Venenivibrio stagnispumantis gen. nov., sp. nov., a thermophilic hydrogen-oxidizing bacterium isolated from Champagne Pool, Waiotapu, New Zealand

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A novel thermophilic, hydrogen-oxidizing bacterium, designated strain CP.B2^T, was isolated from a terrestrial hot spring in Waiotapu, New Zealand. Cells were motile, slightly rod-shaped, non-spore-forming and Gram-negative. Isolate CP.B2^T was an obligate chemolithotroph, growing by utilizing H₂ as electron donor and O₂ as corresponding electron acceptor. Elemental sulfur (S⁰) or thiosulfate (S₂O₃²⁻) was essential for growth. Microbial growth occurred under microaerophilic conditions in 1.0–10.0 % (v/v) O₂ [optimum 4–8 % (v/v) O₂], between 45 and 75 °C (optimum 70 °C) and at pH values of 4.8–5.8 (optimum pH 5.4). The DNA G + C content was 29.3 mol%. 16S rRNA gene sequence analysis demonstrated that strain CP.B2^T belonged to the order *Aquificales*, with a close phylogenetic relationship to *Sulfurihydrogenibium azorense* (94 % sequence similarity to the type strain). However, genotypic and metabolic characteristics differentiated the novel isolate from previously described genera of the *Aquificales*. Therefore, CP.B2^T represents a novel species in a new genus, for which the name *Venenivibrio stagnispumantis* gen. nov., sp. nov. is proposed. The type strain of *Venenivibrio stagnispumantis* is CP.B2^T (=JCM 14244^T =DSM 18763^T).

The Taupo Volcanic Zone in New Zealand is the terrestrial extension of the Tonga–Kermadec subduction system (Hedenquist, 1986), exhibiting geothermal activities such as fumaroles, steaming grounds, mud pools, geysers and hot springs. Within the Taupo Volcanic Zone, Champagne Pool is one of the largest hot springs. It is around 65 m in diameter and is located in a 900-year-old hydrothermal eruption crater (Lloyd, 1959). The name Champagne Pool derives from the constant flow of CO₂, visible as gas bubbles, buffering the spring water at a pH of around 5.5. Champagne Pool discharges siliceous geothermal fluid rich in arsenic and antimony at approximately 75 °C (Jones *et al.*, 2001). Although a few studies have described microbial activity in Champagne Pool in recent years (Ellis *et al.*, 2005; Jones *et al.*, 2001; Mountain *et al.*, 2003; Phoenix *et al.*, 2005), the successful isolation of a micro-organism has not yet been reported. Previous investigations applying culture-independent approaches, such as denaturing gradient gel

electrophoresis analysis and construction of clone libraries based on the bacterial 16S rRNA gene, have indicated that isolate CP.B2^T, a member of the order *Aquificales*, is one of the few dominant micro-organisms present in Champagne Pool (Hetzer *et al.*, 2007).

Currently, the order *Aquificales* comprises the following genera: *Aquifex* (Huber *et al.*, 1992), *Hydrogenivirga* (Nakagawa *et al.*, 2004), *Hydrogenobacter* (Kawasumi *et al.*, 1984), *Hydrogenobaculum* (Stöhr *et al.*, 2001) and *Thermocrinis* (Huber *et al.*, 1998) of the family *Aquificaceae*; *Hydrogenothermus* (Stöhr *et al.*, 2001), *Persephonella* (Götz *et al.*, 2002) and *Sulfurihydrogenibium* (Takai *et al.*, 2003a) of the family *Hydrogenothermaceae*; and *Balnearium* (Takai *et al.*, 2003b), *Desulfurobacterium* (L'Haridon *et al.*, 1998) and *Thermovibrio* (Huber *et al.*, 2002) of the family *Desulfurobacteriaceae* (Euzéby, 1997). Representatives are thermophilic to hyperthermophilic bacteria that commonly colonize geothermally heated environments. Most members of the *Aquificales* have in common the ability to use the Knallgas reaction (2H₂ + O₂ → 2H₂O) to yield energy for biosynthesis, therefore using H₂ as electron donor and O₂ as electron acceptor. On account of their chemolithotrophic metabolism, these organisms are regarded as primary producers of bacterial biomass within high-temperature ecosystems (Huber & Eder, 2006).

Abbreviation: CTAB, cetyltrimethylammonium bromide.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *polA* gene sequences of strain CP.B2^T are DQ989208 and EF581124.

A graph showing the effect of temperature on growth of cells of strain CP.B2^T is available as supplementary material with the online version of this paper.

In the present study, the novel thermophilic bacterium CP.B2^T, isolated from Champagne Pool, was characterized and shown to differ from previously described strains of the order *Aquificales*.

Geothermal fluid (30 cm below the spring water surface) and sediment (10 cm below the water surface) were sampled in September 2005 at the north-east side of Champagne Pool. Samples were collected in sterile 2 l glass bottles (Schott) carefully avoiding trapping any air. Within 2 h, samples were transferred into enrichment medium.

Enrichments were performed in a modified version of MSH medium (Aguiar *et al.*, 2004). The medium contained the following components (per litre anaerobic water): 0.15 g NaOH, 0.50 g KCl, 1.36 g MgCl₂·6H₂O, 7.00 g MgSO₄·7H₂O, 2.00 g Na₂S₂O₃·5H₂O, 0.40 g CaCl₂·2H₂O, 0.20 g NH₄Cl, 0.25 g K₂HPO₄, 1.95 g MES and trace minerals, containing (final amounts per litre medium) 5.00 mg disodium EDTA dihydrate, 1.50 mg CoCl₂·6H₂O, 1.00 mg MnCl₂·4H₂O, 1.00 mg FeSO₄·7H₂O, 1.00 mg ZnCl₂, 0.40 mg AlCl₃·6H₂O, 0.30 mg Na₂WO₄·2H₂O, 0.20 mg CuCl₂·2H₂O, 0.20 mg NiSO₄·6H₂O, 0.10 mg Na₂SeO₃, 0.10 mg H₃BO₃ and 0.10 mg Na₂MoO₄·2H₂O. The pH value of the medium was adjusted to 5.5. Aliquots of 40 ml and 8 ml were autoclaved under a CO₂ atmosphere in 160 ml serum bottles and 30 ml test tubes, respectively. After inoculation using a 5 % inoculum, the initial gas phase was exchanged

with 79 % H₂/16 % CO₂/5 % O₂ at 170 kPa. Enrichment cultures were incubated at 70 °C. After 2 days incubation without agitation, the medium became turbid due to microbial growth. Serial dilution series on MSH medium solidified by 0.6 % (w/v) Gelrite gellan gum and 0.06 % (w/v) MgSO₄·7H₂O led to the isolation of CP.B2^T. The purity of the novel isolate was routinely checked by microscopy and 16S rRNA gene sequence analysis.

For scanning electron microscopy, cells were captured on a 0.22 µm filter and fixed using 2.5 % glutaraldehyde. The filter was exposed to four changes of 0.1 M sodium cacodylate buffer, rinsed in water and dehydrated in increasing concentrations of ethanol (50, 75 and 90 %), followed by four changes of absolute ethanol. The filter was critical-point-dried, sputtered with platinum and viewed using a Hitachi S-4100 field emission scanning electron microscope. The bacterial cells occurred predominantly singly or in pairs. Large cell aggregates were formed in the late-stationary growth phase, macroscopically visible as whitish flocks (Fig. 1a). Cells of isolate CP.B2^T appeared to have a slightly curved, rod-shaped morphology (Fig. 1b–d), with a mean length of 1.30 ± 0.26 µm and a mean width of 0.37 ± 0.04 µm. Cells were motile and stained Gram-negative using standard methods (Hucker & Conn, 1927) with cells of *Escherichia coli* JM109 as a control. Sporulation was not observed during growth, in 6-month-old cultures or at temperatures above the maximum growth temperature.

Fig. 1. Macroscopic (a), phase-contrast (b) and scanning electron (c, d) micrographs of isolate CP.B2^T. (a) Large cell aggregates were formed after 2 days incubation in serum bottles; bar, 1 cm. (b) Cells occurred predominantly as single cells or in pairs; bar, 2 µm. (c, d) Electron micrographs showing vibrio (c) and biofilm-like (d) structures; bars, 500 nm.

Physiological and metabolic characteristics are summarized in Table 1 and in the genus and species descriptions and were determined as follows. The influence of pH on growth was determined at 70 °C at pH 4.5–6.5 using 10 mM MES, PIPES, MOPS and HEPES. The pH value of the medium was checked at the incubation temperature prior to inoculation and after the growth experiments. NaCl and O₂ requirements and tolerance were determined at 70 °C and pH 5.5 at 0–2 % (w/v) NaCl and 0–20 % (v/v) O₂, respectively. The ability of CP.B2^T to metabolize different substrates was investigated using a basal MSH medium without Na₂S₂O₃·5H₂O and with only 4.00 g MgSO₄·7H₂O to provide a sulfur source for assimilation. The basal medium was supplemented with 80 % (v/v) H₂, 10 mM Na₂S₂O₃ or 1 % (w/v) S⁰ as electron donor and 10 mM NaNO₂, 10 mM NaNO₃, 10 mM Na₂SO₃, 10 mM Na₂SO₄, 5 mM Na₂SeO₃, 5 mM Na₂SeO₄, 5 mM NaAsO₂, 5 mM Na₂HAsO₄ or O₂ as complementary electron acceptor. Further experiments were performed using either 1 mM or 5 mM Na₂HAsO₄ and NaAsO₂ as electron acceptor and donor, respectively. Casamino acids, sodium pyruvate, starch, trypticase peptone, yeast extract (final concentration 0.2 %, w/v) or CO₂ were provided as carbon source. Isolate CP.B2^T grew chemolithotrophically under microaerobic conditions utilizing H₂, sulfur compounds and CO₂ as sole carbon source up to a maximum concentration of 10⁹ cells ml⁻¹, with a doubling time of 230 min (Supplementary Fig. S1, available in IJSEM Online). H₂ could not be replaced by other electron donors such as S⁰ and S₂O₃²⁻ under anaerobic (either 100 % N₂ supplemented with organic substrates or 80 % N₂/20 % CO₂) or microaerobic (either 94 % N₂/6 % O₂ with organic carbon sources or 79 % N₂/16 % CO₂/5 % O₂) conditions. However, growth of CP.B2^T was dependent on

either S⁰ or S₂O₃²⁻ in the medium. Spectrophotometric determination of H₂S (Cline, 1969; Gilboa-Garber, 1971) after 24 and 48 h growth revealed no significant reduction in the quantities of sulfur compounds (<9 µM sulfide) indicating that S⁰ and were not involved in dissimilative metabolism. The strain was not capable of using any of the nine tested electron acceptors in sulfur-limited basal MSH medium providing around 16 mM for assimilation. None of the tested organic compounds were utilized. In all growth experiments, *Sulfurihydrogenibium azorense* Az-Fu1^T cultures were used as controls to verify that all solutions and methods were working correctly.

In toxicity experiments, the novel bacterium was tested for tolerance to elevated arsenic and antimony ion levels. Modified MSH medium supplemented with defined concentrations of Na₂HAsO₄·7H₂O, NaAsO₂ or KSbO·C₄H₄O₆·0.5H₂O was inoculated using a 2.5 % inoculum of a freshly grown culture. The MIC was defined as the absence of growth of the species in 30 ml test tubes after 7 days. The isolate displayed growth in the presence of up to 8 mM NaAsO₂ and 15 mM KSbO·C₄H₄O₆·0.5H₂O and in the presence of more than 20 mM Na₂HAsO₄·7H₂O. Those concentrations are considerably higher than the levels found in Champagne Pool spring water, but lower than levels found in the sediment (Jones *et al.*, 2001): 5.3 p.p.m. (70 µM) As and 3.5 p.p.b. (29 nM) Sb in the pool water and 16 700 p.p.m. (223 mM) As and 15 600 p.p.m. (128 mM) Sb in the orange sediment. However, growth was not observed when Na₂HAsO₄ and NaAsO₂ were provided as the sole electron donor and acceptor pair.

Genomic DNA of CP.B2^T was isolated using a modification of the cetyltrimethylammonium bromide (CTAB) method

Table 1. Comparison of properties of strain CP.B2^T (*Venenivibrio stagnispumantis* gen. nov., sp. nov.) with those of related species

Strains: 1, strain CP.B2^T (*Venenivibrio stagnispumantis*); 2, *Sulfurihydrogenibium yellowstonense* SS-5^T (data from Nakagawa *et al.*, 2005); 3, *Sulfurihydrogenibium azorense* Az-Fu1^T (Aguir *et al.*, 2004); 4, *Sulfurihydrogenibium subterraneum* HGMK-1^T (Takai *et al.*, 2003a); 5, *Persephonella guaymasensis* EX-H2^T (Götz *et al.*, 2002); 6, *Hydrogenothermus marinus* VM1^T (Stöhr *et al.*, 2001). Optimal growth conditions are given in parentheses.

Characteristic	1	2	3	4	5	6
Source	Terrestrial hot spring	Terrestrial hot spring	Terrestrial hot spring	Subsurface gold mine	Hydrothermal vent	Hydrothermal area
Growth conditions						
Temperature (°C)	45–75 (70)	55–78 (70)	50–73 (68)	40–70 (65)	60–80 (75)	45–80 (65)
pH	4.8–5.8 (5.4)	6.0–8.0 (7.5)	5.5–7.0 (6.0)	6.4–8.8 (7.5)	4.7–7.5 (6.0)	5.0–7.0
NaCl (% w/v)	0.0–0.8 (0.4)	0.0–0.6 (0.0)	0.0–0.25 (0.1)	0.0–4.8 (0.5)	1.0–4.5 (2.5)	0.5–6.0 (2–3)
Electron donor(s)	H ₂	S ⁰ , S ₂ O ₃ ²⁻	H ₂ , S ⁰ , S ₂ O ₃ ²⁻ , SO ₃ ²⁻ , Fe ²⁺ , AsO ₃ ³⁻	H ₂ , S ⁰ , S ₂ O ₃ ²⁻	H ₂ , S ⁰ , S ₂ O ₃ ²⁻	H ₂
Electron acceptor(s)	O ₂ (up to 10 %)	O ₂ (up to 18 %)	O ₂ (up to 9 %), S ⁰ , S ₂ O ₃ ²⁻ , Fe ³⁺ , SeO ₄ ²⁻ , AsO ₄ ³⁻	O ₂ , NO ₃ ⁻ , Fe ³⁺ , SeO ₃ ²⁻ , O ₂ (up to 11 %), SeO ₄ ²⁻ , AsO ₄ ³⁻	O ₂ (up to 11 %), NO ₃ ⁻	O ₂ (up to 8 %)
DNA G + C content (mol%)	29.3	32	33.6	31.3	37.4	43

described previously (Dempster *et al.*, 1999). A 40 ml bacterial suspension was harvested by centrifugation at 1850 *g* (JouanCR4.11) for 10 min. The cell pellet was resuspended in 1 ml CTAB buffer [100 mM Tris/HCl, 1.4 M NaCl, 20 mM EDTA, 2 % (w/v) CTAB, 1 % (w/v) polyvinylpyrrolidone (average molecular mass 360 000 Da) (pH 8.0) and freshly prepared 0.4 % (w/v) 2-mercaptoethanol]. The mixture was incubated at 100 °C for 20 min followed by extraction with an equal volume of phenol and then with chloroform/isoamyl alcohol (24:1, v/v). Nucleic acids were precipitated with an equal volume of 2-propanol and 0.1 vols 3 M sodium acetate (pH 5.5) at -20 °C overnight and then washed twice in 70 % ethanol and air-dried.

The G+C content of the DNA was determined by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) according to the method of Mesbah *et al.* (1989). The G+C content of DNA isolated from strain CP.B2^T was 29.3 mol%.

PCR amplification of the 16S rRNA gene sequence between the corresponding *E. coli* positions 9 and 1522 was performed using forward primer 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse primer 5'-AAGGAGGTGATCCARCCGCA-3' (Johnson, 1994). PCR mixtures consisted of PCR buffer (10 mM Tris/HCl, 50 mM KCl, pH 8.3), 1.5 mM MgCl₂, 0.2 mM dNTPs (dATP, dCTP, dGTP, dTTP), 1.0 μM of each amplification primer, 5.0 % (w/v) DMSO and 1.0 U *Taq* polymerase (Roche). The following PCR conditions were applied: an initial denaturation step at 94 °C for 210 s, two cycles of annealing at 50 °C for 30 s, extension at 72 °C for 120 s and denaturation at 94 °C for 30 s, followed by 31 cycles applying an annealing temperature of 48 °C for 120 s. The final extension step was 300 s. PCR products purified by agarose gel electrophoresis were used in cloning experiments applying the pGEM-T Easy vector system (Promega) as recommended by the manufacturer. DNA sequencing of the cloned inserts was undertaken by the Waikato DNA Sequencing Facility based at the University of Waikato in Hamilton, New Zealand, using the MegaBase capillary analysis system (Amersham Biosciences). Sequences of the inserts were obtained by using primer set M13pUC forward 5'-CCCAGTCACGACGTTGTAACACG-3' and M13pUC reverse 5'-AGCGGATAACAATTTTCACACAGG-3'. PCR experiments were carried out targeting the DNA polymerase I (*polA*) gene, which is distinctive in species of the order *Aquificales* (Griffiths & Gupta, 2006). DNA extracted from *S. azorensis* Az-Fu1^T acted as a control.

The BLAST computer algorithm was used to search the sequence database of the National Center for Biotechnology Information (NCBI; <http://www.ncbi.nlm.nih.gov>) for sequence similarities. Phylogenetic analysis and alignment of 16S rRNA gene sequences were performed using the ARB software package (Ludwig *et al.*, 2004). The phylogenetic positions of the sequences were

determined using the PHYLIP package with analysis of sequences undertaken using the programs DNADIST, DNAML, DNAPARS, FITCH, NEIGHBOR and SEQBOOT (Felsenstein, 1993). Phylogenetic analysis on the basis of 16S rRNA gene sequences indicated that CP.B2^T is closely related to previously described species of the order *Aquificales* (Fig. 2). This evidence was supported by sequencing of the *polA* gene of strain CP.B2^T; when the sequence was compared with those in GenBank using BLASTX, it showed the highest similarity to *PolA* from *Hydrogenothermus marinus* DSM 12046^T (sequences of the *polA* gene of other members of the family *Hydrogenothermaceae* were not present in the database). Bootstrap analysis of 16S rRNA gene sequences supported the clustering of the novel isolate and members of the genus *Sulfurihydrogenibium* within the family *Hydrogenothermaceae* (bootstrap value 100), and clustering of members of the families *Hydrogenothermaceae* (bootstrap value 77) and *Aquificaceae* (bootstrap value 95) within the order *Aquificales* (Fig. 2). Within the order *Aquificales*, CP.B2^T displayed the greatest 16S rRNA gene sequence similarity (94 %) to *S. azorensis* Az-Fu1^T (Aguilar *et al.*, 2004). However, the novel isolate differed markedly in genotypic and metabolic properties from members of the genus *Sulfurihydrogenibium* and other genera of the order *Aquificales*. Although the physiological properties of the novel isolate largely resembled those of members of the genus *Sulfurihydrogenibium* (Table 1), the pH range was slightly limited and the optimal pH value was lower. This might be explained by adaptation of strain CP.B2^T to its environment. Champagne Pool spring water is buffered by

Fig. 2. Phylogenetic tree based on analysis of the 16S rRNA gene sequences of strain CP.B2^T and closely related species of the *Aquificales* showing the position of the novel isolate. The dendrogram was produced with DNADIST (neighbour-joining) analysis using 1158 bp of aligned sequence. The phylogenetic tree was rooted to *Methanococcus jannaschii* JAL-1^T (GenBank accession no. M59126; not shown). Bootstrap values are shown from 1000 replicates. Bar, 10 % sequence divergence.

a constant flow of CO₂, keeping the pH stable at around 5.5. The low G + C content distinguishes it from previously described species of the genera *Sulfurihydrogenibium*, *Persephonella* and *Hydrogenothermus*. To our knowledge, this is the lowest DNA G + C content reported for a species belonging to the order *Aquificales*. Strain CP.B2^T grew chemolithotrophically under microaerobic conditions utilizing H₂ as electron donor, O₂ as electron acceptor and CO₂ as carbon source. Growth was dependent on S⁰ or S₂O₃²⁻, but no growth occurred when the sulfur compounds replaced H₂ as potential electron donor or O₂ as potential electron acceptor. *Sulfurihydrogenibium* species (Aguiar *et al.*, 2004; Nakagawa *et al.*, 2005; Takai *et al.*, 2003a), *Persephonella marina* and *Persephonella guaymasensis* (Götz *et al.*, 2002) are able to use sulfur compounds either as electron donors or acceptors. The hydrogen-oxidizing bacterium *H. marinus* requires elemental sulfur for growth as a source for biosynthesis of sulfur-containing compounds (Stöhr *et al.*, 2001) but, like CP.B2^T, cannot use it as a potential electron donor. However, *H. marinus* can be clearly separated from CP.B2^T. As the name indicates, *H. marinus* is of marine origin and therefore adapted to higher NaCl concentrations (Table 1).

The occurrence of the novel isolate might not be limited to Champagne Pool in Waiotapu. A 16S rRNA gene sequence similarity of 98 % was obtained between CP.B2^T and a 16S rRNA gene clone bacteriap28 amplified from an environmental DNA sample. The DNA sample was extracted from a hot spring in Rotorua, New Zealand (Sunna & Bergquist, 2003). Rotorua and Waiotapu are both located in the geothermally active Taupo Volcanic Zone, indicating that CP.B2^T and close relatives might be distributed within this zone. Water samples obtained from a hot spring on Fountain Flat (44° 33.671' N 110° 49.989' W) in Yellowstone National Park, USA, which features similar chemophysical properties (75 °C, pH 6.2) to Champagne Pool, were used for enrichment experiments in modified MSH medium. Morphological characteristics of the enrichment cultures differed from those of cells of strain CP.B2^T, indicating that CP.B2^T was not present. However, it cannot be ruled out that the novel isolate might be abundant outside New Zealand.

Description of *Venenivibrio* gen. nov.

Venenivibrio (Ve.ne'ni.vi'bri.o. L. neut. n. *venenum* poison; N.L. masc. n. *vibrio* that which vibrates; N.L. masc. n. *Venenivibrio* the vibrio of poison).

Cells are slightly curved rods, motile and stain Gram-negative. No sporulation. Microaerophilic. Thermophilic. Strictly chemolithotrophic. Able to utilize molecular hydrogen as electron donor and oxygen as electron acceptor. Either elemental sulfur or thiosulfate is an essential growth requirement. NaCl is not required for growth. Genomic DNA G + C content is around 29 mol%. On the basis of 16S rRNA gene sequence analysis, closely related to members of the genus *Sulfurihydrogenibium*.

Species of the genus *Venenivibrio* have been detected in terrestrial geothermally heated freshwater systems. The type species is *Venenivibrio stagnispumantis*.

Description of *Venenivibrio stagnispumantis* sp. nov.

Venenivibrio stagnispumantis (stag.ni.spu.man'tis. L. n. *stagnum* pool; L. part. adj. *spumans* foaming, frothing; N.L. gen. n. *stagnispumantis* from a frothing pool, referring to Champagne Pool).

Exhibits the following properties in addition to those described for the genus. Slightly curved rods with a mean length of 1.30 ± 0.26 µm and a mean width of 0.37 ± 0.04 µm. Grows at 45–75 °C and pH 4.8–5.8, with optimum growth at 70 °C and pH 5.4. Grows in 0–0.8 % (w/v) NaCl; optimum growth occurs at 0.4 % (w/v) NaCl. Tolerates oxygen in the range 1–10 % (v/v); oxygen is essential for growth. The 16S rRNA gene sequence exhibits 94 % similarity to that from the type strain of *S. azorensis*.

The type strain is CP.B2^T (=JCM 14244^T =DSM 18763^T), isolated from the terrestrial hot spring Champagne Pool in Waiotapu, New Zealand. The DNA G + C content of the type strain is 29.3 mol%.

Acknowledgements

The authors wish to thank Christopher J. Daughney for research funding provided by the New Zealand Foundation of Research, Science & Technology (contract number C05X0303: Extremophilic Micro-organisms for Metal Sequestration from Aqueous Solutions). We are grateful to Richard Fulton for permission to take spring water and sediment samples from Champagne Pool, to Helen M. Turner for the electron microscopy preparation, to Anna-Louise Reysenbach for providing samples of *S. azorensis* and to Craig Cary for spring water samples obtained from Yellowstone National Park (permit issued by the National Park Service with support from the National Geographical Society). The help of Hans G. Trüper with naming the novel isolate is greatly appreciated. A.H. thanks his beloved wife for her patience and encouragement. The editor and the two anonymous reviewers offered comments and suggestions that greatly improved this manuscript.

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