Taxonomic Distinction of Saccharolytic Thermophilic Anaerobes: Description of *Thermoanaerobacterium xylanolyticum* gen. nov., sp. nov., and *Thermoanaerobacterium saccharolyticum* gen. nov., sp. nov.; Reclassification of *Thermoanaerobium brockii*, *Clostridium thermosulfurogenes*, and *Clostridium thermohydrosulfuricum* E100-69 as *Thermoanaerobacter brockii* comb. nov., *Thermoanaerobacterium thermosulfirigenes* comb. nov., and *Thermoanaerobacter thermohydrosulfuricus* comb. nov., Respectively; and Transfer of *Clostridium thermohydrosulfuricum* 39E to *Thermoanaerobacter ethanolicus*

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Two thermophilic, anaerobic, xylan-degrading bacteria, strains B6A-RIT (T = type strain) and LX-11T, were isolated from Frying Pan Springs in Yellowstone National Park. These organisms grew chemooorganotrophically by utilizing xylan and starch but not cellulose, as well as a number of di- and monosaccharides, including glucose and xylose. Both organisms had the same optimum temperature and pH for growth (60°C and pH 6.0). The fermentation products included acetate, ethanol, lactate, CO₂, and H₂. Both organisms were rod shaped and deposited sulfur on their cells. The major difference between the two isolates was in spore formation; strain LX-11T sporulated, whereas strain B6A-RIT did not. Strains LX-11T and B6A-RIT were compared with other thermophilic, anaerobic, xylan-degrading bacteria by performing DNA-DNA hybridizations and total protein analyses in order to determine the relationships of these organisms. Three different groups were identified, and new taxonomic assignments are proposed. *Clostridium thermocellum* LQRI was least closely related to the other seven strains studied and is placed in group I, retaining its original taxonomic assignment. *Clostridium thermosulfurogenes* 4B and new isolates B6A-RIT and LX-11T are closely related and fall into group II, for which the new genus *Thermoanaerobacterium* is proposed. Isolate LX-11T is designated *Thermoanaerobacterium xylanolyticum* sp. nov., and isolate B6A-RIT is designated *Thermoanaerobacterium saccharolyticum* sp. nov. *Thermoanaerobacterium thermosulfurogenes* 4B (originally *C. thermosulfurogenes*) is the type strain of the type species of the genus. Group III strains are placed in the genus *Thermoanaerobacter*; this group includes *Thermoanaerobacter ethanolicus* JW200T, *Clostridium thermohydrosulfuricum* 39E and E100-69T, and *Thermoanaerobium brockii* HTD4T. *Thermoanaerobacterium brockii* HTD4T is renamed *Thermoanaerobacter ethanolicus* JW200T, and these organisms are considered members of the same species. Therefore, *C. thermohydrosulfuricum* 39E is renamed *Thermoanaerobacter ethanolicus* 39E; strain JW200 is the type strain of *Thermoanaerobacter ethanolicus*.

In recent years, our understanding of the biology of thermophilic microorganisms has been greatly advanced. Thermophilic bacteria, which are found in both the *Archaeabacteria* and the *Eubacteria*, have been found in association with all types of thermal habitats, including hot springs (3). Intensive studies on the biology of anaerobic thermophiles have been initiated in the last 15 years in order to learn more about the function of these organisms in nature and because of their potential use in biotechnology (3, 40). Most studies of cubacterial thermophilic anaerobes have focused on saccharolytic bacteria that form ethanol and lactate because of their potential for producing chemicals and fuels from plant biomass (14). The saccharolytic, thermophilic, anaerobic cubacteria include species belonging to the genera *Thermoanaerobacter*, *Thermocellum*, *Thermobacteroides*, and *Clostridium*. Although several strains belonging to these groups have been isolated and characterized, the taxonomic position of these organisms is uncertain.

Little is known about the microbial transformation of hemicellulose in thermooanaerobic habitats. Xylan, a major polysaccharide in hemicellulose, can be hydrolyzed by microbial enzymes to xylose (2). Thermostable xylanolytic enzymes have potential applications in the pulp and paper
industry (25, 26), and so thermophilic microorganisms have attracted considerable attention as sources of thermostable xylanases. Wiegelt et al. (35, 38) studied a variety of thermophilic, anaerobic, saccharolytic bacteria, including *Thermoanaerobacterium* *ethanolicus*, *Thermobacteroides acetethylicus*, *Thermoanaerobacterium brockii*, and *Clostridium thermocellum*, all of which ferment xylan, albeit at very slow rates. *Thermoanaerobacterium* sp. strain B6A, an organism isolated from an algat mat present in Big Spring, Thermopolis, Wyo. (34), has been shown to degrade xylan extensively (33). In this paper we describe the general morphological, cellular, and metabolic characteristics of two new xylanolytic species isolated from Frying Pan Springs in Yellowstone National Park, Wyo., by selective enrichment with xylan as the energy source for growth. This site was chosen because of its low pH (less than 6.0) as we were interested in isolating xylan-degrading anaerobes which grow at low pH values and may have industrial importance. We also compare these strains with previously described thermophilic saccharolytic anaerobes which have xylanolytic activity to investigate the taxonomic relationships among these organisms. The organisms which we studied include *Thermoanaerobacterium* *ethanolicus* (36), *Thermoanaerobacterium* *brockii* HTD4T (T = type strain), (42), C. *thermocellum* LQRI (15), Clostridium *thermodhysulfuricum* E100-69T and 39E (9, 41), Clostridium *thermosulfurogenes* 4BT (29), and *Thermoanaerobacterium* sp. strain B6A (34).

Although morphological and biochemical characteristics are useful, DNA-DNA hybridization techniques have been especially successful in resolving taxonomic relationships among closely related organisms (at the species level and below). In this study we performed DNA-DNA hybridization and total protein analyses to investigate the taxonomic relationships of some xylanolytic, thermophilic, anaerobic species. The results of our protein profile and DNA-DNA relatedness studies indicate that the saccharolytic thermophilic anaerobes which we studied are members of three independent groups. These results are supported by biochemical and physiological data, as well as the results of the partial 16S rRNA sequence studies of Bateson et al. (1). On the basis of our results, we propose new taxonomic assignments for these organisms. In addition, we propose that the genera *Thermoanaerobacter* and *Thermoanaerobacterium*, like the genus *Clostridium*, should be recognized as genera which can include sporeforming species.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** The strains used in this study are listed in Table 1. The stringent anaerobic culture techniques described previously (41) were used for medium preparation and cultivation of organisms. Cultures were grown in crimp-sealed anaerobic tubes (Belco Glass, Vineland, N.J.) that contained 10 ml of medium supplemented with 0.5% (wt/vol) substrate and N₂ as the headspace gas. Mass cultures to obtain cells for DNA isolation were grown in 1-liter round-bottom flasks that contained 500 ml of medium under an N₂ gas phase. *C. thermocellum* LQRI was grown on GS medium (24) supplemented with 0.5% (wt/vol) cellobiose. All other strains were grown on TYE medium (41) containing either glucose or xylose (0.5%, wt/vol) as the substrate or on LPBB medium (41). CM5 medium lacking yeast extract but containing a vitamin solution (34) and glucose was used to induce spore formation of strain LX-11T. All cultures were incubated at 60°C without shaking. Xylanolytic colonies were detected by adding the chromogenic substrate 4-O-methyl-Q-glucurono-d-xylan-Ramozol Brilliant Blue R (Sigma Chemical Co., St. Louis, Mo.) to the agar medium.

**Isolation and enrichment.** Sediments from the Frying Pan thermal acid spring in Yellowstone National Park were used for enrichment of xylanolytic thermoaerobes. After several transfers in TYE medium (41) supplemented with 0.5% (wt/vol) oat spelt xylan, the enrichment culture was plated onto homologous medium containing 2.5% (wt/vol) agar and 0.2% (wt/vol) 4-O-methyl-Q-glucurono-d-xylan-Ramozol Brilliant Blue R-xylan as the substrate in an anaerobic glove box (Coy Laboratory Products, Ann Arbor, Mich.). The plates were incubated anaerobically at 60°C as previously described (10). After 3 days, the colonies which produced clear halos were transferred to liquid media in the anaerobic glove box.

**Morphological and biochemical studies.** Bacterial morphology and sulfur formation were investigated by using a model BHS phase-contrast microscope (Olympus). Agar-coated glass slides (27) were used for photomicroscopy. To prepare specimens for transmission electron microscopy, cells were harvested by centrifugation at 5,000 × g for 10 min, fixed overnight in a solution containing 2.5% (wt/vol) glutaraldehyde in 0.1 M Na₂HPO₄-KH₂PO₄ (pH 7.2) buffer at 4°C, and treated as described previously (17). Growth was determined by measuring the increase in turbidity at 660 nm with a Spectronic 20 spectrophotometer (Bausch & Lomb, Rochester, N.Y.). Fermentation products were measured directly in liquid or gas samples removed from the culture tubes by gas chromatography as described previously (8). Routine biochemical tests were performed by the standard methods of Smibert and Krieg (32).

**Preparation of high-molecular-weight DNA.** The cells were harvested at the early exponential phase of growth and were washed with saline-EDTA (0.15 M NaCl, 1 mM EDTA; pH 8.0). DNA was isolated and purified by the method of

**TABLE 1. Strains used in this study**

<table>
<thead>
<tr>
<th>Name as received</th>
<th>Strain</th>
<th>Other designation(s)</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. thermocellum</em></td>
<td>LQRI</td>
<td>ATCC 35609, DSM 2360</td>
<td>Farm soil</td>
<td>15</td>
</tr>
<tr>
<td>New isolate</td>
<td>B6A-R1T</td>
<td>ATCC 49915T, DSM 7060T</td>
<td>Thermal spring</td>
<td>This study</td>
</tr>
<tr>
<td>New isolate</td>
<td>B6A</td>
<td>None</td>
<td>Thermal spring</td>
<td>This study</td>
</tr>
<tr>
<td><em>Thermoanaerobacter</em></td>
<td>B6A</td>
<td>None</td>
<td>Thermal spring</td>
<td>34</td>
</tr>
<tr>
<td><em>C. thermosulfurogenes</em></td>
<td>4BT</td>
<td>ATCC 33743T, DSM 2229T</td>
<td>Thermal spring</td>
<td>29</td>
</tr>
<tr>
<td><em>Thermoanaerobacterium</em> <em>brockii</em></td>
<td>HTD4T</td>
<td>ATCC 33075T, DSM 1457T</td>
<td>Thermal spring</td>
<td>42</td>
</tr>
<tr>
<td><em>Thermoanaerobacter</em> <em>ethanolicus</em></td>
<td>JW200T</td>
<td>ATCC 31550T, DSM 2246T</td>
<td>Thermal spring</td>
<td>36</td>
</tr>
<tr>
<td><em>C. thermohydrosulfuricum</em></td>
<td>39E</td>
<td>ATCC 33223</td>
<td>Thermal spring</td>
<td>41</td>
</tr>
<tr>
<td><em>C. thermohydrosulfuricum</em></td>
<td>E100-69T</td>
<td>ATCC 35045T, DSM 567T</td>
<td>Farm soil</td>
<td>9</td>
</tr>
</tbody>
</table>

* ATCC, American Type Culture Collection, Rockville, Md.; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany.
Marmur (20). The purity of the DNA was determined spectrophotometrically from the ratio of $A_{260}$ to $A_{280}$.

**Determination of DNA base compositions.** The midpoint thermal denaturation temperatures were determined as described by Mandel and Marmur (19), using a Gilford Response spectrophotometer equipped with a thermal program (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). The guanine-plus-cytosine (G+C) contents were calculated by using the thermal program and the equation described by Mandel and Marmur (19). DNA from Escherichia coli B (51.0 mol% G+C; Sigma) was used as the reference DNA.

**DNA-DNA hybridizations.** DNAs were labeled by nick translation with a commercial kit (Bethesda Research Laboratories, Gaithersburg, Md.), using $[1',2',5-3H]dCTP (NEN). The average specific activity obtained with this procedure was $5 \times 10^{9}$ cpm/µg of DNA. The labeled DNAs were denatured before hybridization by being heated at 100°C for 5 min and then placed on ice. The S1 nuclease procedure described by Johnson (11) was used for the DNA homology experiments. Each reassociation reaction mixture contained 10 µl of heat-denatured labeled DNA (0.03 to 0.05 µg), 50 µl of denatured unlabeled DNA (20 µg), 25 µl of 5.28 M NaCl in 1 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) (pH 7.0), and 25 µl of deionized formamide. The mixtures were reassociated at 53°C (25°C below the thermal melting point in this buffer system) for 24 h. The S1-resistant duplexes and 50 µg of yeast tRNA were coprecipitated by adding 10% trichloroacetic acid and were collected on glass fiber filters (Whatman type GF/C). Radioactivity was measured with a Packard gamma scintillation counter. Sheared salmon sperm DNA was hybridized against radiolabeled DNA in each experiment to determine the background level of reannealing of the test DNA.

**Polycrylamide gel electrophoresis of soluble proteins.** For total cellular protein analysis, 10-ml portions of late-exponential-phase cultures were harvested by centrifugation at 4,000 $\times$ g for 15 min. The cells were washed with saline-EDTA (pH 7.0) and were suspended in 0.3 to 0.5 ml of deionized water and sonicated on ice for 5 min with an ultrasonicator (Kontes, Vineland, N.J.). The cell debris was removed by centrifugation at 15,000 $\times$ g for 5 min, and the protein concentration of the supernatant was determined by the method of Lowry et al. (18). Samples containing 50 µg of protein from each strain were used for gel electrophoresis. Sodium dodecyl sulfate-polycrylamide gel electrophoresis in which the discontinuous buffer system of Laemmli (13) was used was performed with a model Protean I1 vertical slab gel unit (Bio-Rad Laboratories, Richmond, Calif.). The molecular weights of the denatured proteins were estimated by using both high-range molecular weight standards (Bio-Rad), including phosphorylase b (97,400), bovine serum albumin (66,200), and ovalbumin (45,000), and low-range molecular weight standards (Bio-Rad), including bovine serum albumin (66,000), ovalbumin (43,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400). The separating gel contained acrylamide at a final concentration of 7.5% (wt/vol), and the stacking gel contained 4% (wt/vol) acrylamide. The gel was stained with Coomassie blue (0.125% Coomassie blue R-250, 50% [vol/vol] methanol, 10% [vol/vol] acetic acid) and was destained in a solution containing 50% (vol/vol) methanol and 10% (vol/vol) acetic acid.

**Enzyme assays.** Xylanase activity was determined by incubating a 1-ml reaction mixture containing 1% (wt/vol) oat spelt xylan (Sigma) and culture supernatant in 50 mM acetate buffer (pH 5.5) for 1 h at 65°C. The reducing sugar formed was determined by the dinitrosalicylic acid method (21), using p-xylene as the standard; 1 U of xylanase activity was defined as the activity that released 1 µmol of xylose in 1 min.

### RESULTS

**Enrichment and isolation.** Frying Pan Springs are small, shallow hydrothermal areas located in the Sylvan Springs thermal area in Yellowstone National Park. Samples were taken during the summer, and at this time the pH of these springs ranged from 4.5 to 6.0 and the temperature was around 60°C. Samples were collected by passing a jar close along the surface of the bottom of a spring and collecting the sediment. The two isolates studied in detail were strains LX-11T and B6A-R1T.

**Morphology and ultrastructure.** From light microscopy results, morphological differences between strains LX-11T and B6A-R1T were very apparent. The former organism forms short rods that occur either singly or in pairs (Fig. 1a), whereas strain B6A-R1T cells are much longer rods which occur in chains of varying lengths (Fig. 1b). Both strains utilize thiosulfate as an electron acceptor and convert thiosulfate to elemental sulfur, which is deposited on the cell and in the medium (Fig. 1a and b). The organisms also differ in their growth patterns. In the stationary phase, cells of B6A-R1T become more elongated and tend to aggregate and eventually settle to the bottom of culture vessels; this does not occur with LX-11T. Whole-cell transmission electron microscopy showed that the cytoplasm of Thermoanaerobacter sp. strain B6A-R1T is very granular compared with LX-11T cytoplasm (Fig. 2). The most striking morphological difference is that LX-11T forms spores (Fig. 3), whereas despite numerous attempts under various conditions (e.g., different media, carbon limitation, different temperatures), B6A-R1T has not been found to sporulate. Approximately 20% of strain LX-11T cells contained spores, which were located at the terminal part of each cell. The mature spores remained within the mother cells rather than free in solution.

**Metabolism.** In order to determine the taxonomic assignments of strains B6A-R1T and LX-11T, the characteristics of these organisms were compared with the characteristics of other thermophilic anaerobes. The organisms examined are listed in Table 1. Strains LX-11T, B6A-R1T, and B6A utilized the same range of substrates, growing well on xylan and starch, but were not able to degrade cellulose (Table 2). Growth occurred on a number of disaccharides, including maltose, lactose, sucrose, and cellobiose; of the monosaccharides tested, glucose, xylose, galactose, mannose, fructose, and the sugar alcohol mannitol supported growth (Table 2). The fermentation products formed by strains LX-11T and B6A-R1T included acetate and ethanol in approximately equal amounts, as well as hydrogen and carbon dioxide (data not shown). The substrates utilized by most of the other thermophilic anaerobic strains were identical to the substrates utilized by strains B6A-R1T and LX-11T; the exceptions were C. thermosulfurogenes 4B1, which could not utilize lactose, and Thermoanaerobacter ethanolicus JW200T, which could not ferment mannitol. The metabolic properties of strain B6A-R1T and Thermoanaerobacter sp. strain B6A were identical. C. thermocellum LQR1 was the only cellulolytic species among this group of bacteria.

Strains LX-11T and B6A-R1T grew on xylan, and the xylanase activities of these organisms were determined and compared with the xylanase activities of some other xylan-
FIG. 1. Phase-contrast photomicrographs of sulfur-depositing cultures of *Thermoanaerobacter* spp. strains LX-11$^T$ (a) and B6A-RIT$^T$ (b) grown on LPBB medium containing 0.5% (wt/vol) glucose, 0.1% (wt/vol) yeast extract, and 20 mM Na$_2$S$_2$O$_3$. Note that phase-bright sulfur accumulated in the medium and on the cells. Bar = 3 μm.

utilizing, thermophilic, anaerobic bacteria (Table 3). Strains B6A-RIT$^T$ and LX-11$^T$ produced high levels of xylanase during growth on xylose, compared with most of the other organisms; the exception was *C. thermocellum* LQRI, which has an active xylanase system and an active cellulase system (22).

**Molecular taxonomic analyses.** In order to characterize strains LX-11$^T$ and B6A-RIT$^T$ further and to compare these two new isolates with other xylanolytic, thermophilic, anaerobic eubacteria, additional studies were performed in which G+C content, DNA-DNA hybridization, and total protein pattern analyses were carried out.

**DNA base compositions.** The G+C content of both strain B6A-RIT$^T$ and strain LX-11$^T$ was 36 mol% (Table 4), which was identical to the value obtained for *C. thermosulfuro-

FIG. 2. Electron micrographs of thin sections of cells of *Thermoanaerobacter* spp. strains B6A-RIT$^T$ (A) and LX-11$^T$ (B). Bar = 0.5 μm.
FIG. 3. Electron micrograph of a thin section of an endospore of *Thermoanaerobacter* sp. strain LX-11T. The exosporium (EX) surrounds the spore coat, which encloses the cortex (CX) and the cell wall (CW). Bar = 0.2 μm.

genes 4BT and *C. thermohydrosulphuricum* E100-69T. The G+C contents of most of the other organisms were very similar, ranging from 34 to 36 mol%; the exception was *C. thermocellum* LQRI, which had a G+C content of 43 mol% (Table 4).

**DNA-DNA hybridization.** The results of DNA-DNA hybridization studies (Table 4) clearly show that *C. thermocellum* LQRI is not closely related to the other thermophilic anaerobes, as the relative levels of DNA-DNA homology were 13% or less; thus, *C. thermocellum* LQRI was placed in a separate group. The high levels of homology among the remaining organisms gave rise to two other groups. A significant level of similarity was observed between strain LX-11T and *C. thermostaurigenes* 4BT (89% homology), which together with new isolate B6A-RII formed group II. *C. thermohydrosulphuricum* 39E and *Thermoanaerobacter ethanolicus* JW200T were very similar (level of homology, 96%) and formed group III together with *Thermoanaerobacter brockii* HTD4T and *C. thermohydrosulphuricum* E100-69T. The large differences in reciprocal homology values are due to the differences in genome size between the different
organisms. More closely related organisms have genomes that are similar in size and produce similar reciprocal homology values. In unrelated organisms a difference in genome size results in a higher value for binding when the DNA from the larger genome is labeled and a lower value for binding when the smaller genome is labeled.

Electrophoretic analysis of whole-cell proteins. Cellular protein patterns obtained by gel electrophoresis are useful for differentiating species and have been shown to have a close relationship to the level of DNA-DNA homology (6, 7). The soluble protein patterns of the eight strains which we studied are shown in Fig. 4. A considerable difference in protein banding patterns was evident between C. thermocellum LQRI and the other organisms. The proteins from new isolates B6A-RIT and LX-11T and C. thermosulfurogenes 4B T were similar, as were the proteins from Thermoanaerobacter brockii HTD4T, Thermooanaerobacter ethanolicus JW200T, and C. thermohydrosulfiricum 39E and E100-69T.

**DISCUSSION**

In this paper we describe two new thermophilic anaerobic bacteria, strains B6A-RIT and LX-11T, which were isolated from Frying Pan Springs in Yellowstone National Park. These organisms were compared with other xylanolytic thermophilic anaerobes in order to determine their taxonomic affiliations. Traditionally, morphological and biochemical characteristics have been considered important in the classification of bacteria. At present, spore formation is considered a valuable taxonomic criterion to distinguish between organisms, but often sporulation can be observed only under very specialized laboratory conditions and is often misleading for taxonomic assessment of anaerobic bacteria as some organisms originally thought to be non-sporoformers have been found to sporulate (5, 17).

Although classification of bacteria based on similarities in phenotypic characteristics has been successful in the past, this approach is not precise enough for differentiating between superficially similar organisms or for determining phylogenetic relationships among bacteria. More and more organisms are being classified on the basis of differences at the molecular level with respect to G+C content, 16s rRNA sequences, and DNA-DNA hybridization, all characteristics which cannot be influenced by conditions of growth in laboratories.

In this study we compared new isolates B6A-RIT and LX-11T with other thermophilic xylanolytic anaerobes. Strain B6A-RIT appeared to be identical in metabolic and physiological and morphological properties to Thermooanaerobacter sp. strain B6A (34) but differed from strain LX-11T, which formed spores. The physiological and biochemical properties of these saccharolytic thermophilic anaerobic strains indicated that the previous taxonomic assignments and nomenclature of these strains based on phenotypes were not adequate and that these organisms should be provided with new taxonomic assignments. Thus, we reexamined the taxonomic positions of some xylanolytic Clostridium species and compared these organisms with some Thermooanaerobacter and Thermooanaerobium species on the basis of differences at the molecular level.

**TABLE 3. Xylanase activities of various thermophilic anaerobes**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth (460)</th>
<th>Final pH</th>
<th>Activity (U/ml)</th>
<th>Protein concn (mg/ml)</th>
<th>Sp act (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>New isolate B6A-RIT T</td>
<td>1.40</td>
<td>5.1</td>
<td>0.108</td>
<td>1.66</td>
<td>0.065</td>
</tr>
<tr>
<td>New isolate LX-11T T</td>
<td>1.30</td>
<td>4.2</td>
<td>0.067</td>
<td>2.00</td>
<td>0.033</td>
</tr>
<tr>
<td>C. thermohydrolysulfuricum 39E</td>
<td>1.15</td>
<td>5.8</td>
<td>0.029</td>
<td>1.98</td>
<td>0.015</td>
</tr>
<tr>
<td>C. thermosulfurogenes 4B T</td>
<td>1.05</td>
<td>5.5</td>
<td>0.023</td>
<td>2.25</td>
<td>0.010</td>
</tr>
<tr>
<td>Thermooanaerobacter ethanolicus JW200 T</td>
<td>0.85</td>
<td>5.9</td>
<td>0.017</td>
<td>2.99</td>
<td>0.006</td>
</tr>
<tr>
<td>Thermooanaerobium brockii HTD4 T</td>
<td>1.02</td>
<td>5.5</td>
<td>0.006</td>
<td>2.40</td>
<td>0.003</td>
</tr>
<tr>
<td>C. thermohydrolysulfuricum E100-69 T</td>
<td>0.92</td>
<td>6.5</td>
<td>0.206</td>
<td>1.50</td>
<td>0.137</td>
</tr>
</tbody>
</table>

**TABLE 4. Levels of DNA homology between thermophilic anaerobic strains that have xylanase activity**

<table>
<thead>
<tr>
<th>Strain</th>
<th>G+C content (mol%)</th>
<th>% Relative binding with labeled DNA from:*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain LQRI</td>
<td>Strain B6A-RIT T</td>
<td>Strain LX-11T T</td>
</tr>
<tr>
<td>Strain HTD4 T</td>
<td>Strain 4B T</td>
<td>Strain HTD4 T</td>
</tr>
<tr>
<td>Strain JW200 T</td>
<td>Strain 39E</td>
<td>Strain E100-69 T</td>
</tr>
<tr>
<td>Group I strain C. thermocellum LQRI</td>
<td>43</td>
<td>100*</td>
</tr>
<tr>
<td>Group II strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B6A-RIT T</td>
<td>36</td>
<td>30</td>
</tr>
<tr>
<td>LX-11T T</td>
<td>36</td>
<td>35</td>
</tr>
<tr>
<td>C. thermosulfurogenes 4B T</td>
<td>36 (33)*</td>
<td>40</td>
</tr>
<tr>
<td>C. thermohydrolysulfuricum 39E</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group III strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thermoanaerobium brockii HTD4 T</td>
<td>34 (30-31)*</td>
<td>45</td>
</tr>
<tr>
<td>Thermooanaerobacter ethanolicus JW200 T</td>
<td>35 (32)*</td>
<td>40</td>
</tr>
<tr>
<td>C. thermohydrolysulfuricum 39E</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. thermohydrolysulfuricum E100-69 T</td>
<td>36 (36)*</td>
<td>34</td>
</tr>
</tbody>
</table>

* Comparisons were made in triplicate.
+ The levels of binding between homologous strains were arbitrarily defined as 100%.
+ The value in parentheses was obtained from reference 39.
+ The values in parentheses were obtained from reference 42.
+ The value in parentheses was obtained from reference 36.
+ The value in parentheses was obtained from reference 37.
was 89%, the former organism powd on pectin and produces pectinase (30), whereas LX-11 does not use this substrate.

The G+C contents of all of the organisms were very similar and did not permit separation of groups. On the basis of DNA-DNA homology data and protein banding patterns, we placed the strains in this study into three different groups; below we propose new taxonomic assignments in which sporeformers and nonsporeformers are placed in the same genus and species (Table 5). C. thermocellum LQRI stands out as the organism that is least closely related to the other seven strains. This organism forms group I, and we suggest that it should retain its original taxonomic assignment without any change.

C. thermosulfurogenes 4B\textsuperscript{T} and new xylanolytic isolates LX-11\textsuperscript{T} and B6A-RT\textsuperscript{T} are closely related and form group II, for which the new genus Thermoanaerobacterium is proposed. There were sufficient differences among these organisms to warrant different species names. Although the level of DNA-DNA homology between strains 4B\textsuperscript{T} and LX-11\textsuperscript{T} was 89\%, the former organism grows on pectin and produces pectinase (30), whereas LX-11\textsuperscript{T} does not use this substrate. Although strains LX-11\textsuperscript{T} and B6A-RT\textsuperscript{T} exhibit high levels of DNA-DNA homology and produce similar protein banding patterns, they differ in their ability to form spores. Strain LX-11\textsuperscript{T} is placed in Thermoanaerobacterium xylanolyticum sp. nov. because of its xylanolytic properties. Strain B6A-RT\textsuperscript{T} is named Thermoanaerobacterium saccharolyticum sp. nov. Both strain B6A-RT\textsuperscript{T} and Thermoanaerobacter sp. strain B6A are saccharolytic organisms that produce amylases and grow on starch, as well as on other substrates (16, 28). Thermoanaerobacterium thermosulfurogenes 4B\textsuperscript{T} is the type strain of the type species of the genus Thermoanaerobacterium.

Group III includes Thermoanaerobacter ethanolicus JW200\textsuperscript{T}, C. thermohydrodsulfuricum 39E and E100-69\textsuperscript{T}, and Thermoanaerobacterium brockii HTD4\textsuperscript{T}, which are closely related as determined by DNA-DNA homology experiments. The members of this group are placed in the genus Thermoanaerobacter. Thermoanaerobacterium brockii HTD4\textsuperscript{T} is re-named Thermoanaerobacter brockii comb. nov., and C. thermohydrodsulfuricum E100-69\textsuperscript{T} is renamed Thermohydrodsulfuricum thermohydrodsulfuricum comb. nov. C. thermohydrodsulfuricum 39E, as described by Zeikus et al. (41), is nearly identical to Thermoanaerobacter ethanolicus JW200\textsuperscript{T}, and thus these two strains are considered to be members of the same species. Perhaps, Thermoanaerobacter ethanolicus JW200\textsuperscript{T} is an asporogenous mutant of strain 39E. Therefore, strain 39E is renamed Thermohydrodsulfuricum ethanolicus 39E; strain JW200\textsuperscript{T} is the type strain of Thermoanaerobacter ethanolicus. A taxonomic key for preliminary identification of saccharolytic, ethanologenic thermoanaerobes is given below.

A. Does not reduce thiosulfate, ferments cellulose 1. Clostridium thermocellum

B. Does reduce thiosulfate, does not ferment cellulose

1. Reduces thiosulfate to elemental sulfur
   - Thermoanaerobacterium thermosulfurogenes
   - Thermoanaerobacterium saccharolyticum
   - Thermoanaerobacterium xylanolyticum

2. Reduces thiosulfate to H\textsubscript{2}S
   - Thermoanaerobacter brockii
   - Thermoanaerobacter ethanolicus
   - Thermoanaerobacter thermohydrodsulfuricum

Thermophilic, anaerobic, saccharolytic, sporeforming bacteria were first recognized as members of two genera, Clostridium and Desulfotomaculum, on the basis of sulfate reduction. The members of the genera Thermoanaerobacter and Thermohydrodsulfuricum form spores and use thiosulfate but not sulfate as an electron acceptor.

Our proposed taxonomic arrangement of these organisms is supported by the results of previous studies performed by other workers. Wiegel first suggested that Thermoanaerobacterium brockii, Thermoanaerobacter ethanolicus, and C. ther-

![Electrophoretic comparison of cellular proteins from thermoanaerobic strains that have xylanase activity.](image)

**FIG. 4.** Electrophoretic comparison of cellular proteins from thermoanaerobic strains that have xylanase activity. Lane A, C. thermocellum LQRI; lane B, Thermohaerobacter sp. strain B6A-RT\textsuperscript{T}; lane C, new isolate LX-11\textsuperscript{T}; lane D, C. thermosulfurogenes 4B\textsuperscript{T}; lane E, Thermoanaerobacterium brockii HTD4\textsuperscript{T}; lane F, Thermohaerobacterium xylanolyticum JW200\textsuperscript{T}; lane G, C. thermohydrodsulfuricum 39E; lane H, C. thermohydrodsulfuricum E100-69. Molecular weight markers are in the outer lanes.

<table>
<thead>
<tr>
<th>Group</th>
<th>Proposed name</th>
<th>Former name(s)</th>
<th>Strain(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Clostridium thermocellum</td>
<td>Clostridium thermocellum</td>
<td>LQRI</td>
</tr>
<tr>
<td>II</td>
<td>Thermoanaerobacterium thermosulfurogenes</td>
<td>Clostridium thermosulfurogenes</td>
<td>4B\textsuperscript{T}</td>
</tr>
<tr>
<td></td>
<td>Thermoanaerobacterium xylanolyticum</td>
<td>Clostridium thermosulfurogenes</td>
<td>LX-11\textsuperscript{T}</td>
</tr>
<tr>
<td>III</td>
<td>Thermoanaerobacter brockii</td>
<td>Thermoanaerobacter brockii</td>
<td>E100-69\textsuperscript{T}</td>
</tr>
<tr>
<td></td>
<td>Thermoanaerobacter thermohydrodsulfuricus</td>
<td>Thermoanaerobacter ethanolicus</td>
<td>PJ200\textsuperscript{T}</td>
</tr>
<tr>
<td></td>
<td>Thermoanaerobacter ethanolicus</td>
<td>Clostridium thermohydrodsulfuricum</td>
<td>39E</td>
</tr>
</tbody>
</table>
mohydroxysulfurificum 39E could be close taxonomic relatives because they have similar substrate ranges and temperature ranges for growth and produce similar biphasic growth curves (39). Kondratieva et al. (12) recognized the similarity of Thermoanaerobium lactoethylicum to both Thermoanaerobacterium brockii and Thermoanaerobacter ethanolicus and suggested that the genus name Thermoanaerobacter, not the genus name Thermoanaerobacterium, should be used for these species. Bateson et al. (1) have reported that the 16S rRNA sequences of Thermoanaerobacterium brockii, Thermoanaerobacter ethanolicus, and C. thermohydrodsulfurificum are nearly identical, but significantly different from the sequences of C. thermosulfurogenes. The 16S rRNA sequence studies of Cato and Stackebrandt (4) showed that sporulation is not a taxonomic characteristic that is sufficient to distinguish between organisms, as spore-forming clostridia were found to form not one phylogenetically homologous family but six sublines which included both spore-forming and nonspore-forming species.

The genus Thermoanaerobacterium (36) and Thermoanaerobacterium (42) included the first thermophilic, anaerobic, rod-shaped, gram-positive, non-sporulating bacteria that produce ethanol and lactate as principal saccharide fermentation products. The genus Thermoanaerobacterium is listed under the irregular, nonsporeforming, gram-positive rods in Bergey's Manual of Systematic Bacteriology (39). Until now, the genus Thermoanaerobacterium has included two species, Thermoanaerobacterium ethanolicus (36) and Thermoanaerobacterium fennii (31). Strain JW200 has been designated the neotype strain of Thermoanaerobacterium ethanolicus.

The genus Thermoanaerobacterium also includes only two previously described species, Thermoanaerobacterium brockii (42) and Thermoanaerobacterium lactoethylicum (12); Thermoanaerobacterium brockii HDT4 is the neotype strain. Previously, organisms belonging to the genera Thermoanaerobacterium and Thermoanaerobacterium were considered to be strictly anaerobic, motile or nonmotile, rod-shaped, gram-positive to gram-negative, nonsporeforming, cellulose- and formed ethanol and/or lactate as major end products, with as well as lower levels of H₂-CO₂ and acetate. Consequently, it was not possible to adequately distinguish between Thermanaerobacterium and Thermoanaerobacterium strains on the basis of these morphological, cellular, and nutritional properties alone. Using the criteria described in this paper for distinguishing among organisms on the basis of differences at the molecular level, we propose the classification described below for the genera Thermoanaerobacterium and Thermoanaerobacterium.

**Description of Thermanaerobacterium gen. nov.** Thermanaerobacterium (Ther.mo.an.er.o.bac.te.ri.um. Gr. n. thermos, hot; Gr. pref. an, not; Gr.n. aer, air; Gr.n. bacterium, a small rod; M.L. neut. n. *Thermanaerobacterium*, rod which grows in the absence of air at high temperatures).

**Cellular characteristics.** Straight rods (0.5 by >15 μm). Forms long filaments. Cells are motile and peritrichous. Gram-negative cell wall. Catalase negative. Reduces thiosulfate to elemental sulfur. Deposits elemental sulfur on cells when cultivated with thiosulfate. H2S is produced but could not be detected. Surface colonies are about 0.5 to 3 mm in diameter. The optimum temperature for growth is ≥60°C. The G+C content of the DNA is 33 to 36 mol%. The principal products of carbohydrate fermentation are ethanol, acetate, lactate, H₂, and CO₂. The type species is Thermoanaerobacterium thermosulfurigenes comb. nov.

**Description of Thermanaerobacterium thermosulfurigenes.** Thermanaerobacterium thermosulfurigenes (ther.mo.sul.fur.i.ge.nes. Gra. adj. thermos, hot; L.n. sulfur, brimstone; Gra. suff. genes, born from; N.L. neut. adj. thermosulfurigenes, releasing sulfur in heat). (29).

**Cellular characteristics.** Straight rods (0.5 by >2 μm). Gram negative. Exponential-phase cells are motile and peritrichous. Forms long filaments and deposits yellow elemental sulfur on cells and in the medium when cells are grown with thiosulfate. Neither sulfitone nor sulfide is detected. Swollen, white, refractile, spherical endospores are formed. No outer wall membranous layer is present in thin sections. Sporulation occurs in xyllose- or pectin-containing media. Agar-embedded colonies are fluffy, 0.5 to 1.5 mm in diameter, and not pigmented. The DNA base composition is 32.6 ± 0.04 mol% G+C. Cytochromes are not detected. Catalase negative.

**Growth characteristics.** The optimum temperature for growth is ≥60°C; the maximum temperature is 75°C; and the minimum temperature is 55°C. The optimum pH range for growth is 5.5 to 6.5; the minimum pH is 4.0; and the maximum pH is <7.6. Obligately thermophilic and anaerobic. Growth is completely inhibited by penicillin (100 μg ml⁻¹), streptomycin (100 μg ml⁻¹), cycloserine (100 μg ml⁻¹), tetracycline (100 μg ml⁻¹), chloramphenicol (100 μg ml⁻¹), sodium azide (500 μg ml⁻¹), and O₂ (0.203 × 10⁹ Pa). Growth is inhibited by 2% NaCl and by sulfite.

**Metabolic characteristics.** Chemoorganotroph. Utilizes a wide variety of carbohydrates as energy sources, including xylan, pectin, arabinose, cellulose, polygalacturonose, rhamnose, amygdalin, salicin, inositol, mannitol, xylose, galactose, glucose, mannose, maltose, starch, melibiose, sucrose, trehalose, and esculin. No growth occurs on H₂-CO₂, lactose, cellulose, tartrate, lactate, pyruvate, methanol, or glycerol. The fermentation products from glucose are ethanol, H₂, CO₂, lactate, and acetate. Methanol and isopropanol are formed when cultures are grown on pectin. Sulfate, sulfite, and nitrate are not reduced. Pectin methyl-esterase and polygalacturonase hydrolysis are produced.

**Habitat.** The habitat is algal-bacterial mat ecosystems associated with thermal, volcanic springs. Strain 4BT was isolated from Octopus Spring, Yellowstone National Park, Wyo.

**Type strain.** The type strain of Thermanaerobacterium thermosulfurigenes is strain 4BT (= ATCC 33743 = DSM 2229).

**Description of Thermanaerobacterium xylanolyticum sp. nov.** Thermanaerobacterium xylanolyticum (xy.lan.o.lyti.cum. Gr. n. xylanosum, xylan; Gra. adj. lyticus, dissolving; N.L. adj. xylanolyticum, xylan dissolving).

**Cellular characteristics.** Rods (approximately 0.8 to 1.0 by 2.0 to 7.0 μm). Spores are spherical and terminal. Cells are motile. Gram negative. Surface colonies are circular with smooth edges cloudy to white, and about 2 to 5 mm in diameter; the surface texture is rough. The G+C content of the DNA is 36.1 mol%. Catalase negative.

**Growth characteristics.** Anaerobic. Growth occurs in an N₂-gassed medium lacking a chemical reducing agent (cysteine or Na₂S). The pH range for growth is 5.0 to 7.5; the optimum pH is about 6.0. The optimum temperature for growth is 60°C; the growth temperature range is 45 to 70°C. Growth is inhibited by penicillin G (200 μg/ml), neomycin sulfate (100 μg/ml), ampicillin (100 μg/ml), streptomycin sulfate (100 μg/ml), rifampin (100 μg/ml), polymyxin B (100
Brock, named after Thomas Dale Brock, who pioneered glucose or xylan include ethanol, acetic acid, lactic acid, H2,\textsuperscript{+} (Yellowstone National Park and Thermopolis areas) and Nevada (Steamboat area).

Metabolic characteristics. Utilizes xylan, mannose, sucrose, cellobiose, arabinose, rhamnose, galactose, fructose, maltose, pyruvate, lactose, and glucose. Cellulose, melibiose, melezitose, xylitol, ribose, raffinose, and lactate are not fermented. The fermentation end products include ethanol, acetic acid, H2, and CO2. Lactate is not detected in fermentation broth.

Habitat. The habitat is geothermal areas of Yellowstone National Park, Wyo.

Type strain. The type strain of Thermoanaerobacterium xylanolyticum is strain LX-11 (= ATCC 49914 = DSM 7097).

Description of Thermoanaerobacterium saccharolyticum sp. nov. Thermoanaerobacterium saccharolyticum (sac.cha.ro.ly'ti.cum. Gr. n. sacchar, sugar; Gr. adj. ly'ticus, dissolving; N.L. neut. adj. saccharolyticum, sugar dissolving).

Cellular characteristics. Rods (approximately 0.8 to 1.0 by 3.0 to 15 \textmu m; some cells are occasionally as long as 30 \textmu m). Elongated cell morphology occurs during nutrient limitation or stationary phase. The cell walls contain three electron-dense layers that are 5 nm thick and alternate with electron-light layers of similar thickness. Gram-negative, but the distinct outer membrane characteristic of many gram-negative bacteria is not observed. Cell division proceeds by formation of well-defined division septa, often producing daughter cells of unequal lengths. Cells are motile and peritrichous. Cells survive heating at 85°C for 15 min but not heating at 90°C for 5 min. Cells contain a 1.5-MDa plasmid. Surface colonies on agar plates are soft, tan, circular, and convex with hollow centers ("donut" shape). Colony diameters range from 0.5 to 4.0 mm after 4 days of growth at 55°C. The G+C content of the DNA is 36 mol\% (as determined by the thermal denaturation method). Catalase negative.

Growth characteristics. Anaerobic. Growth occurs in an N2-gassed medium lacking chemical reducing agents. Addition of reducing agents (cytochrome or Na2S) does not stimulate growth. The optimum pH for growth is 6.0, and the minimum and maximum pH values for growth initiation are 5.0 and less than 7.5, respectively. The maximum temperature for growth is 68 to 70°C; the optimum temperature is 60°C; and the minimum temperature is 45°C. Growth is inhibited by penicillin G (200 \textmu g/ml), chloramphenicol (100 \textmu g/ml), neomycin (100 \textmu g/ml), or O2 (0.2 atm [ca. 20.26 kPa]). Resistant to 2% NaCl. Yeast extract stimulates growth.

Metabolic characteristics. Ferments a wide variety of carbohydrates, including glucose, fructose, mannose, galactose, maltose, cellobiose, sucrose, lactose, trehalose, xylose, starch, rhamnose, raffinose, and xylan. Cellulose, ribose, melibiose, melezitose, xylitol, and sorbitol are not fermented. No growth occurs in the absence of a fermentable carbohydrate. The fermentation products from either glucose or xylan include ethanol, acetic acid, lactic acid, H2, and CO2. L-Rhamnose is fermented to equimolecular amounts of 1,2-propanediol and a mixture of ethanol, acetic acid, lactic acid, H2, and CO2.

Habitat. The habitat is geothermal sites in Wyoming (Yellowstone National Park and Thermopolis areas) and Nevada (Steamboat area).

Type strain. The type strain of Thermoanaerobacterium saccharolyticum is strain B6A-RI (= ATCC 49915 = DSM 7060).

Description of Thermoanaerobacter brockii comb. nov. Thermoanaerobacter brockii (brock'i.i. M.L. gen. n. brockii, of Brock, named after Thomas Dale Brock, who pioneered studies on physiological ecology of extreme thermophiles (42).

Cellular characteristics. Rods (1.0 by 2 to 20 \textmu m). Cells are frequently uneven in length (minicells) and occur in chains, pairs, and filaments. Gram positive. Round, terminal endospores are formed. The DNA base composition is 30.0 to 31.4 (±1) mol\% G+C. Cytochrome pigments and catalase are absent. Monolayered cell wall architecture without an outer wall membrane.

Colony characteristics. Colonies are uniformly round, mucoid, nonpigmented, and flat and grow to a diameter of 0.2 to 0.3 cm in 48 h.

Growth characteristics. Obligately anaerobic. The optimum temperature for growth is 65 to 70°C; the maximum temperature is <85°C; and the minimum temperature >35°C. The optimum pH for growth is ~7.5; no growth occurs above pH 9.5 or below pH 5.5.

Metabolic characteristics. Chemoorganotroph. Utilizes a variety of saccharides as energy sources, including starch, maltose, glucose, lactose, sucrose, and cellobiose. Growth is inhibited by air, penicillin, cycloserine, streptomycin, tetraacycline, and chloramphenicol. The fermentation end products are ethanol, lactic acid, acetic acid, hydrogen, and carbon dioxide. Reduces thiosulfate to hydrogen sulfide.

Habitat. The habitat is anaerobic thermal features associated with volcanic activity, including springs, decomposing photosynthetic biomass, and sediments.

Type strain. The type strain of Thermoanaerobacter brockii is strain HTD4 (= ATCC 33075 = DSM 1457). This strain was isolated from a Washburn thermal springs edge sediment located in Yellowstone National Park, Wyo.

Description of Thermoanaerobacter thermohydrosulfuricus comb. nov. Thermoanaerobacter thermohydrosulfuricus (ther.mo.hy.dro.sul.fur'i.cus. M.L. masc. adj. thermohydrosulfuricus, pertaining to hydrogen sulfide; M.L. masc. adj. thermohydrosulfuricus, indicating that the organism grows at high temperatures and reduces sulfite to H2S).

Cellular characteristics. Rods (approximately 0.3 to 0.6 by 2.0 to 13.0 \textmu m). Cells occur singly or in short chains; in some strains the cells occur in long filamentous groups. Cells are motile and peritrichous. Spores are spherical and terminal; the sporangia swell the cells. Sporulating cultures contain thinner, more elongated cells than nonsporulating cultures. Gram variable. The cell wall is composed of two layers. The outer layer consists of hexagonally shaped particles. The center-to-center distance between adjacent particles is 13.5 nm; the particles are composed of a glycoprotein containing glucose, galactose, mannose, and rhamnose, which has a molecular weight of approximately 140,000. The outer layer completely covers the cell and is resistant to digestion by proteolytic enzymes. The cell walls contain meso-diaminopimelic acid. The G+C content is 35 to 37 mol\% (as determined by the thermal denaturation method). Catalase negative.

Growth characteristics. Obligately anaerobic. The optimum temperature is 67 to 69°C; the maximum temperature at which growth occurs is 76 to 78°C. Growth at 37°C is poor; no growth occurs at 28°C. Growth occurs at pH 5.5 to 9.2; optimum growth occurs at pH 6.9 to 7.5. Growth is inhibited by H2 in the gas phase and by lactate.

Metabolic characteristics. H2 and CO2 are produced in media containing liver infusion. H2S is produced from tryptophan, peptone, and yeast extract. Sulfite and thiosulfate are reduced to H2S; sulfate is not reduced. Acetyl methyl carbinol is not produced. Fructose, galactose, glucose, man-
nose, xylose, cellobiose, maltose, sucrose, trehalose, pectin, esculin, and salicin are fermented. Fermentation of dextrin, potato starch, mannitol, dulcitol and sorbitol and coagulation of litmus milk are variable. Inositol, erythritol, glycerol, lactate, tartrate, and cellulose are not fermented; nitrite but not nitrate is reduced. Coagulated albumin is not hydrolyzed; indole is not produced. The products of metabolism in PYG broth are acetate, lactic acids, ethanol, CO₂, H₂; formic, butyric, isovaleric, and isoacproic acids, propanol, and isopropanol may be detected. Methanol is a major metabolic end product if cultures are grown in the presence of pectin. Reduces thiosulfate to hydrogen sulfide.

**Habits.** Isolated from extraction juices from beet sugar factories; from mud and soil; from hot springs in Utah and Wyoming; and from a sewage plant in Georgia.

**Neotype strain.** The neotype strain of *Thermoanaerobacter thermohydrosulfuricus* strain E100-69 (= DSM 567 = NCIB 10956).

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