Thermocrinis jamiesonii sp. nov., a thiosulfate-oxidizing, autotropic thermophile isolated from a geothermal spring

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An obligately thermophilic, chemolithotrophic, microaerophilic bacterium, designated strain GBS1^T, was isolated from the water column of Great Boiling Spring, Nevada, USA. Thiosulfate was required for growth. Although capable of autotrophy, growth of GBS1^T was enhanced in the presence of acetate, peptone or Casamino acids. Growth occurred at 70–85 °C with an optimum at 80 °C, at pH 6.50–7.75 with an optimum at pH 7.25, with 0.5–8 % oxygen with an optimum at 1–2 % and with $\leq$200 mM NaCl. The doubling time under optimal growth conditions was 1.3 h, with a final mean cell density of $6.2 \pm 0.5 \times 10^7$ cells ml^{-1}. Non-motile, rod-shaped cells 1.4–2.4 × 0.4–0.6 μm in size occurred singly or in pairs. The major cellular fatty acids (>5 % of the total) were $C_{20:1}\omega 9c$, $C_{18:0}$, $C_{16:0}$ and $C_{20:0}$. Phylogenetic analysis of the GBS1^T 16S rRNA gene sequence indicated an affiliation with *Thermocrinis ruber* and other species of the genus *Thermocrinis*, but determination of 16S rRNA gene sequence similarity ($\leq$97.10 %) and *in silico* estimated DNA–DNA hybridization values ($\leq$18.4 %) with the type strains of recognized *Thermocrinis* species indicate that the novel strain is distinct from described species. Based on phenotypic, genotypic and phylogenetic characteristics, a novel species, *Thermocrinis jamiesonii* sp. nov., is proposed, with GBS1^T (=JCM 19133^T=DSM 27162^T) as the type strain.

The genera *Hydrogenobaculum*, *Hydrogenobacter* and *Thermocrinis*, members of the family *Aquificaceae*, are thermophilic bacteria that are found in terrestrial geothermal systems worldwide and may be important primary producers in these environments (Boyd *et al.*, 2009; Spear *et al.*, 2005). The genus *Thermocrinis* comprises, at the time of writing, three formally described species: *Thermocrinis ruber*, isolated from Octopus Spring in Yellowstone National Park, USA (Huber *et al.*, 1998); *Thermocrinis albus*, isolated from an Icelandic hot spring (Eder & Huber, 2002); and *Thermocrinis minervae*, isolated from a Costa Rican hot spring (Caldwell *et al.*, 2010). Four additional *Thermocrinis* strains have been isolated that are affiliated to *T. albus* (strains G3L1B, UZ23L3A and H7L1B) or *T. ruber* (strain P2L2B; Eder & Huber, 2002). All three characterized species are obligate microaerophiles that can use hydrogen, thiosulfate and elemental sulfur as sole electron donors for chemolithoautotrophic growth. While *T. albus* is apparently an obligate autotroph, *T. ruber* can use formate and formamide as sole carbon sources, and *T. minervae* can use a variety of simple and complex organic compounds as carbon sources (Caldwell *et al.*, 2010). Collectively, *Thermocrinis* species are often prominent members of microbial communities inhabiting circumneutral, terrestrial geothermal springs (Blank *et al.*, 2002; Eder & Huber, 2002; Hall *et al.*, 2008; Meyer-Dombard *et al.*, 2005, 2011; Reysenbach *et al.*, 1994; Spear *et al.*, 2005; Takacs *et al.*, 2001), including those in the Great Basin region of the United States (Connon *et al.*, 2008; Costa *et al.*, 2009; Vick *et al.*, 2010). In one such spring, Great Boiling Spring (GBS) near Gerlach,

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Abbreviations: DDH, DNA–DNA hybridization; GBS, Great Boiling Spring; JGI, Joint Genome Institute.

The GenBank/EMBL/DDBJ accession number for the draft genome sequence of strain GBS1^T is NZ_JNIE00000000.

One supplementary table, one supplementary figure and one supplementary file are available with the online Supplementary Material.

Nevada, USA, a single *Thermocrinis* species-level operational taxonomic unit consistently represented >85 % of 16S rRNA gene tag datasets obtained from samples of the spring water column (Cole *et al.*, 2013a). This paper describes the isolation and characterization of a novel strain of *Thermocrinis*, GBS1^T, from GBS spring water.

A water sample from site A (Cole *et al.*, 2013a) on the north side of the GBS source pool was collected in a sterile 50 ml polypropylene tube on 14 March 2012. The water temperature was 81 °C, pH was 7.56 and conductivity was 8.18 mS cm^{-1} at the time of collection, measured using hand-held pH5 and Con5 meters (LaMotte). The water sample was collected without air headspace by capping the tube while submerged. The sample was transported back to the laboratory at ambient temperature, and transferred to an anaerobic chamber (Coy) where it was stored until inoculation (~48 h). A solid medium based on GBS salts medium buffered at pH 7 with 10 mM sodium phosphate was prepared anaerobically as described previously (Dodsworth *et al.*, 2014). The medium was solidified with 0.8 % (w/v) Gelrite gellan gum (Serva Electrophoresis) and 0.4 % (w/v) $MgCl_2.6H_2O$, and contained 1 mM sodium thiosulfate, 1 mM sodium acetate, 0.05 % yeast extract, 1 mM sodium bicarbonate and 1× concentration of a vitamin mixture (Balch *et al.*, 1979), which were added as concentrated, sterile stocks to the autoclaved medium before pouring in the anaerobic chamber. Using a streak plate technique, 20 µl aliquots of the GBS sample were spread onto plates of this solid medium, which were then sealed in a 2 litre anaerobic incubation vessel (Hedlund *et al.*, 2015) with ambient anaerobic chamber headspace (an approx. 90 : 5 : 5 mixture of $N_2/H_2/CO_2$). Vessel headspace was supplemented with 100 ml (1/20 volume) of air after removal from the anaerobic chamber, and vessels were incubated at 75 °C for 7 days. After incubation and inspection in the anaerobic chamber, approximately 200–300 colonies with apparently identical morphology (~0.5 mm in diameter, round and entire, cream in colour) were observed. Four of these colonies were streaked for isolation three times on the same medium. After this isolation procedure, single colonies from each of the four strains (GBS1–GBS4) were inoculated into 5 ml liquid GBS salts medium in 25 ml serum tubes sealed with butyl rubber stoppers (Dodsworth *et al.*, 2014) containing 1 mM sodium thiosulfate, 1 mM sodium bicarbonate and nitrogen headspace supplemented with 1 ml (1/20 volume) air. Tubes were incubated horizontally without shaking at 75 °C and growth, as assessed by faint turbidity and confirmed using phase-contrast microscopy, of rod-shaped cells was observed in 2 days, with a final density of $\sim 2 \times 10^6$ cells ml^{-1} as determined by counting in a Petroff-Hausser counting chamber (Hausser Scientific). For all four strains, growth under chemolithoautotrophic conditions in liquid medium was apparently stimulated by inclusion of 1 mM sodium acetate (typical final density of $6.2 \pm 0.5 \times 10^7$ cells ml^{-1}) but not by 0.01 % yeast extract. Furthermore, addition of the vitamin mixture was not necessary for growth, and no growth was observed with either yeast extract or acetate in the absence of thiosulfate or when hydrogen (1 or 10 ml added to headspace) was included in place of thiosulfate. DNA was extracted from single colonies of each of the four strains using an SDS-NaOH lysis method (Johnson *et al.*, 2001), near-full-length 16S rRNA genes were amplified by PCR using primers 9bF and 1492uR as described by Vick *et al.* (2010), and approximately 810 nt of sequence was obtained using Sanger sequencing with the 9bF primer. The sequence of all four strains was identical over this region, and thus one of the strains, GBS1^T, was selected for further characterization.

Growth conditions for characterization of strain GBS1^T, unless otherwise stated, were in GBS salts medium (5 ml in 25 ml serum tubes) with 1 mM sodium thiosulfate, 1 mM sodium bicarbonate, 1 mM sodium acetate and 1 ml air (~1 % oxygen) at 80 °C and pH 7.25. These conditions were used for routine maintenance of the strain. Treatment of butyl rubber stoppers with sodium sulfide as described by Dodsworth *et al.* (2014) was not necessary for consistent growth. To obtain larger amounts of cells for DNA isolation, electron microscopy and fatty acid analysis, 150 ml cultures in 500 ml bottles with caps containing butyl rubber septa were used (Dodsworth *et al.*, 2014). For long-term storage, 0.5 ml aliquots of culture in 10 % DMSO were frozen on dry ice and remained viable for at least 2 years when stored at −80 °C. Growth under various conditions described below was assessed on triplicate cultures after 1, 2, 4 and 7 days of incubation by the presence of faint turbidity and was confirmed by phase-contrast microscopy in a Petroff-Hausser counting chamber. Positive growth corresponded to a mean of $>5 \times 10^5$ cells in triplicate experiments (Hedlund *et al.*, 2015).

Growth temperature range was tested by incubation at 50–90 °C in 5 °C increments, and growth was observed at 70–85 °C. Generation times, calculated from growth curves using a Petroff-Hausser counting chamber for quantification of cell concentrations, were determined at 75 °C (1.67 h), 80 °C (1.3 h) and 85 °C (2.1 h), indicating a temperature optimum of 80 °C. The pH range for growth was tested at pH values from 6.25–8.00 in 0.25 unit increments, using 10 mM sodium phosphate in the GBS salts base medium as the buffering agent. All pH measurements were performed at room temperature, and were confirmed in the medium after incubation. Growth was observed at pH 6.50–7.75, with an optimum at pH 7.25. Sodium tolerance was determined in medium without sodium acetate and with potassium phosphate as a buffering agent in place of sodium phosphate, where the only sodium present was due to the sodium thiosulfate (2 mM) used as electron donor and NaCl added to various final concentrations (0, 5, 10, 20, 50, 100, 150, 200, 300 and 400 mM). Growth was observed from 2 mM (no added sodium chloride) to 200 mM NaCl (~1.16 %, w/v), but not at 300 mM or above. In the absence of sodium thiosulfate,

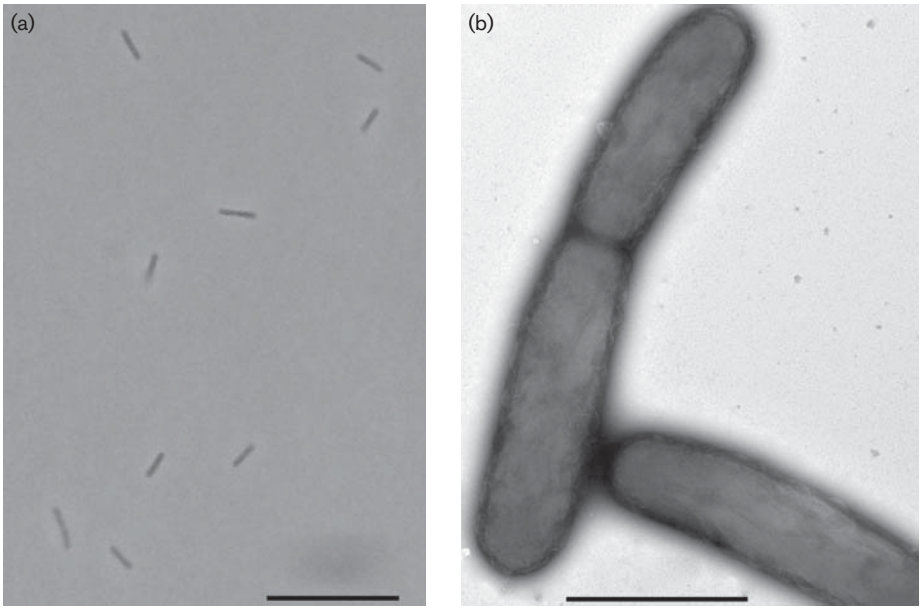

Fig. 1. (a) Phase-contrast photomicrograph and (b) transmission electron micrograph of negatively stained whole cells of strain GBS1^T. Bars, 10 μm (a), 1 μm (b).

none of the following potential electron donors supported growth: 0.05 or 3 % (w/v) elemental sulfur (S^0); 1 mM sodium formate, formaldehyde, formamide, sodium acetate or ferrous sulfate; headspace of 5 or 50 % hydrogen. Growth was observed with 0.5, 1, 2, 4 and 8 % oxygen (optimum growth with 1–2 %), but was not observed with 16 % oxygen or in the absence of oxygen without or with potential alternative electron donors [0.05 or 3 % (w/v) S^0; 2 mM sodium nitrate, sodium nitrite, sodium fumarate or sodium sulfite], suggesting that GBS1^T is an obligate microaerophile. Various potential organic compounds were tested for their ability to serve as sole carbon sources in the absence of sodium bicarbonate and using medium that was not exposed to the anaerobic chamber headspace during preparation to prevent uptake of CO_2. Under these conditions, and only in the presence of sodium thiosulfate, growth was observed with 0.05 % (w/v) peptone, 0.05 % (w/v) Casamino acids and 1 mM sodium acetate, but no growth was observed with 0.05 % (w/v) yeast extract, 1 mM glucose, 1 mM sodium formate, 1 mM formaldehyde or 1 mM formamide. Peptone, Casamino acids and acetate at the concentrations above also resulted in an increase in total growth yield ($4–8 \times 10^7$ cells ml^{-1}) in the presence of sodium bicarbonate in comparison with sodium bicarbonate only ($\sim 2 \times 10^6$ cells ml^{-1}). GBS1^T therefore appears to be an obligate chemolithotroph that can grow autotrophically but will readily assimilate and benefit from exogenous organic carbon if appropriate substrates are present.

Cells of GBS1^T were present as rods or pairs of rods (Fig. 1a) in cultures grown at 80 °C for 2 days without agitation. Individual cells observed by whole-cell transmission electron microscopy with negative staining with Nano-W (Nanoprobes), performed as described previously (Cole *et al.*, 2013b), typically ranged from 1.4 to 2.4 μm in length and 0.4 to 0.6 μm in width (Fig. 1b). Cells were not motile in wet mounts at room temperature, and flagella were not observed by transmission electron microscopy (Fig. 1b). Cells grown in liquid culture for 2 days or on plates for 7 days were negative for the Gram stain reaction. Although similar in overall appearance as described above during initial isolation of GBS1^T, colonies on solid media grown under autotrophic conditions (excluding yeast extract and acetate) were considerably smaller, ~0.1–0.2 mm in diameter, and were typically barely visible to the naked eye.

Fatty acid methyl ester analysis, performed by Microbial ID using the MIDI Sherlock Microbial Identification System, indicated that the major cellular fatty acids (>5 % of the total) identified in cells grown under standard conditions for 2 days were $C_{20:1}\omega 9c$ (44.8 %), $C_{18:0}$ (26.0 %), $C_{16:0}$ (9.9 %) and $C_{20:0}$ (5.4 %). Other cellular fatty acids detected were $C_{18:1}\omega 9c$ (4.0 %), $C_{20:1}\omega 6c$ (2.6 %), $C_{16:4}\omega 3c$ (2.5 %), $C_{18:1}\omega 7c$ (1.5 %), $C_{22:1}\omega 9c$ (0.9 %), $C_{14:0}$ (0.8 %), $C_{18:3}\omega 6c$ (0.7 %), $C_{16:1}\omega 9c$ (0.5 %) and $C_{12:0}$ (0.5 %). The $C_{20:1}$, $C_{20:0}$ and $C_{22:1}$ lipids detected in GBS1^T are considered among a set of 'signature lipids' for members of the order *Aquificales* (Jahnke *et al.*, 2001), and are present in *T. ruber* and *T. albus* (Eder & Huber, 2002). DNA was isolated from a cell pellet obtained by centrifugation of a 150 ml culture of GBS1^T using lysozyme, proteinase K and SDS following a standard protocol at the US

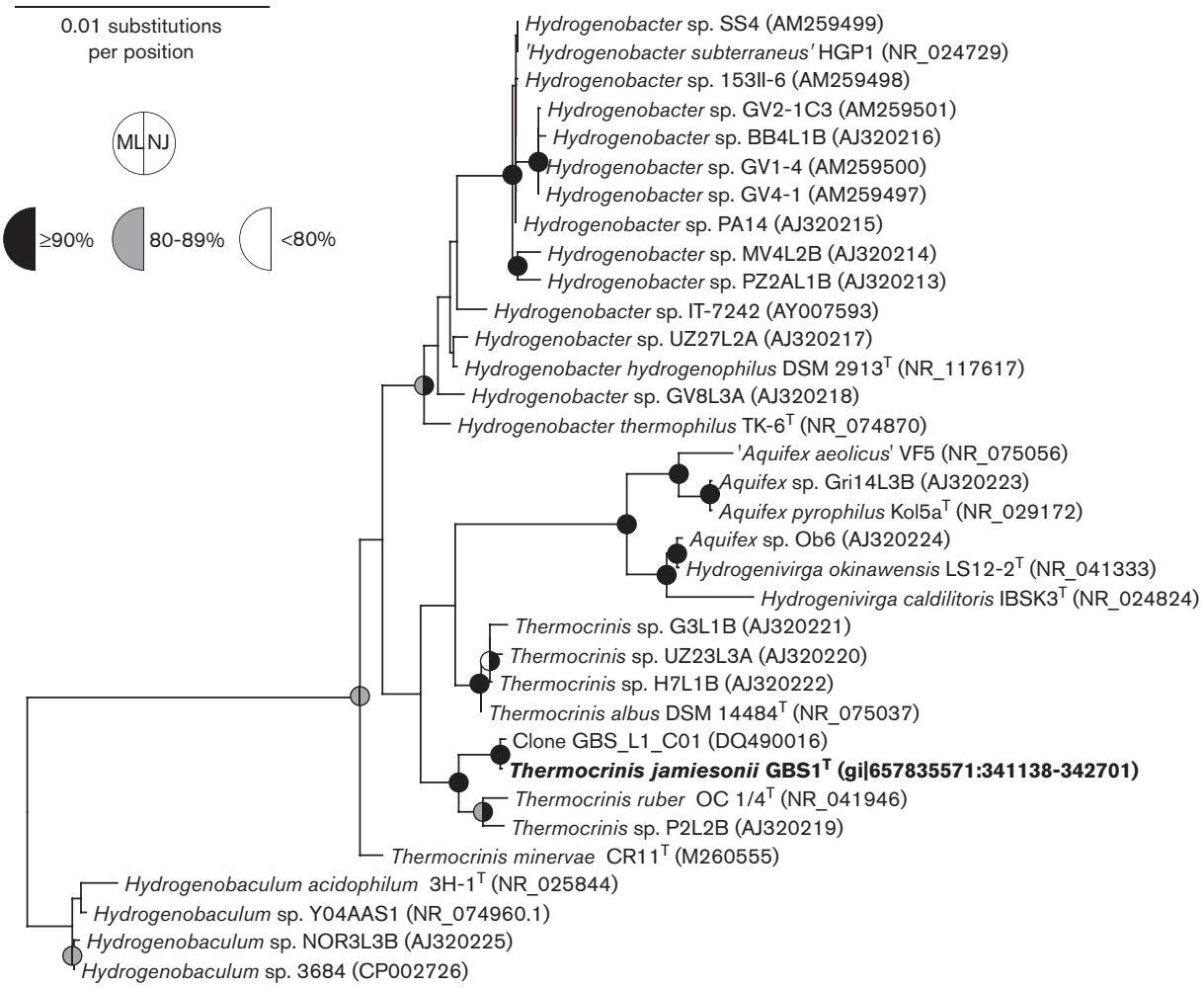

Fig. 2. Relative placement of strain GBS1^T and cloned 16S rRNA gene sequence GBS_L1_C01, obtained from GBS water column (Costa *et al.*, 2009), among cultivated *Thermocrinis* species and various cultivated members of the *Aquificaceae*. Unrooted maximum-likelihood phylogeny was inferred from an alignment of 1391 positions using RaxML (Stamatakis, 2006), and similar topologies were obtained when *Methanocaldococcus jannaschii* or other members of the *Aquificales* were used as outgroups (Hedlund *et al.*, 2015). Filled, grey-shaded and open half-circles indicate bootstrap support for nodes in 100 pseudoreplicates using the maximum-likelihood (ML) and 1000 pseudoreplicates using the distance matrix/neighbour-joining (NJ; using the same alignment, shown in Fig. S1) methods. The alignment used for phylogenetic inference is shown in File S1.

Department of Energy's Joint Genome Institute (JGI) for bacterial genomic DNA isolation using cetyltrimethylammonium bromide (JGI Bacterial DNA isolation CTAB-2012; http://jgi.doe.gov/collaborate-with-jgi/pmo-overview/protocols-sample-preparation-information/), and shotgun genome sequencing and draft genome assembly was performed at JGI. The draft genome sequence (JGI IMG genome ID 2562617198, NCBI accession number NZ_JNIE00000000) will be more thoroughly described in a separate publication, however several relevant features are presented here. A DNA G+C content for GBS1^T of 41.32 mol% was calculated from the draft genome. Several markers diagnostic of a diderm (i.e. Gram-negative-like) cell envelope structure (Sutcliffe, 2011), including BamA/YaeT (Pfams 1103 and 7244), TolC (Pfam 2321), secretin (Pfam0263), and flagellar L- and P-rings (Pfams 2107 and 2119), were encoded in the GBS1^T genome (Table S1, available in the online Supplementary Material). The presence of genes for flagellar L- and P-rings and other flagellar components suggests that GBS1^T may be capable of motility under conditions other than those tested above. Other chemotaxonomic analyses, such as determination of major quinones and cell-wall composition, were not performed due to the relative difficulty in obtaining sufficient amounts of biomass from GBS1^T.

Table 1. Comparison of characteristics of strain GBS1^T and the type strains of recognized *Thermocrinis* species

Strains: 1, GBS1^T; 2, *T. albus* HI 11/12^T (data from Eder & Huber, 2002); 3, *T. minervae* CR11^T (Caldwell *et al.*, 2010); 4, *T. ruber* OC 1/4^T (Huber *et al.*, 1998). ND, Not determined.

	1	2	3	4
Cell size (μm) (length × width)	1.4–2.4 × 0.4–0.6	1–3 × 0.5–0.6	2.4–3.9 × 0.5–0.6	1–3 × 0.4
Temperature range (optimum) (°C)	70–85 (80)	55–89 (ND)	65–85 (75)	44–89 (80)
pH range (optimum)	6.5–7.75 (7.25)	ND (7)	4.8–7.8 (5.9–6.5)	ND (7–8.5)
NaCl range (%, w/v)	≤1.17	≤0.7	≤0.4	≤0.4
Maximum O_2 concentration (%, v/v)	8	ND	16	6
Organic C sources utilized*				
Yeast extract	−	ND	+	−
Peptone	+	ND	+	−
Casamino acids	+	ND	+	ND
Glucose	−	ND	+	ND
Formate	−	−	−	+
Formamide	−	−	−	+
Acetate	+	ND	−	−
Electron donor(s) for chemolithotrophic growth	Thiosulfate	Thiosulfate, sulfur, hydrogen	Thiosulfate, sulfur, hydrogen	Thiosulfate, sulfur, hydrogen
Electron donors for chemo-organotrophic growth	None	None	None	Formate, formamide
DNA G+C content (mol%)				
Determined by HPLC	ND	49.6	40.3	47.2
Genome sequence†	41.32	46.93	NA	45.19

T. minervae can also utilize maltose, succinate, starch and citrate.

†DNA G+C content calculated from the genome sequence, which is not available (NA) for *T. minervae*.

Phylogenetic and genomic comparisons indicate that strain GBS1^T is affiliated with the genus *Thermocrinis* but is not closely related to previously cultivated strains. The complete 16S rRNA gene sequence was identified in the GBS1^T draft genome (JGI gene ID 2563359959; nucleotides 341 138–342 701 of scaffold00001, gi|657835571) and compared with 16S rRNA genes of other *Thermocrinis* species using EzTaxon-e (Kim *et al.*, 2012), displaying 97.10, 94.77 and 94.72 % similarity to the type strains of *T. ruber*, *T. minervae* and *T. albus*, respectively. 16S rRNA gene phylogenies of GBS1^T and other *Aquificaceae* were inferred from alignments of 16S rRNA genes against the SILVA seed alignment (Quast *et al.*, 2013) using MOTHUR v1.20.2 (Schloss *et al.*, 2009). After manual refinement of the alignments, phylogenies were reconstructed using the maximum-likelihood method with RaxML (Stamatakis, 2006) or DNA distance/neighbour-joining method with PHYLIP (Felsenstein, 1989). Although the genus *Thermocrinis* was not monophyletic, as has been observed by others (Caldwell *et al.*, 2010; Hedlund *et al.*, 2015; Wirth *et al.*, 2010), strain GBS1^T and the closely related cloned sequence GBS_L1_C01 recovered from GBS (Costa *et al.*, 2009) formed a monophyletic lineage with *T. ruber* and were closest in overall distance (nucleotide changes per position) to other *Thermocrinis* species (Fig. 2; see also Fig. S1). *In silico* DNA–DNA hybridization (DDH) of the GBS1^T draft genome-to-genome sequences of *T. albus* (NC_013894; Wirth *et al.*, 2010) and *T. ruber* (NZ_CP007028) was calculated using the Genome-to-Genome Distance Calculator 2.0 (http://ggdc.dsmz.de/) at default settings, yielding mean DDH estimates of 18.4 ± 2.26 % with *T. ruber* and 17.70 ± 2.24 % with *T. albus*, both well below the DDH cut-off delineating bacterial species (Meier-Kolthoff *et al.*, 2013). DDH comparison with *T. minervae* could not be performed because genome sequences for this and other *Thermocrinis* strains are not yet available.

Strain GBS1^T shares phenotypic characteristics with members of the genus *Thermocrinis*, but displays some notable differences (Table 1). Similar to *T. ruber*, *T. albus* and *T. minervae*, GBS1^T is autotrophic, obligately microaerophilic and thermophilic, but growth temperature range is more restricted than these previously described species. Also in contrast to *T. ruber*, *T. albus* and *T. minervae*, GBS1^T cannot use either hydrogen or elemental sulfur as sole electron donor for chemotrophic growth, and it has a significantly higher tolerance for NaCl. GBS1^T is further differentiated from *T. ruber* and *T. minervae* by its ability to use acetate as a sole carbon source. In light of the phenotypic, genotypic and phylogenetic comparisons outlined above, we suggest that strain GBS1^T represents a novel species within the genus *Thermocrinis*, for which the name *Thermocrinis jamiesonii* sp. nov. is proposed.

Description of *Thermocrinis jamiesonii* sp. nov.

Thermocrinis jamiesonii (ja.mie.son′i.i. N.L. gen. masc. n. *jamiesonii* of Jamieson, referring to David Jamieson, landowner of GBS, whose generous access to this and neighbouring geothermal springs has greatly facilitated studies of their resident microbes).

Thermophilic. Obligately microaerophilic and chemolithotrophic, requiring thiosulfate as an electron donor. Autotrophic, but capable of chemolithoheterotrophy using peptone, Casamino acids or acetate. Vitamins are not required. Growth occurs at 70–85 °C with an optimum at 80 °C, at pH 6.50–7.75 with an optimum at 7.25, with 0.5–8 % oxygen with an optimum at 1–2 %, and with NaCl concentrations $\leq$200 mM. Negative for the Gram stain reaction, and spores are not formed. Non-motile, rod-shaped cells 1.4–2.4 μm in length and 0.4–0.6 μm in width occur singly or in pairs. Colonies that are 0.5 mm in diameter, round and entire in shape, and cream in colour form after 7 days of incubation under chemolithoheterotrophic conditions. Major (>5 % abundance) cellular fatty acids are $C_{20:1}\omega 9c$, $C_{18:0}$, $C_{16:0}$ and $C_{20:0}$.

The type strain, GBS1^T (=JCM 19133^T=DSM 27162^T), was isolated from the water column of GBS, Nevada, USA. The genomic DNA G+C content is 41.3 mol%.

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