Thermovenabulum gondwanense sp. nov., a thermophilic anaerobic Fe(III)-reducing bacterium isolated from microbial mats thriving in a Great Artesian Basin bore runoff channel

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A strictly anaerobic, thermophilic bacterium, designated strain R270^T, was isolated from microbial mats thriving in the thermal waters (66 °C) of a Great Artesian Basin bore (registered no. 17263) runoff channel. Cells of strain R270^T were straight to slightly curved rods (3.50–6.00×0.75–1.00 µm) that stained Gram-positive, but possessed a Gram-negative cell-wall ultrastructure. Strain R270^T grew optimally in tryptone-yeast extract-Casamino acids medium at 65 °C (growth temperature range between 50 and 70 °C) and at pH 7.0 (growth pH range between 6.0 and 9.0). In the presence of 0.02 and 0.10 % yeast extract, pyruvate and Casamino acids were the only substrates fermented from a wide spectrum of substrates tested. Fe(III), Mn(IV), thiosulfate and elemental sulfur were used as electron acceptors in the presence 0.2 % yeast extract, but not sulfate, sulfite, nitrate, nitrite or fumarate. Growth of strain R270^T increased in the presence of Fe(III), which was reduced in the presence of peptone, tryptone, Casamino acids, amyl media, starch, pyruvate, H₂ and CO₂, but not in the presence of acetate, lactate, propionate, formate, benzoate, glycerol or ethanol. Growth and Fe(III) reduction were inhibited by chloramphenicol, streptomycin, tetracycline, penicillin, ampicillin and 2 % NaCl (w/v). The DNA G + C content of strain R270^T was 41 ± 1 mol% (*T_m*) and phylogenetic analysis of the 16S rRNA gene indicated that this isolate was closely related to *Thermovenabulum ferriorganovororum* DSM 14006^T (similarity value of 96.1 %) within the family 'Thermoanaerobacteraceae', class 'Clostridia', phylum 'Firmicutes'. On the basis of the phylogenetic distance separating the two, together with differences in a number of key phenotypic characteristics, strain R270^T represents a novel species of the genus *Thermovenabulum*, for which the name *Thermovenabulum gondwanense* sp. nov. is proposed; the type strain is R270^T (=KCTC 5616^T=DSM 21133^T).

The Great Artesian Basin (GAB) is the world's largest geothermal subsurface aquifer underlying semi-arid regions comprising more than one-fifth of the Australian continent (Habermehl, 1980). Over 5000 bores have been sunk into the GAB to provide a vital water resource to outback Australian communities. During recent years, there has been a significant drop in the GAB groundwater pressure and volume, largely due to water leakage from corroding bores, and this is a matter of great concern. Our recent isolation of several novel metal-reducing micro-organisms from the GAB (Kanso *et al.*, 2002; Ogg & Patel, 2009a, b, c) suggests that they could be colonizing the metal casing of bores, thereby contributing to bore

corrosion and subsequent complete bore failure. It is widely accepted that metal-reducing micro-organisms have a large impact on the geochemistry of subsurface environments through the cycling of metals and organic matter, and thereby affect water quality and taste (Lovley, 1997). Furthermore, metal-reducing micro-organisms have potential applications in bioremediation, mineral leaching and energy generation processes and are of evolutionary interest as metal reduction is considered to be a very ancient form of respiration (Vargas *et al.*, 1998). In this report, we extend our studies of the GAB environment and describe a strictly anaerobic, thermophilic, metal-reducer, designated strain R270^T, which represents a novel species of the genus *Thermovenabulum*.

The red microbial mat used in this study was collected from the runoff channel of the New Lorne Bore (registered bore no. 17263) situated near Blackall, some 1000 km north-west of Brisbane, Queensland State, Australia (24°

Abbreviation: GAB, Great Artesian Basin.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain R270^T (=KCTC 5616^T=DSM 21133^T) is EU443729.

54° 48' S 145° 08' 18' E). The bore site and its microbiology have been described previously (Spanevello, 2001; Spanevello *et al.*, 2002; Spanevello & Patel, 2004). The bore was initially drilled in 1966 and has a depth of 1613 m. The red microbial mats, which thrive at 66 °C, were collected in sterile glass vessels; the vessels were filled to the brim with water from the runoff channel, capped, transported to the laboratory and stored at 4 °C until used. At the time of sampling, the bore water temperature at the source was 88 °C, the flow rate was 7.6 l s⁻¹, the pH was 8.5 and iron and manganese concentrations were 0.01 mg l⁻¹. The temperature and pH of the bore and the runoff channel have not changed over the 8-year period of our studies.

Enrichment and isolation were performed in anaerobic Patel Laboratory (PL) medium. PL medium was prepared as described previously (Ogg & Patel, 2009a) and contains (l⁻¹ deionized water): 1 g NH₄Cl, 0.6 g K₂HPO₄, 0.3 g KH₂PO₄, 0.1 g MgCl₂·6H₂O, 0.1 g CaCl₂·2H₂O, 1.0 g NaCl, 12.0 g HEPES, 1 ml vitamin solution (Wolin *et al.*, 1963), 1 ml trace element solution (Zeikus *et al.*, 1979), 1 ml 0.2 % resazurin, 2.0 g yeast extract and 2.0 g ammonium Fe(III) citrate (brown). Enrichment cultures were initiated by injecting 500 µl red mat suspension (prepared by gentle inversion) into 9.5 ml PL medium. An enrichment culture that transformed the reddish-brown colour of the Fe(III) to a dark precipitate [Fe(II)] and subsequently resulted in clearing of the medium after 3 days incubation at 70 °C was regarded as positive for Fe(III) reduction. Fe(III) reduction was then confirmed by determining Fe(II) accumulation using the ferrozine method (Sørensen, 1982). For this, 0.1 ml cell-free culture supernatant was mixed with 3 ml ferrozine reagent and absorbance at 562 nm was determined after 1 min. The enrichment culture was then serially diluted, incubated under the same conditions and the tube containing the lowest serial dilution (10⁻³) was selected. The procedure was repeated at least three times before strain R270^T was isolated and selected for further studies. Culture purity was subsequently confirmed by microscopy and 16S rRNA gene sequencing. A pure culture was preserved in a glycerol/PL medium (50:50) mixture at -20 °C.

Cell morphology was determined by phase-contrast microscopy and by electron microscopy (Kanso & Patel, 2003). Cells of strain R270^T were straight to slightly curved rods (3.50–6.00 × 0.75–1.00 µm) that existed singly and in pairs and were peritrichously flagellated. Spores were not observed from cultures that had been grown under suboptimal conditions (pH and temperature) or in the presence of 0.2 % glucose or 0.2 % xylose. In addition, cells of strain R270^T heated at 95 °C for 10 min failed to subculture in PL medium suggesting the absence of heat-resistant bodies such as spores. Cells of strain R270^T stained Gram-positive, although electron micrographs of thin sections revealed a tri-layered cell wall suggestive of a Gram-negative nature (Fig. 1).

Fig. 1. Transmission electron micrograph of a thin section of strain R270^T showing the presence of a cytoplasmic membrane (CM) and a tri-layered cell wall comprising electron-light inner (CWi) and outer (CWo) layers, and an electron-dense middle layer (CW) which is sandwiched between them. Bar, 50 nm.

All growth experiments and nutritional characterization tests were conducted at least twice and cultures were subcultured at least once in the same medium prior to carrying out experiments. Growth studies of strain R270^T were performed in an anaerobic modified low-phosphate-buffered salts (LPBS) medium (Patel *et al.*, 1985a, b) amended with 0.2 % each of tryptone, yeast extract and Casamino acids (designated TYECas). LPBS contained (l⁻¹ deionized water): 0.2 g MgCl₂·6H₂O, 0.9 g NH₄Cl, 0.75 g KH₂PO₄, 1.5 g K₂HPO₄, 1.0 g NaCl, 5 µl 10 % FeSO₄, 9 ml trace element solution (Zeikus *et al.*, 1979), 5 ml vitamin solution (Wolin *et al.*, 1963), 1 ml 0.2 % resazurin and 2 ml 10 % Na₂S·9H₂O. Growth of strain R270^T was tested in TYECas medium at 26–80 °C and pH 4.5–10.0. Growth was measured spectrophotometrically at 580 nm by inserting Hungate culture tubes directly into a modified cuvette holder of a Novaspec LKB spectrophotometer (Pharmacia Biotech). Strain R270^T grew optimally in TYECas medium at 65 °C (range: 50–70 °C) and pH 7.0 (range: pH 6.0–9.0). However, even under optimal growth conditions, growth was poor (increase in turbidity was 0.14 after 72 h incubation). Unless indicated otherwise, all subsequent growth experiments were conducted using optimal growth parameters (65 °C and pH 7.0).

Strain R270^T was tested for growth in medium D (Brock & Freeze, 1969) and in aerobic TYECas medium at 65 °C. No visual turbidity was observed for up to 14 days indicating that the isolate could not grow aerobically.

The effect of soluble Fe(III) on growth of strain R270^T was tested in PL medium and the effect of insoluble Fe(III) was tested in PL medium that lacked ammonium Fe(III) citrate, but instead contained 0.2 % amorphous Fe(III) oxyhydroxide [prepared as described by Lovley & Phillips,

(1988)]. PL medium that lacked a source of Fe(III) was used as a control. Increase in cell numbers was measured over a 4 day incubation period by the MPN technique (Greenberg *et al.*, 1992) using PL medium. Cell numbers were greatest (2.0×10^5) in medium containing soluble Fe(III), followed by medium containing insoluble Fe(III) (1.1×10^5), but an increase in cell numbers was not noted in the Fe(III)-deficient control medium (1.1×10^2).

Substrate utilization tests were performed in sterile LPBS medium amended with 0.02 or 0.1 % yeast extract. Unless otherwise indicated, substrates were added to the medium from sterile, anaerobic stock solutions to a final concentration of 0.2 or 1 %. Growth was measured twice daily at 580 nm until stationary phase was reached or for up to 8 days incubation. Strain R270^T grew on 0.2 % pyruvate and Casamino acids, but not on 0.2 % galactose, fructose, sucrose, glucose, maltose, lactose, mannitol, arabinose, xylose, ethanol, glycerol, acetate, lactate or propionate, or on 1 % yeast extract, peptone, tryptone or starch.

The following alternative electron acceptors, namely, fumarate (20 mM), sodium thiosulfate (20 mM), sodium sulfate (20 mM), sodium sulfite (5 mM), elemental sulfur (1 %), sodium nitrate (20 mM), sodium nitrite (5 mM) and 0.2 % Mn(IV) [prepared as described by Lovley & Phillips (1988)], were tested in Fe(III)-deficient PL medium. Utilization of fumarate as an electron acceptor was determined by measuring growth at 580 nm as described above. Reduction of other alternative electron acceptors was determined as described by Ramamoorthy *et al.* (2006) and Ogg & Patel (2009a). Mn(IV) reduction was inferred by a clearing of the media and was further confirmed using the leuco crystal violet manganese oxide detection technique (Spratt *et al.*, 1994). For this, MnO₂ concentration was determined by mixing 0.2 ml sample with 4 ml leuco crystal violet reagent, which was diluted, and the absorbance at 562 nm was determined. Strain R270^T was able to reduce amorphous Mn(IV), thiosulfate and elemental sulfur, but not sulfate, sulfite, nitrate, nitrite or fumarate.

The ability of strain R270^T to utilize various electron donors in the presence of Fe(III) was tested in PL medium containing a reduced concentration of yeast extract (0.02 %). Substrates were added to the medium from sterile, anaerobic stock solutions to a final concentration of 0.2 % and incubations were performed for up to 14 days. Carbohydrates were excluded as test substrates because Fe(III) reduction can occur spontaneously under sterile conditions (Zavarzina *et al.*, 2002). Fe(III) reduction was determined as described above and was positive for peptone, tryptone, Casamino acids, amyl media (Research Achievement), starch and pyruvate, but not for acetate, lactate, propionate, formate, benzoate, glycerol or ethanol.

The potential for molecular hydrogen to act as an electron donor in the presence or absence of Fe(III) was tested in PL medium containing a reduced concentration of yeast extract (0.02 %). PL medium was prepared essentially as described above except after heating for 10 min, the medium was

cooled under a stream of CO₂ gas and 15 ml was dispensed into 60 ml serum bottles under oxygen-free H₂:N₂ (5:95) gas mixture and the medium was autoclaved. Fe(III) reduction and cell numbers were determined as described above. An increase in cell numbers with a concomitant production of Fe(II) from the reduction of Fe(III) was observed (Fig. 2). No growth or Fe(III) reduction was observed when Fe(III) or yeast extract (0.02 %) was omitted or when H₂ was replaced with N₂.

Antibiotic sensitivity was determined by adding antibiotics from filter-sterilized stock solutions to PL medium to final concentrations of 10 and 100 µg ml⁻¹. Strain R270^T was completely inhibited in the presence of ampicillin, penicillin and chloramphenicol (all at 10 µg ml⁻¹), and streptomycin and tetracycline (both at 100 µg ml⁻¹). The effect of NaCl on growth of strain R270^T was tested in TYECas and PL medium. For this, NaCl was weighed directly into the Hungate tube and the medium was dispensed and sterilized. Strain R270^T was unable to grow or reduce Fe(III) at NaCl concentrations of 2 % (w/v).

The DNA G + C content of strain R270^T was determined by the thermal denaturation method (Marmur & Doty, 1962) in a Cintra20 spectrophotometer (GBC Scientific Equipment) with DNA that had been amplified using a TempliPhi Amplification kit (Amersham Biosciences) as described by Ogg & Patel (2009a). The G + C content of the genomic DNA was calculated to be 41 ± 1 mol%.

16S rRNA gene amplification and sequencing were carried out using the method of Ogg & Patel (2009a). Phylogenetic analysis of the consensus 16S rRNA gene sequence comprising 1423 nt that had been corrected manually for errors indicated that strain R270^T was most closely related to the type strain of *Thermovenabulum ferriorganovorum* (96.1 %), family 'Thermoanaerobacteraceae', class 'Clostridia', phylum 'Firmicutes' (Fig. 3). *T. ferriorganovorum* was isolated from a Russian hot spring and, like strain R270^T, is an anaerobic thermophile that grows optimally at near-neutral pH levels and reduces Fe(III), Mn(IV) and elemental

Fig. 2. Growth (▲) and Fe(II) production (■) by strain R270^T in modified PL medium amended with 0.02 % yeast extract instead of 0.2 % and molecular hydrogen [H₂:N₂ (5:95) gas mixture] as described in the text.

Fig. 3. A dendrogram showing the phylogenetic position of *Thermovenabulum gondwanense* sp. nov. strain R270^T and its closest relatives. GenBank accession numbers are given in parentheses. Clusters of members of the same genus or species are indicated by triangles and include: *Thermoanaerobacter mathranii* DSM 11426^T (GenBank accession no. Y11279), *Thermoanaerobacter siderophilus* DSM 12299^T (AF120479), *Thermoanaerobacter italicus* DSM 9252^T (AJ250846), *Thermoanaerobacter acetoethylicus* ATCC 33265^T (X69336), *Thermoanaerobacter wiegelii* DSM 10319^T (X92513), *Thermoanaerobacter kivui* ATCC 33488^T (L09160), *Thermoanaerobacter thermohydrosulfuricus* ATCC 35045^T (L09161), *Thermoanaerobacter ethanolicus* ATCC 31550^T (L09162), *Thermoanaerobacter brockii* subsp. *brockii* DSM 1457^T (L09165), *Thermoanaerobacter brockii* subsp. *finii* DSM 3389^T (L09166) and *Thermoanaerobacter thermocopriae* JCM 7501^T (L09167) for the genus *Thermoanaerobacter*, and *Caldanaerobacter subterraneus* subsp. *tengcongensis* JCM 11007^T (AF209708) and *Caldanaerobacter subterraneus* subsp. *yonseiensis* DSM 13777^T (AF212925) for the species *Caldanaerobacter subterraneus*. Bootstrap values >95 % are shown. Bar, 5 nt changes per 100 nt.

sulfur, but not sulfate (Zavarzina *et al.*, 2002). Both strains also grow and reduce Fe(III) in the presence of molecular hydrogen and yeast extract (0.02 %), and reduce Fe(III) in the presence of yeast extract, peptone, Casamino acids, starch and pyruvate, but not acetate, lactate, propionate, formate, ethanol or glycerol. Strain R270^T can be differentiated from *T. ferriorganovorum* by its Gram-negative cell-wall ultrastructure, higher DNA G+C content, sensitivity to penicillin, higher pH growth range, lack of heat resistance, inability to form spores and reduce sulfite, nitrate and fumarate, and its restrictive substrate range for growth (e.g. yeast extract, starch, peptone, sucrose, fructose, maltose, ribose and xylose) (Table 1). Based on its close phylogenetic relationship with the genus *Thermovenabulum* (similarity value of 96.1 % to *T. ferriorganovorum* DSM 14006^T) and its considerable phenotypic differences from *T. ferriorganovorum*, it is proposed that strain R270^T represents a novel species in the genus *Thermovenabulum*, *Thermovenabulum gondwanense* sp. nov.

Description of *Thermovenabulum gondwanense* sp. nov.

Thermovenabulum gondwanense (gond.wa.nen'se. N.L. neut. adj. *gondwanense* pertaining to the large land

mass known as Gondwana, which included Australia, Africa, India and South America before they separated).

Cells are strictly anaerobic, straight to slightly curved rods. Gram-reaction is positive, but the cell-wall ultrastructure is Gram-negative. Cells possess peritrichous flagella. No spores are detected. Growth occurs between 50 and 70 °C (optimum temperature 65 °C), and between pH 6.0 and 9.0 (optimum pH 7.0). Ferments pyruvate and Casamino acids, but not yeast extract, peptone, tryptone, starch, galactose, fructose, sucrose, glucose, maltose, lactose, mannitol, arabinose, xylose, ethanol, glycerol, acetate, lactate or propionate. Fe(III), Mn(IV), thiosulfate and elemental sulfur are used as electron acceptors, but not sulfate, sulfite, nitrate, nitrite or fumarate. Growth is increased in the presence of Fe(III), which is reduced in the presence of yeast extract, peptone, tryptone, starch, pyruvate, Casamino acids, amyl media, H₂ and CO₂, but not acetate, lactate, propionate, formate, benzoate, glycerol or ethanol. Sensitive to streptomycin, tetracycline, penicillin, ampicillin, chloramphenicol and NaCl concentrations of 2 % and above.

Table 1. Differential characteristics of strain R270^T and its closest relative, *Thermovenabulum ferriorganovororum* DSM 14006^T

Strains: 1, R270^T; 2, *T. ferriorganovororum* DSM 14006^T (Zavarzina *et al.*, 2002). +, Positive; –, negative.

Characteristic	1	2
Source	Microbial mats from a thermal aquifer bore runoff channel	Hot spring
Geographical location	Great Artesian Basin, Australia	Uzon caldera, Kamchatka peninsula, Russia
Cell width (µm)	0.75–1.0	0.5–0.6
Cell length (µm)	3.5–6.0	1.5–7.0
Cell-wall ultrastructure	Gram-negative	Gram-positive
Spores observed	–	+
Heat resistant	– (95 °C for 10 min)	+ (121 °C for 90 min)
pH growth range	6.0–9.0	4.8–8.2
Salinity range for growth [% (w/v) NaCl]	0–1	0–3.5
Growth inhibition by 100 µg penicillin ml ^{–1}	+	–
Substrate utilization:		
Yeast extract	–	+
Peptone	–	+
Starch	–	+
Sucrose	–	+
Fructose	–	+
Maltose	–	+
Ribose	–	+
Xylose	–	+
Electron acceptors:		
Sulfite	–	+
Nitrate	–	+
Fumarate	–	+
DNA G+C content (mol%; T _m method)	41	36
Similarity to 16S rRNA gene sequence of strain R270 ^T (%)	100	96.1

The type strain is R270^T (=KCTC 5616^T=DSM 21133^T), isolated from a microbial mat sample from the outflow of a Great Artesian bore (the New Lorne Bore) in Queensland, Australia. The genomic DNA G+C content is 41 ± 1 mol%. Phylogenetically, related to *Thermovenabulum ferriorganovororum* DSM 14006^T (96.1 % similarity).

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