Caldicellulosiruptor owensensis sp. nov., an anaerobic, extremely thermophilic, xylanolytic bacterium

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An anaerobic, extremely thermophilic, xylanolytic, non-spore-forming bacterium was isolated from a sediment sample taken from Owens Lake, California, and designated strain OL^T (T = type strain). Strain OL^T had a Gram-negative reaction and occurred as short rods which sometimes formed long chains containing a few coccoid cells. It grew at 50–80 °C, with an optimum at 75 °C. The pH range for growth was 5·5–9·0 with an optimum at about pH 7·5. When grown on glucose at optimal conditions, its doubling time was 7·3 h. In addition to glucose, the isolate utilized sucrose, xylose, fructose, ribose, xylan, starch, pectin and cellulose. Yeast extract stimulated growth on carbohydrates but was not obligately required. The end products from glucose fermentation were lactate, acetate, ethanol, H$_2$ and CO$_2$. The G+C content of strain OL^T was 36·6 mol%. The 16S rDNA sequence analysis indicated that strain OL^T was a member of the subdivision containing Gram-positive bacteria with DNA G+C content of less than 55 mol% and clustered with members of the genus *Caldicellulosiruptor*. Because strain OL^T is phylogenetically and phenotypically different from other members of this genus, it is proposed to designate this isolate *Caldicellulosiruptor owensensis* sp. nov. Strain OL^T is the type strain (= ATCC 700167^T).

INTRODUCTION

Thermophilic and extremely thermophilic micro-organisms have gained a great deal of attention recently (3). Enzymes from these micro-organisms are of special interest since they are not usually denatured by high temperatures and are even active at elevated temperatures (3, 35). Furthermore, members of the thermoanaerobic saccharolytic group may be commercially useful for producing chemicals and fuels such as ethanol and lactate from plant biomass (15).

Xylan is a major component of plant hemicellulose; after cellulose, it is the next most abundant renewable polysaccharide in nature. Several species of xylanolytic, thermophilic anaerobes have been isolated and identified as members the genera *Clostridium*, *Thermoanaerobacter*, *Thermoanaerobacterium* and *Caldicellulosiruptor* (4, 16, 33). 16S rRNA sequence analysis of these four genera showed individual coherent clusters

in the subdivision containing Gram-positive bacteria with a DNA G+C content of less than 55 mol% (26). The newly described genus, *Caldicellulosiruptor*, is assigned to the phylogenetic cluster 'D' and is the least studied. The seven strains in cluster D were isolated exclusively from hot springs and all degrade cellulose. Of these, only *Caldicellulosiruptor saccharolyticus* (formerly designated *Caldocellum saccharolyticus*) was taxonomically validated (25).

We describe in this paper, a new extremely thermophilic, xylanolytic anaerobic bacterium isolated from sediments of Owens Lake, California, USA. Phenotypic and phylogenetic characteristics indicate that it is a new species of the genus *Caldicellulosiruptor*. We designated it *Caldicellulosiruptor owensensis* sp. nov.

METHODS

Sampling procedure. Sediment samples were collected from a small freshwater pond located within the dry Owens Lake bed in California. The samples were kept under anaerobic conditions in serum bottles, transported to the laboratory at UCLA at ambient temperatures, and stored at 4 °C until
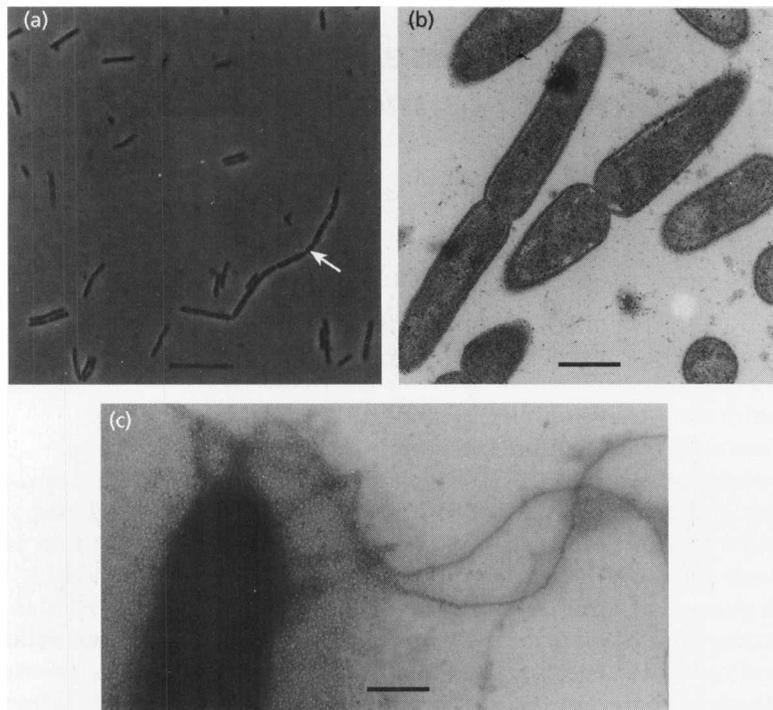
Fig. 1. (a) Phase-contrast photomicrograph of cells from exponential growth phase of strain OL^T. Cocci denoted by arrow. Bar, 10 µm. (b) Transmission electron micrograph of a thin section of whole cells of strain OL^T. Bar, 1 µm. (c) Transmission electron micrograph of negatively stained strain OL^T showing the presence of lophotrichous flagella. Bar, 1 µm.

used. The temperature and pH of the sample site was 32 °C and 9·0, respectively.

Enrichment and isolation. An enrichment culture medium modified from Angelidaki et al. (2) designated CBM was used. CBM consisted of (per litre distilled water) yeast extract (Difco), 1 g; xylan, 5 g; NH$_4$Cl, 1 g; NaCl, 0·1 g; MgCl$_2$.6H$_2$O, 0·1 g; CaCl$_2$.2H$_2$O, 0·05 g; K$_2$HPO$_4$, 0·4 g; resazurin, 0·0005 g; 1 ml vitamin mixture, and 10 ml trace mineral solution (10). The vitamin solution contained (mg l^{-1}): biotin, 2; folic acid, 2; pyridoxine hydrochloride, 10; thiamine hydrochloride, 5; riboflavin, 5; nicotinic acid, 5; DL-calcium pantothenate, 5; vitamin B$_{12}$, 0·1; p-amino-benzoic acid, 5; and lipoic acid, 5. The trace mineral solution contained (per litre): H$_2$SeO$_3$, 0·01 g; MnCl$_2$.4H$_2$O, 0·1 g; FeSO$_4$.7H$_2$O, 0·1 g; CoCl$_2$.6H$_2$O, 0·15 g; ZnCl$_2$, 0·1 g; H$_3$BO$_3$, 0·01 g; Na$_2$MoO$_4$.2H$_2$O, 0·01 g; CuCl$_2$.2H$_2$O, 0·02 g; NiSO$_4$.6H$_2$O, 0·02 g; AlCl$_3$.6H$_2$O, 0·04 g; NaWO$_4$, 0·03 g; and disodium EDTA dihydrate, 0·5 g. CBM was boiled and 3·0 g NaHCO$_3$ was added after cooling under a stream of oxygen-free N$_2$-CO$_2$ (70:30, % v/v) gas. The medium was dispensed in 10 ml aliquots into culture tubes under a stream of N$_2$-CO$_2$ (70%:30%), stoppered with rubber septa, sealed with aluminium cap seals (Bellco Glass) and autoclaved at 140 °C for 20 min. Prior to use, Na$_2$S.9H$_2$O was injected from a sterile stock solution of 25 g l^{-1} into each tube to give a final concentration of 2·5 mg l^{-1}.

For initiating enrichments, 0·5 ml of sample sediment slurry was added to the CBM medium. Tubes were incubated at 75 °C for up to 1 week without shaking until xylan solubilization and an increase in microbial numbers was observed. Such enrichment cultures were subcultured four more times and serially diluted in roll tubes containing CBM fortified with 2% agar (Difco). The roll tubes were incubated at 60 °C for up to 1 week. Single colonies were picked using sterile Pasteur pipettes and inoculated into fresh CBM medium. This process was repeated four times before the

isolate was considered pure as judged by uniform colony morphology and microscopic appearance.

Physiological tests. The modified Hungate anaerobic technique (12, 18) was used throughout these studies. Unless indicated, all tests were performed in triplicate.

For pH and temperature optima studies, CBM with xylose instead of xylan, was used. The effect of pH on growth was determined in xylose medium containing four different buffers at a final concentration of 50 mM: Walpole's Acetate Buffer (31), pH range 3·7–5·1; MES sodium salt, pH range 5·5–6·5; Tris (Trizma base), pH range 6·5–7·5; and Na$_2$CO$_3$, pH range 7·5–9·5. A stream of N$_2$ instead of N$_2$/CO$_2$ (70:30) was used while dispensing medium. The pH values of the medium after sterilization were 3·8–9·5. Cultures were incubated in the optimal temperature.

Gram reaction was determined using a Difco Gram Stain kit according to the manufacturer's recommended protocol (Difco). The effect of sodium chloride (0–3%) on growth was determined in CBM containing xylose.

To determine substrate utilization, other carbohydrates replaced xylan in the CBM-based medium. All carbohydrates were added from sterile anaerobic stock solutions to a final concentration of 0·5%. Growth was positive if the optical density was higher than the control carbohydrate-free CBM tubes. The ability of the isolate to utilize glucose or xylose as the sole carbon source was tested in CBM lacking xylan and yeast extract. The type strain C. saccharo-lyticus (ATCC 43494) used to compare the substrate spectrum was obtained from the American Type Culture Collection. Generation time of the isolate was determined at the optimum pH and temperature in CBM containing glucose (final concentration of 0·5%).

For antibiotic inhibition studies, CBM containing xylose was used. The ability of the isolate to grow aerobically was determined in CBM which lacked xylan, sodium sulfide and

$N_2:CO_2$, but contained glucose and air. Aerobic cultures were incubated in Triple Baffled Nephelo culture flasks (Bellco Glass) at 75 °C and 200 r.p.m. in an Environ-shaker (Lab-Line Instruments). The reduction of nitrate (10 mM), sulfate (10 mM), sulfite (5 mM), and thiosulfate (20 mM) by the isolate was determined in xylan-free glucose CBM medium under anaerobic conditions.

Spore formation was induced using the methods of Schink & Zeikus (29) and Cook *et al.* (6). Endospore presence was determined from 1-week- and 1-month-old cultures using the staining method described by Schaeffer & Fulton (30).

Analytical methods. Growth was measured either by inserting culture tubes directly into a Perkin–Elmer Junior model 35 spectrophotometer and measuring OD_{560} or by counting cells using a Petroff Hausser counting chamber. Volatile fatty acids were analysed using a Hewlett-Packard 5890A gas chromatograph equipped with a flame ionization detector and a Supelco 10% SP-1000, 1% H_3PO_4 on 100/120 Chromosorb W-AW packed 6 ft × 2 mm i.d. glass column maintained at 150 °C. Alcohols were also analysed by using a gas-liquid chromatograph equipped with a flame ionization detector and a Supelco 5% Carbowax 20M on 60/80 Carbopack B glass column at 120 °C. Lactate was measured enzymically (11). H_2 and CO_2 were measured using a Carle AGC series 100 gas chromatograph equipped with an 80/100 silica gel-packed stainless steel column (Supelco) and a thermal conductivity detector. Sulfide was estimated using the method described by Cord-Ruwisch (5).

Electron microscopy. Cells were fixed with cold 2.5% glutaraldehyde in 0.1 M Na_2HPO_4-KH_2PO_4 (pH 7.2) buffer overnight and post-fixed with 1% osmium tetroxide (OsO_4). They were then dehydrated with ethanol and embedded in Spurr. Approximately 1000 Å (100 nm) thick sections were stained with uranyl acetate and lead citrate and examined under a JEOL JEM-100 CX electron microscope at an accelerating voltage of 80 kV. Negative staining of cells for electron microscopy was achieved with 1% (w/v) uranyl acetate.

DNA base composition. DNA was extracted by the method of Pitcher *et al.* (23). The buoyant density of purified DNA was measured by ultracentrifugation in a CsCl density gradient (24). The G + C content of the DNA was calculated by using the formula of Schildkraut *et al.* (28).

16S rDNA sequence studies. Purification of genomic DNA, amplification and purification of the 16S rRNA gene (16S rDNA) from strain OL^T were performed by previously described techniques (17, 27). The purified PCR product was sequenced directly on an ABI automated DNA sequencer by using a Prism Dyedeoxy Terminator Cycle Sequencing kit and protocols recommended by the manufacturer (Applied Biosystems). Ten sequencing primers were used to obtain the sequence which covered approximately 80% of both strands (17, 27). Using the sequence editor, ae2, the 16S rDNA sequence of strain OL^T was aligned with the 16S rRNA gene sequences of various members of the bacterial phyla obtained from the Ribosomal Database Project (19) and from EMBL. Positions of sequence and alignment uncertainty were omitted from the analysis, and pairwise evolutionary distances for 1280 nucleotides were computed. Phylogenetic analysis was performed using programs which form part of the PHYLIP package and include DNADIST (Jukes & Cantor option), NEIGHBOR-JOINING and DNAPARS (9). Tree topology was re-examined by using 100 boot-strapped data sets for which a script file with the following PHYLIP programs was used: SEQBOOT, DNADIST, FITCH and CONSENSE. Programs in the phylogenetic package MEGA (14) were also used. PHYLIP

programs were run on a Sun SPARC workstation and MEGA was run on a Compaq notebook (Contura model 410CX).

RESULTS

Colony and cell morphology

Enrichment cultures were obtained after 1 week at 75 °C. A population of heterogeneous rods and filaments were observed in the enrichment cultures. In roll tubes, 0.5–2 mm colonies developed after 1 week at 60 °C. Zones of clearance were observed around the colonies indicating that xylan solubilization had occurred. A single colony in the final serial dilution was picked and designated strain OL^T. Cells of strain OL^T were straight rods measuring 0.5–0.8 μm in diameter and 2–5 μm in length, and occurred singly, in pairs, or in chains (Fig. 1a). Small coccoid cells were consistently observed during exponential growth, perhaps because of unequal cell division. Colonies of strain
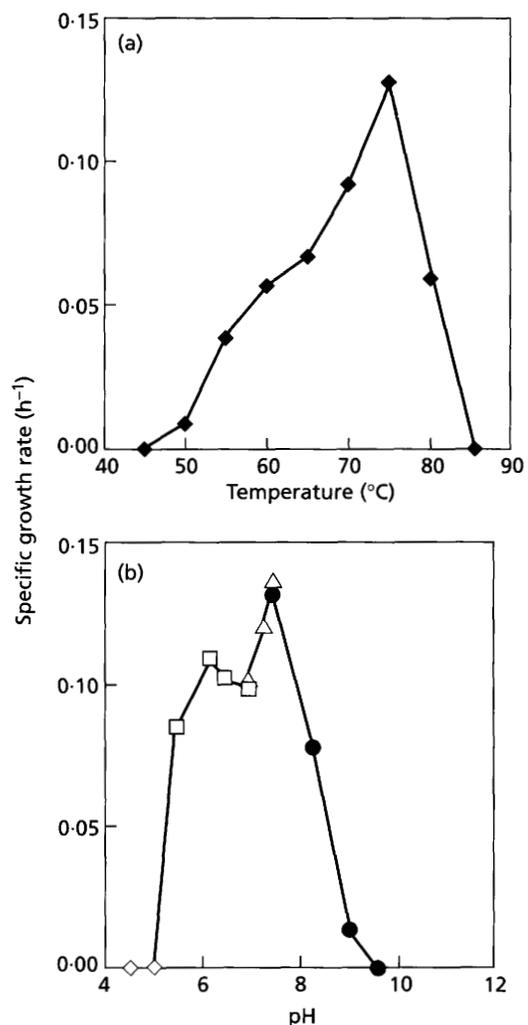

Fig. 2. (a) Effect of temperature on growth of strain OL^T. (b) Effect of pH on growth of strain OL^T. ($\diamond$) Walpole's acetate buffer; ($\blacksquare$) MES buffer; ($\triangle$) Tris buffer; ($\bullet$) sodium carbonate buffer.

Cellulolytic thermophile str. SP83
Cellulolytic thermophile str. COMPB1
Caldicellulosiruptor saccharolyticus str. TP8T.6331
Caldicellulosiruptor owensensis str. OL^T
Cellulolytic thermophile str. R12B1
Cellulolytic thermophile str. Wai35
'*Anaerocellum thermophilum*' str. Z-1320
'*Thermoanaerobacter cellulolyticus*' str. NA10
Cellulolytic thermophile str. Rt8B7
Cellulolytic thermophile str. Rt8B15
Cellulolytic thermophile str. Ok9B1
Dictyoglomus thermophilum str. H-6-12
Thermoanaerobacter thermohydrosulfuricus str. E100-69
Thermoanaerobacter thermocopriae str. JT-3
Thermoanaerobacter thermosulfurigenes str. 4B
Moorella thermoacetica str. JW 701/3
Syntrophomonas wolfei DSM 2245
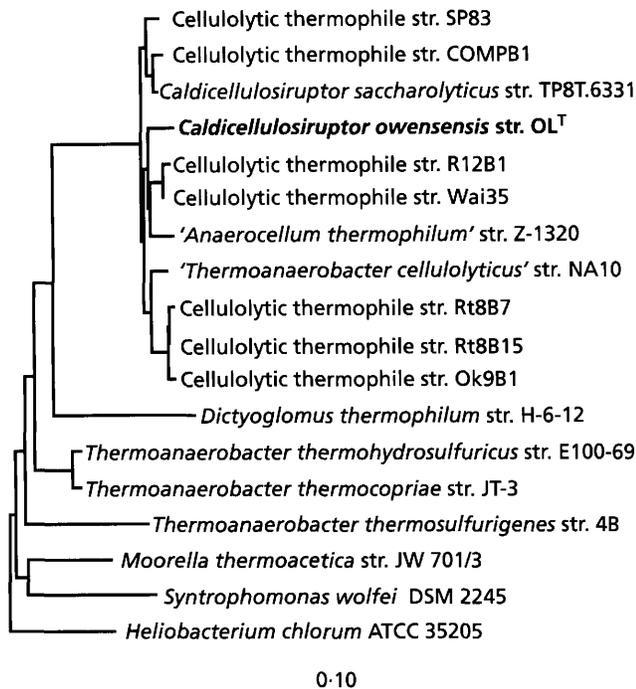*Heliobacterium chlorum* ATCC 35205

0·10

Fig. 3. Phylogenetic dendrogram based on 16S rDNA sequence data indicating the position of strain OL^T within the radiation of members of the genus of *Caldicellulosiruptor* and related taxa. All the sequences used in the analysis, with the exception of the sequence for the cellulolytic strain SP83 (EMBL accession no. X93020) (22), were obtained from the Ribosomal Database Project, version 5.0 (19). Scale bar indicates evolutionary distance.

OL^T in roll tubes were circular with smooth edges, up to 2 mm in diameter, opaque, yellowish and were convex. Strain OL^T was non-motile and had a Gram-negative reaction, but the cell wall was typical of Gram-positive bacteria as seen by electron microscopy (Fig. 1b). Electron microscopic examination of negatively stained young cells showed the presence of lophotrichous flagella (Fig. 1c). Spores were not observed under any conditions tested.

Growth characteristics and physiology

Strain OL^T was a thermophilic, strictly anaerobic, chemoorganotrophic bacterium. The optimal growth temperature of this strain was 75 °C and no growth was observed below 50 °C or above 80 °C (Fig. 2a). Strain OL^T had a broad pH range for growth, 5·5–9·0, with an optimum of 7·5 (Fig. 2b). When grown under optimal conditions in xylan-free CBM-glucose medium, strain OL^T had a doubling time of 7·3 h and lowered the pH by 0·5 units. Presence of either yeast extract or vitamins was not required but stimulated growth.

Strain OL^T could grow on a wide variety of carbon sources including arabinose, cellulose, cellobiose, dextrin, fructose, galactose, glucose, glycogen, inositol, lactose, mannitol, mannose, maltose, pectin, raffinose, rhamnose, ribose, starch, sucrose, tagatose, xylan and xylose but not acetate, arbutin, trypticase peptone, erythritol, glycerol, lactate, melibiose, methanol, pyruvate, sorbitol and trehalose. Autotrophic growth on H_2/CO_2 (80:20, %) was not observed. Yeast extract could also serve as a sole carbon and energy source.

The end products of glucose fermentation by strain

OL^T were acetate, lactate, ethanol, CO_2 and H_2. Nitrate, sulfate, sulfite and elemental sulfur were not reduced.

Strain OL^T was resistant to D-cycloserin (100 µg ml^{-1}), erythromycin (200 µg ml^{-1}), and tetracycline (100 µg ml^{-1}). Growth was inhibited by penicillin G, streptomycin, chloramphenicol, ampicillin at a concentration of 100 µg ml^{-1}. The strain tolerated 0·5 % but was inhibited by 1 % NaCl.

DNA base composition. The genomic DNA base composition of strain OL^T as determined by the buoyant density of purified DNA in a CsCl density gradient was 36·6 mol% G+C.

16S rDNA sequence analysis. Using ten primers, 1539 nucleotides corresponding to *E. coli* positions 8–1542 according to the nomenclature of Winker & Woese (34), were sequenced. Phylogenetic analysis indicated that strain OL^T was a member of the low-G+C-content sub-branch of the Gram-positive group. Further analysis indicated that strain OL^T was specifically related to the 11 members of the *Caldicellulosiruptor* cluster with a mean similarity of 94%. Currently the only taxonomically validated member of the cluster is *C. saccharolyticus*, with which strain OL^T was related at a similarity level of 94%. Fig. 3 is a dendrogram that was generated by the neighbour-joining method from the evolutionary distance matrix and shows this relationship.

DISCUSSION

Most thermophilic microbes were isolated from geothermal environments associated with volcanic activity (8, 13, 35, 36). However, other thermally heated

Table 1. Substrate spectrum of strain OL^T and other *Caldicellulosiruptor* species

Strains OL^T and *C. saccharolyticus* were grown on CBM medium supplied with each substrate tested. Data for *C. lactoaceticus* is from (20). +, Positive; −, negative; ND, not determined.

Substrate	Strain OL^T	C. saccharolyticus	C. lactoaceticus
Acetate	−	−	−
Cellobiose	+	+	+
Cellulose	+	+	+
CM-cellulose	+	+	ND
Sigmacell 100	+	+	ND
Fructose	+	+	−
Galactose	+	+	−
Glucose	+	+	−
Inositol	+	−	ND
Lactose	+	+	+
Mannitol	+	−	−
Pyruvate	−	−	−
Raffinose	+	−	−
Ribose	+	−	−
Sucrose	+	+	−
Trehalose	−	+	−
Xylose	+	+	+

environments including the deep subsurface aquifers, man-made thermal environments and naturally solar heated environments are also reported to harbour thermophilic bacteria (1, 21). Strain OL^T was isolated from a shallow freshwater pond located in the Owens Lake bed area. Owens Lake bed is a largely dry, evaporite deposit at the eastern base of Sierra Nevada in California. Prior to 1910, the lake was fed by excessive run-off and melted snow from the nearby mountains. After 1910, water was diverted by the Los Angeles aqueduct, and by 1921 the lake entered its present state of periodic flooding and drying. Like the rest of the lake, the sampling site is subjected to periods of drying. It may therefore reach high temperatures during summer, creating a favourable environment for growth of thermophilic microbes.

Strain OL^T is an extreme thermophile and is a member of the domain *Bacteria* based on 16S rRNA sequence analysis (8). Furthermore, strain OL^T degraded xylan and hence could be a member of the genera *Clostridium*, *Thermoanaerobacter*, *Thermoanaerobacterium* or *Caldicellulosiruptor*. Strain OL^T was not a spore-former and hence could not be included as a member of the genera *Clostridium* or *Thermoanaerobacter* (6, 7, 16). Strain OL^T did not produce sulfur from thiosulfate and hence could be excluded from the genus *Thermoanaerobacterium* (16). Strain OL^T degraded cellulose and hence possess a similar trait in common with members of the *Caldicellulosiruptor* group (25). In addition, strain OL^T had other similarities to this

group, including G + C content and fermentation end-products. The taxonomic nature of this cluster is still ill-defined and with the exception of *C. saccharolyticus*, none of the other members has been taxonomically validated (25).

Another phylogenetically closely related species to strain OL^T is *Anaerocellum thermophilum* (32). Strain OL^T differed in carbon utilization from *A. thermophilum* and *C. saccharolyticus*. Sequencing of the 16S rRNA gene and phylogenetic analysis confirmed the placement of strain OL^T as a member of the *Caldicellulosiruptor* cluster. Comparison of the phenotypic traits of strain OL^T with the widely studied members of *Caldicellulosiruptor*, namely *C. saccharolyticus* and '*C. lactoaceticus*' (25, 20), indicated that strain OL^T was nutritionally more versatile and that *C. lactoaceticus* was the least versatile (Table 1). Based on this study we propose that strain OL^T is the type strain of a new species of the genus *Caldicellulosiruptor*, *Caldicellulosiruptor owensensis*.

Description of *Caldicellulosiruptor owensensis*

Caldicellulosiruptor owensensis (o.wen.sen'sis. N.L. adj. *owensensis* from Owens Lake, CA, USA).

Cells are non-motile straight rods that are 2–5 µm by 0·5–0·8 µm, and occur singly, in pairs, or in chains. Lophotrichous flagella. Gram staining reaction was negative. Endospores were not found in any tested conditions. Colonies (diameter, ≤ 2 mm) are circular with smooth edges, convex, opaque and yellowish. Growth occurred over the temperature range 50–80 °C with an optimum of 75 °C. No growth was detected at and below 45 °C or above 80 °C. Alkalitolerant. pH range, 5·5–9·0 with an optimum at about pH 7·5. Growth factors found in either yeast extract or vitamin solutions were not required for growth. Growth was inhibited by penicillin G, streptomycin, chloramphenicol and ampicillin at 100 µg ml^{-1}. Cells are resistant to D-cycloserine (100 µg ml^{-1}), erythromycin (200 µg ml^{-1}) and tetracycline (100 µg ml^{-1}). Growth strictly anaerobic. Chemoorganotrophic. Growth with arabinose, cellobiose, cellulose, dextrin, fructose, galactose, glucose, glycogen, inositol, lactose, mannitol, mannose, maltose, pectin, raffinose, rhamnose, ribose, starch, sucrose, tagatose, xylan, xylose and yeast extract. Does not grow on acetate, amygdalin, arbutin, erythritol, glycerol, lactate, melezitose, melibiose, methanol, pyruvate, sorbitol, trehalose, trypticase peptone or H$_2$/CO$_2$. The fermentation products from glucose are acetate, lactate, ethanol, H$_2$ and CO$_2$. The bacterium does not reduce nitrate, sulfate, sulfite and thiosulfate. The G + C content is 36·6 mol% (as determined by buoyant density of purified DNA by CsCl gradient centrifugation). Type strain OL^T (= ATCC 700167) was isolated from the Owens Lake in California, USA.

ACKNOWLEDGEMENTS

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