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Isolation and characterization of *Caldicellulosiruptor lactoaceticus* sp. nov., an extremely thermophilic, cellulolytic, anaerobic bacterium

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**Abstract** An anaerobic, extremely thermophilic, cellulolytic, non-spore-forming bacterium, strain 6A, was isolated from an alkaline hot spring in Hveragerði, Iceland. The bacterium was non-motile, rod-shaped (1.5–3.5 × 0.7 μm) and occurred singly, in pairs or in chains and stained gram-negative. The growth temperature was between 50 and 78°C with a temperature optimum near 68°C. Growth occurred between pH 5.8 and 8.2 with an optimum near 7.0. The bacterium fermented microcrystalline cellulose (Avicel) and produced lactate, acetate and H₂ as the major fermentation products, and CO₂ and ethanol occurred as minor fermentation products. Only a restricted number of carbon sources (cellulose, xylan, starch, pectin, cellobiose, xylose, maltose, and lactose) were used as substrates. During growth on Avicel, the bacterium produced free cellulases with carboxymethylcellulase and avicelase activity. The G + C content of the cellular DNA of strain 6A was 35.2 ± 0.8 mol%. Complete 16S rDNA sequence analysis showed that strain 6A was phylogenetically related to *Caldicellulosiruptor saccharolyticus*. It is proposed that the isolated bacterium be named *Caldicellulosiruptor lactoaceticus* sp. nov.

**Key words** Anaerobic bacterium · Thermophilic bacterium · Cellulose · Cellulase · Hot spring · Chemoorganotrophic

**Introduction**

The vast majority of thermophilic cellulolytic anaerobic bacteria are spore-formers and belong to the genus *Clostridium* (Wiegel 1992). In the last 7 years a number of non-spore-forming, cellulolytic, thermophilic anaerobic bacteria have been isolated from thermal areas (Sissons et al. 1987; Taya et al. 1988; Hudson et al. 1990; Svetlichny et al. 1990). Numerical phenetic study, based on comparison of phenetic data for 51 anaerobic, cellulolytic, thermophilic, rod-shaped bacteria, sharply separated the anaerobic, cellulolytic, non-spore-forming thermophiles represented in “NT clusters A, B, C and D” from “NT cluster E,” which also consists of the spore-forming, anaerobic, cellulolytic thermophilic Clostridia (Rainey et al. 1993b). The results of a study based on complete sequencing of 16S rDNA showed that the extremely thermophilic, non-spore-forming, cellulose-degrading bacteria are closely related phylogenetically and are therefore grouped into “cluster D” (Rainey et al. 1993a). Because they are closely related phylogenetically, the assignment of a new genus was indicated (Rainey et al. 1993b). Rainey et al. (1994) have therefore renamed strain TP8T.6331, formerly *Caldocellum saccharolyticum*, *Caldicellulosiruptor saccharolyticus* gen. nov., sp. nov.

The present paper describes the isolation and the phenotypic and phylogenetic characterization of *Caldicellulosiruptor lactoaceticus* sp. nov., a new extremely thermophilic, anaerobic, non-sporulating, cellulose-degrading bacterium different from previously described cellulolytic anaerobes.

**Materials and methods**

**Media and cultivation methods**

The basal medium used for enrichment, isolation and cultivation was as previously described (Angelidakis et al. 1990) with the following modifications. The medium for the enrichment cultures contained only 10% of the normal concentrations of stock solutions A and B. Cysteine was omitted, and the concentration of sulfide was increased to 0.5 g/l; the vitamin solution, 10 ml/l, was that of medium no. 141 of the German Collection of Microorganisms and Cell Cultures (DSM). Furthermore, 0.2 g/l Avicel (FMC International, Little Island, Cork, Ireland) was used as the carbon and energy source, and the medium was supplemented with 0.1 g/l yeast extract (Difco, Detroit, Mich., USA). The medium for routine cultivation of the pure culture contained 4 g/l Avicel and 0.75 g/l yeast extract. The media were autoclaved at 140°C.
for 20 min. The culture was normally incubated without shaking at 68°C.

Isolation was done by using the anaerobic roll-tube technique (Hungate 1969; Bryant 1972). The medium was solidified with Gelrite (Merck, Rahway, NJ, USA) and 8 g/l Avicel was added as a substrate. Yeast extract, 0.75 g/l, was added. Individual colonies were obtained by preparing serial dilutions of enrichment culture samples in roll tubes containing the solidified medium. Colonies were picked with sterile Pasteur pipettes and transferred at least two times on solidified medium after only one colony type was present.

The ability of the bacterium to utilize various carbon compounds as substrates was determined by inoculating growing cells at 68°C for 20 min. The culture was normally incubated without shaking the new conditions before the pH and temperature response was determined by measuring the culture optical density as described above. The effect of nitrate and sulfuroxy anions on cell growth was determined by measuring the culture optical density as described above.

Growth measurement

Growth was determined by measuring optical density at 578 nm in media with cellodextrin as substrate. In media with Avicel as substrate, cell number was determined by using a Petroff-Hauser counting chamber.

Different pH values in media were obtained by varying the concentrations of HCO$_3^-$ and CO$_3^{2-}$ in the medium and the CO$_2$ content of the headspace gas. Cultures were grown at the new pH or temperature three times to allow for adaptation of the culture to the new conditions before the pH and temperature response was measured.

The ability of the bacterium to utilize various carbon compounds as substrates was determined by inoculating growing cells (10% V/V) into medium containing the substrates to be tested. Substrates were filter-sterilized and added from stock solutions to the autoclaved medium at a concentration of 4 g/l.

Analytical methods

Fermentation products, such as short-chain fatty acids and alcohols, were quantified using gas chromatography with flame ionization detection. H$_2$ and CO$_2$ were analyzed by gas chromatography with thermal conductivity detection. High-performance liquid chromatography (HPLC) with refractive index detection was used to measure lactate, formate, glucose and celllobiose. Residual Avicel was measured gravimetrically by trapping it on filters, washing with formic acid and drying to constant weight. Sulfide was measured according to the method of Cord-Ruwisch (1985). Protein was measured using the method of Bradford (1976), with bovine serum albumin as a standard. The guanine + cytosine (G + C) ratio of the cellular DNA of the isolate was measured using HPLC (Mesbah et al. 1989) at the DSM. The complete sequence of the 16S-rDNA gene was determined at DSM by direct sequencing of the PCR-amplified 16S rDNA, and the sequence data were compared with over 200 currently available sequences of Clostridia and related taxa.

Enzyme assays

Enzyme activity was measured as release of reducing sugars from the substrate tested. Reducing sugars were quantified by the dinitrosalicylic acid method (Miller 1959) using glucose as standard. Activities are given in units/milligram protein where 1 unit (U) of enzyme activity corresponds to 1 μg-equivalent of reducing sugar released per minute.

Carboxymethylcellulase (CMCase) assays were carried out by adding 2 ml of crude supernatant to 6 ml of buffer containing 13.3 g/l low viscosity CM cellulose (Sigma, St. Louis, Mo., USA). The enzyme and substrate mixtures were incubated at 70°C for 2.5 h. All measurements were carried out in triplicate. Parallel controls with heat-inactivated enzymes (140°C for 20 min) were used in each assay. CMCase activity was tested in a pH range from 3.0 to 10.0 using various buffers. Mixtures of 20-40 mM citrate and 20–30 mM phosphate buffer were chosen for pH 3.0-6.0; 50 mM phosphate buffer for pH 7.0; 25 mM 3-(cyclohexylamino)-1-propanesulfonic acid buffer (CAPSO, Sigma) for pH 9.0; and 3-(cyclohexylamino)-1-propanesulfonic acid buffer (CAPS, Sigma) for pH 10.0. The pH of the buffer containing the substrate was checked and adjusted at the test temperatures before the experiments were started.

Thermostability of CMCase was measured after incubation of crude supernatant at various temperatures for the periods shown. The residual CMCase activity was measured with the assay described above.

Filter paper activity was measured in buffer with pH 6.0 at 70°C according to the method of Mandels et al. (1976). Avicelase activity was measured in buffer with pH 6.0 at 70°C according to the method of Sissons et al. (1987). Xylanase activity was determined according to the method of Mathrani and Ahring (1992) in buffer with pH 6.0 at 70°C.

Average values from all experiments are reported and error bars in figures represent the range of at least three values from a typical experiment.

Results

Samples for enrichment, enrichment cultures and isolation

Strain 6A was isolated from liquid samples containing sediment and biomat material collected from a hot spring in the Hveragerði area on Iceland. The in situ temperature and the pH were 74°C and 8.0, respectively. Five milliliters of sample from the hot spring was diluted with 5 ml of liquid medium in nitrogen atmosphere. From this dilution, 0.1 ml was used as an inoculum for enrichment cultures in media with pH 7.0 or pH 9.0. Tubes were incubated at 68, 78 and 90°C. After a period of 1 week, positive enrichments were obtained at incubation 68 and 78°C in media with pH 7.0. Growth was indicated by solubilization of Avicel and microscopic examination of the cultures compared to uninoculated controls and controls without substrate. All enrichments were negative at pH 9.0 and at 90°C in accordance with previous findings for cellulosytic anaerobic bacteria (Hudson et al. 1990; Mathrani et al. 1993). The enrichments were diluted and transferred to anaerobic roll tubes containing Avicel and yeast.