**Fervidobacterium gondwanense** sp. nov., a New Thermophilic Anaerobic Bacterium Isolated from Nonvolcanically Heated Geothermal Waters of the Great Artesian Basin of Australia

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A new thermophilic, carbohydrate-fermenting, obligately anaerobic bacterial species was isolated from a runoff channel formed from flowing bore water from the geothermally heated aquifer of the Great Artesian Basin of Australia. The cells of this organism were nonsporulating, motile, gram negative, and rod shaped and generally occurred singly or in pairs. The optimum temperature for growth was 65 to 68°C, and no growth occurred at temperatures below 44°C or above 80°C. Growth was inhibited by 10 µg of lysozyme per ml, 10 µg of penicillin per ml, 10 µg of tetracycline per ml, 10 µg of phosphomycin per ml, and NaCl concentrations greater than 0.2%. The optimum pH for growth was 7.6, and no growth occurred at pH 5.5 or 8.5. The DNA base composition was 35 mol% guanine plus cytosine, and denaturation of the end products of glucose fermentation were lactate, acetate, ethanol, CO₂, and H₂. Sulfur, but not thiosulfate, sulfite, or sulfate, was reduced to sulfide. Phase-contrast microscopy of whole cells and an electron microscopic examination of thin sections of cells revealed the presence of single terminal spheroids, a trait common in members of the genus *Fervidobacterium*. However, a phylogenetic analysis of the 16S rRNA sequence revealed that the new organism could not be assigned to either of the two previously described *Fervidobacterium* species. On the basis of these observations, we propose that the new organism should be designated a new *Fervidobacterium* species, *Fervidobacterium gondwanense*. The type strain of this species is strain AB39 (= Australian Collection of Microorganisms strain ACM 5017).

The most favored ecosystems for isolating thermophilic bacteria are naturally occurring volcanic marine and terrestrial hot springs (19). However, some thermophilic bacteria have been isolated from man-made heated environments, such as hot water storage tanks (15), and from mesophilic environments, such as lake sediments (14). Waters of the deep subsurface aquifers, which are heated geothermally because of the temperature increase of 2.13°C/100 m of depth (3), are also an important potential source of phylogenetically novel thermophilic bacteria (2, 9, 13). The Great Artesian Basin of Australia is one of the largest deep subsurface aquifers and covers approximately one-fifth of the Australian continent. Some 5,000 bore holes have been sunk into the Great Artesian Basin in order to tap this water resource, and the water is distributed on the surface by runoff channels. The water temperature at the bore source can be as high as 100°C, and temperature gradients form in the runoff channels (5). Microbiological investigations of samples collected directly from bore source and runoff channels have revealed that there is great microbial diversity (2, 9, 13). In this paper we describe the isolation and characterization of a new species of the genus *Fervidobacterium*. The results of a preliminary study of the fatty acid composition of this organism have been described previously (13).

**MATERIALS AND METHODS**

**Bacterial cultures.** Strain AB39T (T = type strain) was isolated from a runoff channel of a bore hole containing geothermal water from the Great Artesian Basin. *Fervidobacterium nodosum* Rt1-BT (= ATCC 35602T) was obtained from H. W. Morgan (Thermophile Research Unit, Waikato University, Hamilton, New Zealand), and *Fervidobacterium islandicum* H12T (= DSM 5753T) was obtained from B. Ollivier (ORSTOM Laboratoire de Microbiologie, Marseille, France), who had obtained it from the Deutsche Sammlung von Mikroorganismen und Zellkulturen. All three strains were routinely cultured by using previously described methods (11, 12).

**Sample source and collection.** Samples were collected directly from bore sources and from runoff channels of the Great Artesian Basin of Australia. Water was collected directly in sterile containers from the bore source or a mixture of water and sediments, or microbial mats were collected from the runoff channels by scooping material into sterile glass containers. In all cases, the containers were completely filled and capped tightly. Temperatures were determined in situ during sample collection. A total of 44 samples, whose temperatures ranged from 31 to 88°C, were collected. The samples were transported to our laboratory at ambient temperatures and stored until they were used.

**Enrichment and isolation.** To initiate enrichment cultures, 0.1- to 0.5-ml portions of samples were inoculated into 10-ml portions of prereduced Trypticase-yeast-extract-glucose (TYEG) medium, and the preparations were incubated at 68°C without agitation for up to 3 days, during which time they were examined for growth with a microscope (11, 12). Positive enrichment cultures were subcultured at least three times in the same prereduced medium to purify cultures. Cultures were purified by using the end point dilution technique and TYEG medium supplemented with 2% (wt/vol) agar. A single colony from the most dilute tubes was picked, and the process of end point dilution was repeated at least twice before the culture was considered pure and used in subsequent characterization tests. The pure culture was stored in a glycerol-TYEG medium (30:50) mixture at −20°C.

**pH, temperature, and NaCl concentration ranges for growth.** All experiments were performed in duplicate. Growth was measured by determining the change in turbidity at 600 nm and also by determining the change in pH. To measure changes in absorbance, Belloco tubes were inserted directly into a Novaspec LKB spectrophotometer (Pharmacia) which had been zeroed with uninoculated medium or water. To determine the pH range for growth, prereduced TYEG medium was adjusted to pH values between 5.0 and 10.0 by injecting appropriate volumes of sterile HCl or NaOH. TYEG medium adjusted to the optimal pH for growth was used to determine the temperature range for growth. TYEG medium at pH 7.0 and an incubation temperature of 65°C was used to determine the NaCl requirement.

**Antibiotic susceptibility.** Antibiotics were added from filter-sterilized stock solutions to sterile prereduced TYEG medium under a stream of oxygen-free nitrogen gas to give final concentrations of 10 and 100 µg/ml. Carbohydrate utilization and end product formation. Carbohydrate utilization was determined in prereduced TYEG medium (TYEG medium lacking glucose). Soluble carbohydrates (prepared as 10% filter-sterilized stock solutions) were dispensed into tubes, and insoluble carbohydrates (final concentration, 0.5%) were weighed directly in tubes before prereduced TYE medium was dispensed.
Growth was measured spectrophotometrically and also by determining the change in pH.

The fermentation end products were determined by gas-liquid chromatography. A Shimadzu model GC8 gas chromatograph equipped with a thermal conductivity detector was used for CO2 and H2 analysis. The gases were separated on a Carbopack B (80/100) column by using N2 as the carrier gas. A Shimadzu model GC14 gas chromatograph equipped with a flame ionization detector was used to analyze volatile fatty acids and lactate. The acids were separated on a Chromosil 100 (60/80) column by using a flame at a flow rate of 8 ml/min as the carrier gas.

The fermentation end products were determined by gas-liquid chromatography. The oven temperature and the injector temperature were 180°C and 200°C, respectively. The amounts of the gases and acids were determined as described previously (12), except that the Delta computer software analysis package (Digital Solutions, Ltd., Brisbane, Queensland, Australia) was used to integrate the peaks.

Light microscopy and electron microscopy. Light microscopy and electron microscopy were performed as described previously (12).

Results

Enrichment and isolation. Of the 44 samples examined, 21 gave rise to positive enrichment cultures after 48 h of incubation at 68°C. Eight of these enrichment cultures contained organisms that had conventional rod and filament morphologies, whereas the remaining 13 contained rods that had single terminal swellings. The organisms that exhibited the latter morphotype were isolated from bore source samples, as well as from sediment and microbial mat samples. The temperatures of the sites from which these samples were obtained ranged from 31 to 75°C. Organisms that exhibited this morphotype were not found in enrichment cultures prepared with samples that had been collected from sites where the temperatures were 76 to 88°C. Pure isolates were obtained from enrichment cultures by using the end point dilution technique and were designated strain AB39T.

Growth of strain AB39T was studied further. Growth of strain AB39T was inhibited in TYEG medium culture tubes when the N2 gas phase was replaced with air or in TYEG medium which lacked a reductant, indicating that strain AB39T was an obligate anaerobe.

Morphology and cell structure. Strain AB39T cells were rods and occurred singly, in pairs, or in short chains. They were motile, gram negative, 0.5 to 0.6 μm in diameter, and 4 to 40 μm long. Protuberances or swellings were usually present at the ends of the bacteria and were observed at all growth temperatures. Only one swelling was present in each cell. The swellings ranged from 1 to 4 μm in diameter (Fig. 1a). Occasionally, giant spheres consisting of membrane-bound structures containing two or more cells and ranging in diameter from 5 to 8 μm were also observed. Phase-contrast microscopy of cultures grown at different temperatures and pH values did not reveal any spores.

Electron microscopy of thin sections of strain AB39T revealed a typical gram-negative two-layer cell wall structure (Fig. 1c). The outer layer protruded to form the characteristic swollen structure, whereas the inner layer remained tightly associated with the cell membrane (Fig. 1b and c).

Optimal growth conditions. NaCl was not required for growth, and concentrations of NaCl greater than 0.2% were inhibitory. Strain AB39T grew optimally at 65 to 68°C, and no growth occurred at temperatures of 44°C or below or above 80°C. The optimum pH for growth was 7.0, and no growth occurred at pH 5.5 or 8.5. The generation time of strain AB39T in TYEG medium at pH 7.0 and 65°C was 79 min.

Substrate utilization, sulfate production, and fermentation end products. The substrates that supported growth of strain AB39T included α-cellobiose, amylopectin, maltose, starch, dextrin, xylose, glucose, pyruvate, lactose, fructose, and mannose (Table 1). Carboxymethyl cellulose and galactose were fermented slowly, whereas Casamino Acids, gelatin, l-sorbosone, chitin, dextran, arabinose, ribose, raffinose, and cellulose did not support growth.

The end products formed during glucose fermentation included ethanol (2 mM), acetate (7 mM), lactate (15 mM), CO2, and H2. Sulfate, but not thiosulfate, sulfate, or sulfate, was reduced to sulfide.

Antibiotic susceptibility. Growth of strain AB39T was completely inhibited by 10 μg of streptomycin per ml, 10 μg of penicillin per ml, 10 μg of novobiocin per ml, 10 μg of phosphomycin per ml, 10 μg of tetracycline per ml, 10 μg of vancomycin per ml, 100 μg of polymyxin B per ml, 100 μg of chloramphenicol per ml, or 100 μg of rifampin per ml.

DNA base composition. The DNA base composition of strain AB39T was 55 mol% G+C. The E. coli reference DNA used had a melting temperature of 76.6°C, corresponding to a G+C content of 51.2 mol%.

16S rRNA sequence analysis. Using eight primers, we determined the sequence of 1,478 bases from position 27 to position 1540 (E. coli numbering of Winker and Woese [21]) of the 16S rRNA gene of strain AB39T. The G+C content of this 16S rRNA gene was 59.63 mol%. A phylogenetic analysis performed with members of the order Thermotogales indicated that strain AB39T is closely related to the type strains of the two previously described Ferrobacterium species, F. nodosum R17-B1 and F. islandicum H21 (levels of similarity, 94 and 95%, respectively. Figure 2 is a dendrogram that was generated by the neighbor-joining method from the
evolutionary distance matrix and shows this relationship. A bootstrap analysis of the data revealed a modest relationship between strain AB39T and F. islandicum H21T (73%), although the confidence level obtained for F. nodosum and the other two Fervidobacterium species was 100%.

DISCUSSION
Numerous genera and species of gram-negative, thermophilic, motile, anaerobic, carbohydrate-fermenting, rod-shaped bacteria have been described. However, as strain AB39T produces terminal protuberances or swellings, its morphology is clearly similar to the morphology of members of the order Thermotogales. In a previous study Patel et al. found high levels of saturated, normal fatty acids (78% of the total fatty acids) and that fatty acids with even numbers of carbon atoms predominated; these findings also indicated that strain AB39T is a member of the order Thermotogales (13). In addition, the protuberances of strain AB39T cells closely resemble the spheroids of Fervidobacterium strains (12) rather than the sheath-like structures called “togas” that are characteristic of Thermotoga, Petrotoga, and Geotoga species (1, 16, 19). Further evidence of the relationship of strain AB39T to F. nodosum Rt17-B1T and F. islandicum H21T was also obtained from a phylogenetic analysis of the 16S rRNA gene. Fervidobacterium species, including F. nodosum and F. islandicum, have been isolated previously only from volcanic hot springs (6, 12). This paper is the first report of the isolation of a Fervidobacterium strain from a nonvolcanic, geothermally heated water source (viz., the Great Artesian Basin of Australia). The presence of Fervidobacterium isolates in the aquifer was not rare, as morphologically similar strains were isolated from another 12 of the 44 enrichment samples examined. As Fervidobacterium strains were isolated from water samples collected from bore holes that were up to 1,500 m deep, we
speculate that the primary habitat of these bacteria is the geothermal waters of the deep aquifer and that they are seeded into the runoff channels, where they adapt to the new environment. The water of the Great Artesian Basin aquifer is meteoric in origin, and the rate of water flow underground is approximately 5 m/year (5); therefore, this water could provide a conducive environment for microbial growth. The modest confidence value (73%) between F. nodosum or F. islandicum and strain AB39\(^T\) indicates that there may be Fervidobacterium species that remain to be identified. Some Fervidobacterium isolates obtained from the Great Artesian Basin that are currently being investigated in our laboratory differ markedly in their ability to reduce thiosulfate or sulfur. Sequencing of the 16S rRNA genes of these isolates for phylogenetic identification is currently in progress.

Strain AB39\(^T\) can be differentiated from F. nodosum R17-B1\(^T\) and F. islandicum H21\(^T\) on the basis of its fast growth rate and its greater nutritional versatility. Strain AB39\(^T\) also has a lower G+C content (35 mol%) than F. islandicum H21\(^T\) (41 mol%) (Table 1). Phylogenetically, strain AB39\(^T\) is more closely related to F. islandicum H21\(^T\) (level of similarity, 95%) than to F. nodosum R17-B1\(^T\) (level of similarity, 94%). Recently, Stackebrandt and Goebel (18) concluded that strains belonging to the same genus that exhibit less than 97% 16S rRNA sequence similarity should be considered members of different species. Thus, it is clear that strain AB39\(^T\) is a member of a new Fervidobacterium species, for which we propose the name Fervidobacterium gondwanense.
REFERENCES


