Clostridium sufflavum sp. nov., isolated from a methanogenic reactor treating cattle waste

Tomomi Nishiyama, Atsuko Ueki, Nobuo Kaku and Katsuji Ueki

Correspondence

Atsuko Ueki

uatsuko@tds1.tr.yamagata-u.ac.jp

Faculty of Agriculture, Yamagata University, Wakaba-machi 1-23, Tsuruoka, Yamagata 997-8555, Japan

A strictly anaerobic, mesophilic, cellulolytic bacterial strain, designated CDT-1^T, was isolated from rice-straw residue from a methanogenic reactor treating waste from cattle farms. The isolation was performed using enrichment culture with filter paper as a substrate. Cells stained Gram-negative, but reacted Gram-positively in the KOH test. Cells were slightly curved rods and were motile by means of peritrichous flagella. The strain produced yellow pigment when grown on filter-paper fragments. Although spore formation was not confirmed microscopically, thermotolerant cells were produced when the strain was grown on filter paper. The optimum temperature for growth was 33 °C and the optimum pH was 7.4. Oxidase, catalase and nitrate-reducing activities were absent. The strain utilized xylose, fructose, glucose, cellobiose, xylooligosaccharide, cellulose (filter-paper fragments and ball-milled filter paper) and xylan. The major fermentation products were acetate, ethanol, H₂ and CO₂. The major cellular fatty acids were iso-C_{15:0}, iso-C_{14:0} and C_{16:0} DMA. The cell-wall peptidoglycan contained *meso*-diaminopimelic acid as the diagnostic diamino acid. The genomic DNA G + C content was 40.7 mol%. On the basis of 16S rRNA gene sequence similarities, strain CDT-1^T could be placed in cluster III of the genus *Clostridium*, being closely related to type strains of *Clostridium hungatei* (96.6% sequence similarity), *Clostridium termitidis* (96.2%) and *Clostridium papyrosolvens* (96.1%). On the basis of the cellular, physiological and phylogenetic differences between CDT-1^T and its close relatives, this strain represents a novel species of the genus *Clostridium*, for which the name *Clostridium sufflavum* sp. nov. is proposed. The type strain is CDT-1^T (=JCM 14807^T=DSM 19573^T).

In anaerobic conditions, cellulose, the most abundant type of organic matter present in plant biomass, is hydrolysed by cellulolytic micro-organisms. The soluble sugars produced are then converted to various organic acids, alcohols, H₂ and CO₂ by fermentative micro-organisms. Finally, methane is produced by methanogens, which use acetate or H₂ + CO₂ as major substrates. Thus, the hydrolysis of cellulose is a key reaction for the efficient methanogenesis of waste that is predominantly made up of plant biomass.

Because of their industrial and ecological importance, many cellulolytic, anaerobic bacteria have been isolated and the properties of their cellulolytic enzymes have been extensively investigated. Some species of the genus *Clostridium*, members of which are anaerobes that produce cellulosomes (highly active cellulolytic and xylanolytic complexes), are classified within *Clostridium* cluster III on the basis of their 16S rRNA gene sequences (Collins *et al.*, 1994). In this report, the isolation and characterization

of a novel anaerobic, cellulolytic strain belonging to *Clostridium* cluster III is described.

Strain CDT-1^T was isolated from a sample of rice-straw residue obtained from a methanogenic reactor treating waste collected from cattle farms (comprising up to 1000 cattle in total) in Betsukai-machi in Hokkaido, Japan. The reactor was a vertical cylindrical type (1500 m³) operated at a mesophilic temperature. Rice-straw used as matting for the cattle was thrown into the reactor, together with cattle faeces and urine, and subjected to waste treatment.

The strain was cultivated anaerobically at 30 °C (unless otherwise indicated) by using peptone/yeast extract (PY) medium as the basal medium and O₂-free mixed gas (N₂/CO₂, 95:5) as the headspace, as described by Ueki *et al.* (2006a). PY medium supplemented with (l⁻¹) 0.25 g each of glucose, cellobiose, maltose and soluble starch, as well as 15 g agar (Difco), was designated PY4S agar and used for maintenance of the strain in agar slants. PY liquid medium supplemented with 10 g glucose l⁻¹ (PYG medium) was used for the cultivation of the strain for various physiological tests and chemotaxonomic analyses of the cells, unless indicated otherwise (Holdeman *et al.*, 1977). For enrichment culture and isolation of cellulolytic

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CDT-1^T is AB267266.

bacteria, the concentrations of both peptone and yeast extract in the basal medium were decreased to one-tenth of those in PY medium (1/10PY medium). Media were usually adjusted to pH 7.3–7.4 with 1 M NaOH.

Anaerobic sludge samples obtained from the reactor were filtered through a mesh (2 mm pore size) and rice-straw residue remaining on the mesh was collected. The rice-straw samples obtained were washed several times with sterile, anoxic diluent and homogenized in a Waring blender (10 000 r.p.m., 10 min) under N₂ gas (Kaku *et al.*, 2000). The homogenized samples were successively diluted anaerobically and the enrichment culture was started by inoculating 1 ml 10-fold-diluted samples into 9 ml 1/10PY-p liquid medium (medium containing filter-paper fragments at 2 g l⁻¹). When the filter-paper fragments in the medium had completely disintegrated, 1 ml enrichment culture was transferred to fresh 1/10PY-p medium (9 ml). After three subcultures of the cellulolytic enrichment, the diluted enrichment cultures were inoculated to anaerobic roll tubes containing 1/10PY-c agar medium (medium containing ball-milled filter paper at 5 g l⁻¹, as opposed to the paper fragments used in 1/10PY-p medium). After incubation of the roll tubes for 2–3 weeks, clear zones, indicating decomposition of ball-milled filter paper, appeared in the agar. Some colonies that generated clear zones were picked; strain CDT-1^T was finally obtained after purification using the anaerobic roll-tube method (Hungate, 1966).

Growth of the strain under aerobic conditions was examined as described previously (Ueki *et al.*, 2007). The KOH test was performed as described by Wallace & Gates (1986). Spore formation was assessed by observing cells after Gram-staining as well as by means of phase-contrast microscopy. Production of thermotolerant cells was investigated by assessing the growth (in PYG medium) of cells previously exposed to a temperature of 80 °C for 10 min. The motility of the cells was examined using phase-contrast microscopy and flagella staining was carried out according to Blenden & Goldberg (1965). Catalase, oxidase and nitrate-reducing activities were determined according to the methods described by Akasaka *et al.* (2003a, b). Utilization of carbon sources was tested in PY liquid medium with each substrate (monosaccharides, disaccharides, oligosaccharides and sugar alcohols) being added at 10 g l⁻¹. Other substrates (polysaccharides and glycosides) were added at 5 g l⁻¹. Utilization of each substrate was determined from growth measured using OD₆₆₀ values and also by assessing changes to the pH of the medium after cultivation. Fermentation products were analysed by GC as described previously (Ueki *et al.*, 1986; Akasaka *et al.*, 2003a). Other characteristics were determined using the methods described by Holdeman *et al.* (1977) and Ueki *et al.* (2006a, b).

Whole-cell fatty acids were converted to methyl esters according to the method of Miller (1982) and were analysed by GC (Hewlett Packard HP6890 or Hitachi

G-3000) equipped with an HP Ultra 2 column (Hewlett-Packard). Whole-cell fatty acids were identified from equivalent chain-lengths (Miyagawa *et al.*, 1979; Ueki & Suto, 1979) according to the protocol of TechnoSuruga Co., Ltd (Shimizu, Japan) (Moore *et al.*, 1994; Ueki *et al.*, 2007). Isoprenoid quinones were extracted and purified as described by Komagata & Suzuki (1987) and identified by using a mass spectrometer (JMS-SX102A; JEOL). Genomic DNA extracted according to the method described by Akasaka *et al.* (2003b) was digested with P1 nuclease by using a Yamasa GC kit (Yamasa shoyu) and the G + C content was measured by means of HPLC (L-7400; Hitachi) using a µBondapak C18 column (3.9 × 300 mm; Waters).

PCR amplification of the almost-complete 16S rRNA gene sequence was carried out using a primer set consisting of 27f and 1492r (Akasaka *et al.*, 2003a). The PCR-amplified 16S rRNA gene was sequenced using a Thermo Sequenase cycle sequencing kit (USB Corp.) and a DNA sequencer (4000L; Li-COR). Multiple alignments of the sequences with reference sequences in GenBank were performed with the BLAST program (Altschul *et al.*, 1997). A phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei, 1987) with the CLUSTAL W program (Thompson *et al.*, 1994). All gaps and unidentified base positions in the alignments were excluded before sequence assembly.

After about 20 days incubation of a homogenized rice-straw sample inoculated into 1/10PY-p liquid medium, the filter-paper fragments in the medium became light yellow and the fibres began to disperse slowly. After further incubation for about 20 days, the filter paper was completely solubilized. The culture was transferred to fresh medium and an enrichment culture capable of degrading the filter paper completely within 20 days incubation was obtained after two more subcultures. Strain CDT-1^T was finally obtained from the culture by picking a colony that produced a clear zone in 1/10PY-c agar medium with the anaerobic agar roll-tube method.

Cells of strain CDT-1^T were slightly curved rods with oval ends, approximately 0.5–0.6 µm in diameter and 2.0–5.0 µm in length. Cells occurred singly or in pairs (Fig. 1a). Cells of strain CDT-1^T stained Gram-negative, but reacted Gram-positively in the KOH test. Cells were motile, as observed under phase-contrast microscopy, and staining showed that the cells had peritrichous flagella (Fig. 1b). Colonies on PY4S agar after 3–4 days anaerobic incubation were white, translucent and irregular with smooth surfaces. Cells of strain CDT-1^T did not grow in air. Although spore formation of the cells was not confirmed microscopically (Fig. 1a) and cells grown in PYG medium and treated at 80 °C for 10 min did not grow, cells grown on filter-paper fragments in PY-p medium (PY medium containing filter-paper fragments at 5 g l⁻¹) did grow after heat treatment. Thus, we concluded that cells of strain CDT-1^T produce thermotolerant spores.

Fig. 1. Photomicrographs of cells of strain CDT-1^T: (a) phase-contrast photomicrograph of cells on an agar slant containing PY4S medium; (b) photomicrograph of flagella-stained cells. Bar, 10 μ m.

Cells were found to be negative for catalase and oxidase activities. The strain utilized xylose, fructose, glucose, cellobiose, xylooligosaccharide, cellulose (fragments of filter paper and filter-paper powder) and xylan as growth substrates. Weak growth was observed on ribose, carboxymethylcellulose and sorbitol. When the strain was cultivated in PY-p medium, filter-paper fragments became yellowish after 7–10 days, at which point visible degradation of the filter paper started. After about 12 days incubation, the filter-paper fragments were completely dispersed in the medium and the pH of the medium fell to 6.5. After a further 20 days incubation, the final pH was 5.7. The final pH of the culture in PYG liquid medium was 5.6. The highest growth rate obtained from among the soluble substrates tested was achieved with cellobiose ($\mu=0.064$ h⁻¹), while the growth rate on xylose was rather low ($\mu=0.035$ h⁻¹). The strain did not use arabinose, galactose, mannose, rhamnose, lactose, maltose, melibiose, sucrose, trehalose, melezitose, raffinose, glycogen, inulin, pectin, inositol, mannitol, amygdalin or salicin. The major products of glucose metabolism (10 g l⁻¹) after 4 days incubation were acetate (11.2 mmol l⁻¹), ethanol (4.0 mmol l⁻¹), H₂ (14.7 mmol l⁻¹) and CO₂ (11.4 mmol l⁻¹). The strain produced 13.9 mmol acetate l⁻¹ and 8.0 mmol ethanol l⁻¹ from cellobiose (10 g l⁻¹), and produced 9.7 mmol acetate l⁻¹ and 3.4 mmol ethanol l⁻¹ from filter-paper fragments (5 g l⁻¹) (30 days incubation). The strain did not reduce nitrate. Aesculin and starch were not hydrolysed. Indole, hydrogen sulfide, lecithinase and lipase were not produced. The strain did not change milk and did not grow in chopped-meat broth.

The temperature range for growth was 20–33 °C, with an optimum at 33 °C. The pH range for growth was 5.9–8.2, with an optimum at pH 7.4. The NaCl tolerance of strain CDT-1^T was low: it did not grow in PYG liquid medium containing NaCl at 5 g l⁻¹. The growth rate in PYG liquid medium under the above-mentioned optimum conditions (33 °C, pH 7.4) was 0.073 h⁻¹.

The G + C content of the genomic DNA was 40.7 mol%. Saturated branched-chain fatty acids and dimethyl acetals were detected as major components of the whole-cell fatty

acids of strain CDT-1^T, which consisted of iso-C_{15:0} (16.1 %), iso-C_{14:0} (14.6 %), C_{16:0} DMA (11.5 %), anteiso-C_{15:0} (8.7 %), C_{14:0} (7.7 %), iso-C_{15:0} DMA (7.2 %), C_{16:0} (4.8 %), C_{14:0} DMA (3.7 %), iso-C_{16:0} (3.2 %) and C_{16:0} ALD (2.4 %). The cell-wall peptidoglycan of strain CDT-1^T contained *meso*-diaminopimelic acid. Respiratory quinones were not detected.

Analysis of the almost-complete 16S rRNA gene sequence (1436 bp) of strain CDT-1^T indicated that the strain should be assigned to the phylum *Firmicutes* (*Clostridia*, *Clostridiales*, *Clostridiaceae*). The closest relative of strain CDT-1^T found in GenBank was *Clostridium hungatei* ATCC 700212^T (Monserate *et al.*, 2001), one of the cellulolytic clostridia constituting *Clostridium* cluster III (Collins *et al.*, 1994), with a 16S rRNA gene sequence similarity of 96.6 %. The next most closely related species were also cellulolytic members of *Clostridium* cluster III, namely *Clostridium termitidis* DSM 5398^T (sequence similarity of 96.2 %) (Hethener *et al.*, 1992), *Clostridium papyrosolvans* DSM 2782^T (96.1 %) (Madden *et al.*, 1982), *Clostridium josui* FERM P-9684^T (95.9 %) (Sukhumavasi *et al.*, 1988) and *Clostridium cellulolyticum* ATCC 35319^T (95.5 %) (Petitdemange *et al.*, 1984) (Fig. 2).

Some characteristics of strain CDT-1^T and the three most closely related species are compared in Table 1. Although strain CDT-1^T resembled the other related cellulolytic species morphologically and physiologically, it had some distinctly different characteristics. Cells of strain CDT-1^T had five to eight peritrichous flagella, but cells of *C. hungatei* have only one or two subpolar flagella (Monserate *et al.*, 2001) and the cells of *C. termitidis* have only two to three peritrichous flagella (Hethener *et al.*, 1992). Strain CDT-1^T did not grow at temperatures above 35 °C, but *C. hungatei*, *C. termitidis* and *C. papyrosolvans* grow at temperatures up to 45, 48 and 37 °C, respectively. The substrates utilized by strain CDT-1^T differed from those utilized by related species (Table 1).

Although cells of strain CDT-1^T appeared to have the Gram-positive type of cell wall on the basis of the KOH test, cells of strain CDT-1^T and of the related species (except *C. termitidis*) stain Gram-negative. Cells of strain CDT-1^T and of the related species (except *C. papyrosolvans*)

Fig. 2. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships of strain CDT-1^T and related species in the genus *Clostridium* (group III). Bootstrap values (based on 1000 replicates) are shown at the branch points. The sequence of *Bacillus subtilis* NCDO 1769^T, which belongs to the class *Bacilli*, was used as the outgroup. Bar, estimated difference of 2% in nucleotide sequence positions.

are slightly curved rods. Thus, these cellular characteristics (Gram-negative, slightly curved rods) seem to be properties that are common to the cellulolytic species in *Clostridium* cluster III. Although cells of *Clostridium* species generally produce spores and spore formation in species closely

related to strain CDT-1^T has been also confirmed microscopically, we could not observe spores for strain CDT-1^T (Fig. 1a) and cells grown in PYG medium did not tolerate a temperature of 80 °C for 10 min. The novel strain may form spores only rarely during growth on soluble saccharides, although thermotolerant cells did occur in cultures grown on filter paper. The rare formation of spores has also been reported for *C. cellulolyticum* (Petitdemange *et al.*, 1984).

It has been observed that cellulose degradation by *Clostridium thermocellum*, a thermophilic member of *Clostridium* cluster III, is accompanied by the production of a pigment called yellow affinity substance (Ljungdahl *et al.*, 1983). It has been suggested that this pigment may facilitate the binding of endoglucanase (produced by the cells) to cellulose, operating as a signal substance helping the cells to attach to cellulose fibres (Ljungdahl *et al.*, 1983). Yellow pigment formation by another cellulolytic clostridium belonging to *Clostridium* cluster III, i.e. the moderately thermophilic species *Clostridium straminisolvens* (Kato *et al.*, 2004), is known to occur. The pigment is also produced by a species belonging to *Clostridium* cluster IV, namely *Ruminococcus flavefaciens* (Kopečný & Hodrová, 1997; Willems & Collins, 1995).

The 16S rRNA gene sequence similarity between strain CDT-1^T and the most closely related species, *C. hungatei*, is 96.6%, indicating that it is unlikely that these organisms have more than 70% identity in terms of DNA–DNA hybridization (Stackebrandt & Goebel, 1994). Thus, on the basis of cellular and physiological differences between strain CDT-1^T and closely related species, strain CDT-1^T represents a novel species in *Clostridium* cluster III, for which the name *Clostridium sufflavum* sp. nov. is proposed.

Table 1. Some characteristics of strain CDT-1^T and related species

Strains: 1, CDT-1^T; 2, *C. hungatei* ATCC 700212^T (data from Monserrate *et al.*, 2001); 3, *C. termitidis* DSM 5398^T (Hethener *et al.*, 1992); 4, *C. papyrosolvans* DSM 2782^T (Madden *et al.*, 1982). +, Positive; –, negative; w, weak reaction.

Characteristic	1	2	3	4
Isolation source	Methanogenic reactor treating waste from cattle farms	Moist soil rich in decaying plant material	Gut of wood-feeding termite	Estuarine sediments
Cell shape	Slightly curved rods	Slightly curved rods	Slightly curved rods	Straight rods
Flagellation	Peritrichous	Subpolar	Peritrichous	Peritrichous
Growth temperature (°C)				
Optimum	33	30–40	37	25–30
Range	20–33	20–45	20–48	15–37
Gram-staining	–	–	+	–
Aesculin hydrolysis	–	+	+	+
Substrate utilization				
Arabinose	–	–	–	+
Lactose	–	–	+	–
Maltose	–	–	+	–
Mannose	–	+	+	–
Ribose	w	–	+	+
DNA G + C content (mol%)	40.7	40.0	39.2	30.0

Description of *Clostridium sufflavum* sp. nov.

Clostridium sufflavum (suf.flā'vum. L. neut. adj. *sufflavum* light yellow, referring to the production of a yellow pigment).

Cells are strictly anaerobic, peritrichously flagellated, slightly curved rods with oval ends and are 0.5–0.6 µm in diameter and 2.0–5.0 µm in length. Cells occur singly or in pairs. Colonies on PY4S agar are white, translucent and irregular with smooth surfaces. When grown on filter paper as an insoluble cellulose substrate, cells produce a yellow pigment. Cells stain Gram-negative, but react Gram-positively in the KOH test. Although spore formation is not observed for cells grown in PYG liquid and in PY4S agar-slant cultures and cells do not grow after treatment at 80 °C for 10 min, thermotolerant cells do occur when growth takes place in media with filter-paper fragments. Growth occurs at 20–33 °C (optimally at 33 °C) and pH 5.9–8.2 (optimally at pH 7.4). NaCl tolerance is very low: growth does not occur in PYG liquid medium containing NaCl at 5 g l⁻¹. Negative for oxidase, catalase and nitrate-reducing activities. Xylose, fructose, glucose, cellobiose, xylooligosaccharide, cellulose (filter-paper fragments and ball-milled filter paper) and xylan are utilized as growth substrates. Ribose, carboxymethylcellulose and sorbitol are weakly utilized. Arabinose, galactose, mannose, rhamnose, lactose, maltose, melibiose, sucrose, trehalose, melezitose, raffinose, glycogen, inulin, pectin, inositol, mannitol, amygdalin and salicin are not utilized. Acetate, ethanol, H₂ and CO₂ are produced as major fermentation products from the saccharides used. Aesculin and starch are not hydrolysed. Indole, hydrogen sulfide, lecithinase and lipase are not produced. Milk is unchanged and no growth occurs in chopped-meat broth. The cell-wall peptidoglycan contains *meso*-diaminopimelic acid. The major cellular fatty acids are iso-C_{15:0}, iso-C_{14:0} and C_{16:0} DMA. No respiratory quinones are present.

The type strain, CDT-1^T (=JCM 14807^T=DSM 19573^T), was isolated from rice-straw residue collected from a methanogenic reactor treating waste originating in cattle farms in Japan. The genomic DNA G+C content of the type strain is 40.7 mol%.

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