Anaerobaculum thermoterrenum gen. nov., sp. nov., a Novel, Thermophilic Bacterium Which Ferments Citrate

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A thermophilic anaerobic bacterium designated strain RWcit2^T (T = type strain) was isolated from the production water of a petroleum reservoir. The cells of this organism are straight to slightly curved rods that are gram negative and nonmotile. Spore formation has not been demonstrated. Growth occurs at temperatures ranging from 28 to 60°C, with optimum growth occurring at 55°C, and at pH values ranging from 5.5 to 8.6, with optimum growth occurring between pH 7 and 7.6. Growth occurs in media containing 0 to 20 g of NaCl per liter, and optimum growth occurs in the presence of 10 g of NaCl per liter. Strain RWcit2^T grows on a range of organic acids, including citrate, pyruvate, malate, fumarate, and tartrate; on protein extracts; and on a limited number of carbohydrates. Sulfur, thiosulfate, and cystine are reduced to hydrogen sulfide. Sulfate, sulfite, and nitrate are not reduced. The DNA base composition is 44 mol% G+C. The 16S ribosomal DNA sequence revealed that strain RWcit2^T is a member of the domain *Bacteria* and forms a branch that is approximately equidistant from *Dictyoglomus thermophilum* and *Thermoanaerobacter* spp. (level of similarity, 82%). Strain RWcit2^T cannot be placed in any previously described taxon based on its phylogenetic and physiological traits and is named *Anaerobaculum thermoterrenum* gen. nov., sp. nov.

It is now recognized that a range of thermophilic and hyperthermophilic microorganisms can be isolated from petroleum reservoir fluids. Complex media containing biological peptones, yeast extract, and carbohydrates have been used successfully for the isolation of these microorganisms (14, 19, 27). Investigations into anaerobic organic acid degradation by petroleum reservoir microorganisms have been confined to oxidation by sulfate-reducing bacteria (3, 16, 20, 28) and acetlastic methanogens (4). In contrast, organic acids, which are known to occur in petroleum reservoirs and are potential substrates, have not been widely reported as compounds that support the growth of thermophilic fermentative bacteria. The reports which have occurred have involved organic acid fermentation by thermophilic microorganisms subsequent to their isolation on other substrates (10).

In this paper we described the isolation and characterization of a thermophilic fermentative microorganism, designated strain RWcit2^T (T = type strain), which is able to degrade organic acids.

MATERIALS AND METHODS

Source of organism. Strain RWcit2^T was isolated from production fluid from the Redwash oil field in Utah. The Redwash oil field has a depth of 1,524 m, a temperature of approximately 52°C, and a salinity of 25 g per liter. River water is injected into the field as part of the secondary oil recovery processes.

Media and culture conditions. Anaerobic procedures used in the preparation of media and cultivation of anaerobic microorganisms have been described previously (25, 28). For enrichment and purification of strain RWcit2^T, brackish bicarbonate-buffered, sulfide-reduced medium (34) was prepared without sodium sulfate to eliminate possible growth of sulfate-reducing bacteria. The additions to the medium were 0.1 g of yeast extract per liter, trace element solution SL-10 (17), and the vitamin solution of Wolin et al. (37). Following purification of strain RWcit2^T, 0.1 g of Na₂SO₄ per liter was included in the culture medium as an inorganic sulfur source. Complex medium MMB was modified from MB medium (27) and contained (per liter) 0.5 g of NH₄Cl, 0.3 g of K₂HPO₄, 0.3 g of KH₂PO₄, 0.2 g of MgCl₂ · 6H₂O, 0.1 g of CaCl₂ · 2H₂O, 5.0 g of NaCl, 0.1 g of KCl, 0.8 g of sodium acetate · 3H₂O, 0.5 g of cysteine-HCl, 5.0 g of yeast extract (Oxoid), 5.0 g of tryptone (Oxoid), 0.001 g of resazurin, and 1 ml of solution

SL-10. The pH of the medium was adjusted to 7, and the medium was dispensed anaerobically under oxygen-free nitrogen. Anaerobic agar plates were prepared by including 2.0 g of agar per liter in MMB medium. Autoclaved agar was cooled and plates were poured in an anaerobic chamber. Inoculated plates were incubated in anaerobic sealed jars at 50°C. Substrate tests were carried out by using the brackish bicarbonate-buffered medium supplemented with 0.1 g of yeast extract per liter in the presence and absence of elemental sulfur (2%, wt/vol). Media containing sulfur were autoclaved at 110°C for 30 min. Cellulose powder, carboxymethyl cellulose, pectin, and soluble starch were each added at a concentration of 2.0 g per liter directly to culture vessels before media were dispensed.

Enrichment, isolation, and purification. Strain RWcit2^T was enriched from Redwash field production water in brackish bicarbonate-buffered medium containing 10 mM citrate and incubated at 50°C. Enrichment cultures were maintained for at least five subcultures in bicarbonate-buffered medium containing citrate. Culture purification was achieved by the agar shake-dilution tube process (34). Purity was confirmed by microscopically examining cultures inoculated into medium containing citrate, pyruvate, and peptone; cooked meat medium (Difco); and thioglycolate broth (Difco).

Cellular characterization. The Gram type was determined by staining (11) and by the KOH lysis method (5). The ability of strain RWcit2^T to produce spores was examined in cooked meat medium (Difco) supplemented with 5.0 g of NaCl per liter, AE sporulation medium (2), xylose medium (7), and mineral media containing 10 mM citrate supplemented with either 2% (vol/vol) soil extract, 0.05 g of MnSO₄ per liter, or 300 mg of thiamine-HCl per liter. All cultures were examined microscopically for spores and then heated at 90°C for 5 min and inoculated into fresh medium. Phase-contrast micrographs were taken by using agar-coated slides (26) and a Leica diaphan microscope. Negative staining and preparation of thin sections for electron microscopy were performed as described previously (24). DNA base composition was determined by the thermal denaturation method (22), using DNA purification procedures described by Owen and Lapage (23). Cytochromes were characterized as described by Janssen and Harfoot (18).

Temperature, salinity, and pH experiments were carried out in 20-ml tubes (Bellco) containing 10 ml of MMB medium supplemented with 10 mM thiosulfate. The optical density was determined between day 4 and day 5 of incubation, which corresponded to mid-growth in optimally growing cells.

For H₂ inhibition experiments, 120-ml serum bottles containing 20 ml of MMB medium and N₂ as the headspace gas were prepared, inoculated, and subjected to 1 atm of overpressure of H₂-CO₂ (80:20) or N₂-CO₂ (80:20). Optical density at 580 nm was determined after 1 week of incubation. All growth experiments were begun with a 1% (vol/vol) inoculum.

Molecular characterization. The PCR product of the 16S rRNA gene was sequenced directly with an ABI automated sequencer by using a prism dideoxy terminator cycle sequencing kit according to the recommendations of the manufacturer (Applied Biosystems, Foster City, Calif.). All of the procedures used for phylogenetic analysis have been described previously (28). A nearly complete sequence consisting of 1,526 bases was obtained, but only 1,189 unambiguous

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FIG. 1. Phase-contrast micrograph of strain RWcit2^T. Bar = 5 μ m. Cells were grown in MMB medium.

FIG. 2. Growth of strain RWcit2^T on citrate. The bicarbonate-buffered medium contained mineral salts, 10 mM citrate, and vitamins (see Materials and Methods). Symbols: □, optical density at 580 nm (OD_{580nm}); △, citrate concentration; ●, acetate concentration.

positions were used in pairwise evolutionary distance calculations with the algorithm of Jukes and Cantor. A phylogenetic tree was drawn by using the neighbor-joining method. A bootstrap analysis was performed by using DBOOT and CONSENSE. All computer programs used for the taxonomic analyses are part of the PHYLIP package (13). The sequence was also analyzed by using SUGGEST TREE, which forms part of the Ribosomal Database Project; SUGGEST TREE implements the program fastDNAmI (21).

Analytical techniques. Growth was determined by removing samples from culture vessels and determining the optical density at 580 nm (1-cm light path) with a Cary 3 spectrophotometer (Varian, Melbourne, Australia). Resazurin was omitted from media on which optical density measurements were made. A Shimadzu gas chromatograph fitted with a type BP21 column (12 m by 0.32 mm; SGE, Melbourne, Australia) and a flame ionization detector was used to quantify short-chain monocarboxylic acids. Samples used for gas chromatographic analysis were acidified in 0.03 M oxalic acid prior to injection. A Waters high-pressure liquid chromatography (HPLC) system fitted with a Bio-Rad type HPX-87H column and UV and refractive index detectors also was used to detect and quantify organic compounds. The column was maintained at 50°C, and a mobile phase (0.005 M H₂SO₄) at a flow rate of 0.5 ml per min was used. Sulfide was measured as described by Trüper and Schlegel (33). Nitrate reduction was monitored as described by Smibert and Krieg (31).

Nucleotide sequence accession number. The 16S ribosomal DNA sequence of strain RWcit2^T has been deposited in the GenBank database under accession number U50711.

RESULTS

Enrichment and isolation of strain RWcit2^T. Enrichment cultures containing citrate grew within 3 to 5 days of inoculation. The primary enrichment culture contained microorganisms with diverse morphologies, but subsequent subcultures were dominated by small rod-shaped cells. A pure culture of these cells was designated RWcit2^T. Colonies in agar shake dilution tubes were white and lens shaped. Colonies grown on agar plates for 3 weeks were 1 to 2 mm in diameter, circular with entire margins, smooth with shiny surfaces, and white with opaque sectors.

Cellular characteristics. Strain RWcit2^T cells grown on citrate were generally 0.75 by 2 μ m (Fig. 1). Sheath-like material extending past the cell poles was evident in cells grown on complex MMB medium. No such material was evident in cells grown on citrate. The cells stained gram negative, but their reaction in the KOH lysis test was intermediate between the reactions of *Escherichia coli* and *Staphylococcus aureus*. Spores were never observed in any of the sporulation test media, and the cultures did not survive heat treatment. The cells were nonmotile, and flagella were not observed in negatively stained electron microscope preparations (data not shown).

Physiological characteristics. Strain RWcit2^T grew in defined medium supplemented with vitamins and with citrate as the sole carbon and energy source (Fig. 2). When strain RWcit2^T grew on citrate, acetate was produced, and the molar ratio of amount of acetate produced to amount of citrate degraded was approximately 1:1. HPLC elution traces of culture supernatant also revealed that another metabolite was produced during growth on citrate, but the retention time of this metabolite did not correspond to the retention times of known end products of citrate fermentation. Further analysis demonstrated that succinate, isocitrate, *trans*-aconitate, tricarballic acid, glyoxylate, and oxalate were not present. The identity of the other metabolite remains unknown and is being investigated.

The compounds that strain RWcit2^T was able to use for growth were citrate (10 mM), fumarate (10 mM), malate (10 mM), pyruvate (10 mM), glutamate (10 mM), α -ketoglutarate (10 mM), tartrate (10 mM), tryptone (2 g/liter), Biological peptone (Oxoid catalog no. L-37) (2 g/liter), starch (2 g/liter), pectin (1 g/liter), glucose (5 mM), fructose (5 mM), mannose (5 mM), inositol (1 g/liter), and glycerol (1 g/liter). No growth occurred on xylose (5 mM), galactose (5 mM), lactose (2 mM), sucrose (2 mM), maltose (2 mM), rhamnose (2 mM), raffinose (1 mM), cellulose (2 g/liter), carboxymethyl cellulose (2 g/liter), gum arabic (2 g/liter), malonate (10 mM), succinate (10 mM), glutarate (10 mM), and lactate (10 mM).

Strain RWcit2^T grew in complex medium MMB (Fig. 3). When elemental sulfur was included in the medium, the growth of strain RWcit2^T was biphasic. Initially, growth was slightly faster in the presence of sulfur compared to controls without sulfur. After approximately 70 h of incubation, the cells in the medium without sulfur were in a death phase, whereas the cells in the medium containing sulfur showed an increase in growth. A marked increase in sulfide production occurred concomitant with this increased growth. Cystine and thiosulfate also could be reduced to hydrogen sulfide by strain RWcit2^T. Thiosulfate reduction proceeded directly to sulfide, without formation of elemental sulfur. Sulfate and nitrate (both at a concentration of 10 mM) were not used as electron acceptors and did not affect the growth of strain RWcit2^T. Sulfite was not reduced, and concentrations greater than 1 mM inhibited growth.

Strain RWcit2^T produced a range of carboxylic acids when it was grown on MMB medium and glucose (Table 1), and sig-

FIG. 3. Growth of strain RWcit2^T on MMB medium in the presence and absence of 10 mM sulfur. Symbols: ○, optical density at 580 nm (OD_{580nm}) in the presence of sulfur; ■, optical density in the absence of sulfur; △, sulfide production in the presence of sulfur; ●, sulfide production in the absence of sulfur.

nificantly higher end product concentrations occurred in the presence of elemental sulfur. Butyrate, lactate, and ethanol were not produced when strain RWcit2^T grew on glucose. A 1-atm overpressure of H₂-CO₂ applied to cultures caused almost complete inhibition of growth compared to controls pressurized with N₂-CO₂. An overpressure of N₂-CO₂ had no effect on the growth of strain RWcit2^T.

The optimum temperature for growth was 55°C, and the maximum temperature for growth was 60°C. Cultures incubated at 28°C grew very slowly and after 2 weeks had an optical density of 0.07. Growth occurred in media containing 0 to 20 g of NaCl per liter, and optimum growth occurred in medium containing 10 g of NaCl per liter. Growth occurred at pHs ranging from 5.5 to 8.6, and the optimum pH was between 7 and 7.6.

Strain RWcit2^T was able to grow in anoxic MMB medium without a reducing agent. No growth occurred in medium that remained aerobic throughout the incubation period.

Biochemical characteristics. Strain RWcit2^T was catalase negative. Cells did not hydrolyze gelatin or produce indole from tryptophan. The base composition of DNA isolated from strain RWcit2^T was 44 mol% G+C. Cytochromes were not detected in dithionite-reduced-minus-air-oxidized difference spectra.

Phylogeny. The dendrogram in Fig. 4 shows the phylogenetic position of strain RWcit2^T among related bacteria. An analysis of its 16S rRNA gene sequence showed that strain RWcit2^T formed a branch that was approximately equidistant from *Dictyoglomus thermophilum* (level of similarity, 81.7%) and *Thermoanaerobacter brockii* (level of similarity, 82.3%). The boot-

FIG. 4. Phylogenetic position of strain RWcit2^T within the radiation of *D. thermophilum*, *Thermoanaerobacter* spp., and reference organisms. Bar = evolutionary distance of 0.1.

strap analysis results obtained with *Thermoanaerobacter brockii* were robust (level of relatedness, 100%), whereas the level of relatedness with *D. thermophilum* was 72%. The levels of relatedness may increase as additional sequence data are obtained from new strains.

DISCUSSION

Strain RWcit2^T is an anaerobic, thermophilic bacterium with an optimum growth temperature of 55°C. This strain is able to ferment several organic acids, as well as protein extracts and a limited number of carbohydrates. Extensive organic acid degradation by thermophilic fermentative bacteria has not been reported previously.

A phylogenetic analysis based on 16S ribosomal DNA comparisons demonstrated that strain RWcit2^T is almost equidistant from *Thermoanaerobacter* spp. and *D. thermophilum* and forms a distinct line of descent. The current lack of sequence data for this region of the phylogenetic tree means that the exact position of the branch cannot be determined precisely. The exact position will become clearer as new sequence data become available.

Strain RWcit2^T shares some characteristics with its phylogenetic relatives, but there are also significant differences. The end products formed from peptone degradation by strain RWcit2^T are similar to the end products formed by several thermophilic fermentative bacteria, including *Thermoanaerobacter* spp., as these organisms produce acetate, isovalerate, and isobutyrate as major products and propionate as a minor product during peptide fermentation (12, 35). The end products produced by *Thermoanaerobacter* spp. from glucose fermentation are acetate, ethanol, and lactate, and the actual products and product ratios are species dependent (6). Strain RWcit2^T differed from *Thermoanaerobacter* species in that it did not produce lactate or ethanol. In general, carbohydrate degradation by strain RWcit2^T is significantly more restricted than carbohydrate degradation by *Thermoanaerobacter* species. Thiosulfate reduction as a means of eliminating inhibition by hydrogen has been described in *Thermoanaerobacter* spp. (12) and members of the *Thermotogales* (19); however, some *Thermotoga* spp. are inhibited by elemental sulfur (19, 27, 36). Strain RWcit2^T could reduce sulfur, thiosulfate, and cystine. Cystine reduction has been described in *Thermotoga subterranea* (19).

Strain RWcit2^T has a significantly higher G+C content than *D. thermophilum* (44 and 29 mol%, respectively). Strain RWcit2^T can degrade starch but in general differs from *D.*

TABLE 1. End products formed by RWcit2^T growing on complex medium containing tryptone and yeast extract

Medium	End product concn (mM)				
	Acetate	Propionate	Isobutyrate	Isovalerate	Sulfide
MMB	4.7	1.1	ND ^a	1.4	ND
MMB + glucose	11.0	0.9	ND	0.8	ND
MMB + sulfur	15.3	4.4	2.1	4.9	25.9
MMB + glucose + sulfur	14.0	2.0	1.0	3.1	18.7

^a ND, not detected (detection limit, 0.1 mM).

thermophilum in its substrate utilization patterns and the end products formed from glucose fermentation (29). *Dictyoglomus* spp. cells form large bundles, termed rotund bodies, in the late log phase, whereas members of the *Thermotogales*, *Geotoga* spp., and *Petrotoga* spp. always produce a "sheath" (9). Sheath-like material was observed in strain RWcit2^T, but this was an intermittent phenomenon. Rotund bodies have never been observed in strain RWcit2^T.

Several pathways for citrate degradation have been found in mesophilic bacteria. *Ruminococcus pasteurii* and *Formivibrio citricus* produce acetate plus formate (30, 32); *Acidaminococcus fermentans* produces acetate, butyrate, and H₂ (8); *Clostridium sphenoides* produces acetate plus ethanol (1); and *Streptococcus diacetylactis* produces a mixture of acetate, diacetyl, acetoin, 2,3-butandiol, and α -acetoacetate (15). At present, the metabolites formed during citrate degradation by strain RWcit2^T have not been fully elucidated, but they clearly differ from the metabolites described previously for certain mesophilic microorganisms. This suggests that citrate metabolism by strain RWcit2^T may occur via a different pathway than the pathways described previously. Citrate degradation is the key physiological difference between strain RWcit2^T and all other thermophilic microorganisms that have been described. The characteristics of strain RWcit2^T are not consistent with the physiology or phylogeny of any previously described taxon. We propose the name *Anaerobaculum thermoterrenum* gen. nov., sp. nov. for this organism.

Description of *Anaerobaculum* gen. nov. *Anaerobaculum* (An.ae.ro.ba'cu.lum. Gr. pref. *an*, not; Gr. n. *aer*, air; L. neut. n. *baculum*, small stick; M. L. n. *Anaerobaculum*, rod growing in the absence of air). Chemoorganotrophic anaerobe. Non-motile, straight to slightly curved rods. In complex media, cells grow with sheath-like material extending past the cell poles. Endospores are not produced. Cells stain gram negative. Ferments a range of organic acids, protein extracts, and a limited number of carbohydrates. Belongs to the domain *Bacteria* and forms a branch approximately equidistant from *D. thermophilum* and *Thermoanaerobacter* spp. (level of similarity, approximately 82%).

Description of *Anaerobaculum thermoterrenum* sp. nov. *Anaerobaculum thermoterrenum* (ther.mo.ter.re'num. Gr. adj. *thermus*, warm, hot; L. adj. *terrenum*, earthen, belonging to the earth; M. L. adj. *thermoterrenum*, from hot earth, describing the site of isolation).

Cells are 0.75 by 2 μ m and occur singly or in pairs. Obligately anaerobic. Grows very slowly at 28°C, and the maximum temperature for growth is 60°C; the optimum temperature is 55°C. Growth occurs in medium containing 0 to 20 g of NaCl per liter; the optimum salinity is about 10 g/liter. Growth occurs at pHs ranging from 5.5 to 8.6, and the optimum pH is between 7 and 7.6. Ferments citrate, fumarate, malate, pyruvate, glutamate, α -ketoglutarate, tartrate, starch, pectin, glucose, fructose, mannose, inositol, glycerol, protein extracts, and Casamino Acids. Compounds that are not used are xylose, galactose, lactose, sucrose, maltose, rhamnose, raffinose, cellulose, carboxymethyl cellulose, gum arabic, malonate, succinate, glutarate, and lactate. Sulfide is produced when the organism is grown in the presence of elemental sulfur, thiosulfate, and cystine. Sulfate, sulfite, and nitrate are not reduced. Growth is inhibited by H₂. The DNA base composition is 44 mol% G+C (as determined by the thermal denaturation method). Cytochromes are not present.

Isolated from production fluid of a petroleum reservoir.

The type strain is RWcit2, which has been deposited in the Australian Collection of Microorganisms (University of Queensland, Brisbane, Australia) as strain ACM 5076.

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