Characterization of a New Obligately Anaerobic Thermophile, *Thermoanaerobacter wiegeli* sp. nov.

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An obligately anaerobic, extremely thermophilic *Thermoanaerobacter* species was isolated from a freshwater pool formed from a geothermally heated (56 to 69°C) water outlet in Government Gardens, Rotorua, New Zealand. This organism was a spore-forming, gram-negative, rod-shaped bacterium. Strain R8.B17 (= DSM 10319T) (T = type strain) fermented a wide variety of mono-, di-, and polysaccharides and produced ethanol, acetate, lactate, propionate, and hydrogen. Sugar alcohols were also fermented, and amino acids were not utilized. On the basis of its morphological characteristics, DNA G+C content, obligately anaerobic, thermophilic, polysaccharolytic nature, and levels of 16S rRNA sequence homology, we propose that strain R8.B17 should be classified in the genus *Thermoanaerobacter* as a new species, *Thermoanaerobacter wiegeli*.

The thermophilic, anaerobic, saccharolytic bacteria include species that belong to the genera *Clostridium*, *Thermoanaerobacterium*, *Thermoanaerobacter*, and *Thermoanaerobacterium* (18, 34). Until recently, the genus *Thermoanaerobacterium* contained only two species, *Thermoanaerobacterium ethanolicus* (type strain, strain JW200) (35) and *Thermoanaerobacterium finnii* (30). Type strain JW200 of the type species of this genus, *Thermoanaerobacterium ethanolicus* (37), was isolated from hot springs located in Yellowstone National Park by Wiegel and Ljungdahl (35). This organism was described as a gram-variable, rod-shaped, non-spore-forming, obligately anaerobic thermophile with a DNA G+C content of 37 to 39 mol% (35). On the basis of the results of DNA-DNA hybridization and total-cell-protein analyses, Lee et al. (18) redefined the genus *Thermoanaerobacter* so that it included *Thermoanaerobacterium brockii* (40) (formerly *Thermoanaerobacterium brockii* HTD4), as well as *Thermoanaerobacterium thermohydrosulfuricum* E100-69 (12) and *Thermoanaerobacterium finnii* E100-69 (39) (formerly *Clostridium thermohydrosulfuricum* E100-69 and 39E, respectively).

The results of a phylogenetic analysis of the majority of the thermophilic anaerobic bacteria indicated that *Thermoanaerobacterium ethanolicus*, *Thermoanaerobacterium brockii*, *Thermoanaerobacterium thermohydrosulfuricum*, and *Thermoanaerobacterium finnii* formed a distinct phylogenetic cluster which also included *Thermobacteroides acetethylicus*, *Acetogenium kivui*, and *Clostridium thermocloacae* (29). *Thermobacteroides acetethylicus* was subsequently transferred to the genus *Thermoanaerobacter* as *Thermoanaerobacter acetethylicus* (28). On the basis of the 16S ribosomal RNA (rDNA) data of Rainey et al. (29), Collins et al. (4) included *Thermoanaerobacter thermopropionicum* (formerly *Clostridium thermopropionicum* [14]) and *Thermoanaerobacter kivui* (formerly *Acetogenium kivui* [19, 20]) in this genus.

In this paper we describe the characteristics of an obligately anaerobic, extremely thermophilic strain, strain R8.B17 (T = type strain). Strain R8.B17 differs from other *Thermoanaerobacter* species in the following characteristics: spore formation, ability to ferment sugar alcohols, ability to produce propionate from xylose, ability to ferment cellulose, pH range for growth, and antibiotic susceptibility. The results of a 16S rDNA sequence analysis also showed that this organism differs from other *Thermoanaerobacter* species. In this paper we propose the name *Thermoanaerobacter wiegeli* sp. nov. for strain R8.B17.

**MATERIALS AND METHODS**

**Sampling procedure.** Samples from a freshwater pool formed by a water outlet of a heat exchanger in Government Gardens, Rotorua, New Zealand, were collected in sterile 30-ml universal bottles. Each collection vessel was completely filled, sealed with a gas-tight septum, and tightly capped. All samples were transported to the laboratory and stored at ambient temperature for future use. The pool water had a pH range of 8.3 to 9.1, and the temperature ranged from 56 to 69°C.

**Medium and culture conditions.** The new isolate strain R8.B1 was maintained by fortnightly subculture on a modified Typtrcase peptone-yeast extract medium of Zekus et al. (40). The yeast extract and Typtrcase peptone concentrations were reduced to 1 and 2 g/liter, respectively. The medium was autoclaved at 121°C for 20 min. For solidified media 200 g of purified Oxoid agar (Unipath, Basing-stoke, Hampshire, England) per liter was added.

Carbon sources were prepared anaerobically in distilled water as concentrated stock solutions and were sterilized by autoclaving. Each carbon source was added to sterile media to a final concentration of 5 g/liter. All of the sugars were D isomers unless indicated otherwise. Antibiotics were added at concentrations of 1 to 100 μg/ml, and the resulting growth was compared with the growth of control preparations (with no antibiotic added) after 24 h of incubation. The preparations were incubated at 65°C unless indicated otherwise. Plates were incubated in stainless steel anaerobic jars under an N2 gas atmosphere.

**Cellular characterization.** The methods used to prepare cells for thin sectioning and electron microscopic examination were the methods described by Patel et al. (23). Thin sections were stained with 1% (wt/vol) uranyl acetate and then examined with a Philips EM 400 electron microscope at an accelerating voltage of 80 kV.

**16S rDNA sequence analysis.** Genomic DNA was isolated, and the 16S rDNA was amplified as described previously (27). PCR products were sequenced by using a Taq Dye Deoxy Terminator cycle sequencing kit (Applied Biosystems, Foster City, Calif.) as recommended by the manufacturer. Purified sequence reaction mixtures were electrophoresed by using an Applied Biosystems model 373A DNA sequencer. The 16S rDNA sequence of strain R8.B1 was manually aligned with previously published 16S rDNA sequences of representatives of the genus *Clostridium* and related taxa. The method of Jukes and Cantor (15) was used to calculate evolutionary distances, from which a phylogenetic dendrogram was constructed by using the algorithm of De Soete (10).

**G+C content.** The guanine-plus-cytosine (G+C) content of the DNA was determined by high-performance liquid chromatography (HPLC) as described by Mesbah et al. (21).

**Other analyses.** Fermentation product and substrate concentrations in medium samples were determined by HPLC as described previously (9). Sodium succinate was used as an internal standard. Gaseous end products were analyzed by gas chromatography by using a model PU 4500 gas chromatograph (Pye Unicam, Cambridge, England) equipped with a thermal conductivity detector as...
RESULTS

Enrichment and isolation. Trypticase peptone-yeast extract-glucose (TYEG) medium (40) was used for enrichment of strain Rt8.B1\textsuperscript{T}. Primary enrichment cultures were prepared by adding 0.2 ml of pool water to 10 ml of prereduced TYEG medium under an N\textsubscript{2} atmosphere. After 1 to 3 days at 70°C, gas production and visible turbidity were observed, indicating substrate utilization was occurring. All positive cultures were transferred and incubated at least three times in the same media, after which they were purified in TYEG agar deeps (2% [wt/vol] agar). Pure cultures of strain Rt8.B1\textsuperscript{T} were then stored at -20°C in a glycerol-medium mixture (5:50).

Cellular features. Cells grown on solid TYEG medium produced nonpigmented colonies (diameter, 0.5 to 2.0 mm) that were smooth and uniformly round. Cells obtained from isolated colonies were gram-negative rods that were 0.4 to 0.6 \mu m wide by 4 to 10 \mu m long. The cells occurred singly, in pairs, or (less frequently) in chains. Cells grown on TYEG medium did not sporulate. When the organisms were grown in a minimal medium (5), the cells were long and filamentous and spores were produced. The spores were round and terminal, distended the cells, and were brightly refractile as determined by phase-contrast microscopy (24). Cultures grown on minimal medium at 65°C (conditions that induced sporulation) survived more than 80 min of exposure to 115°C, thus confirming that they were heat resistant.

Electron micrographs of ultrathin sections of strain Rt8.B1\textsuperscript{T} are shown in Fig. 1. Figure 1a shows a single cell with distinct cell envelope layers. The cells appeared to divide where two membrane invaginations arose from displaced positions to give an S-shaped appearance. Figure 1b shows cell division at a latter stage in the cycle. Cell division appeared to occur via a septation mechanism, and a high percentage (70%) of the cells exhibited this type of division. A higher magnification of the cell wall revealed a two-layer structure (Fig. 1c). The inner layer, which was adjacent to the cytoplasmic membrane, stained intensely, whereas the outer layer was less dense. The cells were sluggishly motile, and flagella were peritrichous (24).

Antibiotic susceptibility. Cephalosporin C, erythromycin, bacitracin, tetracycline, or polymyxin B completely inhibited growth at a concentration of 10 \mu g/ml of medium. Trimethoprim, rifampin, amphotericin B, d-cycloserine, penicillin G, streptomycin sulfate, chloramphenicol, and ampicillin did not inhibit growth at concentrations up to 100 \mu g/ml.

The metabolic inhibitors monensin (100 \mu M), 2,4-dinitrophenol (500 \mu M), tetrachlorosalicylanilide (10 \mu M), N,N-dicyclohexylcarboimide (500 \mu M), and iodoacetate (500 \mu M) all inhibited growth when they were added to cultures that were growing exponentially on glucose. Sodium azide, sodium fluoride, potassium cyanide, and sodium arsenate completely stopped growth at 65°C when they were added to a final concentration of 5 mM. Oxygen completely inhibited growth.

Growth conditions of strain Rt8.B1\textsuperscript{T}. Strain Rt8.B1\textsuperscript{T} did not grow on TYEG medium lacking sodium sulfide reductant or cysteine-HCl, indicating that anaerobic conditions were required for growth. Hydrogen sulfide was produced from sodium sulfite and sodium thiosulfate. Growth occurred at pH 5.5 to 7.2, but not at pH 5.0 or 7.25 in pH-controlled batch cultures. The optimum pH for growth was 6.8. The temperatures at which growth occurred ranged from 38 to 78°C, and the optimum temperature was between 65 and 68°C. No growth occurred at 34 or 80°C.

Growth substrates. Strain Rt8.B1\textsuperscript{T} did not exhibit an absolute growth requirement for yeast extract or Trypticase, and both of these growth supplements could be replaced by vitamin-free Casamino Acids (5 g/liter) and vitamins (5). No growth occurred in Trypticase peptone-yeast extract medium in the absence of a fermentable carbon source. Glucose, xylose, maltose, lactose, cellobiose, raffinose, glucosamine, galactose, fructose, mannose, sucrose, glycerol, soluble starch, peptin, and chitin were used as fermentable substrates. Sorbitol, mannitol, and trehalose were also fermented, but ethanol, D-lactate, sodium citrate, sodium succinate, transaconitate, malonate, glutamate, glutamine, sodium pyruvate, 2-deoxyglucose, a-methyl-glucoside, L-arabinose, a-L-rhamnose, dulcitol, m-inositol, ribose, a-L-fucose, and L-sorbitose were not fermented. Growth did not occur via reduction of nitrate, oxygen, sulfate, or sulfur when glucose was the sole carbon source. Indole was not formed from L-tryptophan. Escculin and gelatin were not hydrolyzed. Cells did not accumulate anthrone-reactive material when they were grown on glucose.

Phylogenetic position. We determined an almost complete 16s rDNA sequence for strain Rt8.B1\textsuperscript{T} (>95% of the Esche-
Moorella thermoacetica
Moorella thermoautotrophica
with the type strain of the type species of the genus
anaerobacter, Thermoanaerobacter ethanolicus
Clostridium butyricum
Clostridium stercorarium
Clostridium thermocellum
Caldicellulosiruptor saccharolyticus
Thermoanaerobacterium saccharolyticum
Thermoanaerobacterium thermosulfurigenes
Thermoanaerobacter thermohydrosulfuricus
Thermoanaerobacter finnii
Thermoanaerobacter thermocopriae
Thermoanaerobacter kivui
Thermoanaerobacter brockii
Thermoanaerobacter ethanolicus
Thermoanaerobacter acetoethylicus
Thermoanaerobacter ethanolicus

The 16S rDNA sequence of strain Rt8.BlT exhibited levels of similarity ranging from 95.2 to 98.2% with the sequences of the other and related taxa. Scale bar (Table 1). The highest level of sequence similarity (98.2%) was with the type strain of the type species of the genus Thermoanaerobacter, Thermoanaerobacterielanasacricus JW200.

**DISCUSSION**

Until recently, the descriptions of most extremely thermo-
philic (optimum growth temperature, 65 to 70°C) anaerobic
cultures were based solely on phenotypic characteristics and
DNA G+C contents. Spore formation was used as a key tax-
onomic marker, and isolates in which spore formation had not
been observed were described as members of the genera Thermo-
aerobacterium, Thermoanaerobacter, and Thermobacteroides.
On the basis of DNA-DNA hybridization, total-cell-protein,
and 16S rRNA gene sequence data (4, 18), the genus Thermo-
aerobacter was redefined so that it included Thermoanaer-
obacter brockii, Thermoanaerobacter thermohydrosulfuricus E100-69, Thermoanaerobacter thermocapitae, and Thermo-
aerobacter kivui.

Strain Rt8.BlT was initially considered a Clostridium ther-
mosulfurisricus strain because of its phenotypic character-
istics (6, 24) and was considered a Thermoanaerobacter
termosulfurisricus strain in subsequent publications (7-9)
in accordance with the proposed nomenclature of Lee et al.
(18). Thermoanaerobacter wiegeli R8.BT metabolizes glucose
via the Embden-Meyerhof-Parnas pathway and exhibits het-
eryo-ethanol fermentation (9). NADP-activated alcohol dehydrogenases have been found previously in Thermobacteroides,
Thermoanaerobacter thermohydrosulfuricus, and Thermobacteroides
strains Rb. (38). Pentoses are fermented via the pentose phosphate pathway (8). The major end products of glucose fermentation by strain Rb. are from pyruvate via the following three routes: (i) a route to lactate via a pH-dependent fructose-1,6-diphosphate-activated lactate de-
hydrogenase; (ii) a route to acetate via acetate kinase; and (iii) a route to ethanol via an irreversible NADP-linked alcohol dehydrogenase (9). NADP-activated alcohol dehydrogenases and fructose-1,6-diphosphate-activated lactate dehydrogenases have been found previously in Thermobacteroides fimnii (30), Thermobacteroides ethanolicus (2), Thermobacteroides brockii (1, 16, 17), and Thermobacteroides thermohydrosulfuricus (11). Strain R8.BlT lacks a phosphoenolpyruvate-dependent phos-
Thermoanaerobacter wiesteili Rt8.B1T grows on a wide range of carbohydrates. An ethanol yield of up to 1.1 mol of ethanol per mol of substrate utilized was obtained with single substrates (9), but values of 1.56 to 1.7 mol of ethanol per mol of substrate utilized were obtained when substrates were supplied in combinations and were fermented simultaneously (9). These values are in line with our recent isolation of polyphosphate kinase and xylulokinase, respectively (7, 8).

Thermoanaerobacter wiesteili Rt8.B1T was susceptible to the antibiotics cephalosporin C, erythromycin, bacitracin, tetracycline, and polymyxin B but not to penicillin, ampicillin, or chloramphenicol. Tetracycline has been shown to inhibit the growth of Thermoanaerobacter brockii (40) and Thermoanaerobacter finnii (30) but not the growth of Thermoanaerobacter ethanolicus (35). Penicillin G inhibited the growth of Thermoanaerobacter thermohydrosulfuricus (25), Thermoanaerobacter brockii (40), and Thermoanaerobacter finnii (30), but had no effect on Thermoanaerobacter wiesteili Rt8.B1T.

Thermoanaerobacter wiesteili Rt8.B1T ferments some sugar alcohols and glucosamine, but does not ferment ribose or arabinose. Both ribose and arabinose are fermented by Thermoanaerobacter thermohydrosulfuricus (36); however, glycerol, sorbitol, and glucosamine are not fermented. Strain Rt8.B1T did not have the absolute growth requirement for yeast extract or Trypticase described previously for the genus Thermoanaerobacter (37). Both of these growth supplements could be replaced by high concentrations of a vitamin solution (5) and Casamino Acids. These observations and the results described above support our conclusion that strain Rt8.B1T belongs to a new Thermoanaerobacter species.

**Description of Thermoanaerobacter wiesteili sp. nov. Thermoanaerobacter wiesteili** (wie.gei.l.i. M.L. gen. n. wiesteili, of Wiegel, in recognition of Juergen Wiegel's contributions to our knowledge of thermophilic anaerobes).

**Morphology.** Colonies are 0.5 to 2.0 mm in diameter, smooth, nonpigmented, and uniformly round. Gram-negative rods are 0.4 to 0.6 μm wide by 4 to 10 μm long. Cells occur singly, in pairs, or (less frequently) in chains. Cells grown in rich TYEG medium do not sporulate. When the organism is grown in a minimal medium (5), the cells are long and filamentous and spores are formed. The spores are round and terminal, distend the cells, and are brightly refractile as determined by phase-contrast microscopy.

**Cellular characteristics.** The G + C content of the DNA is 35.6 ± 1.2 mol% (as determined by the HPLC method). An inner cell wall layer and an outer cell wall layer are present (a monolayer cell wall coated by an inner layer and an outer layer having a globular structure).

**Growth characteristics.** Obligately anaerobic. The optimum temperature is 65 to 68°C; the maximum temperature at which growth occurs is 76 to 78°C. Growth at 38°C is poor; no growth occurs at 28°C. Growth occurs at pH 5.5 to 7.2; optimum growth occurs at pH 6.8. The doubling time in glucose-containing medium is 72 min.

**Metabolic characteristics.** Chemoorganotroph. Utilizes various mono-, di-, and polysaccharides, including glucose, xylose, maltose, lactose, cellobiose, raffinose, glucosamine, galactose, fructose, mannose, sucrose, glycerol, soluble starch, pectin, and chitin. Sorbitol, mannitol, and trehalose are also fermented, but ethanol, D-lactate, sodium citrate, sodium succinate, transacinitate, malonate, glutamate, glutamine, sodium pyruvate, 2-deoxyglucose, α-methyl-glucoside, l-arabinose, dulcitol, m-inositol, ribose, α-1-fucose, α-1-rhamnose, and l-sorbose are not fermented. The products of metabolism during growth on glucose are ethanol, lactate, acetate, CO₂, and H₂. Propionate is formed during growth on xylose or cellobiose in pH-controlled (pH 7) batch cultures. Growth on glucose is inhibited by oxygen, cephalosporin C, erythromycin, bacitracin, tetracycline, polymyxin B, and various metabolic inhibitors.
Habitat. The habitat is neutral to alkaline freshwater (56° to 69°C) in a geothermally heated water source in Government Gardens, Rotorua, New Zealand.

Type strain. Thermoclostridium wiikeyi Rkt.8.B1T was isolated from a freshwater runoff pool from a geothermally heated water source in Rotorua, New Zealand, and has been deposited in the Deutsche Sammlung von Mikroorganismen as strain DSM 10319. Strain Rkt.8.B1T is the only strain that has been isolated so far.

We propose that on the basis of its morphological characteristics, DNA G+C content, relatively narrow pH range for growth (pH 5.5 to 7.2), antibiotic susceptibility, ability to ferment some sugar alcohols, growth in the absence of yeast extract, formation of propionate when it is grown on xylose or cellobiose, and levels of 16S rRNA sequence homology, strain Rkt.8.B1T should be included in the genus Thermoclostridium as a new species, Thermoclostridium wiikeyi.

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REFERENCES