Thermoanaerobacter sulfurigignens sp. nov., an anaerobic thermophilic bacterium that reduces 1 M thiosulfate to elemental sulfur and tolerates 90 mM sulfite

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Two anaerobic thermophilic bacteria, designated strains JW/SL824 and JW/SL-NZ826^T, were isolated from an acidic volcanic steam outlet on White Island, New Zealand. Cells were rod-shaped, spore-forming, motile and Gram-stain negative, but contained Gram-type positive cell wall. Strain JW/SL-NZ826^T utilized various carbohydrates including xylose and glucose. The fermentation end products produced from glucose in the absence of thiosulfate were lactate, ethanol, acetate, CO₂ and H₂. The temperature range for growth was 34–72 °C, with an optimum at 63–67 °C. The pH^{60 °C} range for growth was 4.0–8.0, with an optimum at 5.0–6.5. The doubling time of strain JW/SL-NZ826^T under optimal growth conditions was 2.4 h. The DNA G + C content was 34–35 mol% (HPLC). The two strains reduced up to 1 M thiosulfate to elemental sulfur without sulfide formation, which is a trend typically observed among species belonging to the genus *Thermoanaerobacterium*. Sulfur globules containing short and long sulfur chains but no S₈-ring sulfur were produced inside and outside the cells. Up to 90 mM sulfite was tolerated. This tolerance is assumed to be an adaptation to the geochemistry of the environment of White Island. The 16S rRNA gene sequence analysis, however, indicated that the two strains belonged to the genus *Thermoanaerobacter*, with similarities in the range 95.6–92.7 %. Therefore, strains JW/SL-NZ824 and JW/SL-NZ826^T represent a novel taxon, for which the name *Thermoanaerobacter sulfurigignens* sp. nov. is proposed, with strain JW/SL-NZ826^T (= ATCC 700320^T = DSM 17917^T) as the type strain. Based on this and previous studies, an emended description of the genus *Thermoanaerobacter* is given.

Microbial thiosulfate reduction is a highly significant process in the geochemical cycling of sulfur species in the anaerobic environment (Ravot *et al.*, 1995). Under anaerobic conditions, thiosulfate can be either disproportionated (Jorgensen, 1990) or reduced to produce other inorganic sulfur compounds. As the reduction of thiosulfate increases with sediment depth, anaerobes participate prominently in

dissimilatory thiosulfate reduction. In addition to mesophilic facultative and strict anaerobes (Barrett & Clark, 1987), several thermophilic archaea and bacteria can reduce thiosulfate (Lee *et al.*, 1993; Ravot *et al.*, 1995). However, only a limited number of thermophiles can reduce thiosulfate to either hydrogen sulfide or elemental sulfur, or both. Lee *et al.* (1993) used this characteristic to differentiate between the genera *Thermoanaerobacter* and *Thermoanaerobacterium* in the family 'Thermoanaerobacteriaceae'. Members of the genus *Thermoanaerobacter* reduce thiosulfate to hydrogen sulfide, whereas members of the genus *Thermoanaerobacterium* reduce thiosulfate to elemental sulfur. However, it was reported recently that two species

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain JW/SL-NZ826^T is AF234164.

belonging to *Thermoanaerobacterium* were unable to reduce thiosulfate to elemental sulfur (Cann *et al.*, 2001). Furthermore, three *Thermoanaerobacter* species have been reclassified as species and subspecies of the recently created genus *Caldanaerobacter* (Fardeau *et al.*, 2004). At present, the genus *Thermoanaerobacter*, with the type species *Thermoanaerobacter ethanolicus* JW 200^T, comprises 11 recognized species and three *Thermoanaerobacter brockii* subspecies. All of the species produce sulfide from thiosulfate except *Thermoanaerobacter italicus*, which produces both sulfide and elemental sulfur (Kozianowski *et al.*, 1997). Thus, the former distinguishing feature of the genus (Lee *et al.*, 1993) of reduction of thiosulfate solely to hydrogen sulfide became genus unspecific. In this paper, we report on two *Thermoanaerobacter* isolates from a hot spring on the volcanic White Island off the coast of New Zealand and which, atypically for the genus *Thermoanaerobacter*, reduce thiosulfate (up to a concentration of 1 M) to elemental sulfur only without the expected formation of sulfide and can tolerate the high concentration of up to 90 mM sodium sulfite.

A mixed water and sediment sample was collected from an acidic hot spring on White Island (New Zealand) in 1993. White Island exhibits a highly reductive environment due to high fumarolic gas discharges all over the island (Giggenbach, 1987; Hedenquist *et al.*, 1993). More recently, an environmental 16S rRNA gene sequence analysis was conducted that showed the presence of uncultured *Firmicutes* in the hydrothermal waters (Donachie *et al.*, 2002). The pH^{60 °C} at the site was 2.5–3.5 and the temperature was 45–80 °C. The pH of the sample collected was immediately, i.e., before the samples could cool down to ambient temperature, adjusted to 5.0 by the addition of bicarbonate. Except for the time of travel, the sample was stored at 4–7 °C for a total of about 1 month (Liu, 1995).

Initial enrichment was performed at 60 °C under anaerobic conditions by using the modified Hungate technique (Ljungdahl & Wiegel, 1986). Samples (~0.5 g/50 ml) were inoculated into phosphate-buffered basal medium (pH^{60 °C} 4.0–5.0). The phosphate-buffered basal medium contained: 11.6 mM Na₂HPO₄, 3.7 mM KH₂PO₄, 17.0 mM NaCl, 3.8 mM (NH₄)₂SO₄, 9.3 mM NH₄Cl, 0.2 mM MgCl₂, 0.3 mM CaCl₂, 0.5 mM Na₂S, 0.5 mM cysteine acid, 0.1 % (w/v) resazurin and 5.0 ml l⁻¹ of a trace element solution and 0.5 ml l⁻¹ of a vitamin solution (Freier *et al.*, 1988). Unless stated otherwise, the medium was supplemented with 0.5 % (w/v) yeast extract and 0.5 % (w/v) xylose as carbon sources and the pH was adjusted to 4.5 before degassing. Thiosulfate (Na₂S₂O₃, 20 mM) was added to the medium for isolation of the target bacteria, *Thermoanaerobacterium*. Subsequently, colonies on thiosulfate-containing agar that contained cells with internal refractive sulfur globules were purified using the agar-shake-roll-tube method with the above medium supplemented with 2.0 % (w/v) agar. Pure cultures were obtained by repeated isolation of single colonies. Two strains from two separate enrichments were chosen for further investigation.

The colonies of the two isolates were creamy white and circular, and 1–2 mm in diameter after incubation for 3–4 days. No pigmentation was observed. The morphology of the isolates was studied by using a PM-10AD phase-contrast microscope (Olympus Optical Co.) and a Zeiss field-emission scanning electron microscope (DSM982 Gemini). Cells used for negative staining (Valentine *et al.*, 1968; Beuscher *et al.*, 1974) were from either early exponential or stationary growth phase. The cells of the two strains were rod-shaped in all growth phases in both liquid and agar media. The size of the cells during exponential growth ranged from 0.3 to 0.8 µm in diameter and 1.2 to 4.0 µm in length. The mean length of the cells increased in the stationary growth phase, and cells were elongated up to 35 µm without any indication of septation. When the two strains were grown on thiosulfate (20 mM), elemental sulfur was observed to be deposited inside (data not shown) and outside the cells (Fig. 1). The cells were motile and peritrichously flagellated. Spores detected by microscopy were spherical and terminal with a diameter of 0.45–0.85 µm and were usually found during the late exponential or early stationary growth phase in both liquid and agar media. The cells stained Gram-negative regardless of the growth phase and growth conditions. However, the test for the formation of a lipopolysaccharide–polymyxin B complex (Wiegel & Quandt, 1982) gave no indication of the presence of lipopolysaccharides and the electron micrographs of the cell wall indicated that the cells possessed a Gram-positive cell wall, thus the strains are Gram-type positive (Wiegel, 1981; see also the 16S rRNA gene sequence analysis). Both isolates were able to survive for more than 2 years in liquid medium at room temperature. Liquid cultures mixed with anaerobic, sterile glycerol at a 1 : 1 ratio remained viable after more than 4 years at –70 °C.

The temperature range for growth was determined from 28 to 80 °C by using a temperature-gradient incubator (Scientific Industries Inc.) with the medium described above (pH^{25 °C} = 6.5). Strain JW/SL-NZ826^T grew at 34–72 °C at pH^{25 °C} 6.5, with an optimum at 63–67 °C. No growth was observed at or below 32 °C or at or above 74 °C (Fig. 2a). The pH range for growth was determined at 60 °C with an

Fig. 1. Scanning electron micrograph of a cell of strain JW/SL-NZ826^T showing sulfur globules. Bar, 1 µm.

Fig. 2. Growth dependence of strain JW/SL-NZ826^T on incubation temperature at pH²⁵ °C 6.5 (a) and on pH⁶⁰ °C (b). 1/t_d, Inverse of doubling time.

825-MP pH meter (Fisher Scientific) equipped with a combination of a pH electrode (Sensor) and a temperature probe. The pH was kept constant (± 0.1 pH unit) by periodic titration using anaerobic 1 M NaOH. The pH⁶⁰ °C range for growth was 4.0–8.0, with an optimum at 5.0–6.5 (Fig. 2b). No growth was observed at or below pH 3.8 or at or above pH 8.2. A similar broad pH optimum, as well as the biphasic response to growth temperature, has been observed with other *Thermoanaerobacter* species, in particular the type species, *Thermoanaerobacter ethanolicus* JW 200^T (Wiegel & Ljungdahl, 1981; Wiegel, 1990, 1998).

The range of substrate utilization was determined using the medium described above with 0.3 % (w/v) yeast extract and 0.5 % of the filter-sterilized substrate at 65 °C (pH²⁵ °C 6.5). The inocula comprised 1.0 % (v/v) exponentially growing cells that were subcultured three times in medium containing only 0.01 % yeast extract. Utilization was judged to be positive when the optical density (OD₆₀₀) of the culture

increased by 0.15 or more, accompanied by a decrease in pH of two units. A control culture containing only yeast extract gave a final OD₆₀₀ of less than 0.1. To confirm the utilization of a specific substrate, cells were subcultured with the same substrate twice. Strains JW/SL-NZ824 and JW/SL-NZ826^T both grew well on xylose, glucose, starch, galactose, fructose, lactose, maltose, mannose, sucrose, cellobiose, raffinose, pyruvate, methanol and mannitol in the presence of 0.3 % (w/v) yeast extract. The two strains showed no growth on ribose, arabinose, dextran, xylan, cellulose, glycerol, xylitol, formate, fumarate, gluconate, succinate, peptone, tryptone, casein hydrolysate or Casamino acids (0.5 %, w/v). Both strains required yeast extract for growth. There was no indication of growth either under aerobic conditions or chemolithoautotrophic conditions with H₂/CO₂ (80:20, v/v). The fermentation end products produced by strain JW/SL-NZ826^T from 0.5 % glucose in the presence of 0.3 % yeast extract were ethanol, acetate, lactate, CO₂ and H₂ (see equation 1), which are common fermentation end products of *Thermoanaerobacter* species (R. Onyenwoke & J. Wiegel, unpublished data).

Thiosulfate was reduced by both strains, resulting in the production of sulfur globules, regardless of the presence of sulfide/cysteine.HCl as a reducing agent. The onset of sulfur granule formation was dependent upon the initial concentration of thiosulfate used: a higher concentration led to an earlier onset during the exponential growth phase. Sulfur globules were observed inside and outside the cells. Although S⁰ started to form during the late exponential growth phase (thiosulfate, 20 mM), the majority of the sulfur globules appeared at the end of the stationary growth phase.

The two strains grew in the presence of and reduced sodium thiosulfate without inhibition at concentrations of around 700 mM and tolerated sodium thiosulfate up to 1 M with lower growth rates. Concentrations at or above 1.2 M sodium thiosulfate prevented growth. No sulfate reduction was detected in the presence of 2–30 mM sulfate in medium containing glucose, xylose, acetate or lactate as a carbon source, supplemented with 0.15 % yeast extract. Sodium sulfate concentrations above 500 mM were inhibitory. Strain JW/SL-NZ826^T also tolerated the presence of up to 90 mM sodium sulfite whereas most bacteria are inhibited by sulfite concentrations above 5 mM. In contrast to sodium sulfate and sodium thiosulfate, 100 mM NaCl (about 0.6 %, w/v) was inhibitory for strain JW/SL-NZ826^T. No growth was observed at or above 150 mM NaCl (around 1 %, w/v). We speculate that the high tolerance of these strains to thiosulfate, sulfate and sulfite was an adaptation to an environment with abundant sulfur and sulfoxy species present (due to frequent outbursts of SO₂ caused by mining of the sulfur on the island).

The formation of sulfur globules from thiosulfate, by cells grown in sulfate-free medium but supplemented with 50 and 500 mM sodium thiosulfate, was confirmed using X-ray absorption spectroscopy as described in detail by Prange *et al.* (1999, 2002). Similar sulfur-chain structures have been observed for the sulfur in the sulfur globules of phototrophic sulfur bacteria (Prange *et al.*, 1999; Dahl & Prange, 2006). Comparative analysis of the sulfur globules produced by *Thermoanaerobacterium thermosulfurigenes*, under conditions similar to those for strain JW/SL-NZ826^T, yielded similar results (data not shown), and no differences were observed.

Biochemical features of the isolates were tested using the An-Ident Strip system (API Analytab Products). Positive results were obtained for indolyl-acetate, leucine aminopeptidase, β -glucosidase and arginine utilization. Assays for the following enzymes were negative: *N*-acetylglucosaminidase, α -glucosidase, α -arabinosidase, α -fructosidase, phosphatase, α -galactosidase, β -galactosidase, proline aminopeptidase, pyroglutamic acid arylamidase, tyrosine aminopeptidase, arginine aminopeptidase, alanine aminopeptidase, histidine, phenylalanine aminopeptidase, glycine aminopeptidase, catalase and indole production.

The susceptibility to antibiotics was determined by transferring 1.0 % (v/v) of an exponentially growing culture into fresh medium containing 0.3 % (w/v) yeast extract, 0.5 % (w/v) xylose and either 10 or 100 $\mu\text{g ml}^{-1}$ of the filter-sterilized antibiotic (Sigma). The two strains were resistant to kanamycin, streptomycin, cycloheximide and cycloserine at 100 $\mu\text{g ml}^{-1}$ and to vancomycin, bacitracin, tetracycline and ampicillin at 10 $\mu\text{g ml}^{-1}$. Both strains were sensitive to 10 μg gramicidin ml^{-1} and 100 μg neomycin or chloramphenicol ml^{-1} . However, these results should be considered in the light of the previously reported instability of the antibiotics under the growth and test conditions used (Peteranderl *et al.*, 1990).

DNA was isolated from cells in exponential growth phase according to the methods of Wilson (1987) and Marmur

(1961) by using CsCl gradient ultracentrifugation. The DNA G+C content was determined by HPLC as described previously (Whitman *et al.*, 1986; Mesbah *et al.*, 1989). The DNA G+C content of strain JW/SL-NZ826^T was 34.5 mol%.

Genomic DNA was isolated and the 16S rRNA gene was amplified as described previously (Rainey *et al.*, 1996). The sequences of the PCR products were determined using an ABI 373A DNA sequencer with a *Taq*DyeDeoxy Terminator cycle sequencing kit (Applied Biosystems), as recommended by the manufacturer. The 16S rRNA gene sequences of strains JW/SL-NZ826^T and JW/SL-NZ824 were aligned manually with previously published 16S rRNA gene sequences of representatives of the genus *Thermoanaerobacter* and related taxa. The model of Jukes & Cantor (1969) was used to calculate the evolutionary distances and phylogenetic trees were inferred by using the neighbour-joining method (Saitou & Nei, 1987) and Fitch–Margoliash distance-based method (Fitch & Margoliash, 1967), with the phylogenetic analysis package PHYLIP v3.6a2.1 (Felsenstein, 2001).

An almost complete 16S rRNA gene sequence of strain JW/SL-NZ826^T was determined, comprising 1501 nucleotides (29–1529, based on *Escherichia coli* ATCC 11775^T numbering). Based on the 16S rRNA gene sequence analysis, strains JW/SL-824 and JW/SL-NZ826^T were identical and were distantly related to recognized species of the genus *Thermoanaerobacter*, with *Thermoanaerobacter brockii* subsp. *brockii* (95.6 % sequence similarity) as the closest relative (Fig. 3).

The main difference between strain JW/SL-NZ826^T and other species of *Thermoanaerobacter* was the production of sulfur globules instead of hydrogen sulfide when grown in medium containing thiosulfate. This feature more resembles species of the genus *Thermoanaerobacterium*. The 16S rRNA gene sequence is the main characteristic that differentiates strain JW/SL-NZ826^T from species of the genus *Thermoanaerobacterium* (only 87.1 % similarity to the type species

Fig. 3. Phylogenetic dendrogram based on 16S rRNA gene sequences showing the position of strain JW/SL-NZ826^T amongst members of the family 'Thermoanaerobacteriaceae'. Numbers at nodes are percentage bootstrap values (based on 1000 replicates); only values above 80 % were considered significant and shown. Bar, 2 substitutions per 100 nucleotides.

Table 1. Characteristics that differentiate strain JW/SL-NZ826^T and related species of *Thermoanaerobacter* and *Thermoanaerobacterium*

Strains: 1, strain JW/SL-NZ826^T (*Thermoanaerobacter sulfurigignens* sp. nov.; data from this study); 2, *Thermoanaerobacter italicus* DSM 9252^T (Kozianowski *et al.*, 1997); 3, *Thermoanaerobacter ethanolicus* JW 200^T (Wiegel & Ljungdahl, 1981); 4, *Thermoanaerobacterium thermosulfurigenes* 4B^T (Schink & Zeikus, 1983). NR, Not reported; ND, not determined.

Characteristic	1	2	3	4
Source	Acidic hot spring	Thermal spa	Alkaline hot spring	Hot spring
Cell size (µm)	0.3–0.8 × 1.2–4.0	0.4–0.75 × 2–6	0.3–0.8 × 4–8	0.5 × > 2
Temperature range (optimum) (°C)	34–72 (63–67)	45–78 (70)	37–78 (69)	35–75 (60)
pH range (optimum)	4.0–8.0 (5.0–6.5)	NR (7.0)	4.4–9.9 (5.8–8.5)	4.0–7.6 (5.5–6.5)
DNA G + C content (mol%)	34.5	34.4	32	32.6
Gram stain	–	–	Variable	–
Spore formation	+	+	–	+
Product from S ₂ O ₃ ^{2–} reduction	S ⁰	S ^{2–} , S ⁰	S ^{2–}	S ⁰
Toleration of 1 M thiosulfate and reduction to S ⁰	+	ND	–	–
Toleration of 90 mM sulfite	+	ND	–	–

Thermoanaerobacterium thermosulfurigenes), although there are minor differences in substrate spectrum, susceptibility to antibiotics, growth response to temperature and pH, and the limited growth in the absence of yeast extract. Other morphological and physiological differences among related species are summarized in Table 1. Despite the fact that strain JW/SL-NZ826^T branched off most distantly and was between the recently created genus *Caldanaerobacter* and all other species of *Thermoanaerobacter*, it was decided to place the strain within the genus *Thermoanaerobacter* and to designate it as representing the type strain of a novel taxon, *Thermoanaerobacter sulfurigignens* sp. nov.

Members of the genus *Thermoanaerobacter* have been described as producing hydrogen sulfide exclusively from thiosulfate (Lee *et al.*, 1993). *Thermoanaerobacter italicus* produces both hydrogen sulfide and elemental sulfur (Kozianowski *et al.*, 1997), whereas strain JW/SL-NZ826^T reduces thiosulfate exclusively to elemental sulfur, forming sulfur globules, and does not form sulfide. Thus the description of the genus has been amended.

Emended description of the genus *Thermoanaerobacter*

Reduce thiosulfate to either hydrogen sulfide (majority) or elemental sulfur, or both.

Description of *Thermoanaerobacter sulfurigignens* sp. nov.

Thermoanaerobacter sulfurigignens (sul.fur.i'gig.nens. L. n. sulfur brimstone, sulfur; L. part. adj. gignens producing; N.L. part. adj. sulfurigignens sulfur-producing).

Cells are rod-shaped, 0.3–0.8 µm in diameter and 1.2 (exponential growth phase) to 35 µm (stationary growth phase) in length. Cells are motile by means of peritrichous flagella, stain Gram-negative but possess Gram-type positive cell wall. Belongs to the Gram-type positive (Wiegel, 1981)

Firmicutes. Spherical terminal spores are observed. Temperature range for growth is 34–72 °C (pH^{25 °C} 6.5), with an optimum at around 65 °C. pH^{60 °C} range for growth is 4.0–8.0 with an optimum at 5.0–6.5. In the presence of 0.3 % yeast extract, xylose, glucose, starch, fructose, galactose, lactose, maltose, mannose, sucrose, cellobiose, raffinose, pyruvate, methanol and mannitol serve as carbon and energy sources. Up to 1 M thiosulfate is reduced to elemental sulfur mainly at the end of the exponential growth phase (deposited in sulfur chains without indication of the presence of S₈-ring sulfur species and the formation of sulfide). No indication of sulfate reduction, but tolerates up to 500 mM sulfate and 90 mM sulfite. Growth does not occur at or above 150 mM (~1 %, w/v) NaCl. Resistant to kanamycin, streptomycin, cycloheximide, cycloserine, vancomycin, bacitracin, tetracycline and ampicillin, but sensitive to gramicidin, neomycin and chloramphenicol. The DNA G + C content is 34–35 mol% (HPLC).

The type strain, JW/SL-NZ826^T (= ATCC 700320^T = DSM 17917^T), was isolated from an acidic volcanic steam outlet on White Island, New Zealand.

Acknowledgements

This study was partially supported by a grant to J.W. from the US Department of Energy (DE-FG05-95ER-20199). We would like to thank H. Morgan and R. Daniel for organizing the helicopter flight to White Island, New Zealand. We thank Jean P. Euzéby for his help with the nomenclature. The corresponding author is highly indebted to his former student Shu-Ying Liu for the isolation of the two strains; unfortunately, we do not have her present contact address, thus her name had to be removed from the author line.

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