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Thermocrinis minervae sp. nov., A Hydrogen and Sulfur-oxidizing, Thermophilic Member of the Aquificales from a Costa Rican Terrestrial Hot Spring

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Thermocrinis minervae sp. nov., a hydrogen and sulfur-oxidizing, thermophilic *Aquificales* from a Costa Rican terrestrial hot spring

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Running title: *Thermocrinis minervae* sp. nov.

Graphs showing the effect of temperature and pH on the growth of *Thermocrinis minervae* CR11^T are available as supplementary material in IJSEM online.

The GenBank accession number for the partial 16S rRNA gene sequence of strain CR11^T is AM260555.

Summary

A thermophilic bacterium, designated strain CR11^T, was isolated from a filamentous sample collected from a terrestrial hot spring on the southwestern foothills of the Rincón Volcano, Costa Rica. The Gram-negative cells are approximately 2.4-3.9 µm long, 0.5-0.6 µm wide, and are motile rods with polar flagella. Strain CR11^T grows between 65 °C and 85 °C (optimum 75 °C, doubling time 4.5 h) and between pH 4.8 and 7.8 (optimum between 5.9 and 6.5). The isolate grows chemolithotrophically with S^o, $S_2O_3^{2-}$, or H_2 as the electron donor, and with O_2 (up to 16 % v/v) as the sole electron acceptor. The isolate can grow on mannose, glucose, maltose, succinate, peptone, Casamino acids, starch, citrate, and yeast extract in the presence of oxygen (4 %) and S°. Growth only occurs at NaCl concentrations below 0.4 % (v/w). The G+C content of strain CR11^T is 40.3 mol%. Phylogenetic analysis of the 16S rRNA gene places the strain as a close relative of *Thermocrinis ruber* (95.7 % sequence similarity). Based on phylogenetic and physiological characteristics, we propose the name *Thermocrinis minervae* for the new isolate with CR11^T as the type strain (=DSM 19557^T, =ATCC BAA-1533^T).

Introduction

The order *Aquificales*, represented by the *Aquificaceae, Hydrogenothermaceae* and *Desulfurobacteriaceae*, are thermophilic Bacteria that are widely distributed in hydrothermal systems and include microaerophilic chemolithotrophs and heterotrophs. Phylogenetic analysis of 16S rRNA gene sequences places the *Aquificales* as one of the deeply-branching lineages within the Bacteria (Burggraf *et al*., 1992; Pitulle *et al*., 1994; Di Giulio, 2003 a, b, c; Barion *et al*., 2007).

The *Aquificaceae* includes the genera *Aquifex*, *Thermocrinis*, *Hydrogenobacter*, *Hydrogenivirga*, and *Hydrogenobaculum*. *Aquifex pyrophilus*, originally isolated from a submarine hydrothermal vent system in Iceland (Huber *et al*., 1992), was the first described representative of this family, although numerous described *Hydrogenobacter* strains (Kryukov *et al*., 1983; Kawasumi *et al*., 1984; Kristjansson *et al*., 1985; Nishihara *et al*., 1990) also grouped within the *Aquificaceae* once their 16S rRNA sequences were determined (Burggraf *et al*., 1992; Pitulle *et al*., 1994). Another clade within the *Aquificaceae* was reported from a culture-independent, molecular phylogenetic assessment of the diversity associated with the pink filamentous streamers from Octopus Spring, Yellowstone National Park (Reysenbach *et al*., 1994). Following this study, Huber *et al*. (1998) isolated the dominant *Aquificales* from this hot spring on a medium containing organic acids and named it *Thermocrinis ruber*. Subsequently, numerous new *Thermocrinis* isolates, including *T. albus*, were obtained from hot springs in Russia, Iceland, and Yellowstone National Park (Eder and Huber, 2002).

Cultivation studies and associated geochemical analyses (Huber *et al*., 1998; Eder and Huber, 2002; Blank *et al*., 2002; Hall *et al*., 2008; Connon *et al*., 2008) suggest that the metabolic activities of this group may contribute significantly to biogeochemical cycling in certain hydrothermal systems. Like the other members of the *Aquificales, T. ruber* and *T. albus* are dominant primary producers (Blank *et al*., 2002; Eder and Huber, 2002) in many ecosystems where photosynthesis is limited by high temperatures. Their ability to oxidize sulfur in laboratory cultures (Huber *et al*., 1998; Eder and Huber, 2002) suggests that these bacteria may also contribute to sulfur cycling in these environments. Additionally, 16S rRNA gene studies of a biofilm associated with As (III) oxidation in the Alvord Hot Spring, Oregon, indicated the presence of bacteria related to *Thermocrinis* and other *Aquificales* genera (Connon *et al*., 2008).

T. ruber and *T. albus* share similar physiological properties, including pH, temperature, and salinity optima (Huber *et al*., 1998; Eder and Huber, 2002). Both isolates are able to oxidize hydrogen, sulfur, and thiosulfate with oxygen as the sole electron acceptor, and have similar distributions of fatty acids and glycerol monoethers (Jahnke *et al*., 2001). However, *T. ruber* can also grow heterotrophically with formate and formamide, while *T. albus* appears to be a strict chemolithoautotroph (Huber *et al*., 1998; Eder and Huber, 2002). Based on 16S rRNA gene sequences, these isolates are somewhat distantly related (5.1 % sequence difference; Eder and Huber, 2002).

Here we report the isolation and characterization of a new species of *Thermocrinis.* This strain is the first member of the *Aquificales* isolated from Costa Rica and is capable of using a relatively

large number of organic carbon sources, further expanding the geographic range and metabolic diversity of this group.

Sample collection, enrichment, and isolation

Filamentous biomass samples were collected aseptically from a thermal spring (93 °C, pH 7.0) on the southwestern foothills of the Rincón de la Vieja Volcano, Costa Rica. A sub-sample was inoculated into 5 ml modified MSH medium (pH 6.2) under a gas phase of CO_2:H_2 (20:80; Aguiar *et al*., 2004). Prior to inoculation, O_2 (4 %) was added to the medium. Enrichments were incubated at 80 °C, without agitation, until the tubes became turbid and contained motile rods under phase contrast microscopy. These cultures were immediately transferred into the same media and purified by multiple dilution-to-extinction serial transfers. Purity of the isolate was determined by 16S rRNA gene sequencing. The resulting pure culture was designated strain $CR11^T$ and was characterized further. The isolate grew better with S° than with H_2 as the electron donor. Therefore, unless otherwise noted, all subsequent growth experiments used a modified MSH medium (Aguiar *et al*., 2004) supplemented with approximately 0.06 % (w/v) S° and 4 % (v/v) O_2.

Morphology

Cells were routinely monitored under phase-contrast microscopy using an Olympus BX60 microscope. Electron microscopic examination was performed as previously described (Nakagawa *et al*., 2005). Thin sections were prepared by treating fixed cells with 2 % (w/v) osmium tetroxide and en block staining with 2 % uranyl acetate as described in Beveridge *et al*. (1994). Cells were then dehydrated in ethanol and embedded in LR White. Sectioned cells were

mounted on carbon- and Formvar-coated 200-mesh grids and stained with uranyl acetate and lead citrate. To create negative stains, grids were coated with a thin cell suspension, dried, and stained with 2 % uranyl acetate.

CR11^T cells are motile, Gram-negative rods that vary in length from approximately 2.4 to 3.9 µm and from 0.5 to 0.6 µm in width (Fig. 1C). Cells did not form filaments during growth although we did not try to stimulate filament formation as reported by Huber et al.1998 . The Gram-negative envelope has only an outer membrane as the surface component (Fig. 1A). Approximately 5 % of the cells observed by transmission electron microscopy also have cytoplasmic structures with unknown function (Fig. 1B). These structures have been reported previously in other *Aquificales* (Götz *et al*., 2002: Aguiar *et al*., 2004; Flores *et al*., 2008). Transmission electron micrographs of negatively stained cells show amphitrichous flagella (Fig. 1C).

Growth characteristics

Growth of the isolate was determined by direct cell counts using a Petroff-Hauser counting chamber and a phase-contrast microscope (Olympus BX60). All experiments were performed in triplicate at optimum temperature and pH unless otherwise noted.

The isolate grew between 65 °C and 85 °C with optimum growth occurring at about 75 °C (doubling time 4.5 h; supplementary Fig. 1 in IJSEM Online). This growth range is below the temperature measured in the spring during sample collection. It is well-estalished that that growth under laboratory conditions may not directly reflect the growth conditions in the

environment or alternatively, a lower temperature variant was selected for in this study. The effect of pH on growth was determined by adjusting the media to different pH values using 10 mM acetate / acetic acid buffer (pH 4-5), MES (pH 5-6.5), HEPES (pH 7), PIPES (pH 7-7.5), and Tris (pH 7.5-8.0). CR11^T grew between pH 4.8 and 7.8 and optimally between 5.9 and 6.5 (supplementary Fig. 1B in IJSEM Online). No growth occurred below pH 4.8 or above 7.8. NaCl requirements for growth of the isolate were determined from 0 to 1 % NaCl (w/v) in modified MSH medium. The isolate grew in media containing 0-0.4 % NaCl.

Electron donors and acceptors were added to modified MSH medium without $S_2O_3^{2-}$ (since it may be used as an electron donor) and containing a reduced concentration of $MgSO_4 \cdot 7H_2O$ (4 g l^{-1}). Electron couples were added aseptically after autoclaving and at concentrations reported in Aguiar *et al*. (2004). Media used for determining growth of CR11^T with H_2 as the electron donor were prepared with H_2:CO_2 (80:20) as the gas phase and with S°, $S_2O_3^{2-}$, NO_3^-, SO_3^{2-}, SO_4^{2-}, arsenate (as $Na_2HAsO_4 \cdot 7H_2O$), arsenite (as $NaAsO_2$), selenate (as Na_2SeO_4), or selenite (as Na_2SeO_3) as the electron acceptor. Growth with all other electron couples was determined using media with a gas phase of N_2:CO_2 (80:20). Electron donors and acceptors were added to this media as follows: $S_2O_3^{2-}$ / Fe^{3+} (as ferric citrate), Fe^{2+} (as $FeCl_2$) / O_2, Fe^{2+} (as $FeCl_2$) / NO_3^-, $S_2O_3^{2-}$ / NO_3^-, NH_4^+ / O_2, NH_4^+ / NO_2^-, $S_2O_3^{2-}$ / SO_4^{2-}, SO_3^{2-} / O_2, $S_2O_3^{2-}$ / arsenate (as $Na_2HAsO_4 \cdot 7H_2O$), arsenite (as $NaAsO_2$) / O_2, $S_2O_3^{2-}$ / selenate (as Na_2SeO_4), S° / selenite (as Na_2SeO_3), $S_2O_3^{2-}$ / selenite. CR11^T grew with H_2, S°, and $S_2O_3^{2-}$ as electron donors and with O_2 as the sole electron acceptor. The isolate grew with O_2 concentrations between 2 % and 16 % (v/v). However, growth of the isolate was weak below 4 % and above 13 % (v/v) oxygen.

Heterotrophic growth of CR11^T was determined by adding carbon sources at concentrations reported in Aguiar *et al*. (2004) to modified MSH medium containing no CO_2 or $S_2O_3^{2-}$. Growth was monitored in the presence of O_2 as an electron acceptor and S° as an electron donor. Cultures were also incubated in the absence of O_2 and S° to test for fermentative growth. Cultures were transferred (5%) at least twice in the same substrate combinations to ensure that the cultures were not growing on the carried-over media. CR11^T grew with 0.1 % mannose, glucose, maltose, succinate, Bacto peptone, Casamino acids, starch, citrate, and yeast extract as carbon sources with S° as the electron donor and O_2 (4 % v/v) as the electron acceptor. No growth was detected under anaerobic conditions or in the absence of S°. Growth did not occur with sucrose, fructose, lactate, malate, oxalate, acetate, formaldehyde, propionate, sorbitol, methanol, tartaric acid, formamide, formate, or 2-propanol as the sole carbon sources.

DNA composition and phylogenetic analysis

DNA base composition (mol% G+C) was determined by thermal denaturation of genomic DNA (Marmur and Doty, 1962). DNA was extracted from a pure culture of CR11^T (1 l) using the Qiagen Genomic-tip 100/G DNA extraction kit following the manufacturer's protocol. The G+C content of CR11^T is 40.3 mol%. This value is lower than the values reported for other *Thermocrinis* isolates (Table 1), but it is within the range of G+C content reported for other members of the *Aquificaceae* (the lowest reported value is for *Hydrogenobaculum acidophilum*, 35 mol%, Shima and Suzuki, 1993).

The 16S rRNA gene sequence was amplified by PCR and sequenced as described in Ferrera *et al*. (2007). The near full-length sequence of the 16S rRNA gene was assembled using

AutoAssembler (Applied Biosystems Inc.) and compared, using a BLAST search against the NCBI non-redundant database. The 16S rRNA gene sequence was manually aligned using the ARB program (Ludwig *et al*., 2004; http://www.mikro.biologie.tu-muenchen.de) based on the constraints of the secondary structure of the 16S rRNA molecule. The similarities in 16S rRNA gene sequences of CR11^T and the more closely-related members of the *Aquificaceae* were calculated in ARB using 1469 homologous nucleotides within the *Thermocrinis*-*Hydrogenobacter* group. Phylogenetic trees were constructed in PAUP* (Swofford *et al*., 2003) using representative sequences of all members of the *Aquificales* and including only unambiguously aligned nucleotides (1370 nt). Neighbor-joining (NJ; 1000 bootstrap replications) and maximum-likelihood (ML; 100 bootstrap replications) analyses were performed in ARB and PAUP* as previously described (Ferrera *et al*., 2007). Since the NJ and ML tree topologies are nearly identical, only the ML tree is shown in Fig. 2.

Comparison to related species

The 16S rRNA gene sequence analysis places strain CR11^T as a new species within the genus *Thermocrinis* (70 % ML bootstrap value). CR11^T is most closely related to the environmental clone RIN3BA4 obtained from the same hot spring (Fig. 2). The closest described isolate to CR11^T is *T. ruber* (95.7 % similar in 16S rRNA gene sequence). Strain CR11^T and RIN3BA4 form a separate lineage from *T. ruber* and related strains. Together, these two lineages form a clade with *Hydrogenobacter* (80 % ML bootstrap value), while the *T. albus*-like group forms a separate monophyletic lineage (Fig. 2). Strain CR11^T is > 95 % similar in the 16S rRNA gene to most sequences of the *T. ruber*-like clade. For example, CR11^T is 95.1 % similar to the clone sequence EM17 (Reysenbach *et al*., 1994) and 95.2 % similar to *Thermocrinis* sp. P2L2B (Eder

and Huber, 2002). The new isolate and clone RIN3BA4 are less than 95 % similar in the 16S rRNA gene (below the cutoff for genus level; Stackebrandt and Goebel, 1994) to all sequences within the *T. albus*-like clade (94.8 % similarity between CR11^T and *T. albus*). The phylogenetic distance between these groups is similar to the previously reported distance of 5.1 %, based on maximum parsimony analysis, between *T. ruber* and *T. albus* (Eder and Huber, 2002).

The strains of *Thermocrinis* (including CR11^T) are physiologically similar with respect to growth temperature ranges, low NaCl tolerances, and the electron donor/acceptor pairs used for chemolithotrophic growth (Table 1). However, they differ significantly in their ability to use organic carbon sources. Among these strains,, CR11^T appears to be metabolically more similar to *T. ruber* than to *T. albus*, in that CR11^T is also capable of growing on organic carbon sources. However, CR11^T grows on a greater diversity of organic carbon sources than *T. ruber* (Table 1). Furthermore, CR11^T has a lower G+C (mol%) content than either *T. ruber* or *T. albus*. Therefore, based on physiological and phylogenetic characteristics, we propose the new species, *Thermocrinis minervae*, with CR11^T as the type strain (=DSM 19557^T, =ATCC BAA-1533^T).

Description of *Thermocrinis minervae* sp. nov.

Thermocrinis minervae (mi.ner'vae L. n. gen. fem. *minervae*, from Minerva, a Roman goddess, also known as Pallas Athena in Greek mythology, considered to be the virgin goddess of science, medicine, and wisdom).

Motile, Gram-negative rods with length approximately 2.4 to 3.9 µm and width 0.5-0.6 µm. Cells occur singly. Growth occurs between 65 and 85 °C (75 °C optimum), pH 4.8 to 7.8 (5.9-6.5

optimum), and NaCl concentrations from 0 to 0.4 % (w/v). Grows chemolithoautotrophically with H_2, S°, and $S_2O_3^{2-}$ as electron donors and with only O_2 (up to 16% v/v) as the electron acceptor. Able to use yeast extract, mannose, glucose, maltose, succinate, peptone, Casamino acids, starch, citrate, and CO_2 as carbon sources. The G+C content of genomic DNA is 40.3 mol%. Isolated from a terrestrial hot spring on the southwestern foothills of the Rincón de la Vieja Volcano in Costa Rica. The GenBank accession number for the partial 16S rRNA gene sequence of strain CR11^T is AM260555. The type strain is *Thermocrinis minervae* CR11^T (=DSM 19557^T, =ATCC BAA-1533^T).

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Table 1. Comparison of physiological properties and DNA base composition between CR11^T and other described representatives of *Thermocrinis*. NR = not reported.

Character	**CR11^T**	***Thermocrinis ruber* OC 1/4^T**	***Thermocrinis albus* HI 11/12^T**
Origin	Terrestrial hot spring, Costa Rica	Octopus Spring, Yellowstone National Park	Terrestrial hot spring, Iceland
Cell size (µm; length x width)	2.4-3.9 x 0.5-0.6	1-3 x 0·4	1-3 x 0·5-0·6
Temperature range (opt.) [°C]	65-85 (75)	44-89 (80)	55-89 (NR)
pH range (opt.)	4.8-7.8 (5·9-6·5)	Range NR (7 and 8·5)	Range NR (7)
NaCl range (opt.) [v/w]	0-0·4 % (NR)	0-0·4 % (NR)	<0·7 % (NR)
Electron donors	H_2, S°, $S_2O_3^{2-}$	H_2, S°, $S_2O_3^{2-}$	H_2, S°, $S_2O_3^{2-}$
Electron acceptors	O_2 (up to 16 % v/v)	O_2 (up to 6 % v/v)	O_2 (limit NR)
Organic carbon sources	Yeast, mannose, glucose, maltose, succinate, peptone, Casamino acids, starch, and citrate	Formate, formamide	None
G+C content (mol%)	40·3	47·2	49·6

Figure Legends

Fig. 1. Transmission electron micrographs of thin sections (A, B, and D) and a negatively stained cell (C) of CR11^T. The arrow in B is pointing to the cytoplasmic structures with unknown function. The scale bar in A, B, and D is 0.5 µm; the scale bar in C is 2 µm.

Fig. 2. Maximum likelihood phylogenetic tree inferred from 16S rRNA gene sequences (1370 nt) showing the relative position of CR11^T within the *Aquificaceae*. Bootstrap values correspond to 100 replicates. The tree topology was confirmed by the neighbor-joining algorithm.

Supplementary Fig. 1. Effects of temperature (A) and pH (B) on the growth of *Thermocrinis minervae* CR11^T.

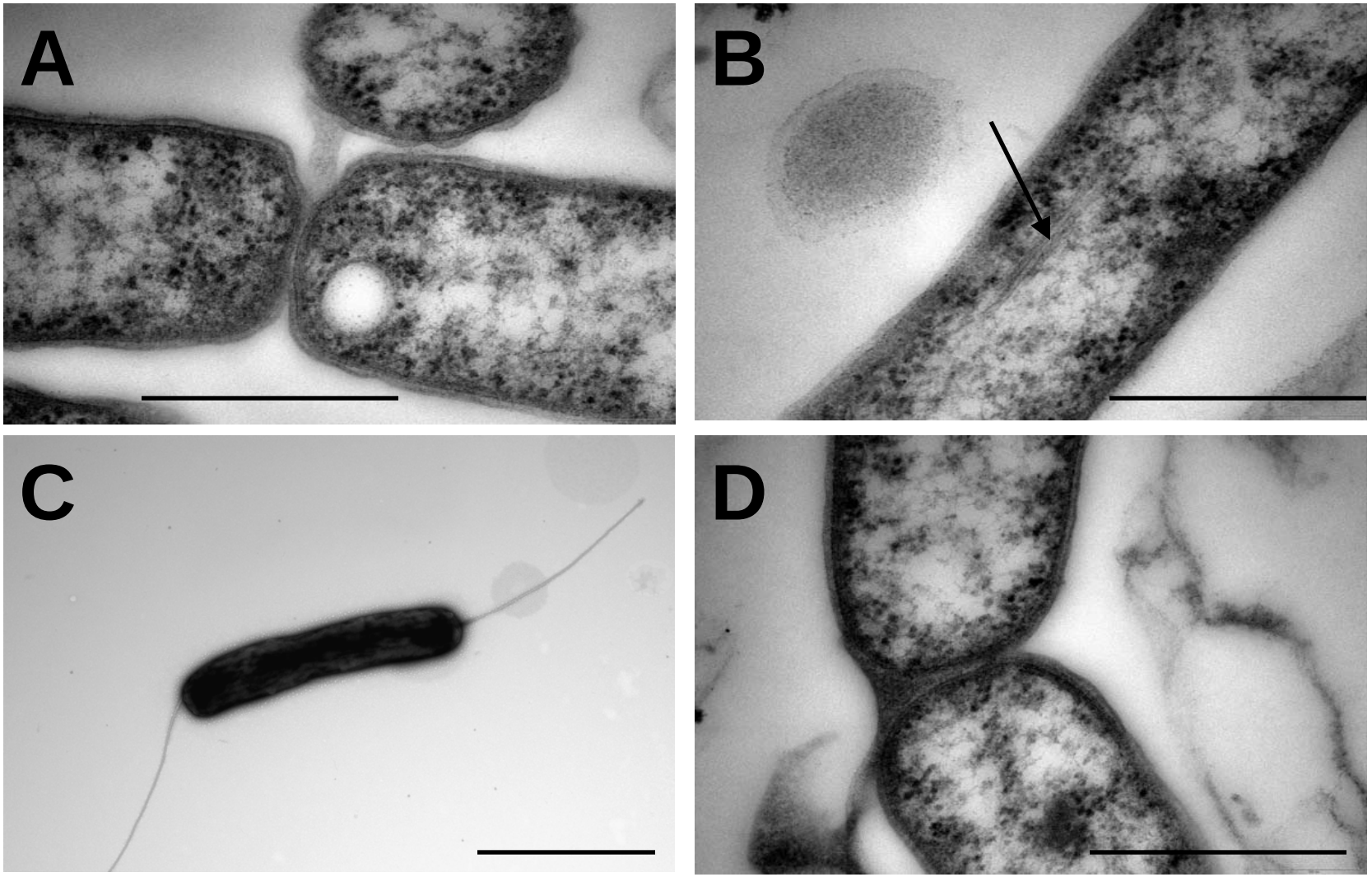


Figure 1

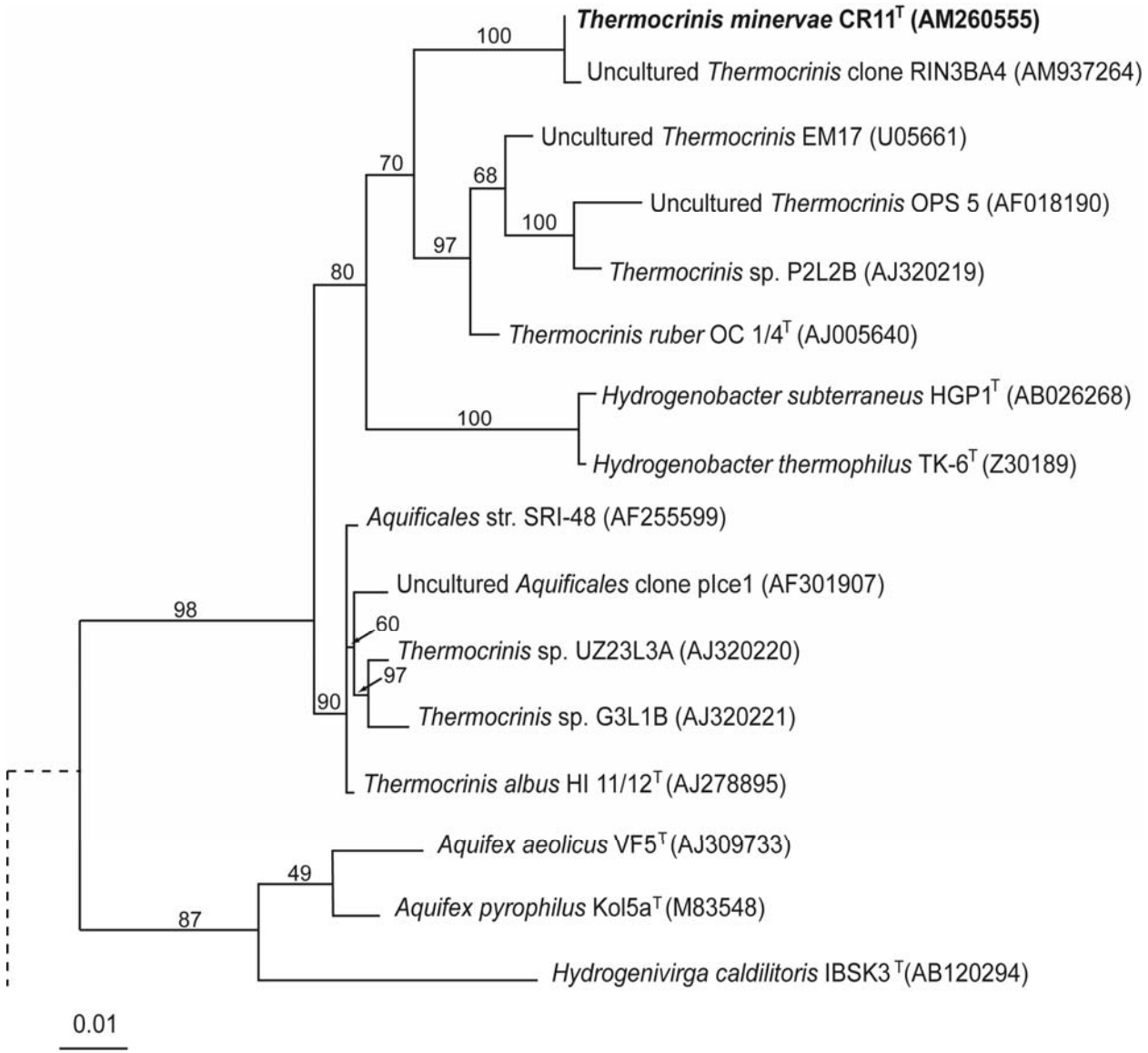
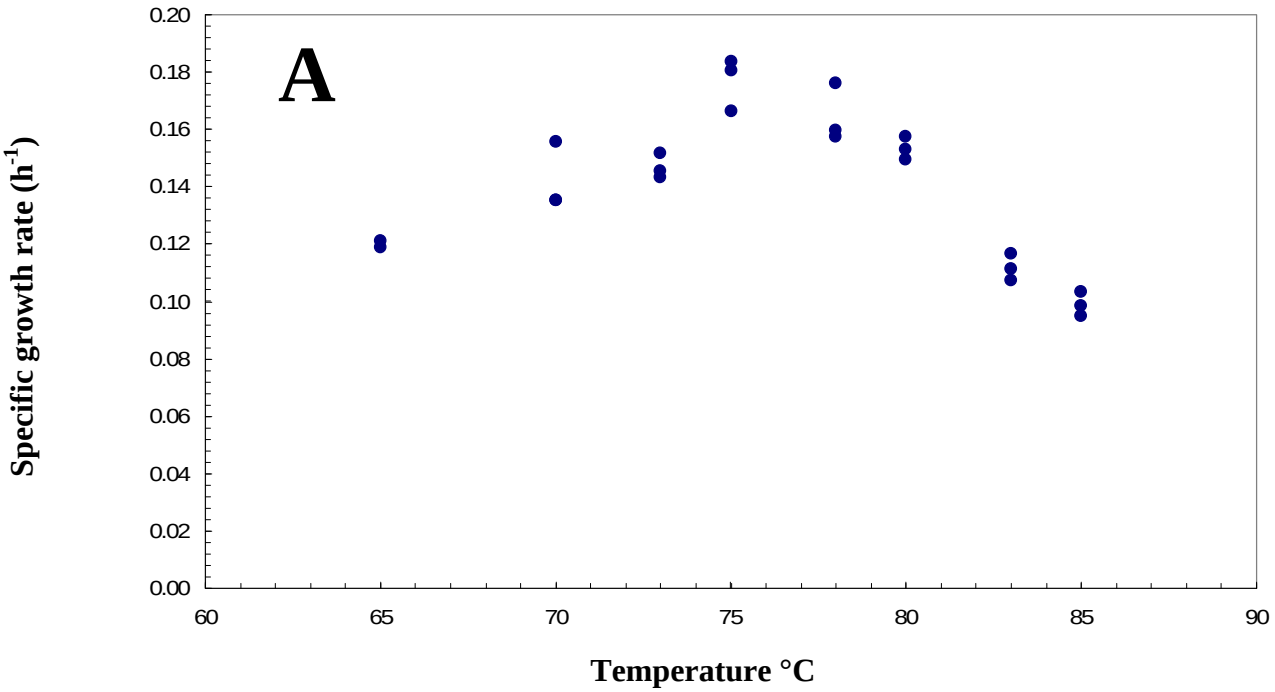
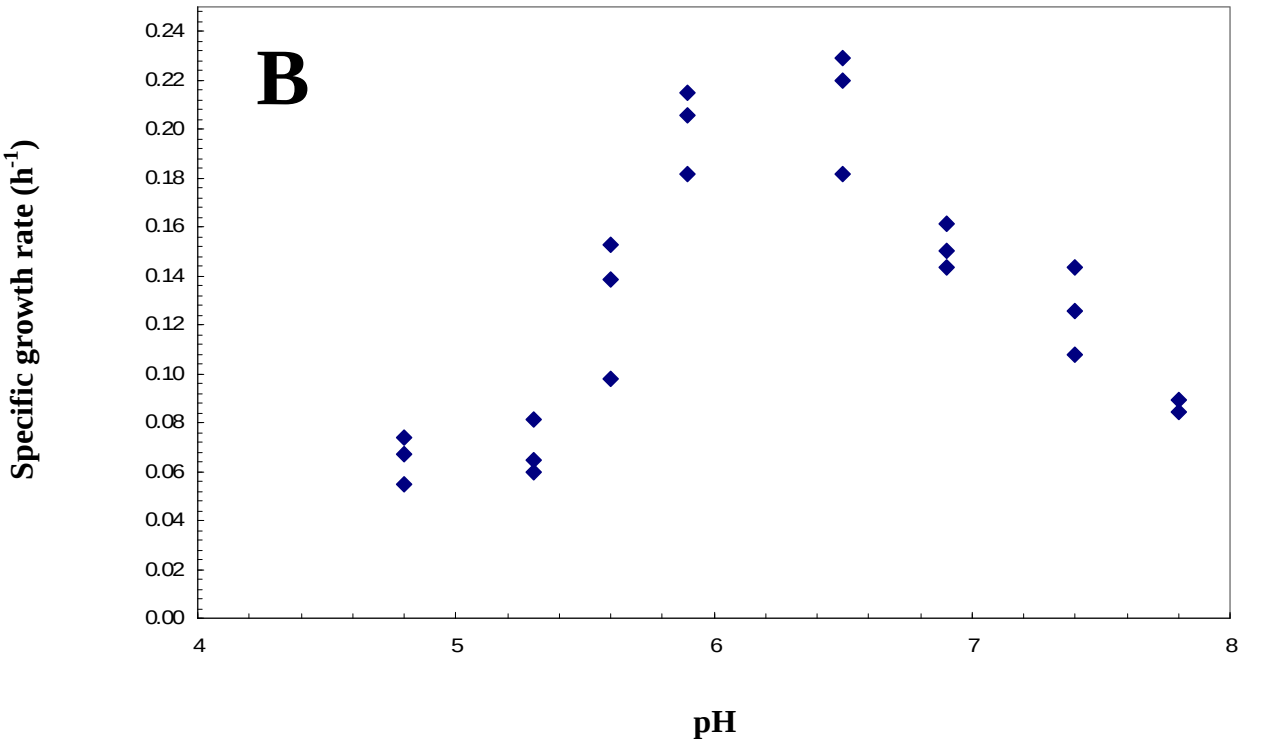

***Thermocrinis minervae* CR11^T (AM260555)**
Uncultured *Thermocrinis* clone RIN3BA4 (AM937264)
Uncultured *Thermocrinis* EM17 (U05661)
Uncultured *Thermocrinis* OPS 5 (AF018190)
Thermocrinis sp. P2L2B (AJ320219)
Thermocrinis ruber OC 1/4^T (AJ005640)
Hydrogenobacter subterraneus HGP1^T (AB026268)
Hydrogenobacter thermophilus TK-6^T (Z30189)
Aquificales str. SRI-48 (AF255599)
Uncultured *Aquificales* clone pIce1 (AF301907)
Thermocrinis sp. UZ23L3A (AJ320220)
Thermocrinis sp. G3L1B (AJ320221)
Thermocrinis albus HI 11/12^T (AJ278895)
Aquifex aeolicus VF5^T (AJ309733)
Aquifex pyrophilus Kol5a^T (M83548)
Hydrogenivirga caldilitoris IBSK3^T (AB120294)
100
70
68
100
97
80
100
98
60
97
90
49
87
0.01

Figure 2





Supplementary Figure 1