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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A thermophilic bacterium, designated strain CR11^T, was isolated from a filamentous sample collected from a terrestrial hot spring on the south-western foothills of the Rincón volcano in Costa Rica. The Gram-negative cells are approximately 2.4–3.9 µm long and 0.5–0.6 µm wide and are motile rods with polar flagella. Strain CR11^T grows between 65 and 85 °C (optimum 75 °C, doubling time 4.5 h) and between pH 4.8 and 7.8 (optimum pH 5.9–6.5). The isolate grows chemolithotrophically with S⁰, S₂O₃^{2–} or H₂ as the electron donor and with O₂ (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 S⁰. Growth occurs only at NaCl concentrations below 0.4% (w/v). The G+C content of strain CR11^T is 40.3 mol%. Phylogenetic analysis of the 16S rRNA gene sequence places the strain as a close relative of *Thermocrinis ruber* OC 1/4^T (95.7% sequence similarity). Based on phylogenetic and physiological characteristics, we propose the name *Thermocrinis minervae* sp. nov., with CR11^T (=DSM 19557^T =ATCC BAA-1533^T) as the type strain.

The members of the order *Aquificales*, represented by the families *Aquificaceae*, *Hydrogenothermaceae* and *Desulfurobacteriaceae*, are thermophilic bacteria that are widely distributed in hydrothermal systems and include micro-aerophilic 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, 2003a, b, c; Barion *et al.*, 2007).

The family *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 gene 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 in Yellowstone National Park (Reysenbach *et al.*, 1994). Following this study, Huber *et al.* (1998) isolated the dominant member of the *Aquificales* from this hot spring on a medium containing organic acids and named it *Thermocrinis ruber*. Subsequently, numerous new *Thermocrinis* isolates, including a strain assigned to the novel species *Thermocrinis albus*, were obtained from hot springs in Russia, Iceland and Yellowstone National Park (Eder & Huber, 2002).

Cultivation studies and associated geochemical analyses (Huber *et al.*, 1998; Eder & 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 & Huber, 2002) in many ecosystems where

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

Graphs showing the effect of temperature and pH on growth of strain CR11^T are available as supplementary material with the online version of this paper.

photosynthesis is limited by high temperatures. Their ability to oxidize sulfur in laboratory cultures (Huber *et al.*, 1998; Eder & Huber, 2002) suggests that these bacteria may also contribute to sulfur cycling in these environments. Additionally, 16S rRNA gene sequence studies of a biofilm associated with As(III) oxidation in the Alvord hot spring in Oregon indicated the presence of bacteria related to *Thermocrinis* and other genera of the *Aquificales* (Connon *et al.*, 2008).

T. ruber and *T. albus* share similar physiological properties, including pH, temperature and salinity optima (Huber *et al.*, 1998; Eder & Huber, 2002). Isolates of both species 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 & Huber, 2002). Based on 16S rRNA gene sequences, these isolates are somewhat distantly related (5.1 % sequence difference; Eder & Huber, 2002).

Here, we report the isolation and characterization of a strain that represents a novel 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 geographical 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 south-western foothills of the Rincón de la Vieja volcano in Costa Rica. A subsample was inoculated into 5 ml modified MSH medium (pH 6.2) under a gas phase of CO₂/H₂ (20:80) (Aguiar *et al.*, 2004). Prior to inoculation, O₂ (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. Samples of these cultures were transferred immediately into the same medium 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 S⁰ than with H₂ 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) S⁰ and 4 % (v/v) O₂.

Morphology

Cells were routinely monitored under phase-contrast microscopy using an Olympus BX60 microscope. Electron microscope examination was performed as described previously (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 by 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.

Cells of strain CR11^T 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 of unknown function (Fig. 1b). These structures have been reported previously in other members of the *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 and 85 °C, with optimum growth occurring at about 75 °C (doubling time 4.5 h; Supplementary Fig. S1a, available in IJSEM Online). This growth range is below the temperature measured in the spring during sample collection. It is well-established that growth under laboratory conditions may not directly reflect the growth conditions in the environment; alternatively, a lower-temperature variant was selected for in this study. The effect of pH on growth was determined by adjusting the medium 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). Strain CR11^T grew between pH 4.8 and 7.8 and optimally between pH 5.9 and 6.5 (Supplementary Fig. S1b). No growth occurred below pH 4.8 or above pH 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 medium containing 0–0.4 % NaCl.

Electron donors and acceptors were added to modified MSH medium without S₂O₃²⁻ (since it may be used as an electron donor) and containing a reduced concentration of MgSO₄·7H₂O (4 g l⁻¹). Electron couples were added aseptically after autoclaving and at concentrations reported by Aguiar *et al.* (2004). Medium used for determining growth of CR11^T with H₂ as the electron donor was prepared with H₂/CO₂ (80:20) as the gas phase and with S⁰,

Fig. 1. Transmission electron micrographs of thin sections (a, b, d) and a negatively stained cell (c) of strain CR11^T. The arrow in (b) indicates an example of the cytoplasmic structures with unknown function. Bars, 0.5 µm (a, b, d) and 2 µm (c).

$\text{S}_2\text{O}_3^{2-}$, NO_3^- , SO_3^{2-} , SO_4^{2-} , arsenate (as $\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$), 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 medium with a gas phase of N_2/CO_2 (80:20). Electron donors and acceptors were added to this medium as follows: $\text{S}_2\text{O}_3^{2-}/\text{Fe}^{3+}$ (as ferric citrate), Fe^{2+} (as FeCl_2)/ O_2 , Fe^{2+} (as FeCl_2)/ NO_3^- , $\text{S}_2\text{O}_3^{2-}/\text{NO}_3^-$, NH_4^+/O_2 , $\text{NH}_4^+/\text{NO}_2^-$, $\text{S}_2\text{O}_3^{2-}/\text{SO}_4^{2-}$, $\text{SO}_3^{2-}/\text{O}_2$, $\text{S}_2\text{O}_3^{2-}$ /arsenate (as $\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$), arsenite (as NaAsO_2)/ O_2 , $\text{S}_2\text{O}_3^{2-}$ /selenate (as Na_2SeO_4), S^0 /selenite (as Na_2SeO_3) and $\text{S}_2\text{O}_3^{2-}$ /selenite. Strain CR11^T grew with H_2 , S^0 and $\text{S}_2\text{O}_3^{2-}$ as electron donors and with O_2 as the sole electron acceptor. The isolate grew at 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 by Aguiar *et al.* (2004) to modified MSH medium containing no CO_2 or $\text{S}_2\text{O}_3^{2-}$. Growth was monitored in the presence of O_2 as an electron acceptor and S^0 as an electron donor. Cultures were also incubated in the absence of O_2 and S^0 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 medium. Strain CR11^T grew with 0.1 % mannose, glucose, maltose, succinate, Bacto peptone, Casamino acids, starch, citrate and yeast extract as carbon sources with S^0 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^0 . 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 source.

DNA composition and phylogenetic analysis

DNA base composition (mol% G + C) was determined by thermal denaturation of genomic DNA (Marmur & 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 35 mol%, for *Hydrogenobaculum acidophilum*; Shima & Suzuki, 1993).

Table 1. Comparison of physiological properties and DNA base composition between CR11^T and other described representatives of *Thermocrinis*

All three strains use only H₂, S⁰ and S₂O₃²⁻ as electron donors and O₂ as electron acceptor. Optimum NaCl concentrations for growth have not been reported for any of the strains. NR, Not reported.

Property	Strain CR11 ^T	<i>T. ruber</i> OC 1/4 ^T	<i>T. albus</i> HI 11/12 ^T
Origin	Terrestrial hot spring, Costa Rica	Octopus Spring, Yellowstone National Park	Terrestrial hot spring, Iceland
Cell size (µm) (length × width)	2.4–3.9 × 0.5–0.6	1–3 × 0.4	1–3 × 0.5–0.6
Temperature range (optimum) (°C)	65–85 (75)	44–89 (80)	55–89 (NR)
pH range (optimum)	4.8–7.8 (5.9–6.5)	NR (7–8.5)	NR (7)
NaCl range (% w/v)	0–0.4	0–0.4	<0.7
Maximum O ₂ concentration (% v/v)	16	6	NR
Organic carbon sources	Yeast, mannose, glucose, maltose, succinate, peptone, Casamino acids, starch, citrate	Formate, formamide	None
DNA G + C content (mol%)	40.3	47.2	49.6

The 16S rRNA gene was amplified by PCR and sequenced as described by Ferrera *et al.* (2007). The nearly full-length sequence of the 16S rRNA gene was assembled using AutoAssembler (Applied Biosystems) and compared using a BLAST search against the NCBI non-redundant database. 16S rRNA gene sequences were aligned manually using the ARB program (Ludwig *et al.*, 2004; <http://www.arb-home.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, 2003) using representative sequences of all members of the *Aquificales* and including only unambiguously aligned nucleotides (1370 nt). Neighbour-joining (NJ; 1000 bootstrap replications) and maximum-likelihood (ML; 100 bootstrap replications) analyses were performed in ARB and PAUP* as described previously (Ferrera *et al.*, 2007). Since the NJ and ML tree topologies are nearly identical, only the ML tree is shown in Fig. 2.

Comparison with related species

The 16S rRNA gene sequence analysis places strain CR11^T within a novel species of 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* OC 1/4^T (95.7 % similar in 16S rRNA gene sequence). Strain CR11^T and clone RIN3BA4 form a lineage separate from *T. ruber* OC 1/4^T 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 & Huber, 2002). The new isolate and clone RIN3BA4 are less than 95 % similar in the 16S rRNA gene (below the cut-off for relatedness at the genus level; Stackebrandt & Goebel, 1994) to all sequences within the *T. albus*-like clade (94.8 % similarity between CR11^T and *T. albus* HI 11/12^T).

Fig. 2. Maximum-likelihood phylogenetic tree inferred from 16S rRNA gene sequences (1370 nt) showing the relative position of strain CR11^T within the *Aquificaceae*. Bootstrap values correspond to 100 replicates. The tree topology was confirmed by the neighbour-joining algorithm. Bar, 0.01 substitutions per nucleotide position.

The phylogenetic distance between these groups is similar to the previously reported distance of 5.1%, based on maximum-parsimony analysis, between *T. ruber* OC 1/4^T and *T. albus* HI 11/12^T (Eder & Huber, 2002).

The strains of *Thermocrinis* (including CR11^T) are physiologically similar with respect to growth temperature range, low NaCl tolerance and 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 content than either *T. ruber* or *T. albus*. Therefore, based on physiological and phylogenetic characteristics, we propose the novel species *Thermocrinis minervae* sp. nov. to accommodate strain CR11^T.

Description of *Thermocrinis minervae* sp. nov.

Thermocrinis minervae (mi.ner'vae. L. fem. gen. n. *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, approx. 2.4–3.9 µm long and 0.5–0.6 µm wide. Cells occur singly. Growth occurs at 65–85 °C (optimum 75 °C), pH 4.8–7.8 (optimum pH 5.9–6.5) and 0–0.4% (w/v) NaCl. Grows chemolithoautotrophically with H₂, S⁰ and S₂O₃²⁻ as electron donors and with only O₂ (up to 16%, v/v) as the electron acceptor. Able to use yeast extract, mannose, glucose, maltose, succinate, peptone, Casamino acids, starch, citrate and CO₂ as carbon sources. The G+C content of genomic DNA of the type strain is 40.3 mol%.

The type strain, CR11^T (=DSM 19557^T =ATCC BAA-1533^T), was isolated from a terrestrial hot spring on the south-western foothills of the Rincón de la Vieja volcano in Costa Rica.

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