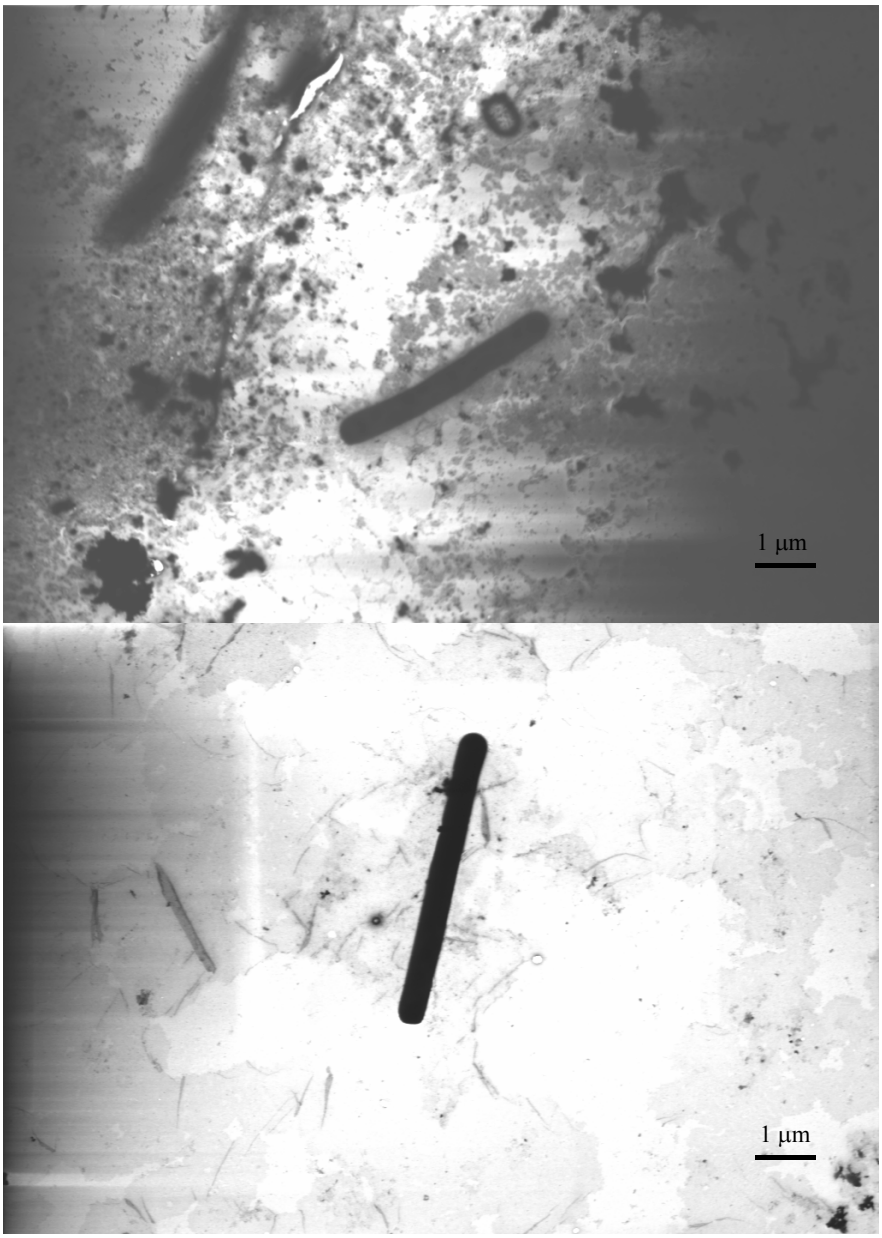

Fig. 2 Transmission electron micrographs of strain CBA1501^{T}

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

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

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

*Data with media reduced with $Na_2S{\cdot}9H_2O$

2.3.3 16S rRNA gene sequence and phylogenetic analysis

Partial 16S rRNA gene sequences were assembled and a nearly full-length 16S rRNA gene sequence (1,441 bp) was obtained. 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%), '*V. moutnovskia*' 768-28 (96.8%), *Caldivirga maquilingensis* IC-167^T (94.6%), *Pyrobaculum islandicum* GEO3^T (93.5%), and *P. organotrophum* H10^T (93.5%). The homology of 16S rRNA between 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. 3).

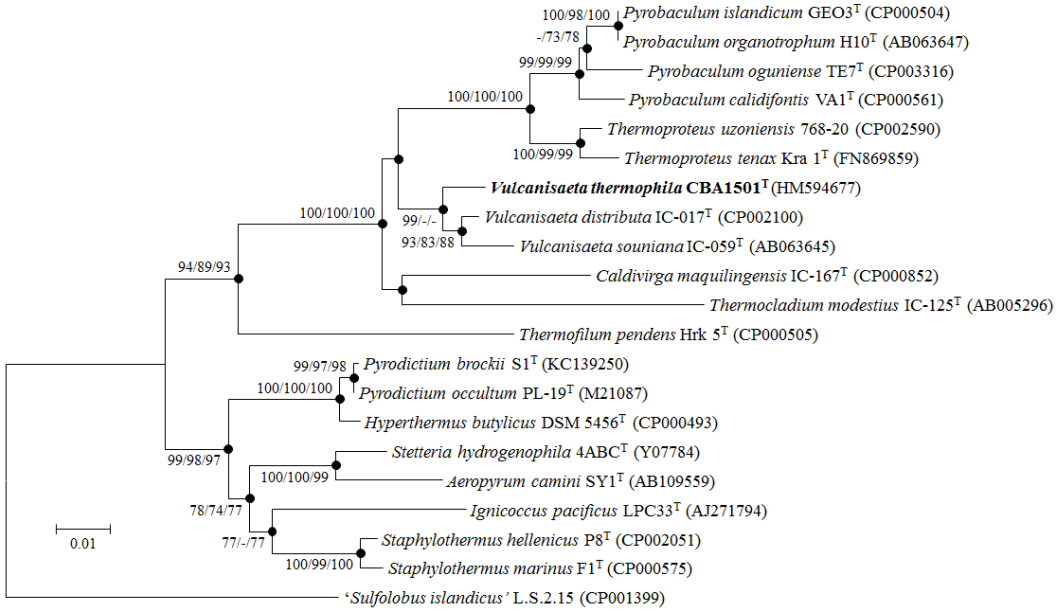

Fig. 3 Phylogenetic tree based on the neighbour-joining (NJ) algorithm for the 16S rRNA gene sequences of strain CBA1501^T and the type strains of closely related species

The numbers on the nodes indicate the bootstrap values (>70%) calculated using the NJ, maximum-parsimony (MP), and maximum-likelihood (ML) algorithm probabilities. Closed 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.

2.3.4 DNA G+C content and DNA-DNA hybridization

The DNA G+C content of strain CBA1501^T was 43.1 mol%. This value is slightly lower than the reported ranges of 44–46 mol% for other members of the genus *Vulcanisaeta* (Itoh *et al.*, 2002). The DNA–DNA homology values of strain CBA1501^T with the validly named species in the genus *Vulcanisaeta*: *V. distributa* DSM 14429^T and *V. souniana* DSM 14430^T were 42.6% and 20.3%, respectively (Table 2). Based on DDH relatedness of less than 70% indicated that strain CBA1501^T represents a distinct genospecies (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994).

Table 2. DNA-DNA hybridization values for strain CBA1501^T and the reference species

species	DNA-DNA hybridization (%) with:		
	CBA1501^T	*V. distributa* DSM 14429^T	*V. souniana* DSM 14430^T
V. thermophila CBA1501^T	100	42.6	20.3

2.4 Description of *Vulcanisaeta thermophila* sp. nov

Cells are anaerobic, hyperthermophilic, acidophilic, obligately chemoorganotrophic, 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 0–1.0% (w/v) NaCl (optimum, 0%). 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 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%.

3. *Thermoproteus thermophilus* sp. nov., a hyperthermophilic crenarchaeon isolated from solfataric soil

3.1 Introduction

The genus *Thermoproteus* is a hyperthermophilic archaeon growing optimally above 80 °C and belonging to the family *Thermoproteaceae* in the order *Thermoproteales* of the phylum *Crenarchaeota*. Microorganisms belonging to the order *Thermoproteales* are isolated from hot springs and hydrothermal systems and grow chemoorganotrophically (Zillig *et al.*, 1981; Burggraf *et al.*, 1997). The genus *Thermoproteus* proposed by Zillig *et al*. (1981) had included three species: *T. tenax* (Zillig *et al.*, 1981), *T. neutrophilus* (Stetter & Zillig, 1989) and *T. uzoniensis* (Bonch-Osmolovskaya *et al.*, 1990) in the List of Prokaryotic Names with Standing in Nomenclature (http://www.bacterio.net/thermoproteus.html). However, *Thermoproteus neutrophilus* has been recently reclassified to the genus *Pyrobaculum* as *Pyrobaculum neutrophilum*, based on phylogenetic analysis (Chan *et al.*, 2013). In the present study, we determined the phylogenetic, phenotypic and genotypic characteristics of a hyperthermophilic isolate (designated strain CBA1502^{T}) from solfataric soil and propose it as a novel species of the genus *Thermoproteus*.

3.2 Materials and Methods

3.2.1 Archaeal strains and culture conditions

Soil sample from a solfataric thermal field was collected as previously described (Yim *et al.*, 2015). A 5-g soil sample was enriched at 80 °C for 1 month using Japan Collection of Microorganisms (JCM) medium number 236 (M236) containing the components as follows: 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 in 1 l salt base solution, prepared anaerobically according to the JCM culture medium guideline. The serial dilution method was applied with growth culture at the highest dilution. This procedure was repeated twice and yielded the same isolate. The purity of the isolated strain, designated CBA1502^T, was validated by light microscopy of its morphology (BA210; Motic), sequencing of its PCR-amplified 16S rRNA gene and phylogenetic analysis. *T. uzoniensis* DSM 5263^T and *T. tenax* DSM 2078^T were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) and used as reference strains for the DNA–DNA hybridisation analyses.

3.2.2 Morphological, physiological and biochemical characterization

Cells of strain CBA1502^T was observed using an electron microscopy (Tecnai G2 Spirit; FEI) as described previously (Lee *et al.*, 2013). Optimum growth conditions for strain CBA1502^T were determined by analysing growth between 65–95 °C at intervals of 5 °C and pH values between pH 3.0–8.0 at intervals of 1.0 pH unit using the M236 medium. The pH was adjusted using the buffers as follows: 1 M acetic acid and sodium acetate, pH 3.0; 10 mM 2-(*N*-morpholino) ethanesulfonic acid (MES), pH 4.0–6.0; and 10 mM *N*-Tris (hydroxymethyl) methyl-3-aminopropanesulfonic acid, pH 7.0 and 8.0. NaCl tolerance was tested using the M236 medium with the modification of adding NaCl (0–3% at intervals of 0.5%, w/v). The following tests were determined by the cell growth in

cultures transferred three times under the optimal growth conditions using the M236 medium (without citrate) with a pH buffer (10 mM MES). For the test of utilization of different energy substrates, potential energy sources (0.5% w/v) were added individually to the medium wherein a minor amount of yeast extract was present in the medium (0.1 g/l). The difference with controls (with yeast extract only) in the cell yield was also confirmed by hydrogen sulfide formation as described by Cui *et al*. (2007b). For the test of utilization of different carbon sources, potential carbon sources (0.5% w/v) were added individually to the medium wherein yeast extract was removed. For the test of possible electron acceptors, strain CBA1502^T was cultured in the medium wherein sulfur was replaced with ferric chloride (10 mM), fumarate (20 mM), malate (20 mM), nitrate (40 mM), oxidized glutathione (2.5 mM), sulfate (20 mM), or thiosulfate (20 mM). When sulfur compounds were used as the electron acceptors, the results were also confirmed by hydrogen sulfide formation. To test antibiotic sensitivity, strain CBA1502^T was inoculated in the medium with the following amounts (100 μg ml^{-1}): erythromycin, novobiocin, rifampicin, ampicillin, chloramphenicol, kanamycin, streptomycin and vancomycin.

3.2.3 16S rRNA gene sequence determination and phylogenetic analysis

The genomic DNA extraction, PCR-amplification and sequencing of the 16S rRNA gene and phylogenetic analysis were carried out as previously described (Yim *et al.*, 2015).

3.2.4 DNA G+C content determination and DNA-DNA hybridization

The DNA G+C content of strain CBA1502^T was performed fluorimetric method (González & Saiz-Jimenez, 2002) using SYBR Green I and a real-time PCR thermocycler. We used photobiotinylated DNA probes as described by Ezaki *et al*. (1989).

3.3 Results and Discussion

3.3.1 Nomenclature

Thermoproteus thermophilus (ther.mo′phi.lus. Gr. n. therme heat; Gr. adj. philos friendly, loving; N.L. masc. adj. *thermophilus* heat-loving, referring to its growth temperature).

3.3.2 Morphological, physiological and biochemical characteristics

Cells of strain CBA1502^T were rod-shaped (mainly 0.3–0.5 μm wide and 2.9–7.7 μm long) (Fig. 4) and motile with peritrichous flagellation. Although cells of *T. uzoniensis* and *T. tenax* were sometimes branching or with spherical protrusions on the ends or at the branching points, and have no flagella (Zillig *et al.*, 1981; Bonch-Osmolovskaya *et al.*, 1990), cells of strain CBA1502^T were straight rods and had flagella. The isolate grew at 75–90 °C and pH 4.0–6.0 (w/v) and in the presence of 0–0.5% (w/v) NaCl, with optimal growth at 85 °C and pH 5.0. The strain grew with doubling time of 14.6 h (under the optimal growth conditions in the M236 medium without citrate) and the culture at stationary phase contained approximately 7.0×10^6 cells ml^{-1}. D-Arabinose, beef extract, Casamino acids, formate, fumarate, peptone, pyruvate, and trimethylamine were utilized as energy substrates, but not acetate, butyrate, citrate, D-fructose, D-galactose, gelatine, D-glucose, lactose, L-malate, D-maltose, D-mannose, methanol, methylamine, starch, succinate, sucrose, or D-xylose. D-Arabinose, formate, and pyruvate were utilized as carbon sources, but not acetate, beef extract, butyrate, Casamino acids, citrate, D-fructose, fumarate, D-galactose, gelatine, D-glucose, lactose, L-malate, D-maltose, D-mannose, methanol, methylamine, peptone, starch, succinate, sucrose, trimethylamine, or D-xylose. When sulfur compounds were used as the electron acceptors, the results were also confirmed by hydrogen sulfide formation. Fumarate, sulfate, and thiosulfate were utilized as electron acceptors, but not ferric chloride, malate, nitrate, or oxidized glutathione. Strain CBA1502^T showed no growth in the medium only with yeast extract in the absence

of other energy substrates, carbon sources and electron acceptors. Strain CBA1502^T showed weak growth in a low-oxygen atmosphere [5.0% (v/v) air in N_2], but no growth in 5.5% (v/v) air in N_2. The strain showed no growth under chemolithotrophic conditions of a H_2/CO_2 (4:1, v/v) gas mixture in the medium without yeast extract. The strain was susceptible to erythromycin, novobiocin, chloramphenicol, kanamycin, streptomycin and vancomycin, but resistant to rifampicin, ampicillin. Differential characteristics between strain CBA1502^T and its close relatives in the genus *Thermoproteus* are shown in Table 3. The maximum and optimal growth temperature of strain CBA1502^T were relatively lower than those of *T. uzoniensis* and *T. tenax* (Bonch-Osmolovskaya *et al.*, 1990; Boone *et al.*, 2001). Strain CBA1502^T differed from *T. uzoniensis* and *T. tenax* by the motility, utilization of formate, and reduction of sulfate.

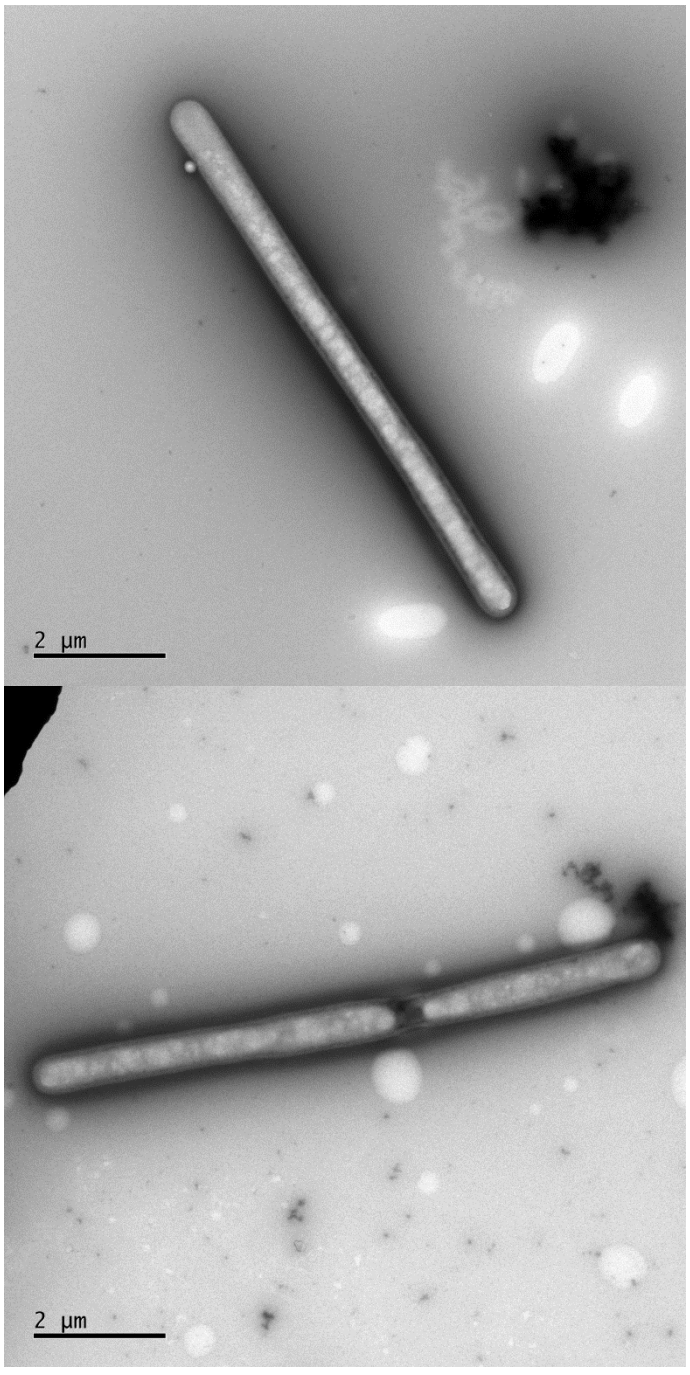

Fig. 4 Transmission electron micrographs of strain CBA1502^{T}

Table 3. Characteristics that differentiate between strain CBA1502^T and each of the type strains of its closest relatives in the genus *Thermoproteus*

Taxa: 1, *Thermoproteus thermophilus* CBA1502^T sp. nov.; 2, *T. uzoniensis* Z-605^T (data from Bonch-Osmolovskaya *et al.*, 1990); 3, *T. tenax* Kra 1^T (Boone *et al.*, 2001). +, Positive; −, negative.

Characteristics	**1**	**2**	**3**
Motility	+	−	−
Temperature range for growth (°C)	75–90	74–102	≤96
Optimum temperature	85	90	90
pH range for growth	4.0–6.0	4.6–6.8	2.5–6.0
Optimum pH	5.0	5.6	5.0
Carbon source			
Formate	+	−	−[a]
Glucose	−	−	+
Electron acceptor			
Malate	−	−	+
Sulfate	+	−	−[a]
Thiosulfate	+	−	+[a]
DNA G+C content (mol%)	62.0	56.5	55.5

[a] data from this study with *T. tenax* DSM 2078^T

3.3.3 16S rRNA gene sequence and phylogenetic analysis

A nearly full-length 16S rRNA gene sequence of strain CBA1502^T (1,435 bp) was obtained. The 16S rRNA gene sequence of strain CBA1502^T was 99.2%, 99.1%, 99.0% and <97.0% identical to the sequences of *T. uzoniensis* DSM 5263^T (982 bp; data from this study), *T. uzoniensis* 768-20, *T. tenax* Kra 1^T and other members of the family *Thermoproteaceae*, respectively. The phylogenetic trees based on the 16S rRNA gene sequences indicated that strain CBA1502^T falls within the cluster of species of the genus *Thermoproteus* of the family *Thermoproteaceae,* with high bootstrap values (99, 98 and 97 % in the neighbour-joining, maximum-parsimony and the maximum likelihood trees, respectively) (Fig. 5).

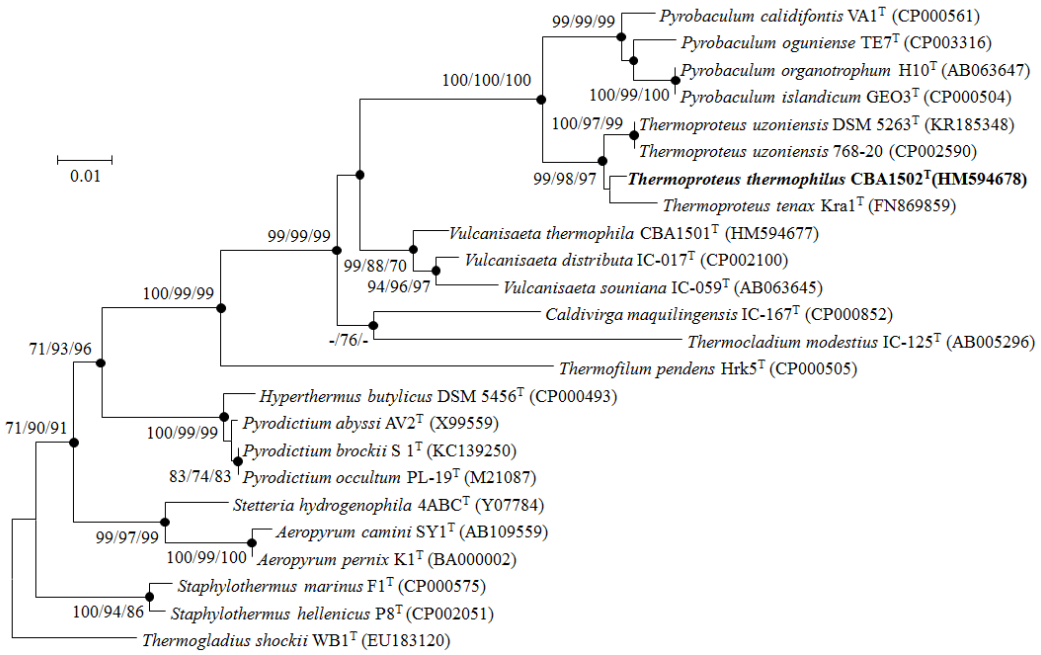

Fig. 5 Phylogenetic tree derived from the 16S rRNA gene sequences from strain CBA1502^T and the closely related species

Branch nodes (filled circles) indicate generic branches present in phylogenetic trees generated by neighbour-joining as well as the maximum-parsimony and the maximum likelihood algorithms. Numbers at nodes indicate bootstrap percentages, calculated by neighbour-joining, maximum-parsimony and maximum likelihood. Bootstrap analyses were performed using 1000 replicates and values greater than 70% are shown at branch points. Bar, 0.01 changes per nucleotide position.

3.3.4 DNA G+C content and DNA-DNA hybridization

The DNA G+C content of strain CBA1502^T was 62.0 mol%. We found that the homology between the genomic DNA of CBA1502^T and that of *T. uzoniensis* DSM 5263^T and *T. tenax* DSM 2078^T was 46.1% and 24.9%, respectively (Table 4). Values less than 70% homology indicate that an isolate represents a distinct genospecies (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994).

Table 4. DNA-DNA hybridization values for strain CBA1502^T and the reference species

species	DNA-DNA hybridization (%) with:		
	CBA1502^T	*T. uzoniensis* DSM 5263^T	*T. tenax* DSM 2078^T
T. thermophilus CBA1502^T	100	42.6	20.3

3.4 Description of *Thermoproteus thermophilus* sp. nov.

Cells are anaerobic, hyperthermophilic, acidophilic, obligately chemoorganotrophic, rod-shaped (0.3–0.5 μm wide and 2.9–7.7 μm long) and motile with peritrichous flagellation. Growth occurs at 75–90 °C (optimum, 85 °C), pH 4.0–6.0 (optimum, pH 5.0) and in media containing 0–0.5% (w/v) NaCl. Under optimal growth conditions, doubling time is 14.6 h. D-Arabinose, beef extract, Casamino acids, formate, fumarate, peptone, pyruvate, trimethylamine, and yeast extract, but not acetate, butyrate, citrate, D-fructose, D-galactose, gelatine, D-glucose, lactose, L-malate, D-maltose, D-mannose, methanol, methylamine, starch, succinate, sucrose, and D-xylose, are utilized as energy substrates. D-Arabinose, formate, pyruvate, and yeast extract, but not acetate, beef extract, butyrate, Casamino acids, citrate, D-fructose, fumarate, D-galactose, gelatine, D-glucose, lactose, L-malate, D-maltose, D-mannose, methanol, methylamine, peptone, starch, succinate, sucrose, trimethylamine, and D-xylose, are utilized as carbon sources. Fumarate, sulfate, sulfur, and thiosulfate are utilized as electron acceptors, but not ferric chloride, malate, nitrate, or oxidized glutathione. Cells tolerate a low level of oxygen [5.0% (v/v) air in N_2]. The DNA G+C content of the type strain is 62.0 mol%.

The type strain is CBA1502^T (= ATCC BAA-2416^T = JCM 17229^T), isolated from solfataric soil from the Mayon volcano on the island of Luzon in the Republic of the Philippines.

4. Genome sequence of the hyperthermophilic and acidophilic crenarchaeon *Vulcanisaeta thermophila* type strain CBA1501^T

4.1 Introduction

Hyperthermophilic archaea isolated from hot terrestrial, subterranean, and submarine environments grow optimally above 80°C (Stetter, 1999; Stetter, 2006; Stetter, 2013). The genus *Vulcanisaeta* belongs to the family *Thermoproteaceae*, order *Thermoproteales*, phylum *Crenarchaeota*. *Vulcanisaeta thermophila* CBA1501^T (= ATCC BAA-2415^T = JCM 17228^T) was isolated from solfataric soil of the Mayon volcano in the Republic of the Philippines (Yim *et al.*, 2015). It is an anaerobic, rod-shaped, hyperthermophilic and acidophilic crenarchaeon, and grew at 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 0% (w/v) NaCl. Hyperthermophilic enzymes are stable and active at high temperatures of >70°C (Vieille *et al.*, 1996). Here, a draft genome sequence of *V. thermophila* CBA1501^T is reported, including sequence information on hyperthermophilic enzymes with high biotechnological value.

4.2 Materials and Methods

Genomic DNA from *V. thermophila* strain CBA1501^{T} was extracted and purified using the G-spin^{TM} total DNA extraction kit (iNtRON Biotechnology, Seongnam, Korea), and sequenced using the Illumina MiSeq system according to the manufacturer's instructions. A total of 6,939,439 reads (with 688-fold coverage) were analyzed using CLC Genomics Workbench 7.0.4 (CLC Bio, Aarhus, Denmark) and deposited in the DNA Data Bank of Japan (DDBJ). Single reads were assembled using the GS *de novo* assembler. RNAmmer 1.2 (Lagesen *et al.*, 2007) and tRNAscan-SE 1.21 (Lowe & Eddy, 1997) were used to identify rRNA and tRNA sequences, respectively. The genome was annotated using Rapid Annotation using Subsystem Technology (RAST) (Aziz *et al.*, 2008) and the CLgenomics program developed by ChunLab (http://www.chunlab.com/genomics). Genetic relatedness was analyzed using the average nucleotide identity (ANI) value (http://www.ezbiocloud.net/ezgenome/ani). Mauve 2.4.0. was used for whole-genome alignments (Darling *et al.*, 2010).

4.3 Results and Discussion

The draft genome of *V. thermophila* CBA1501^T consists of 2,022,594 bp with a G+C content of 49.1% (Fig. 6). The genome is predicted to include 2,214 open reading frames (ORFs), 3 rRNA genes, 41 tRNA genes, and one different RNA gene (Table 5). Based on clusters of orthologous groups (COG) annotations (http://www.ncbi.nlm.nih.gov/COG/), 1,515 genes were divided into functional subcategories of transport and energy production and conversion (164), metabolism of amino acid (157), metabolism of carbohydrate (90), coenzyme (88), lipid (64), and nucleotide (47) (Table 6). The genome of *V. thermophila* CBA1501^T encodes 6 alcohol dehydrogenases, 6 acetyl-CoA acetyltransferases, 2 thiosulfate sulfurtransferases, 4 metallophosphoesterases, 2 fumarate reductases, 2 cellulases, 1 phospholipase. The complete genomes of *V. distributa* DSM 14429^T and *V. moutnovskia* 768-28 showed 71.09% and 70.06% similarity, respectively, indicating showed that *V. thermophila* CBA1501^T was highly similar to *V. distributa* DSM 14429^T and was slightly more distantly related to *V. moutnovskia* 768-28 (Fig. 7). The draft genome of *V. thermophila* CBA1501^T will provide insights into the metabolism of hyperthermophilic archaea and aid in identifying opportunities for biotechnological applications of novel hyperthermophilic enzymes.

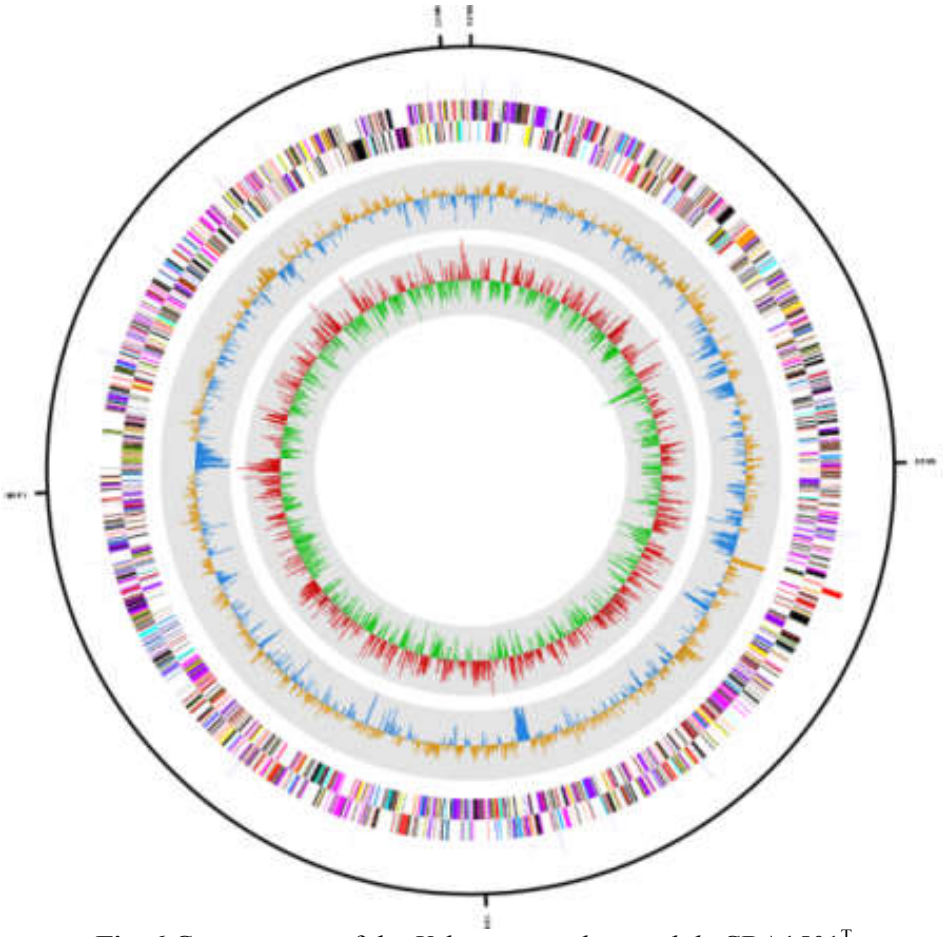

Fig. 6 Genome map of the *Vulcanisaeta thermophila* CBA1501^{T}

Table 5. Features of the *Vulcanisaeta thermophila* CBA1501^T genome

Attribute	Value
Genome size (bp)	2,022,594
G+C content (%)	49.1
Contigs (n)	10
ORFs (n)	2,214
ribosomal-RNA genes (n)	3
transfer-RNA genes (n)	41
Genes assigned to COGs	1,515
Genes with Enzyme (n)	
Alcohol dehydrogenases	6
Acetyl-CoA acetyltransferases	6
Thiosulfate sulfurtransferases	2
Metallophosphoesterases	4
Fumarate reductases	2
Cellulases	2
Phospholipase	1
Reverse gyrase	1
CRISPR repeats	3

Table 6. Functional categories of the *Vulcanisaeta thermophila* CBA1501^T based on COG

COG	Description	Number of Genes	%
J	Translation, ribosomal structure and biogenesis	153	10.10%
K	Transcription	70	4.62%
L	Replication, recombination and repair	62	4.09%
D	Cell cycle control, cell division, chromosome partitioning	11	0.73%
O	Posttranslational modification, protein turnover, chaperones	71	4.69%
M	Cell wall/membrane/envelope biogenesis	61	4.03%
N	Cell motility	7	0.46%
P	Inorganic ion transport and metabolism	49	3.23%
T	Signal transduction mechanisms	22	1.45%
C	Energy production and conversion	164	10.83%
G	Carbohydrate transport and metabolism	90	5.94%
E	Amino acid transport and metabolism	157	10.36%
F	Nucleotide transport and metabolism	47	3.10%
H	Coenzyme transport and metabolism	88	5.81%
I	Lipid transport and metabolism	64	4.22%
Q	Secondary metabolites biosynthesis, transport and catabolism	12	0.79%
R	General function prediction only	232	15.31%
S	Function unknown	155	10.23%
Total		1515	100%

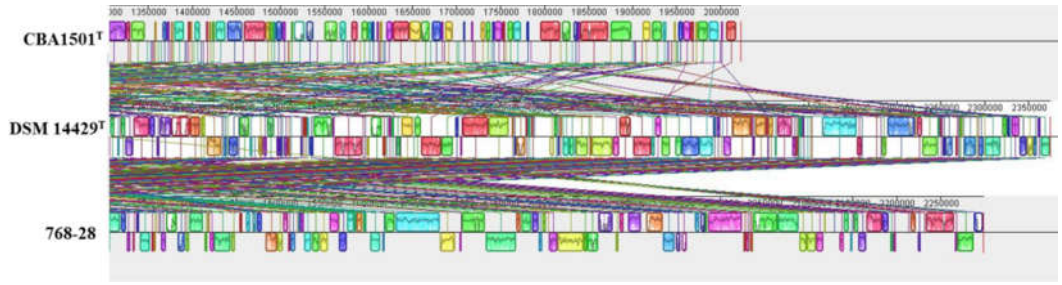

Fig. 7 Whole-genome alignment of the draft genome of *V. thermophila* CBA1501^{T} and the fully sequenced *V. distributa* DSM 14429^{T} and *V. moutnovskia* 768-28 genomes.

The Mauve algorithm was used. The colored blocks represent locally collinear blocks (LCBs) indicating highly homologous regions. The white areas indicate similarity gaps in the sequences. Approximately nine large Indels were identified in the *V. thermophila* CBA1501^{T} sequence. The genomes were drawn to scale based on the *V. distributa* DSM 14429^{T} reference genome.

5. Conclusion

1) These findings raise the possibility of a wider distribution of hyperthermophilic Crenarchaeota strains 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 in the genus *Vulcanisaeta* based on the phylogenetic, phenotypic, and genomic comparisons. On the basis of polyphasic taxonomic analyses, strain CBA1501^T is a novel species of the genus *Vulcanisaeta* in the family *Thermoproteaceae*, for which I propose the name *Vulcanisaeta thermophila* sp. nov.

2) The physiological, biochemical and genomic characteristics of strain CBA1502^T differ from those of other members of the genus *Thermoproteus*. Thus, strain CBA1502^T represents a novel species of the genus *Thermoproteus* in the family *Thermoproteaceae* and I propose the name *Thermoproteus thermophilus* sp. nov.

3) The whole genome of *Vulcanisaeta thermophila* CBA1501^T was sequenced. The genome of strain CBA1501^T was analyzed for finding unique characteristics. Therefore, the enzymes produced by hyperthermophiles can be provided with model systems to comprehend enzyme evolution and mechanisms of stable protein at high temperature and to determine the higher temperature limit for enzyme stability.

This information on hyperthermophile can be applied to industrial applications of extreme enzymes.

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국문 요약

활화산(필리핀 마욘산)의 토양에서 분리된 *Vulcanisaeta thermophila* CBA1501 균주와 *Thermoproteus thermophilus* CBA1502 균주는 혐기성, 초고온성, 호산성이며, 막대 모양의 세포 형태를 보였다. CBA1501 균주는 *Vulcanisaeta* genus 에 속하는 균주로서 *Vulcanisaeta distributa* IC-017 그리고 *Vulcanisaeta souniana* IC-059 균주와 각각 98.5 그리고 97.4%의 16S rRNA 유전자의 상동성을 나타냈다. CBA1501 균주는 너비 0.6–0.7 μm 이고 길이 4.7–9.3 μm 였으며, 75–90 °C (optimum 85 °C), 0–1.0% NaCl (w/v; optimum 0%), 그리고 pH 4.0–6.0 (optimum pH 5.0)에서 성장을 보였다. CBA1501 균주의 DNA G+C content 는 43.1 mol%를 보였다. CBA1502 균주는 *Thermoproteus* genus 에 속하는 균주로서 *Thermoproteus uzoniensis* DSM 5263 그리고 *Thermoproteus tenax* Kra 1 와 각각 99.2 그리고 99.0%의 16S rRNA 유전자의 상동성을 나타냈다. CBA1502 균주는 너비 0.3–0.5 μm 이고 길이 2.9–7.7 μm 였으며, 75–90 °C (optimum 85 °C), 0–0.5% (w/v) NaCl, 그리고 pH 4.0–6.0 (optimum pH 5.0)에서 성장을 보였다. CBA1502 균주의 DNA G+C content 는 46.1 mol%를 보였다. CBA1501 균주와 CBA1502 균주는 생리, 생화학적 특성 분석 및 계통분류학적 분석 통해 각각 *Vulcanisaeta thermophila* 와

Thermoproteus thermophilus 라고 명명되었다. 그리고 *Vulcanisaeta thermophila* CBA1501 의 유전체는 차세대 염기서열 분석을 통해 2,022,594 bp 와 49.1% G+C content 로 확인되었다. CBA1501 균주의 염기서열 정보는 향후 극한효소의 산업적 응용 정보를 제공 할 수 있다.

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벌써 2 년이라는 석사과정 시간이 지났습니다. 2 년이란 시간 동안 저에게 도움을 주신 분들이 많습니다. 부족하지만 학위 논문을 마치면서 그 분들께 감사의 마음을 전하고 싶습니다.

누구보다도 학부 때부터 연구와 배움에 길로 인도해주시고, 참된 연구자의 모습을 가르쳐 주시고, 부족한 점을 언제나 세심하고 꼼꼼한 손길로 지적해주신 노성운 박사님께 진심으로 감사 드립니다. 또한 바쁘신 와중에도 이번 학위논문 심사를 흔쾌히 허락해주시고, 격려와 조언을 해주신 박수제 지도교수님과 최종순 박사님께 감사 드립니다.

항상 저를 많이 도와준 같은 실험실원 준용이형, 혜선이와 박수제 지도교수님 실험실원 고현우씨에게도 고맙다는 말을 전하고 싶습니다.

이제까지 옆에서 저를 지지해준 친구들, 승범이, 동억이, 최훈, 홍석이, 재현이, 경도, 송혁, 지수, 주환이 그리고 항상 저를 응원해준 현진이에게도 고맙다는 말을 전하고 싶습니다.

마지막으로, 저에게 아낌없이 모든 것을 주신 부모님께 감사의 마음을 전하고 싶습니다. 아버지와 같은 분야의 길을 걸을 수 있어 행복하다고 말씀 드리고 싶습니다. 저는 참된 연구자의 모습을 닮기 위해 열심히 노력하겠습니다.

이 외에도 많은 분들께서 저를 응원해 주시고 도와 주셨습니다. 한 분 한 분 다 언급 드리지 못한 점 죄송하게 생각하며, 대신 깊은 감사의 마음을 전합니다.