

Sulfurihydrogenibium yellowstonense sp. nov., an extremely thermophilic, facultatively heterotrophic, sulfur-oxidizing bacterium from Yellowstone National Park, and emended descriptions of the genus *Sulfurihydrogenibium*, *Sulfurihydrogenibium subterraneum* and *Sulfurihydrogenibium azureense*

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A novel thermophilic, sulfur-oxidizing Gram-negative bacterium, designated strain SS-5^T, was isolated from the Calcite Hot Springs in Yellowstone National Park, USA. The cells were motile rods (1.2–2.8 µm long and 0.6–0.8 µm wide). The new isolate was a facultative heterotroph capable of using elemental sulfur or thiosulfate as an electron donor and O₂ (1–18%; optimum 6%, v/v) as an electron acceptor. Hydrogen did not support growth. The isolate grew autotrophically with CO₂. In addition, strain SS-5^T utilized various organic carbon sources such as yeast extract, tryptone, sugars, amino acids and organic acids. Growth was observed between 55 and 78 °C (optimum 70 °C; 3.5 h doubling time), pH 6.0 and 8.0 (optimum pH 7.5), and 0 and 0.6% (w/v) NaCl (optimum 0%). The G + C content of the genomic DNA was 32 mol%. Phylogenetic analysis based on the 16S rRNA gene sequence indicated that the isolate was a member of the genus *Sulfurihydrogenibium*. On the basis of the physiological and molecular characteristics of the new isolate, we propose the name *Sulfurihydrogenibium yellowstonense* sp. nov. with SS-5^T (=JCM 12773^T=OCM 840^T) as the type strain. In addition, emended descriptions of the genus *Sulfurihydrogenibium*, *Sulfurihydrogenibium subterraneum* and *Sulfurihydrogenibium azureense* are proposed.

The order *Aquificales* consists of the genera *Hydrogenobacter*, *Aquifex* and *Hydrogenobaculum* (Reysenbach, 2001). Recently, new genera *Hydrogenothermus* (Stöhr *et al.*, 2001), *Persephonella* (Götz *et al.*, 2002; Nakagawa *et al.*, 2003), *Sulfurihydrogenibium* (Takai *et al.*, 2003; Aguiar *et al.*, 2004) and *Hydrogenivirga* (Nakagawa *et al.*, 2004) have been

reported within the *Aquificales*. Members of the genus *Sulfurihydrogenibium* were detected first by culture-independent molecular analyses from terrestrial hot environments (Hugenholtz *et al.*, 1998; Yamamoto *et al.*, 1998; Reysenbach *et al.*, 2000). Recently, two species of the genus *Sulfurihydrogenibium* were isolated and characterized as strict chemolithoautotrophs capable of using numerous electron donors and acceptors [H₂, thiosulfate, elemental sulfur, sulfite, Fe(II), arsenite and selenite as electron donors and molecular oxygen, nitrate, Fe(III), arsenate and selenate as electron acceptors] (Takai *et al.*, 2003; Aguiar *et al.*, 2004). Members of the genus *Sulfurihydrogenibium* play a significant role in sulfur-cycling (Skirnisdottir *et al.*, 2000; Takai *et al.*, 2002; Nakagawa & Fukui, 2003) and iron mineralization (Reysenbach *et al.*, 1999) in terrestrial hot spring environments. Based on the phylogenetic analysis of 16S

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Graphs showing the effects of temperature, pH, and NaCl and O₂ concentrations on the growth of *Sulfurihydrogenibium yellowstonense* SS-5^T, and sulfate production during growth on mJT50 medium lacking thiosulfate and all sulfate-containing salts are available as supplementary material in IJSEM Online.

rRNA gene sequence retrieved by using culture-dependent or -independent methods, the genus *Sulfurihydrogenibium* consists of two distinct clades. One clade contains both *S. subterraneum* and *S. azorense*, together with environmental clone sequences retrieved from Icelandic and Japanese hot springs (Yamamoto *et al.*, 1998; Skirnisdottir *et al.*, 2000; Takacs *et al.*, 2001). A second clade is distinguished by environmental 16S rRNA gene sequences from hot springs in Yellowstone National Park (Reysenbach *et al.*, 2000). In this paper, we describe the isolation and characterization of the first member of *Sulfurihydrogenibium* from Yellowstone National Park; this bacterium is a facultatively heterotrophic, aerobic, sulfur-oxidizing thermophile.

Sample collection, enrichment and purification

A black filamentous sample (74 °C and pH 8.3) was collected from Calcite Springs in Yellowstone National Park, WY, USA (44° 54.291' N, 110° 24.242' W), and stored in a serum vial until inoculation. Black filamentous biomass (Reysenbach *et al.*, 2000) was inoculated into 5 ml modified MSH medium (Aguir *et al.*, 2004) supplemented with 0.06% (w/v) elemental sulfur under a gas phase of 93% CO₂ and 7% O₂ (100 kPa). The tube became turbid after 2 days incubation at 70 °C, and contained motile rods. To obtain a pure culture, an extinction-to-dilution method was employed at 70 °C and repeated at least seven times (Baross, 1995). The first pure culture was designated strain SS-5^T (=OCM 840^T=JCM 12773^T) and investigated in detail. The purity was confirmed routinely by microscopic observation and by repeated partial sequencing of the 16S rRNA gene using several PCR primers. All subsequent characterization was done using mjTSM medium: 1 g NaHCO₃, 1 g Na₂S₂O₃·5H₂O, 3 g S⁰, 3 g NaCl, 0.34 g MgCl₂, 0.42 g MgSO₄, 0.05 g KCl, 0.14 g K₂HPO₄, 0.25 g NH₄Cl, 0.07 g CaCl₂ and 10 ml trace mineral solution (described below) per litre. Trace mineral solution contained (per litre): 0.5 g Na₂-EDTA·2H₂O, 150 mg CoCl₂·6H₂O, 100 mg MnCl₂·4H₂O, FeSO₄·7H₂O and ZnCl₂, 40 mg AlCl₃·6H₂O, 30 mg Na₂WO₂·2H₂O, 20 mg CuCl₂·2H₂O and NiSO₄·6H₂O and 10 mg H₂SeO₃, H₃BO₃ and NaMoO₄·2H₂O. To prepare mjTSM medium, all the components other than NaHCO₃ and elemental sulfur were dissolved and the pH of the medium was adjusted to around 7.5 with NaOH before autoclaving. After autoclaving, a concentrated solution of NaHCO₃ (filter-sterilized) and elemental sulfur (steam-sterilized three times at 90 °C for 1 h) were added to the medium. The stability of thiosulfate was checked and only trace amounts of sulfite were detected after autoclaving (from 1 mM thiosulfate, only 50 pM sulfite was produced). The pH of the medium was readjusted to 7.5 with NaOH unless otherwise noted. Then the tubes were tightly sealed with butyl rubber stoppers under a gas phase of 93% CO₂ and 7% O₂ (100 kPa).

Morphology

Cells were routinely observed by phase-contrast microscopy (BX60; Olympus). For negative stains the bacteria were

stained with 2% (w/v) uranyl acetate and for thin sections the bacteria were fixed using a 2% (v/v) glutaraldehyde/2% (w/v) osmium tetroxide regimen, then stained with uranyl acetate, ethanol-dehydrated and embedded in LR White plastic. All electron microscopy was performed using a Philips EM400 at 100 kV under standard operating conditions.

The cells were Gram-negative rods with a mean length of 1.2–2.8 µm and a width of approximately 0.6–0.8 µm. Cells

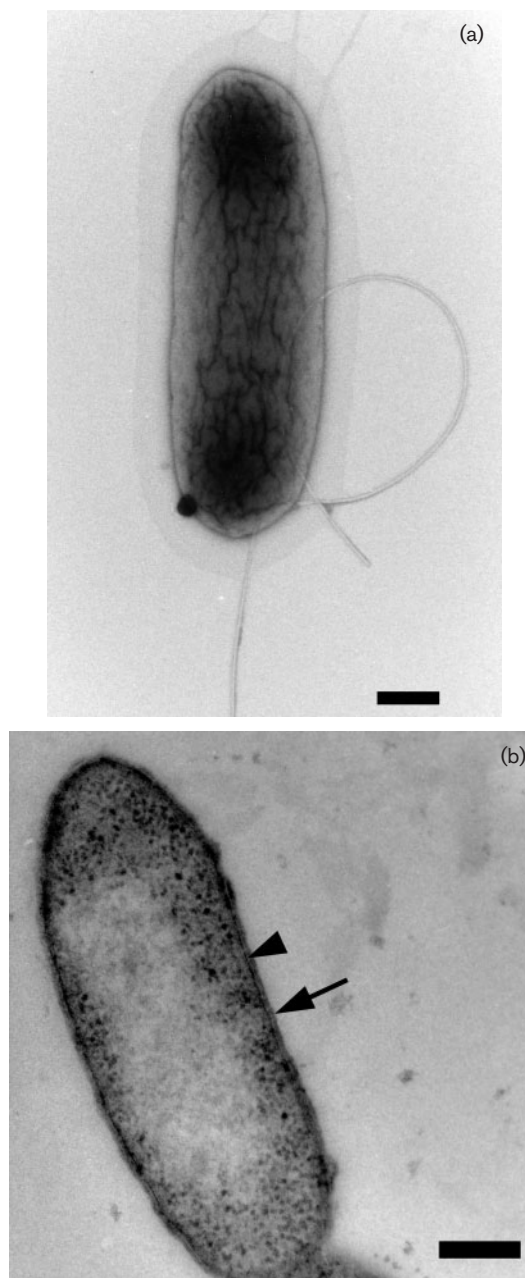


Fig. 1. Electron micrographs of a negatively stained cell (a) and thin section (b) of strain SS-5^T. Arrowhead, cytoplasmic membrane; arrow, outer membrane. Bars, 0.25 µm.

occurred singly or in pairs and no sporulation was observed. The cells were motile when viewed under phase microscopy. One to two flagella were commonly seen emanating from a single pole of each cell when preparations were negatively stained and observed by transmission electron microscopy (Fig. 1a). Electron micrographs of thin sections showed that the isolate had an envelope consisting of a plasma membrane and outer membrane (Fig. 1b). The internal stacked membranes reported in *Persephonella marina* EX-H1^T (Götz *et al.*, 2002) and *S. azorensis* Az-Fu1^T (Aguiar *et al.*, 2004), were not detected in strain SS-5^T. However, these structures were observed in environmental samples from Calcite Springs (Reysenbach *et al.*, 2005).

Growth characteristics

Growth of the new isolate was determined by direct cell counts of DAPI-stained cells (Porter & Feig, 1980). All experiments described below were conducted in duplicate. To determine temperature, pH and NaCl ranges for growth, cultures were grown in 25 ml test tubes containing 10 ml mJTISO medium. Growth conditions for all cultivation tests were 70 °C and pH 7.5, unless otherwise noted.

The isolate grew between 55 and 78 °C, showing optimal growth at 70 °C (supplementary Fig. 1a in IJSEM Online). The generation time and maximum cell density at 70 °C were about 3.5 h and 4.6×10^7 cells ml⁻¹, respectively. No growth was observed below 50 °C or above 80 °C. Growth at different pH values was monitored in mJTISO medium adjusted with the following: 10 mM acetate/acetic acid buffer (pH 4–5), MES (pH 5–6), PIPES (pH 6–7), HEPES (pH 7–7.5) and Tris (pH 8–9.5). pH was measured at room temperature. These buffers had no effect on the growth of the isolate. During growth, the pH was checked and

remained stable (supplementary Fig. 2 in IJSEM Online). Growth of the new isolate occurred between pH 6.0 and 8.0, with optimum growth at about pH 7.5 (supplementary Fig. 1b in IJSEM Online). No growth was observed at or below pH 5.5 or at or above pH 8.5. NaCl requirements for growth were determined from 0 to 3 % NaCl (w/v). The isolate grew in medium with up to 0.6 % NaCl and grew optimally without NaCl (supplementary Fig. 1c in IJSEM Online). No growth was observed above 0.7 % NaCl. The temperature, pH and NaCl ranges for growth of the new isolate were similar to those of *S. subterraneum* HGMK1^T (Takai *et al.*, 2003) and *S. azorensis* Az-Fu1^T (Aguiar *et al.*, 2004) (Table 1).

Oxygen tolerance was determined by injecting defined volumes of O₂ (up to 25 %, v/v) into culture tubes of mJTISO medium as described previously (Nakagawa *et al.*, 2003). The isolate grew in medium containing 0.1–18 % (v/v) O₂, with optimum growth at around 6 % O₂ (supplementary Fig. 1d in IJSEM Online).

In an attempt to examine growth on hydrogen as an electron donor, 80 % H₂ + 20 % CO₂ (300 kPa) was used as the gas phase. Electron acceptors were provided at final concentrations as follows: Na₂S₂O₃·5H₂O and NaNO₃ (0.1 %, w/v), Na₂SO₃ and NaNO₂ (0.01–0.1 %, w/v), arsenate, arsenite, selenate and selenite (5 mM), S⁰ (3 %, w/v) or O₂ (0.09–20 %, v/v). For testing growth with Na₂S₂O₃·5H₂O (0.1 %, w/v), S⁰ (3 %, w/v), sulfide (0.025–0.1 %), arsenite or selenite (5 mM) as electron donors, 80 % N₂ + 20 % CO₂ (300 kPa) was used as the gas phase. NaNO₃ (0.1 %, w/v) or O₂ (7 %, v/v) was provided as an electron acceptor. The isolate could grow on S⁰ and S₂O₃²⁻ as an electron donor and O₂ as an electron acceptor. Although growth was observed on sulfide, sulfide is rapidly converted

Table 1. Comparison of physiological characteristics of strain SS-5^T with members of the genus *Sulfurihydrogenibium*

Character	SS-5 ^T	<i>S. azorensis</i> Az-Fu1 ^T	<i>S. subterraneum</i> HGMK1 ^T
Origin	Terrestrial hot spring, Yellowstone National Park, USA	Terrestrial hot spring, Azores, Portugal	Subsurface gold mine, Hishikari, Japan
Temperature range (opt.) [°C]	55–78 (70)	50–73 (68)	40–70 (65)
pH range (opt.)	6–8.0 (7.5)	5.5–7.0 (6.0)	6.4–8.8 (7.5)
NaCl range (opt.) [%, v/w]	0.0–0.6 (0.0)	0.0–0.25 (0.1)	0.0–4.8 (0.5)
Electron donor	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⁻
Electron acceptor	O ₂ (up to 18 %, v/v)	S ⁰ , Fe ³⁺ , O ₂ (up to 9 %, v/v), SO ₃ ²⁻ , AsO ₄ ³⁻ , SeO ₄ ²⁻	Fe ³⁺ , O ₂ , NO ₃ ³⁻ , HAsO ₄ ²⁻ , SeO ₄ ²⁻ , SeO ₃ ²⁻
Organic carbon source	Yeast extract, Bacto peptone, trypticase peptone, sucrose, glucose, starch, formate, Casamino acids, citrate, propionate, acetate	Yeast extract, Bacto peptone, trypticase xpeptone, Casamino acids	Acetate
Internal structures	Not detected	Stacked membranes	No internal structures reported
G + C content (mol%)	32	33.6	31.3

to thiosulfate and thus we cannot conclude if the organism grows on sulfide. Sulfate was produced when sulfur was the electron donor.

Heterotrophic growth of SS-5^T was examined using mJT_{SO}, where NaHCO₃ and CO₂ were replaced by various organic carbon sources. Under the same cultivation conditions, heterotrophic growth of *S. subterraneum* HGMK1^T (provided by K. Takai) and *S. azorensis* Az-Fu1^T was also tested. Each of the following substrates were added at concentrations of 0.01 and 0.1% (w/v): yeast extract (Difco), Bacto peptone (Difco), trypticase peptone, sucrose, glucose, starch, formate, Casamino acids, formaldehyde, formamide, citrate, propionate, acetate and 2-propanol. The gas phase was 93% N₂ and 7% O₂ (100 kPa). Cultures were transferred at least twice on the same substrate combination to ensure that the growth was not due to the carry-over of substrates from the inoculum. Strain SS-5^T was able to utilize a variety of organic compounds as sole carbon source (yeast extract, Bacto peptone, trypticase peptone, sucrose, glucose, starch, formate, Casamino acids, citrate, propionate, acetate; all at 0.1%, w/v). No growth occurred in mJT_{SO} medium lacking thiosulfate and elemental sulfur under a gas phase of 93% CO₂ and 7% O₂ (100 kPa) and in the presence of an organic carbon substrate, indicating that the isolate is unable to utilize organic substrates as energy sources. Organoheterotrophic growth was also not detected in medium containing a small amount (0.001–0.01%, w/v) of sulfide and cysteine-HCl. In contrast to the previous descriptions by Takai *et al.* (2003) and Aguiar *et al.* (2004), *S. subterraneum* HGMK1^T and *S. azorensis* Az-Fu1^T grew on the following organic substrates as carbon sources: 0.1% (w/v) acetate (*S. subterraneum* HGMK1^T) and 0.1% (w/v) yeast extract, Bacto peptone, trypticase peptone and Casamino acids (*S. azorensis* Az-Fu1^T).

Phylogenetic analyses

Genomic DNA was extracted using the MoBio environmental DNA Extraction Kit (MoBio Laboratories). The 16S rRNA gene was amplified by PCR, purified and sequenced as described previously (Götz *et al.*, 2002). The partial 16S rRNA gene sequence (1447 bp) of strain SS-5^T has been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases under accession number AY686713. The sequence was aligned with a subset of 16S rRNA gene sequences obtained from the DDBJ using the FastAligner utility of the ARB software package (Ludwig *et al.*, 2004). The resulting alignment was verified using secondary structure constraints, and only unambiguously aligned nucleotide positions (1169 bp) were used for phylogenetic analyses with ARB and PAUP* 4.0 beta 10 (Swofford, 2000). The 16S rRNA gene sequence was found to be most closely related to environmental clone sequences from Yellowstone National Park: OPB13 (Hugenholtz *et al.*, 1998; 99.4% similarity) and another strain isolated from Calcite Springs (Reysenbach *et al.*, 2002; 97% similarity). Among the species validly described to date, *S. azorensis* Az-Fu1^T (96.8%) and *S. subterraneum* HGMK-1^T (95.8%) were close relatives of the isolate. Using the recommended sequence similarity to differentiate species (97%; Stackebrandt & Goebel, 1994), our strain is a new species of *Sulfurihydrogenibium*. The phylogenetic tree was inferred by using neighbour-joining analysis (Saitou & Nei, 1987) with the Jukes & Cantor correction (Jukes & Cantor, 1969). Bootstrap analysis was used for 100 trial replications to provide confidence estimates for tree topologies. Phylogenies were confirmed by maximum-likelihood analysis. The phylogenetic analysis revealed that the new isolate was the first cultivated *Sulfurihydrogenibium* forming a separate clade with environmental sequences from Yellowstone National Park (Fig. 2).

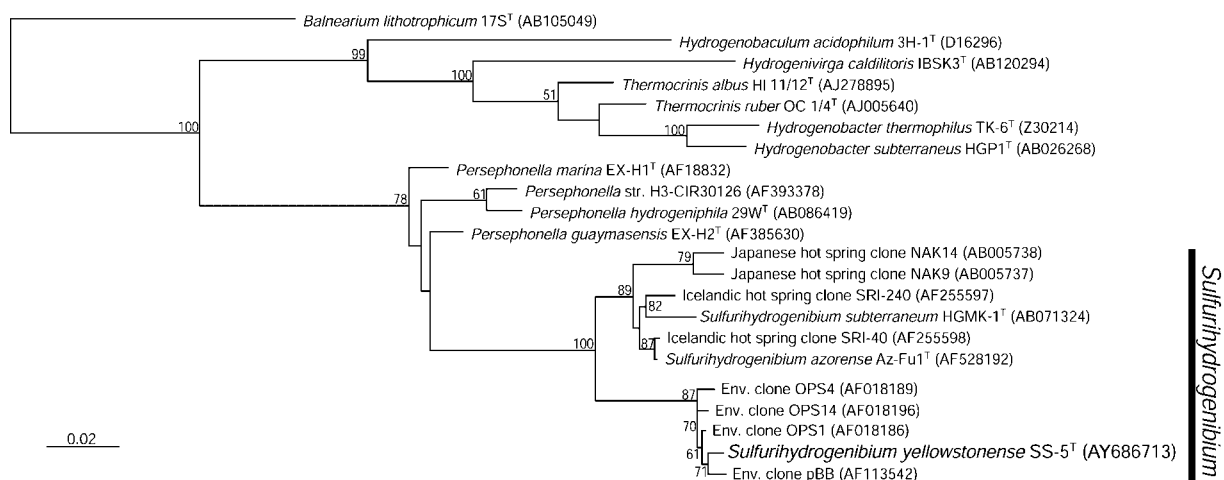


Fig. 2. Neighbour-joining phylogenetic tree of 16S rRNA gene sequence from representative isolates and environmental clones of the order Aquificales. Bootstrap values are based on 100 replicates each and shown for branches with greater than 50% support. The scale bar represents the expected number of changes per nucleotide position. The numbers in parentheses are EMBL/GenBank/DDBJ accession numbers.

Isolation and base composition of DNA

The G+C content of the genomic DNA was determined from the draft genome sequence (TIGR, unpublished). The G+C content of the genomic DNA of strain SS-5^T was 32 mol%, similar to those of *S. subterraneum* HGMK-1^T (31.3 mol%) and *S. azorensis* Az-1^T (33.6 mol%) (Table 1).

Comparison with related species

The phylogenetic analysis based on the 16S rRNA gene sequence indicates that strain SS-5^T is a new species of the genus *Sulfurihydrogenibium* and forms a separate clade with environmental sequences from Yellowstone National Park. Two *Sulfurihydrogenibium* species, *S. subterraneum* HGMK-1^T (Takai *et al.*, 2003) and *S. azorensis* Az-1^T (Aguiar *et al.*, 2004), have been isolated and validly described from a subsurface gold mine and a terrestrial hot spring, respectively. Based on the molecular and physiological characteristics, strain SS-5^T was clearly different (Table 1). The strain is also distinguished from *S. subterraneum* and *S. azorensis* in the ATP citrate lyase B subunit (*aclB*) and ITS sequences (I. Ferrera & A.-L. Reysenbach, unpublished data). Although growth temperature, NaCl and pH ranges of strain SS-5^T were similar to those of other *Sulfurihydrogenibium* species, the ability to utilize a much greater variety of organic carbon sources was unique to the new isolate. In addition, strain SS-5^T was unable to utilize the range of electron donors and acceptors reported to be used by other *Sulfurihydrogenibium* species. In particular, the inability of strain SS-5^T to use hydrogen as an electron donor is unique within the genus *Sulfurihydrogenibium*. On the basis of these molecular and physiological characteristics, we propose a new species called *S. yellowstonense*. The type strain is *S. yellowstonense* SS-5^T (= JCM 12773^T = OCM 840^T).

Description of *Sulfurihydrogenibium yellowstonense* sp. nov.

Sulfurihydrogenibium yellowstonense (yel.low.ston.en'se. N. L. neut. adj. *yellowstonense* from the Yellowstone National Park, the place of isolation).

Motile Gram-negative rods, about 2.5 × 0.6 μm. Growth occurs from 55 to 78 °C (optimum 70 °C). The pH range for growth is 6.0–8.0 (optimum 7.5). Growth occurs in 0–6.0 g NaCl l⁻¹ (optimum 0 g l⁻¹). Thiosulfate or elemental sulfur can be used as electron donors with molecular oxygen as an electron acceptor. The organism utilizes a variety of organic carbon sources. The G+C content of genomic DNA is 32 mol% (genome sequence). The organism was isolated from Calcite Springs in the Yellowstone National Park. The type strain is *S. yellowstonense* SS-5^T (= JCM 12773^T = OCM 840^T).

Emended description of the genus *Sulfurihydrogenibium*

The description remains as given by Takai *et al.* (2003), with the following modifications: facultatively heterotrophic.

Able to utilize sulfur compounds as electron donors and molecular oxygen as an electron acceptor. Some species can use other electron donors and acceptors.

Emended description of *Sulfurihydrogenibium subterraneum*

The description remains as given by Takai *et al.* (2003), with the following modifications: facultatively heterotrophic. Able to utilize acetate as carbon source.

Emended description of *Sulfurihydrogenibium azorensis*

The description remains as given by Aguiar *et al.* (2004), with the following modifications: facultatively heterotrophic. Able to utilize yeast extract, Bacto peptone, tryptic peptone and Casamino acids as carbon source.

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