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.B2T, 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.B2T was an obligate chemolithotroph, growing by utilizing H₂ as electron donor and O₂ as corresponding electron acceptor. Elemental sulfur (S⁰) or thiosulfate (S₂O₃⁻) 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.B2T 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.B2T 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.B2T (≡JCM 14244T =DSM 18763T).

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.B2T, 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).
In the present study, the novel thermophilic bacterium CP.B2<sup>T</sup>, 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 (Aguir et al., 2004). The medium contained the following components (per litre anaerobic water): 0.15 g NaOH, 0.50 g KCl, 1.36 g MgCl<sub>2</sub>.6H<sub>2</sub>O, 7.00 g MgSO<sub>4</sub>.7H<sub>2</sub>O, 2.00 g Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>.5H<sub>2</sub>O, 0.40 g CaCl<sub>2</sub>.2H<sub>2</sub>O, 0.20 g NH<sub>4</sub>Cl, 0.25 g K<sub>2</sub>HPO<sub>4</sub>, 1.95 g MES and trace minerals, containing (final amounts per litre medium) 5.00 mg disodium EDTA dihydrate, 1.50 mg CoCl<sub>2</sub>.6H<sub>2</sub>O, 1.00 mg MnCl<sub>2</sub>.4H<sub>2</sub>O, 1.00 mg FeSO<sub>4</sub>.7H<sub>2</sub>O, 1.00 mg ZnCl<sub>2</sub>, 0.40 mg AlCl<sub>3</sub>.6H<sub>2</sub>O, 0.30 mg Na<sub>2</sub>WO<sub>4</sub>.2H<sub>2</sub>O, 0.20 mg CuCl<sub>2</sub>.2H<sub>2</sub>O, 0.20 mg NiSO<sub>4</sub>.6H<sub>2</sub>O, 0.10 mg Na<sub>2</sub>SeO<sub>3</sub>, 0.10 mg H<sub>3</sub>BO<sub>3</sub> and 0.10 mg Na<sub>2</sub>MoO<sub>4</sub>.2H<sub>2</sub>O. The pH value of the medium was adjusted to 5.5. Aliquots of 40 ml and 8 ml were autoclaved under a CO<sub>2</sub> 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 % H<sub>2</sub>/16 % CO<sub>2</sub>/5 % O<sub>2</sub> 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) MgSO<sub>4</sub>.7H<sub>2</sub>O led to the isolation of CP.B2<sup>T</sup>. 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<sup>T</sup> 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.
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.B2T 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 NaN3O2, 10 mM NaN3O3, 10 mM NaSO4, 5 mM Na2SeO3, 5 mM Na2SeO4, 5 mM Na2AsO2, 5 mM NaHAsO4 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, tryptase peptone, yeast extract (final concentration 0.2%, w/v) or CO2 were provided as carbon source. Isolate CP.B2T 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 S2O2 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-Fu1T 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 K5SO4. 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 K5SO4. 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 16700 p.p.m. (223 mM) As and 15600 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.B2T was isolated using a modification of the cetyltrimethylammonium bromide (CTAB) method.

Table 1. Comparison of properties of strain CP.B2T (Venenivibrio stagnispumantis gen. nov., sp. nov.) with those of related species

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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<tr>
<td>Source</td>
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<td>Terrestrial hot spring</td>
<td>Terrestrial hot spring</td>
<td>Subsurface gold mine</td>
<td>Hydrothermal vent</td>
<td>Hydrothermal area</td>
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<tr>
<td>Temperature (°C)</td>
<td>45–75 (70)</td>
<td>55–78 (70)</td>
<td>50–73 (68)</td>
<td>40–70 (65)</td>
<td>60–80 (75)</td>
<td>45–80 (65)</td>
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<td>pH</td>
<td>4.8–5.8 (5.4)</td>
<td>6.0–8.0 (7.5)</td>
<td>5.5–7.0 (6.0)</td>
<td>6.4–8.8 (7.5)</td>
<td>4.7–7.5 (6.0)</td>
<td>5.0–7.0</td>
</tr>
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<td>NaCl (%), w/v</td>
<td>0.0–0.8 (0.4)</td>
<td>0.0–0.6 (0.0)</td>
<td>0.0–0.25 (0.1)</td>
<td>0.0–4.8 (0.5)</td>
<td>1.0–4.5 (2.3)</td>
<td>0.5–6.0 (2–3)</td>
</tr>
<tr>
<td>Electron donor(s)</td>
<td>H2</td>
<td>S0, S2O32–</td>
<td>H2, S0, S2O32–, SO42–, Fe2+, AsO33–</td>
<td>H2, S0, S2O32–</td>
<td>H2, S0, S2O32–</td>
<td>H2</td>
</tr>
<tr>
<td>Electron acceptor(s)</td>
<td>O2 (up to 10%)</td>
<td>O2 (up to 18%)</td>
<td>O2 (up to 9%), S0, S2O32–, Fe3+, SeO42–</td>
<td>O2, NO3–, Fe3+, SeO42–, O2 (up to 11%), O2, NO3–, S2O32–</td>
<td>O2 (up to 11%), O2, NO3–, S2O32–</td>
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<tr>
<td>DNA G+C content (mol%)</td>
<td>29.3</td>
<td>32</td>
<td>36</td>
<td>31.3</td>
<td>37.4</td>
<td>43</td>
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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 360000 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’-AAGGAGGTGAAGGAT改革创新-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'-CCGATGCAAGGTGTTAAAAAGC-3' and M13pUC reverse 5’-AGGCGATAAATTTCTACACAGG-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. azorense 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 Sulfitirehydrogenibium 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. azorense Az-Fu1 T (Aguir et al., 2004). However, the novel isolate differed markedly in genotypic and metabolic properties from members of the genus Sulfitirehydrogenibium and other genera of the order Aquificales. Although the physiological properties of the novel isolate largely resembled those of members of the genus Sulfitirehydrogenibium (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 Sulfurireducingibium, 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.B2T grew chemolithotrophically under microaerobic conditions utilizing H₂ as electron donor, O₂ as electron acceptor and CO₂ as carbon source. Growth was dependent on S²⁻ or S₂O³⁻, but no growth occurred when the sulfur compounds replaced H₂ as potential electron donor or O₂ as potential electron acceptor. Sulfurireducingibium 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.B2T, cannot use it as a potential electron donor. However, H. marinus can be clearly separated from CP.B2T. 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.B2T and a 16S rRNA gene sequence of strain CP.B2T, indicating that CP.B2T was not present. However, enrichment cultures differed from those of cells of strain MSH medium. Morphological characteristics of the enrichment cultures differed from those of cells of strain CP.B2T, indicating that CP.B2T 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 sporation. Microaerophilic. Thermophilic. Strictly chemolithothrophic. 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 Sulfurireducingibium. 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. azorense.

The type strain is CP.B2T (= JCM 14244T = DSM 18763T), 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%.

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**References**


