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Caldicellulosiruptor obsidiansis sp. nov., an Anaerobic, Extremely Thermophilic, Cellulolytic Bacterium Isolated from Obsidian Pool, Yellowstone National Park[▽]

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A novel, obligately anaerobic, extremely thermophilic, cellulolytic bacterium, designated OB47^T, was isolated from Obsidian Pool, Yellowstone National Park, WY. The isolate was a nonmotile, non-spore-forming, Gram-positive rod approximately 2 μm long by 0.2 μm wide and grew at temperatures between 55 and 85°C, with the optimum at 78°C. The pH range for growth was 6.0 to 8.0, with values of near 7.0 being optimal. Growth on cellobiose produced the fastest specific growth rate at 0.75 h^{-1}. The organism also displayed fermentative growth on glucose, maltose, arabinose, fructose, starch, lactose, mannose, sucrose, galactose, xylose, arabinogalactan, Avicel, xylan, filter paper, processed cardboard, pectin, dilute acid-pretreated switchgrass, and *Populus*. OB47^T was unable to grow on mannitol, fucose, lignin, Gelrite, acetate, glycerol, ribose, sorbitol, carboxymethylcellulose, and casein. Yeast extract stimulated growth, and thiosulfate, sulfate, nitrate, and sulfur were not reduced. Fermentation end products were mainly acetate, H_2, and CO_2, although lactate and ethanol were produced in 5-liter batch fermentations. The G+C content of the DNA was 35 mol%, and sequence analysis of the small subunit rRNA gene placed OB47^T within the genus *Caldicellulosiruptor*. Based on its phylogenetic and phenotypic properties, the isolate is proposed to be designated *Caldicellulosiruptor obsidiansis* sp. nov. and OB47 is the type strain (ATCC BAA-2073).

Cellulosic biomass will likely serve as an important source of stored renewable energy in the future. However, improvements in overcoming the recalcitrance of lignocellulosic materials to enzymatic hydrolysis must be made in order to efficiently convert biomass to liquid fuels (23, 27). Members of the genera *Caldicellulosiruptor* and *Anaerocellum* are obligatory anaerobic, extreme thermophiles within the *Firmicutes* and are known to express heat-stable extracellular enzyme systems for breaking down biomass (4). In addition, both hexose and pentose sugars can be utilized for fermentation (12, 16, 19, 28). Given these properties, recent studies have focused on the use of extreme thermophiles for biomass conversion to fuels, including *Caldicellulosiruptor saccharolyticus* as a biocatalyst for hydrogen production from biomass (10, 26) and *A. thermophilum* (also known as *Caldicellulosiruptor bescii*), which has been evaluated for growth on plant biomass without physical or chemical pretreatment (28, 29).

A number of isolated strains of *Caldicellulosiruptor* have been described thus far, with several organisms originating from Icelandic hot springs (3, 14, 17, 18); the geothermal region of Kamchatka (15, 24); thermal features in New Zealand (19); and solar-heated freshwater ponds in Owens Valley, CA (9). The environmental parameters for growth appear to be fairly uniform for these organisms which prefer circumneutral to slightly alkaline pH and temperatures ranging from 60 to 83°C. None of the described isolates form spores, and all strains are heterotrophic obligate anaerobes which utilize a broad range of carbohydrates for fermentative growth. Complete genome sequences are available for *C. saccharolyticus* (26) and *Anaerocellum thermophilum* (28).

Thus far, no species of *Caldicellulosiruptor* have been isolated and characterized from the major geothermal formations within Yellowstone National Park (YNP), WY. Recent enrichment and isolation attempts revealed a high abundance of cellulolytic organisms from hot springs within the Mud Volcano region of YNP. Using a high-throughput isolation method based on flow cytometry, a total of 53 isolates of *Caldicellulosiruptor*, based on small subunit rRNA analysis, were isolated from Obsidian Pool. Secondary screening of these strains provided an organism with rapid growth kinetics on pretreated biomass substrates as well as crystalline cellulose and xylan at 80°C. Based on small subunit rRNA analysis, genomic sequence comparisons, and phenotypic properties, we propose this organism as *Caldicellulosiruptor obsidiansis* OB47^T sp. nov., which is named in reference to the location from which it was isolated, Obsidian Pool, YNP.

MATERIALS AND METHODS

Microrganisms. The following organisms used for comparison studies were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ): *Caldicellulosiruptor owensensis* DSMZ 13100, *Caldicellulosiruptor saccharolyticus* DSMZ 8903, and *Anaerocellum thermophilum* DSMZ 6725. These organisms were maintained on the media described below.

Environmental sampling procedure. Sediment and water samples were collected from Obsidian Pool (44°36.603′N and 110°26.331′W), which is located in the Mud Volcano area of YNP, WY. The samples were immediately placed in sterile medium bottles and tightly sealed with butyl rubber stoppers. Each sample was reduced using a few milligrams of sodium dithionite and 1 ml of a 2.5% (wt/vol) cysteine-HCl solution (pH 7.2). The sample yielding OB47^T was col-

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lected from the peripheral area of Obsidian Pool, and the *in situ* temperature was 66°C and the pH was 5.0 at the time of sampling. The samples were kept at room temperature during transportation to Oak Ridge National Laboratory (Oak Ridge, TN) and then stored at 4°C.

Enrichment and isolation. The enrichment culture medium for fermentative cellulolytic anaerobes (FCA) consisted of 17 mM NaCl, 2 mM $MgCl_2 \cdot 6H_2O$, 1.5 mM KH_2PO_4, 3.4 mM KCl, 9.4 mM NH_4Cl, 0.7 mM Na_2SO_4, 1× ATCC trace element solution (ATCC, Manassas, VA), 0.5 μg/ml resazurin, 10 mM $NaHCO_3$ (pH 7.3), 0.7 mM $CaCl_2 \cdot 2H_2O$, 1× ATCC anaerobic vitamins, 0.01% (wt/vol) yeast extract (Fisher Scientific, Pittsburgh, PA), 1.0 g/liter carbon source (i.e., Avicel, dilute sulfuric acid-pretreated switchgrass or *Populus*) (22), 1.4 mM cysteine-HCl · H_2O, and 1 mM $Na_2S \cdot 9H_2O$. Medium was prepared anaerobically by a modified Hungate technique (13) and sterilized by autoclaving. Enrichment cultures were established by inoculating 125-ml serum bottles containing 50 ml of FCA medium with 2.5 ml of environmental sample (5% [vol/vol]) and incubating them at 75°C without shaking. Enrichments were monitored for growth by observing turbidity, measuring changes in pH, and end product analyses. Cell densities were determined by using a Thoma cell counting chamber (Blaubrand, Wertheim, Germany) with an Axioskop2 Plus microscope (Zeiss, Thornwood, NY) with phase-contrast illumination. Enrichment cultures displaying positive growth after 3 to 5 consecutive transfers were selected for isolation attempts using flow cytometry. Briefly, 0.5-ml aliquots of FCA medium were dispensed into 48-well plates containing pretreated switchgrass in a Don Whitley MACS 500 anaerobic workstation (Microbiology International, NJ). A fresh enrichment culture was vigorously shaken by hand and then diluted 1:100 in anaerobic, ice-cold phosphate-buffered saline (PBS) solution. Cells were sorted via forward/side scatter into 48-well plates, at one cell per well, using an InFlux flow cytometer (Cytopeia, Seattle, WA), and then the plates were immediately returned to the anaerobic workstation. The plates were incubated in gastight stainless steel cylinders at 75°C filled with a 0.2-bar overpressure of 80% N_2–20% CO_2. After 5 days of incubation, individual wells were screened for growth by adding bromothymol blue (final concentration, 150 μg/ml) to indicate a drop in pH followed by microscopy. Wells displaying growth were marked as putative isolates and transferred to FCA medium in Balch tubes (10 ml/tube) containing 0.5% (wt/vol) pretreated switchgrass. Culture 47 displayed rapid growth on switchgrass and was resorted for isolation and verified to be pure by streaking on medium solidified with 0.8% (wt/vol) Gelrite and by 16S small-subunit rRNA gene sequence analysis.

Growth experiments and controlled cultivation. The basal growth medium for characterizing isolate OB47^T consisted of 4.5 mM KCl, 4.7 mM NH_4Cl, 2.5 mM $MgSO_4$, 1.0 mM NaCl, 0.7 mM $CaCl_2 \cdot 2H_2O$, 0.25 mg/ml resazurin, 2.8 mM cysteine-HCl · H_2O, 2.1 mM Na_2S, 6.0 mM $NaHCO_3$, 1 mM phosphate buffer, 10 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), pH 6.8, 1× ATCC trace minerals, 1× ATCC vitamin supplement, 0.02% (wt/vol) yeast extract (Fisher Scientific, Pittsburgh, PA), and 0.4% (wt/vol) cellobiose. Small-scale batch cultures were grown in 125-ml serum bottles containing 50 ml of media. Unless stated otherwise, all serum bottle growth experiments were performed in quadruplicate and cell growth was monitored microscopically using a Thoma counting chamber. A 2% (vol/vol) inoculum was passed at least 3 times on the same carbon source before determining growth on different substrates after 16 h of incubation. Medium containing cellobiose at a concentration of 0.4% (wt/vol) was used to determine optimal pH and temperature. Cultures were incubated at 55, 60, 65, 70, 75, 80, and 85°C with shaking at 90 rpm to determine optimal growth temperature. Four buffers at a concentration of 10 mM were used separately to study the effects of pH on growth at optimal incubation temperature: acetate for pH 5 to 6, MOPS for pH 6.7, and *N*-Tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS) for pH 7 to 8. The chemicals were purchased from Sigma-Aldrich.

Controlled-growth experiments were performed using a temperature- and pH-controlled custom fermentor which consists of a 10-liter round-bottom flask supported in an electric heating mantle (Southeastern Lab Apparatus, Inc., North Augusta, SC). A 5-liter working volume was used for Avicel, and 6 liters was used for pretreated switchgrass fermentation studies. The basal growth medium was as described above, except resazurin and MOPS buffer were eliminated. Carbon and energy sources consisted of 1.5% (wt/vol) Avicel-101 (Fluka, St. Louis, MO) with 0.25% (wt/vol) yeast extract and 1.0% (wt/vol) pretreated switchgrass with 0.5% (wt/vol) yeast extract. pH was maintained between 6.5 and 7.0 by addition of 5% (wt/vol) sodium bicarbonate using a pH control unit (Cole Parmer). N_2-CO_2 (80:20) gas was streamed through the vessel at a rate of 25 ml/min and exhausted over a condenser coil maintained at 4°C using a refrigerated recirculating water bath. Fermentations were performed in duplicate.

Electron and atomic force microscopy. Cells were grown on a 0.4% (wt/vol) cellobiose with 0.05% (wt/vol) yeast extract. Cells for scanning electron microscopy were prepared by fixing with 2% (vol/vol) glutaraldehyde and 1% (wt/vol) osmium tetraoxide (OsO_4) as previously described (7). Cells for atomic force microscopy were prepared by being immobilized on gelatin-coated mica as described previously (5).

Analytical methods. For protein analysis, a modified Bradford assay was used to determine total protein in the presence of the insoluble substrates Avicel and pretreated switchgrass (2, 30, 31). To each protein sample, 200 μl of H_2O and 2 μl of 1% sodium dodecyl sulfate (SDS) were added and mixed well. The samples were then sonicated with a Branson sonifier 150 (Branson, Danbury, CT) for 1 min at room temperature and centrifuged at 17,000 × *g* for 20 min, and 100 μl was removed for total protein determinations using the Bradford assay (2).

Reduction of total mass for Avicel and switchgrass was determined gravimetrically by drying samples at 85°C for 16 h and then weighing them on an analytical balance to determine total mass of insoluble material remaining.

Volatile fatty acids were tested by filtering (0.2-μm pore size) sample supernatant and were protonated with 1 M phosphoric acid. The samples were then diluted with deionized water before injection into an Agilent 6890N gas chromatograph equipped with a flame ionization detector (FID) and a DB-FFAP (J & W Scientific) capillary column (30-m by 0.53-mm internal diameter by 1.5-μm film thickness) with constant helium flow at 7.0 ml/min. Chromatogram peak identification of the compounds was performed by comparison of elution times with known standards, and identifications were verified by mass ion detection with an Agilent 5975 mass selective detector. Quantification of each compound was calculated against individual linear standard curves. Recovery was determined by adding 10 μl of 0.1 M *n*-butyric acid to each sample as an internal standard.

Lactate production was analyzed by filtering supernatant, and lactate was protonated with 200 mM sulfuric acid (final concentration of 5 mM) before injection into a Hitachi Lachrom Elite high-performance liquid chromatography (HPLC) system (Hitachi High Technologies). Metabolites were separated on an Aminex HPX-87H column (Bio-Rad Laboratories) under isocratic temperature (40°C) and flow (0.5 ml/min) conditions then passed through a refractive index (RI) detector (Hitachi L-2490). Identification was performed by comparison of retention times with known standards. Quantization of the metabolites was calculated against linear standard curves. All standards were prepared in uninoculated culture media to account for interference of salts in the RI detector.

Genomic DNA isolation and 16S rRNA gene sequence analysis. For DNA isolation, the cells of strain OB47^T were grown in 50 ml FCA medium with 0.1% Avicel, washed twice with PBS (pH 7.2), and resuspended in 0.5 ml Tris-EDTA (TE). The cells were treated with lysozyme (1 mg/ml) at 37°C for 2 h, followed by SDS (1% final) treatment at 37°C for 1 h. After RNase A and proteinase K treatment, DNA was precipitated using cetyltrimethylammonium bromide (CTAB) and extracted with chloroform-isoamyl alcohol (24:1). Finally, DNA was precipitated with isopropanol, washed with 70% ethanol, air dried, and dissolved in the nuclease-free water at a concentration of 0.7 mg/ml.

The genomic DNA was amplified using GoTaq Flexi DNA polymerase (Promega, Madison, WI) and *Bacteria*-specific primers 8F and 1492R targeted to *Escherichia coli* 16S rRNA positions 8 to 27 (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1510 to 1492 (5′-GGTTACCTTTTACGACTT-3′), respectively. The thermal PCR profile was as follows: initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, primer annealing at 55°C for 1 min, and elongation at 72°C for 1.5 min. The final elongation step was 7 min at 72°C. PCR product was purified from UltraPure agarose (Invitrogen, Carlsbad, CA) using QIAquick gel extraction kit (Qiagen, Inc., Valencia, CA) and sequenced using the BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) and primers 8F and 1492R (see above) and additional internal primers 515F [5′-GTGCCAGC(A/C)GCCGCGGTAA-3′], 519R [5′-G(A/T)ATTACCGCGGC(G/T)GCTG-3′], 1100F [5′-GGCAACGAGCG(A/C)GACCC-3′], and 1100R (5′-AGGGTTGCGCTCGTTG-3′) to obtain overlapping DNA fragments (11). Sequences were determined by resolving the sequence reactions on an Applied Biosystems 3730 automated sequencer.

For 16S rRNA gene sequence-based identification, the sequence was initially aligned with highly similar sequences in the BLASTN program (1). The 16S rRNA gene sequences from closely related bacteria were retrieved from NCBI GenBank. Multiple sequence alignment was done using the program ClustalX (8). Phylogenetic 16S rRNA gene analyses were performed by the neighbor-joining method (21). Bootstrap values were based on 1,000 replicates generated using the program Mega 4 (25). For determination of the total G+C content, total genomic DNA was sequenced via pyrosequencing on a 454 genome sequencer (454 Life Sciences, Branford, CT).

The average nucleotide identity (ANI) between the whole-genome sequences of *C. obsidiansis* OB47, *C. owensensis* DSMZ 13100, *C. saccharolyticus* DSMZ 8903, and *A. thermophilum* DSMZ 6725 was determined by using the *in silico*

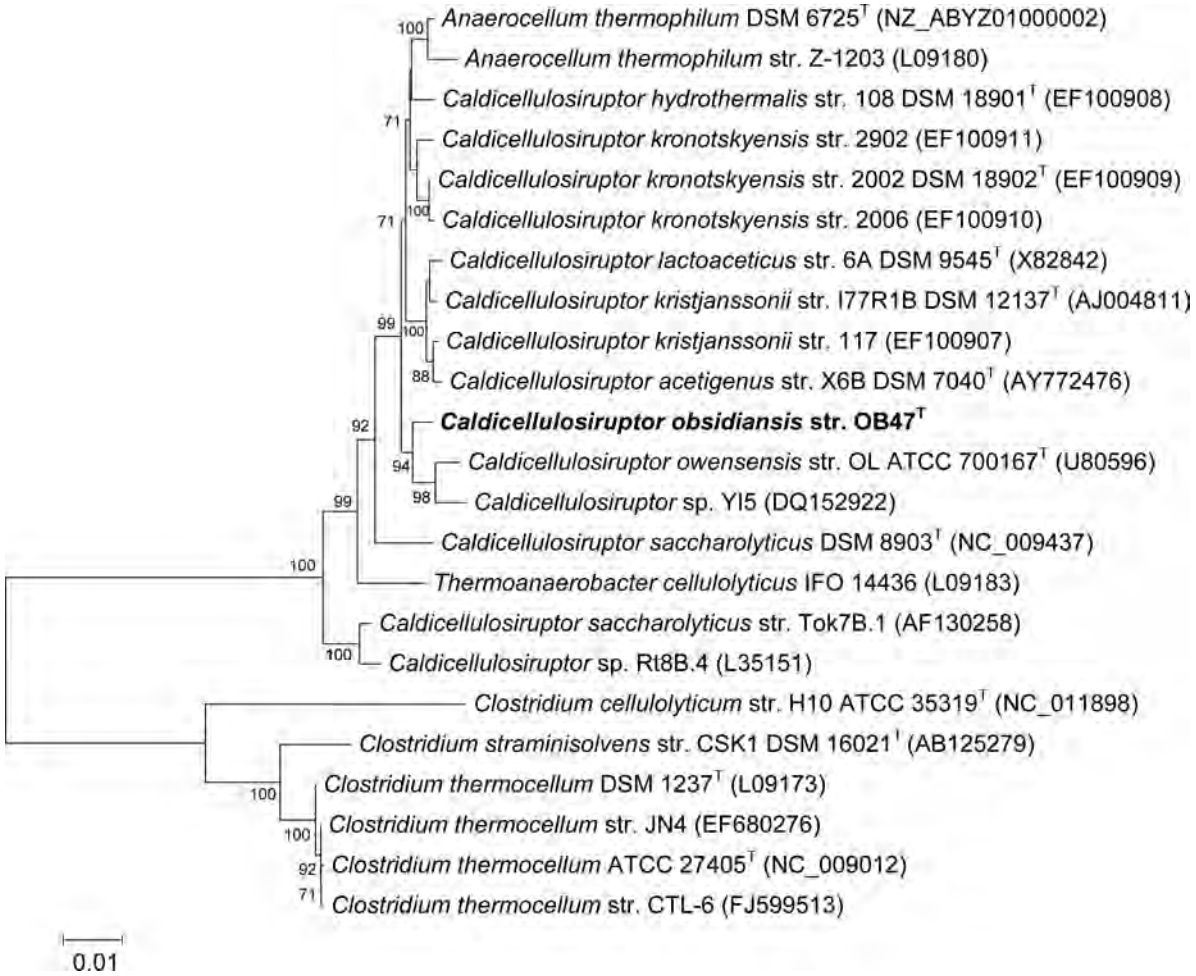

FIG. 1. Neighbor-joining tree based on 16S rRNA gene sequence comparisons of strain OB47^T and selected bacteria. Bootstrap values were based on 1,000 replicates. The scale bar represents 0.01 change per nucleotide position. T, type strain.

DNA-DNA hybridization method of the Jspecies software (http://www.imedea.uib-csic.es/jspecies/about.html) (6, 20). The following ANIb BLAST options were used: −x, 150; −q, −1; −F, F; −e, 1e−15; and −a, 2. The following ANI calculation settings were used: identity, ≥30%; alignment, ≥70%; and length, 1,020 nucleotides. The complete genomes for *C. saccharolyticus* DSMZ 8903 and *A. thermophilum* DSMZ 6725 and the high-quality draft genome (in 38 contigs) for *C. obsidiansis* OB47 are available through the Integrated Microbial Genomes (IMG) website (http://img.jgi.doe.gov). The complete genome for *C. owensensis* DSMZ 13100 was provided by R. M. Kelly (North Carolina State University).

Nucleotide sequence accession numbers. Sequence for 16S rRNA gene of the strain OB47^T has been deposited in the NCBI database under accession no. GQ338764. Accession numbers for sequences used as references are indicated in the phylogenetic tree.

RESULTS

Enrichment and isolation. Isolate OB47^T originated from a 100-ml sample consisting of water, sediment, and grass taken from collection site OB1 (66°C, pH 5) located at the edge of Obsidian Pool, YNP. FCA medium with 1% (wt/vol) pretreated switchgrass was inoculated with the OB1 sample, and after four consecutive transfers in the same medium, a mixed-cell density of approximately 1.0×10^8 cell/ml was obtained. Individual cells were separated by a flow cytometer into six 48-well plates with 0.5 ml of FCA medium containing 0.5% (wt/vol) pretreated switchgrass per well and incubated for 5 days in an anaerobic cylinder placed at 75°C. Several wells showed changes in the pH indicator from blue (pH 7) to yellow (pH 4 to 5), indicating that the pH had dropped due to acid production during growth. A total of 48 wells (17% efficiency) were considered to be positive. The positive wells were then transferred to Balch test tubes with the same FCA switchgrass medium and incubated at 75°C. Culture tube 47 contained an organism that rapidly grew on cellulosic biomass and was designated OB47.

Phylogenetic characterization. Phylogenetic analysis of the 16S rRNA gene sequence indicated that strain OB47^T is a member of the genus *Caldicellulosiruptor*, with identity to other strains within that group ranging from 92.7to 97.7% (Fig. 1), the closest relatives being *C. owensensis* (97.7%). The members of the genus *Caldicellulosiruptor* formed one cluster with a bootstrap value of 100% with *Thermoanaerobacter cellulolyticus* IFO 14436 and 2 strains of *Anaerocellum thermophilum* DSM 6725 and Z-1203. Further analysis showed that strain OB47^T clustered with *C. owensensis* and *Caldicellulosiruptor* sp. strain Y15, with an identity level of 97.2% to both strains. The identity levels of the 16S rRNA gene sequence of strain OB47^T to

TABLE 1. Physiological characteristic of OB47^T and three closely related cellulolytic organisms

Characteristic	Result for organism:			
	OB47^T	*C. owensensis* DSMZ 13100	*C. saccharolyticus* DSMZ 8903	*A. thermophilum* DSMZ 6725
General				
Temp range in °C (optimal)	55–85 (78)[a]	50–80 (75)[b]	45–80 (70)[b]	40–83 (72–75)[b]
pH range (optimal)	5.0–8.0 (7.0)[a]	5.5–9.0 (7.5)[b]	5.5–8.0 (7.0)[b]	5.0–8.3 (7.1–7.3)[b]
Gram stain	−[a]	−[b]	−[b]	−[b]
DNA G+C content (%)	35.2[a]	36.6[b]	37.5[b]	36.7[b]
ANI with genomic DNA of OB47^T (%)	100	92.31	80.33	88.09
Doubling time under optimal conditions (h)	1.6[a,c]	7.3[b]	1.6[a,c]	2.5[a,c]
Motility	No[a]	Yes[b]	No[b]	No[b]
Ethanol production	Yes[a]	Trace[b]	Trace[b]	No[b]
NaCl tolerance (%)	0.5[a]	ND[d]	ND	ND
Ethanol tolerance (%)	1.0[a]	ND	ND	ND
Carbon utilization (carbon source)[a]				
Acetate	−	−	−	−
Arabinogalactan	+	−	+	+
Avicel	+	−	+	+
Arabinose	+	+	+	+
Carboxymethylcellulose	−	−	−	−
Casein	−	−	−	−
Cellobiose	+	+	+	+
Filter paper	+	−	+	+
Fructose	+	+	+	+
Fucose	−	−	+	−
Galactose	+	+	+	+
Gelrite	−	−	−	−
Glucose	+	+	+	+
Glycerol	−	−	−	−
Lactose	+	+	+	+
Lignin	−	−	−	−
Maltose	+	+	+	+
Mannitol	−	−	−	−
Mannose	+	+	+	+
Pectin	+	+	+	+
Pretreated *Populus*	+	−	−	+
Pretreated switchgrass	+	−	+	+
Processed cardboard	+	−	−	+
Ribose	−	+	−	−
Sorbitol	−	−	−	−
Starch	+	+	+	+
Sucrose	+	+	+	+
Trehalose	+	−	+	+
Xylan	+	+	+	+
Xylose	+	+	+	+
Yeast extract	+	+	+	+

[a] Results from this study.
[b] Information acquired from the literature (9, 19, 24, 28).
[c] Medium conditions as described in this study. Optimal doubling times were determined in 125-ml serum bottles with 0.1% (wt/vol) Avicel.
[d] ND, not determined.

A. thermophilum DSM 6725, *A. thermophilum* Z-1203, and *Thermoanaerobacter cellulolyticus* IFO 14436 were 97.3%, 96.5%, and 95.0%, respectively. The GC content of the genomic DNA was 35% per mol, as determined by pyrosequencing, and the GC content of the 16S rRNA gene was 58.9% per mol.

In order to unequivocally determine the species status of the new isolate, a pairwise genomic comparison by the *in silico* DNA-DNA hybridization method of the Jspecies software was performed (Table 1). The ANI percentage of 94% corresponds to the recommended cutoff point of 70% DNA-DNA hybridization for species delineation (6, 20). The ANI values of strain OB47 to all strains studied were below the threshold for species identity (80.33 to 92.31%), suggesting that strain OB47^T is a new species.

Physiological characterization of OB47^T. Cultivation studies were conducted in batch with 125-ml serum bottles containing 50 ml of anaerobic medium with shaking at 90 rpm. Temperature and pH optima were determined with 0.4% (wt/vol) cellobiose and 0.02% (wt/vol) yeast extract. Specific growth rates calculated from the log phase showed that the fastest growth rate of 0.75 h^{-1} was obtained at 78°C (Fig. 2A) and between the pH ranges of 6.7 and 7.0 (Fig. 2B). Substrate utilization was determined by transferring OB47^T, *Caldicellulosiruptor owensensis*, *C. saccharolyticus*, and *A. thermophilum* three times on the substrates listed in Table 1. Strain OB47^T

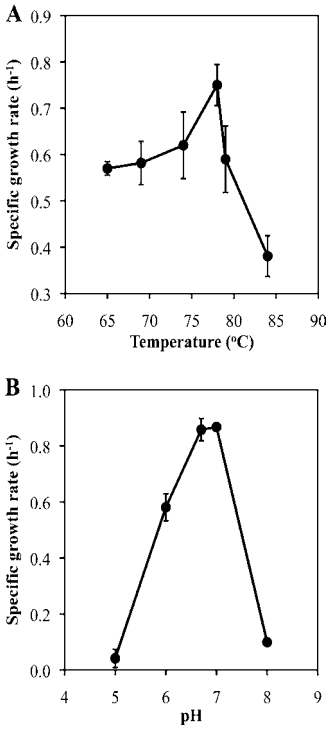

FIG. 2. Specific growth rates for OB47^T at various temperatures (A) and various pHs (B). Each data point represents the mean ± standard deviation calculated from 4 replicate cultures.

displayed growth on cellobiose, glucose, maltose, mannose, arabinose, fructose, starch, lactose, sucrose, galactose, xylose, arabinogalactan, Avicel, xylan, filter paper, processed cardboard, pectin, dilute acid-pretreated switchgrass, and *Populus*. OB47^T was unable to grow on mannitol, fucose, lignin, Gelrite, acetate, glycerol, ribose, sorbitol, carboxymethylcellulose, and casein. The addition of external electron acceptors in the form of thiosulfate, sulfur, sulfate, and nitrate did not stimulate growth. End products produced from the fermentation of cellobiose included acetate, lactate, CO_2, H_2, and ethanol.

Cell and colony morphology. Scanning electron microscopy showed rod-shaped cells approximately 2 μm long by 0.2 μm wide (Fig. 3A). Atomic force microscopy did not reveal the presence of flagella (Fig. 3B). Spore formation was not observed regardless of growth condition. When plated on anaerobic Gelrite plates containing 0.5% (wt/vol) cellobiose and 0.5% (wt/vol) yeast extract, the cells formed flat, opal-colored circular colonies with smooth edges.

Fermentation and mass analysis. OB47^T demonstrated rapid initial growth in bench-scale fermentations with a doubling time of 4.5 h when grown on Avicel and 5.5 h when grown on pretreated switchgrass. In both cases, cell densities reached $>1.0 \times 10^9$ cells/ml in 42 h on Avicel and 44 h on switchgrass, respectively. We observed a decrease in Avicel concentration from 15 g/liter to 4 g/liter during the 234 h of hydrolysis, resulting in 73% of the cellulose consumed (Fig. 4). The end products generated from the Avicel fermentation were 38 mM acetate and 36 mM lactate (Fig. 4). Ethanol was also produced in the reactor medium (approximately 2 mM) and was concentrated in the condenser trap to 60 mM. Fermentation of pre-

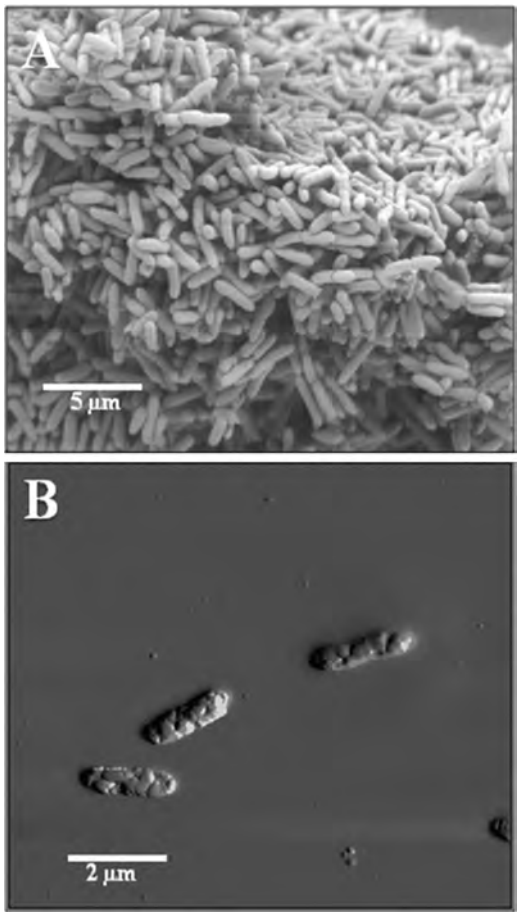

FIG. 3. Microscopy of OB47^T. (A) Scanning electron microscopy at ×5,000; (B) atomic force microscopy deflection signals at 12 μm^2.

treated switchgrass began with 9.7 g/liter, and after 241 h, 4.2 g/liter remained, resulting in 57% of the lignocellulosic biomass consumed (Fig. 5). End products generated during the switchgrass fermentation were 37 mM acetate and 2 mM ethanol, and there was no detectable lactate (Fig. 5). Ethanol

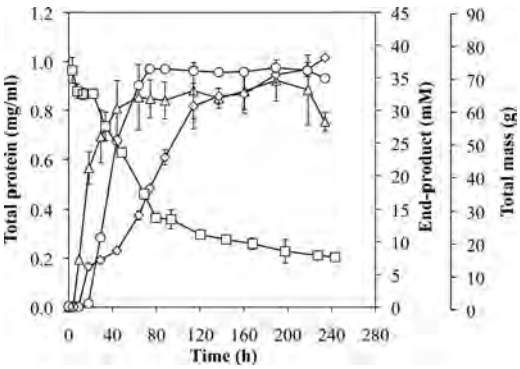

FIG. 4. Growth of OB47^T on 1.5% (wt/vol) crystalline cellulose and end product formation during fermentation. Triangles, total cellular protein accumulation; squares, hydrolysis of crystalline cellulose (Avicel); diamonds, acetate production; and circles, lactate production. Each data point represents the mean ± standard deviation calculated from triplicate samples collected from 2 independent experiments.

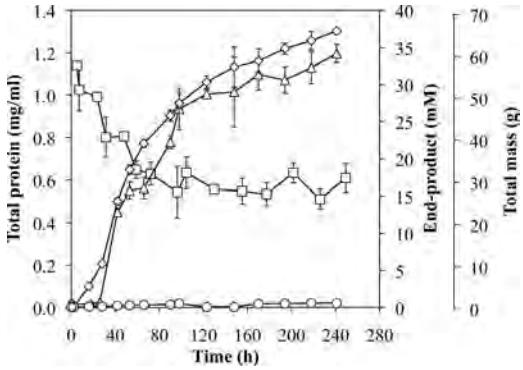

FIG. 5. Growth of OB47^T on 1.0% (wt/vol) dilute acid-pretreated switchgrass and end product formation during the fermentation. Triangles, total cellular protein accumulation; squares, total mass reduction in substrate; diamonds, acetate production; and circles, lactate production. Each data point represents the mean ± standard deviation calculated from triplicate samples collected from 2 independent experiments.

evaporation reached up to 48% and 13% (wt/wt) from the medium into the condenser trap for the Avicel and the pretreated switchgrass fermentations, respectively. Carbon balance analysis for both crystalline cellulose and lignocellulosic biomass fermentations can account for up to 60.2% and 74.0% carbon mass utilized, including predicted concentrations of CO_2 per mol of acetate and ethanol (data not shown).

DISCUSSION

Thermophilic, cellulolytic organisms belonging to the genus *Caldicellulosiruptor* have not been previously described from thermally active sites within YNP. In this study, we provide a description of an extremely thermophilic bacterium from YNP with rapid growth kinetics on a wide range of carbohydrates and polysaccharides. Strain OB47^T was isolated from samples collected at the peripheral edge of Obsidian Pool, where heat-tolerant grasses are present that likely provide a source of lignocellulosic biomass. Detritus in the form of leaf litter and animal dung would also be a source of cellulosic material for growth. Validated strains of the genus *Caldicellulosiruptor* possess a wide range of sugar utilization capacities, and strain OB47^T is able to ferment all of the basic monomeric sugars that compose plant material, including glucose, arabinose, fructose, galactose, mannose, and xylose. Strain OB47^T is near the upper temperature limit of extremely thermophilic, cellulose-hydrolyzing organisms, with optimal growth at 78°C and sustained growth above 80°C (maximum growth temperature, 85°C). Strains *C. obsidiansis* OB47^T and *C. owensensis* are phylogenetically related at the 16S rRNA gene sequence level (97.2%) and share several physiological characteristics; however, distinct differences were observed, including lack of flagella for OB47^T and a greater ability to hydrolyze cellulosic substrates such as Avicel. *C. owensensis* is noted to be a xylanolytic organism and in our studies appeared to prefer this substrate rather than C_6 polymers, whereas OB47^T, *Anaerocellum thermophilum*, and *Caldicellulosiruptor saccharolyticus* readily grew to cell densities greater than 1.0×10^8 cells/ml when provided with Avicel and pretreated biomass. On the bases of *in silico* DNA-DNA hybridization, OB47^T was most closely related to *C. owensensis*; however, the ANI between these two organisms was below the recommended threshold value of 94%, and therefore, the organism represents a new species within the genus *Caldicellulosiruptor*. Substrate utilizations of *A. thermophilum* and OB47^T were identical, with the same doubling time of 2.5 h on pretreated switchgrass; however, the doubling time of OB47^T appeared to be 1.5 times more rapid (1.6 h versus 2.5 h) on Avicel when compared in the same medium at optimal growth temperatures for each strain. In contrast to recent reports describing the breakdown of non-pretreated biomass by *A. thermophilum* (28), OB47^T produced measurable quantities of ethanol from switchgrass and Avicel.

Temperature- and pH-controlled experiments in larger volumes (5 liters) displayed different growth kinetics and end product formation, depending on the type of substrate. The doubling time for OB47^T when grown on Avicel was 4.5 h, yet it was 5.5 h when grown on pretreated switchgrass, which is to be expected due to the more complex composition of the natural substrate and the presence of lignin remaining after pretreatment. Acetate and lactate were the major end products when OB47^T was grown on Avicel, yet when OB47^T was grown on pretreated switchgrass, no lactate production was observed. Presumably, excess carbon was available during growth on Avicel for flux through lactate dehydrogenase (LDH); however, further studies are needed to investigate the carbon flux distribution through LDH and pyruvate ferredoxin oxidoreductase. Ethanol was produced when OB47^T was metabolizing both Avicel and switchgrass, suggesting that excess reducing equivalents were available during growth on both substrates. Due to the elevated incubation temperature, ethanol was recovered by flowing the fermentor headspace gasses over a chilled coil held at 4°C using a recirculating, refrigerated water bath. The boiling temperature of pure ethanol is 78.4°C, although under fermentation conditions, ethanol forms an azeotrope with the aqueous medium. OB47^T exhibited an optimal growth temperature near 80°C, which allowed for partial distillation to occur. The inhibitory effects of ethanol on cell growth may be greatly reduced if the solvent is present primarily in the vapor phase, which would be beneficial if higher production rates could be achieved.

Although the quantities of ethanol produced by *C. obsidiansis* OB47^T are low, recent progress has been made in genetically modifying thermophilic anaerobes to produce knockout mutants capable of homofermentative ethanol production (23). A similar strategy applied to members of the *Caldicellulosiruptor* group such as OB47^T could theoretically yield organisms that could hydrolyze and ferment lignocellulosic biomass (both C_5 and C_6 sugars) directly to ethanol in a consolidated process at temperatures above the boiling point of the product. Organisms that can efficiently hydrolyze recalcitrant biomass, ferment both hexose and pentose sugars, and produce ethanol are of great interest to developing a cost-effective process for cellulosic fuels (27).

Description of *Caldicellulosiruptor obsidiansis*. *Caldicellulosiruptor obsidiansis* (ob.si.dian′sis. N.L. adj. *obsidiansis*, from Obsidian Pool, Yellowstone National Park, WY). Cells are short linear rods approximately 2 μm long by 0.2 μm wide and typically occur singly, in pairs, or can be found as small chains of 6 cells. The cell wall is the Gram-positive type. Cells are nonmotile, and endospore formation was not observed. The optimal growth temper-

ature is 78°C, with a maximum growth temperature of 85°C and a minimum growth temperature near 55°C. The optimal pH is 7.0, with a range of 6.7 to 7.0. Growth on solid media produces round, opalescent colonies with smooth edges. OB47^T is an obligate anaerobe and chemoorganoheterotroph utilizing cellobiose, glucose, maltose, arabinose, fructose, starch, lactose, sucrose, galactose, mannose, xylose, arabinogalactan, Avicel, xylan, Whatman filter paper, processed cardboard, pectin, acid-pretreated switchgrass, acid-pretreated *Populus*, trehalose, and yeast extract. OB47^T was unable to grow on mannitol, fucose, lignin, Gelrite, acetate, glycerol, ribose, sorbitol, carboxymethylcellulose, and casein. Electron acceptors such as sulfur, nitrate, thiosulfate, and sulfate did not stimulate growth. End products are acetate, lactate, CO_2, H_2, and ethanol. G+C content is 35.2% (as determined by pyrosequencing). Type strain OB47^T (ATCC BAA-2073) was isolated from Obsidian Pool, Yellowstone National Park, WY.

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