Clostridium straminisolvens sp. nov., a moderately thermophilic, aerotolerant and cellulolytic bacterium isolated from a cellulose-degrading bacterial community

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A novel anaerobic, thermophilic and cellulolytic bacterium (strain CSK1^T) was isolated from a cellulose-degrading bacterial community. On the basis of 16S rRNA gene sequence similarity, strain CSK1^T was mapped to cluster III of the genus *Clostridium*. Strain CSK1^T is closely related to *Clostridium thermocellum* (96.2%) and *Clostridium aldrichii* (95.1%). Strain CSK1^T is a non-motile, spore-forming, straight or slightly curved rod. The optimum temperature and initial pH for its growth and cellulose degradation are 50–55 °C and pH 7.5. Strain CSK1^T grew under a gas phase containing up to 4% O₂. Phylogenetic and phenotypic analyses support the differentiation of strain CSK1^T from its closest relatives. Strain CSK1^T therefore represents a novel species, for which the name *Clostridium straminisolvens* sp. nov. is proposed, with CSK1^T (= DSM 16021^T = IAM 15070^T) as the type strain.

Cellulosic materials are among the Earth's most abundant, renewable resources, and their degradation and utilization by microbes are considered very important (Coughlan & Mayer, 1992). Micro-organisms that degrade cellulosic materials, and the enzymes involved (e.g. cellulase, xylanase and peroxidase), have been well studied (Fukumori *et al.*, 1989; Hayashi *et al.*, 1999) and several microbial-related applications have been developed for textile, food and paper-pulp processing. However, utilizing these micro-organisms and enzymes to process natural cellulosic materials without pre-treatment and/or sterilization is difficult. In nature, cellulosic materials are degraded by the cooperation of many micro-organisms. It has been reported that a mixed culture comprising a cellulolytic bacterium and a non-cellulolytic bacterium is ideal for degrading cellulose (Odom & Wall 1983; Lewis *et al.*, 1988).

In our laboratory, a bacterial community capable of effectively degrading various cellulosic materials (e.g. filter paper, cotton and rice straw) under aerobic static conditions was constructed by means of a succession of enrichment cultures, as reported in Haruta *et al.* (2002).

This bacterial community degraded natural cellulosic materials without sterilization and had a high degree of stability (the cellulose-degrading efficiency and the composition of the bacteria did not change after more than 20 subcultures). Denaturing gradient gel electrophoresis and 16S rRNA gene sequence analyses indicated the coexistence of aerobic and anaerobic bacteria in the community. Elucidation of the characteristics of each bacterium in the community (especially the cellulolytic bacteria) will lead to greater knowledge of the mechanisms of effective cellulose degradation by cooperation amongst bacteria.

In this article, we report the isolation of a novel strain, CSK1^T, from a cellulose-degrading bacterial community. We characterized this strain further as a novel moderately thermophilic, cellulolytic member of cluster III of the genus *Clostridium*. We propose to assign this bacterium to a novel species of the genus *Clostridium*, namely *Clostridium straminisolvens* sp. nov.

Unless stated otherwise, stringent anaerobic procedures (Holdeman *et al.*, 1977) were used for culturing and for physiological and substrate-utilization tests. All anaerobic media containing reducing agents were pre-reduced by cooling after sterilization inside an anaerobic chamber operated with anaerobic gas mixture (H₂/N₂: 10:90, v/v). Only fully reduced media, as indicated by the colourless state of resazurin in the media, were used for cultivation.

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CSK1^T is AB125279.

The isolation source was the cellulose-degrading bacterial community (Haruta *et al.*, 2002) that was cultured in liquid medium containing the following (per litre distilled water): yeast extract, 1 g; peptone, 5 g; CaCO₃, 2 g; NaCl, 5 g; dried rice straw, 10 g, under static conditions at 50 °C. After rice-straw degradation had been observed, the rice straw was removed and washed four times with anaerobic buffer (PBS with 1%, w/v, L-cysteine hydrochloride) under anaerobic conditions. The washed rice straw was inoculated into isolation medium (Drent *et al.*, 1991) containing the following (per litre distilled water): NaCl, 1.2 g; MgCl₂·6H₂O, 0.4 g; KCl, 0.3 g; CaCl₂·2H₂O, 0.15 g; NH₄Cl, 0.27 g; KH₂PO₄, 0.205 g; Na₂SO₄, 0.1 g; NaHCO₃, 2.52 g; Na₂S·9H₂O, 0.15 g; yeast extract, 25 mg; Casamino acids, 25 mg; filter paper (Advantec quantitative filter paper no. 5A) or ball-milled cellulose [Avicel (Merck), ball-milled for 1 min with a TI-200 vibrating sample mill (CMT)], 10 g; resazurin, 1 mg; trace-element solution SI 6⁺ [containing (per litre distilled water): FeCl₂·4H₂O, 1.5 g; ZnCl₂, 70 mg; MnCl₂·4H₂O, 100 mg; H₃BO₃, 6 mg; CoCl₂·6H₂O, 190 mg; CuCl₂·2H₂O, 2 mg; NiCl₂·6H₂O, 24 mg; Na₂MoO₄·2H₂O, 36 mg], 1 ml; and vitamin solution, 1 ml (Heijthuijsen & Hansen, 1986).

Growth experiments were performed in duplicate, using medium 122 [Saddler & Chan, 1982, and DSMZ List of Media (<http://www.dsmz.de/media/med122.htm>)] containing the following (per litre distilled water): (NH₄)₂SO₄, 1.3 g; MgCl₂·6H₂O, 2.6 g; KH₂PO₄, 1.43 g; K₂HPO₄·3H₂O, 7.2 g; CaCl₂·6H₂O, 0.13 g; FeSO₄·7H₂O, 1.1 mg; sodium β-glycerophosphate, 6.0 g; yeast extract, 4.5 g; filter paper, ball-milled cellulose or cellobiose, 10 g; glutathione (reduced), 0.25 g; and resazurin, 1 mg. The potential for growth and cellulose degradation at various initial pH values ranging from 5.0 to 8.0 (adjusted by addition of 1 M HCl or 1 M NaOH) was determined using medium 122 with cellobiose (0.5%, w/v) or filter paper (1%, w/v) at 50 °C. The potential for growth and cellulose degradation at various temperatures ranging from 37 to 70 °C was determined using medium 122 with cellobiose (0.5%, w/v) or filter paper (1%, w/v) at pH 7.0. The cellulosic substrate degradation rates were determined using medium 122 with filter paper or rice straw (1%, w/v) at 50 °C, pH 7.0. Residual solid cellulosic materials were gravimetrically determined using a method described by Tailliz *et al.* (1989), with uninoculated medium as a negative control. Individual substrate utilization was assessed at 50 °C (pH 7.0) using medium 122 containing each substrate (0.5%, w/v). The substrates tested were glucose, fructose, ribose, mannose, mannitol, melibiose, saccharose, xylose, cellobiose, sucrose, lactose, ball-milled cellulose, filter paper, rice straw, xylan (from beechwood; Sigma), starch (from potato; Kanto Chemical), laminarin (from *Laminaria digitata*; Sigma), pachyman (from *Poria cocos*; ICN Biomedicals), chitin, chitosan (from crab shells; Sigma) and 1,3-β-glucan. Utilization of a substrate was judged from the pH drop of the culture solution after 5 days cultivation. Motility, aesculin hydrolysis, nitrate reduction,

casein digestion, lectinase activity, lipase activity and indole production were tested using methods described by Holdeman *et al.* (1977). The microscopic sample for checking motility was prepared in an anaerobic chamber. Fermentation products were determined for strain CSK1^T grown in medium 122 with ball-milled cellulose (1%, w/v) or cellobiose (0.5%, w/v) at 50 °C for 5 days. Production of H₂ and CO₂ were determined by GC (Küsel & Drake, 1995). Organic acid and alcohol by-products were determined by HPLC (Tanaka *et al.*, 2002). The potential for growth and cellulose degradation of strain CSK1^T and *Clostridium thermocellum* IAM 13660^T under the gas phase with various concentrations of O₂ (0, 1, 2, 4 and 8%, v/v) was determined using medium 122 with ball-milled cellulose (1%, w/v) or cellobiose (0.5%, w/v) at 50 °C (for strain CSK1^T) or 60 °C (for *C. thermocellum*). The gas phase was replaced with a gas mix of N₂ and O₂, prepared in appropriate ratios by using a GB-4C gas blender (Kofloc). Growth was judged from the pH drop of the culture solution after 5 days cultivation.

DNA was extracted by using benzyl chloride methodology (Zhu *et al.*, 1993). The DNA G+C composition of strain CSK1^T was determined by HPLC (Mesbah *et al.*, 1989). DNA–DNA hybridization was performed by incubating target DNA with biotinylated DNA for 2 h at 45 °C in microdilution wells; the amount of hybridization was assessed fluorometrically (Ezaki *et al.*, 1989). Genomic DNA from *Escherichia coli* was used as the negative control.

The 16S rRNA gene sequence of strain CSK1^T was determined by direct sequencing of the purified PCR-amplified 16S rRNA gene fragment. Genomic DNA was used as the PCR template. The PCR was performed with universal bacterial primers complementary to conserved regions of the 5' and 3' ends of the 16S rRNA gene. The primer sequences were 27F (forward; 5'-AGAGTTTGATCCTGG-CTCAG-3', positions 8–27 in *E. coli* numbering) and 1512R (reverse; 5'-ACGGCTACCTTGTTACGACT-3', positions 1512–1493 in *E. coli* numbering) (Devereux & Willis, 1995). The PCR was performed using AmpliTaqGold (Perkin Elmer). After initial denaturation for 10 min at 95 °C, target DNA was amplified for 30 cycles. Each cycle consisted of denaturation for 30 s at 93 °C, annealing for 30 s at 55 °C and extension for 2 min at 72 °C. The final extension was for 5 min at 72 °C. The PCR products were purified with the QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions. The purified 16S rRNA gene was sequenced directly using the ABI PRISM BigDye Terminator cycle sequencing ready reaction kit and an ABI PRISM model 377 genetic analyser (Perkin Elmer). The 16S rRNA gene sequence of strain CSK1^T was compared with those from the DDBJ nucleotide sequence database, using BLAST (Altschul *et al.*, 1997). To determine the phylogenetic position of the isolate, its sequence and the sequences of its close relatives in the *Clostridium* subphylum were aligned using the program CLUSTAL_X (version 1.81) (Thompson *et al.*, 1997). A phylogenetic tree was constructed using the

neighbour-joining method (Saitou & Nei, 1987) with the program MEGA (version 2.1) (Kumar *et al.*, 2001). To evaluate the robustness of the inferred tree, the bootstrap resampling method of Felsenstein (1985) was used, with 1000 replicates.

The bacteria were enriched in batch culture under static conditions using screw-capped test tubes (16 × 100 mm) filled with 5 ml isolation medium and ball-milled cellulose (1 g l⁻¹) under anaerobic conditions using an AnaeroPack pouch bag (Mitsubishi Gas Chemical) with an AnaeroPack oxygen absorber at 50 °C. After 4–5 days cultivation, the colour of the ball-milled cellulose became yellow, indicating that degradation was progressing. Bacteria adhering to the cellulose powder were collected by centrifugation (approx. 300 g for 5 min) and transferred into fresh medium. This enrichment culturing procedure was repeated more than five times. The enriched bacteria were streaked on a sterilized filter paper moistened with anaerobic buffer, covered with isolation medium containing 1.5 % (w/v) agar (without carbohydrates) and then incubated under anaerobic conditions (using the AnaeroPack system) at 50 °C. After 4–5 days cultivation, yellow-coloured colonies appeared on the filter paper. After the colonizing procedures had been repeated, confirmation of the purity of strain CSK1^T was achieved by microscopic observation.

Cells of strain CSK1^T were observed, under a phase-contrast microscope (Axioplan II imaging; Carl Zeiss) (Fig. 1), to be straight or slightly curved rods, 0.5–1.0 µm wide and 3.0–8.0 µm long. Spores were oval, subterminal and 0.5–1.0 × 1.0–1.5 µm. Colonies grown in cellobiose agar medium were tan–yellow, round, entire and 1.0–2.0 mm in diameter.

Strain CSK1^T grew and degraded cellulose (filter paper and ball-milled cellulose) at temperatures ranging from 50 to 60 °C, with an optimum temperature range of 50–55 °C. For strain CSK1^T, the optimum pH for growth and cellulose degradation was 7.5; growth and cellulose degradation occurred between pH 6.0 and 8.5. Older cultures of strain CSK1^T show extensive growth in the culture liquid phase, as does *C. thermocellum*. Strain CSK1^T did not grow in medium 122 without carbohydrates. Cellobiose, laminarin and cellulosic materials (ball-milled cellulose, filter paper

and rice straw) were each utilized as sole carbon and energy sources by strain CSK1^T in medium 122. Other substrates tested were not fermented. Motility was not observed, even under anaerobic conditions. Aesculin was hydrolysed. The following biochemical properties were tested and produced negative results: nitrate reduction, casein digestion, lectinase activity, lipase activity and indole production. In medium 122 with cellobiose or ball-milled cellulose, the major fermentation products formed by strain CSK1^T were acetate, ethanol, hydrogen and carbon dioxide; minor amounts of lactate were detected.

Strain CSK1^T degraded filter paper (62 %) and rice straw (45 %, on a dry organic matter basis) after 8 days cultivation. The degradation rates (especially for rice straw) were apparently lower than those of the original bacterial community, which degrades more than 80 % of rice straw within 8 days (Haruta *et al.*, 2002). In the bacterial community, non-cellulolytic bacteria would help strain CSK1^T degrade cellulosic materials more completely.

The 16S rRNA gene sequence of strain CSK1^T, containing a continuous stretch of 1451 nt (approx. positions 29–1492 according to *E. coli* numbering), was compared with those from the DDBJ nucleotide sequence database. The results of the sequence-similarity calculations indicated that the relatives of strain CSK1^T are *C. thermocellum* DSM 1237^T (96.2 %) and *Clostridium aldrichii* DSM 6159^T (95.1 %). These relatives are reported as being anaerobic, cellulolytic bacteria (Ng *et al.*, 1977; Yang *et al.*, 1990). The phylogenetic tree was constructed using the neighbour-joining method; it indicates that strain CSK1^T belongs to cluster III (Collins *et al.*, 1994) of the genus *Clostridium* (Fig. 2).

DNA–DNA hybridization experiments revealed a 23 % genome sequence similarity between strain CSK1^T and *C. thermocellum* IAM 13660^T when genomic DNA from *E. coli* was used as the negative control. This result confirms that strain CSK1^T should be separated into a different species from its closest relative, *C. thermocellum*. The G + C content of the DNA of strain CSK1^T was 41.3 mol%.

Strain CSK1^T differs from its close relatives, *C. thermocellum* and *C. aldrichii*, in terms of optimum temperature for growth. The optimum temperature for growth of strain CSK1^T was 50–55 °C. In contrast, the optimum temperatures for growth of *C. thermocellum* and that of *C. aldrichii* were reported to be 60–64 °C and 35 °C, respectively (Ng *et al.*, 1977; Yang *et al.*, 1990). Strain CSK1^T did not grow or degrade cellulose at temperatures under 45 °C, and scarcely grew at 60 °C. The bacterial community from which strain CSK1^T was isolated was cultivated at 50 °C, and the optimum temperature for cellulose degradation was between 50 and 55 °C (Haruta *et al.*, 2002). This temperature sensitivity could be one of the reasons why only strain CSK1^T survived within the bacterial community as a cellulolytic bacterium at 50 °C.

Strain CSK1^T also differs from its closest relative, *C.*

Fig. 1. Phase-contrast micrograph of cells of strain CSK1^T, showing subterminal oval spores. Bar, 5 µm.

Fig. 2. Phylogenetic dendrogram indicating the position of strain CSK1^T amongst cluster III of the order *Clostridiales* and related bacteria, based on 16S rRNA gene sequence data. Bootstrap values are given at the branching points. Accession numbers are shown in parentheses. Bar, 2% sequence divergence.

thermocellum, in that it has a higher tolerance of O₂. Strain CSK1^T grew in an atmosphere containing an O₂ concentration of up to 4%. In contrast, *C. thermocellum* (IAM 13660^T) could not grow in an atmosphere containing an O₂ concentration of more than 2%. In the genus *Clostridium*, some species that can grow in an atmosphere containing an O₂ concentration of up to 6% have been reported, and the species have the ability to consume O₂ (Küsel *et al.*, 2001; Karnholz *et al.*, 2002). Colour changing of resazurin in the media indicated that strain CSK1^T consumes O₂ during cultivation in an atmosphere containing O₂. Cellulolytic clostridia with aerotolerance have not been reported previously. This aerotolerance of strain CSK1^T could be advantageous to its survival within the bacterial community cultivated under static conditions that were not strictly anaerobic.

Description of *Clostridium straminisolvens* sp. nov.

Clostridium straminisolvens (stra.mi.ni.sol'vens. L. neut. n. *stramen* straw; L. v. *solvere* to dissolve; N.L. part. adj. *straminisolvens* straw-dissolving).

Cells are anaerobic, non-motile, spore-forming, straight or slightly curved rods, 0.5–1.0 µm wide and 3.0–8.0 µm long, occurring singly or in pairs. The optimum temperature for growth is 50–55 °C, and no growth occurs at or below 45 °C or at 65 °C and above. The optimum initial pH for growth is 7.5, and growth occurs between pH 6.0 and 8.5. Growth occurs under a gas phase containing up to 4% O₂. Cellulose and cellobiose are utilized as sole carbon and energy sources; the fermentation products are acetate, lactate, ethanol, hydrogen and carbon dioxide. Glucose, fructose, ribose, mannose, mannitol, melibiose, saccharose, xylose, sucrose, lactose, xylan and starch were not utilized as sole carbon and energy sources. The G+C content of the DNA of the type strain is 41.3 mol% (HPLC).

The type strain, CSK1^T (= DSM 16021^T = IAM 15070^T), was isolated from a cellulose-degrading bacterial community.

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