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

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

**Abstract**

A strictly anaerobic, mesophilic, cellulolytic bacterial strain (CDT-1$^T$) was isolated from rice-straw residue in a methanogenic reactor treating waste from cattle farms by using enrichment culture with filter paper as a substrate. Cells stained Gram-negative, but reacted Gram-positively by the KOH test. Cells were slightly curved rods and motile by peritrichous flagella. The strain produced yellow pigment when grown on filter paper fragments. Although spore formation was not confirmed by microscopic observation of cells, the strain produced thermotolerant cells when 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 negative. The strain utilized xylose, fructose, glucose, cellobiose, xylooligosaccharide, cellulose (filter paper fragments and ball-milled filter paper) and xylan. Major fermentation products were acetate, ethanol, $H_2$ and $CO_2$. The major cellular fatty acids were iso-$C_{15:0}$, iso-$C_{14:0}$ and $C_{16:0}$ DMA. Cell wall contained *meso*-DAP in amino acids of peptidoglycan. The genomic DNA G+C content was 40·7 mol%. On the basis of 16S rRNA gene sequence similarities, strain CDT-1$^T$ was placed in cluster III of the genus *Clostridium*, and was closely related to *Clostridium hungatei* (96·6%), *Clostridium termitidis* (96·2%) and *Clostridium papyrosolvens* (96·1%). Based on the differences in cellular, physiological and phylogenetic characteristics of strain CDT-1$^T$ from those of closely related species, a novel species, *Clostridium sufflavum* sp. nov., is proposed to accommodate the strain. The type strain is CDT-1$^T$ (=JCM 14807$^T$ =DSM 19573$^T$).

**MAIN TEXT**

In anaerobic condition, cellulose, the most abundant organic matter in plant biomass, is hydrolyzed by cellulolytic microorganisms, and then soluble sugars produced are converted to various organic acids,

alcohols, $H_2$ and $CO_2$ by fermentative microorganisms. Methane is finally produced by methanogens using acetate or $H_2$ + $CO_2$ as major substrates. Thus, the hydrolysis of cellulose is a key reaction for efficient methanogenesis from waste containing plant biomass as a dominant component.

Because of the importance in industry as well as the ecological role, many cellulolytic anaerobic bacteria have been isolated and the properties of the cellulolytic enzymes have been also investigated extensively. Some *Clostridium* species, which represent anaerobes producing a highly active cellulolytic and xylanolytic complex termed cellulosome, are classified into *Clostridium* cluster III according to 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 from rice-straw residue in a methanogenic reactor will be described.

Strain CDT-$1^T$ was isolated from a rice-straw residue sample obtained from a methanogenic reactor treating waste collected from cattle farms (up to 1000 cattle as a total) in Betsukai-machi in Hokkaido, Japan. The reactor was a vertically-cylindrical type (1500 $m^3$) operated at mesophilic temperature. Rice-straw used as matting of the cattle farms was thrown into the reactor together with feces and urine of animals and treated as waste.

The strain was cultivated anaerobically at 30˚C unless otherwise stated by using peptone/yeast extract (PY) medium as basal medium with oxygen-free mixed gas (95% $N_2$/ 5% $CO_2$) 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 $l^{-1}$ of glucose (PYG medium) was used for the cultivation of the strain for various physiological tests and chemotaxonomic analyses of the cells unless otherwise stated (Holdeman *et al*., 1977). For enrichment culture and isolation of cellulolytic 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 1M NaOH.

Anaerobic sludge samples obtained from the reactor were filtrated through a mesh (2 mm of pore size) and rice-straw residue remained on the mesh was collected. The rice-straw samples obtained were washed several times with sterile anoxic diluent and homogenized by a Waring blender (10000 rpm, 10 min.) under $N_2$ gas (Kaku *et al*., 2000). The homogenized samples were successively diluted anaerobically and the enrichment culture was started by inoculating 1 ml of 10-fold diluted samples into 9 ml of 1/10PY-p liquid medium (medium containing 2 g $l^{-1}$ of filter paper fragments). When the filter paper fragments in the medium were completely disintegrated, 1 ml of the 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 the anaerobic roll tubes using 1/10PY-c agar medium (medium containing 5 g $l^{-1}$ of ball-milled filter paper instead of paper fragments in 1/10PY-p medium). After incubation for 2-3 weeks, clear zones indicating decomposition of ball-milled filter paper appeared in the roll tube agar. Some colonies making clear zones were picked up and strain CDT-$1^T$ was finally obtained after purification by the anaerobic roll tube method (Hungate, 1966).

Growth of the strain under the aerobic condition 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 observation of cells after Gram-staining as well as by phase-contrast microscopy. Production of thermotolerant cells was examined by growth in PYG medium of cells previously exposed to 80˚C for 10 min. The motility of the cells was examined by 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) added at 10 g $l^{-1}$. Other substrates (polysaccharides and glycosides) were added at 5 g $l^{-1}$. Utilization of each substrate was determined by growth measured by $OD_{660}$ as well as by change of the pH values of the medium after cultivation. Fermentation products were analyzed by GC as described previously (Ueki *et al*., 1986; Akasaka *et al*., 2003a). Other characterizations were performed according to the methods as described by Holdeman *et al*. (1977) and Ueki *et al*. (2006a, b).

Whole-cell fatty acids (CFAs) were converted to methyl esters according to the method of Miller (1982) and analyzed by GC (Hp6890; Hewlett-Packard or G-3000; Hitachi) equipped with a HP Ultra 2 column. CFAs were identified by equivalent chain-length (ECL) (Miyagawa *et al*., 1979; Ueki & Suto, 1979) according to the protocol of TechnoSuruga Co., Ltd (Shimadu, 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 as described by Akasaka *et al*. (2003b) was digested with P1 nuclease by using a YAMASA GC kit (Yamasa shoyu) and its

G+C content was measured by HPLC (HITACHI L-7400) equipped with a μBondapack C18 column (3·9 × 300 mm; Waters).

PCR amplification of almost full-length of 16S rRNA gene was carried out by using a primer set of 27f and 1492r (Akasaka *et al*., 2003a). PCR-amplified 16S rRNA gene was sequenced by using a Thermo Sequenase Cycle Sequencing kit (USB Corporation) 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 with the neighbour-joining method (Saitou & Nei, 1987) by using 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 of incubation of 1/10PY-p liquid medium inoculated with a homogenized rice-straw sample, the filter paper fragments in the medium became light yellow and the fibers began to disperse slowly. After further incubation for about 20 days, the filter paper was completely solubilized. The culture was transferred to the fresh medium and an enrichment culture decomposing filter paper completely within 20 days of incubation was obtained after more two subcultures. Strain CDT-1$^T$ was finally obtained from the culture by picking up a colony producing a clear zone in 1/10PY-c agar medium by using 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 of strain CDT-1$^T$ occurred singly or in pairs (Fig. 1a). Cells of strain CDT-1$^T$

stained Gram-negative, but reacted Gram-positively by the KOH test. Cells were motile as observed under phase-contrast microscopy, and flagella-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 by microscopy (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 5 g $l^{-1}$ of filter paper fragments) grew after the heat treatment. Thus, we concluded that the cells of the strain produced thermotolerant spores. Both catalase and oxidase activities were not detected.

The strain utilized xylose, fructose, glucose, cellobiose, xylooligosaccharide, cellulose (fragments and powder of filter paper) and xylan as growth substrates. Weak growth was observed on ribose, CMC and sorbitol. When the strain was cultivated in PY-p medium, filter paper fragments became yellowish after 7-10 days, and then visible degradation of filter paper started. After about 12 days of incubation, filter paper fragments were completely dispersed in the medium and the pH value of the medium lowered to 6·5. The pH value finally decreased to pH 5·7 during further 20 days of incubation. The final pH of the culture in PYG liquid medium was 5·6. The highest growth rate ($\mu$ = 0·064 $h^{-1}$) was obtained on cellobiose among the soluble substrates utilized, 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 and salicin. The major products of strain CDT-1$^T$ from glucose (10 g $l^{-1}$) after 4 days incubation were acetate (11·2 mmol $l^{-1}$), ethanol (4·0 mmol $l^{-1}$), $H_2$ (14·7 mmol $l^{-1}$) and $CO_2$ (11·4 mmol $l^{-1}$). The strain produced 13·9 mmol $l^{-1}$ of acetate and 8·0 mmol $l^{-1}$ of

ethanol from cellobiose (10 g $l^{-1}$), and 9·7 mmol $l^{-1}$ of acetate and 3·4 mmol $l^{-1}$ of ethanol from filter paper fragments (5 g $l^{-1}$) (30 days of incubation). The strain did not reduce nitrate. Aesculin and starch were not hydrolyzed. Indole, hydrogen sulfide, lecithinase and lipase were not produced. The strain did not change milk and did not grow in chopped meat broth.

Temperature range for growth was 20-33˚C with an optimum at 33˚C. pH range for growth was 5·9-8·2 with an optimum at pH 7·4. NaCl tolerance of strain CDT-$1^T$ was low and it did not grow in PYG liquid medium containing 5 g $l^{-1}$ of NaCl. The growth rate in PYG liquid medium under the above mentioned optimum condition (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 dimethylacetals (DMA) were detected as major components of CFAs 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 $C_{16:0}$ aldehyde (2·4%). Cell wall of strain CDT-$1^T$ contained *meso*-DAP in amino acids of peptidoglycan. Respiratory quinones were not detected.

Analysis of an almost-complete 16S rRNA gene sequence (1436 bp) of strain CDT-$1^T$ assigned the strain to the phylum *Firmicutes*, *Clostridia*, *Clostridiales*, *Clostridiaceae*. The closest relative of strain CDT-$1^T$ found in GenBank was *Clostridium hungatei* ATCC 700212$^T$ (Monserrate *et al*., 2001), one of members of cluster III of cellulolytic *Clostridium* (Collins *et al*., 1994), with a 16S rRNA gene sequence similarity of 96·6%. The next closely related species of strain CDT-$1^T$ were also cellulolytic members of *Clostridium* cluster III,

*Clostridium termitidis* DSM 5398$^T$ (96·2%) (Hethener *et al*., 1992), *Clostridium papyrosolvens* DSM 2782$^T$ (similarity of 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 as well as physiologically, it had some distinctly different characteristics. Cells of strain CDT-1$^T$ had 5-8 peritrichous flagella, but the cells of *C. hungatei* have only one or two subpolar flagella (Monserrate *et al*., 2001) and the cells of *C. termitidis* also have only 2-3 peritrichous flagella (Hethener *et al*., 1992). Strain CDT-1$^T$ did not grow at the temperature higher than 35˚C, but *C. hungatei*, *C. termitidis* and *C. papyrosolvens* grow at the temperatures up to 45, 48 and 37˚C, respectively. Substrates utilized by strain CDT-1$^T$ differed from those of related species (Table 1).

Although the cells of strain CDT-1$^T$ appeared to have a Gram-positive type of cell wall based on the KOH test, cells of strain CDT-1$^T$ as well as the related species except *C. termitidis* stain Gram-negative. Cell morphology of strain CDT-1$^T$, as well as other related species except *C. papyrosolvens*, is slightly curved rods. Thus, these cellular characteristics (Gram-negative and slightly curved rods) seem to be rather common properties to the cellulolytic species in *Clostridium* cluster III. Although cells of *Clostridium* species generally produce spores and spore formation of the closely related species of strain CDT-1$^T$ has been also confirmed microscopically, we could not observe spores for strain CDT-1$^T$ (Fig. 1a) and the cells grown in PYG medium did not tolerate at 80˚C for 10 min. The strain may rarely form spores during the growth on

soluble saccharides, although thermotolerant cells occurred in the culture grown on filter paper. Similar rare formation of spores is also reported for *C. cellulolyticum* (Petitdemange *et al*., 1984).

Ljungdahl *et al*. (1983) have observed that cellulose degradation by *Clostridium thermocellum*, a thermophilic member of *Clostridium* cluster III, is accompanied by the production of yellow affinity substance (YAS). It has been thought that this pigment facilitates binding of endoglucanase produced by cells to cellulose and operates as a signal substance helping the bacterial cells to attach to cellulose fibers (Ljungdahl *et al*., 1983). Yellow pigment formation by cellulolytic clostridia is also known for another species belonging to *Clostridium* cluster III, moderately thermophilic *Clostridium straminisolvens* (Kato *et al*., 2004), as well as for a species in *Clostridium* cluster IV, *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 the DNA-DNA hybridization (Stackebrandt & Goebel, 1994). Thus, based on the differences in cellular and physiological characteristics between strain CDT-1$^T$ and the closely related species, we propose that strain CDT-1$^T$ should be assigned to a novel species of *Clostridium* cluster III as *Clostridium sufflavum* sp. nov.

**Description of *Clostridium sufflavum* sp. nov.**

*Clostridium sufflavum* (suf. fla' vum. L. neut. adj. *sufflavum* light yellow, referring to its yellow pigment production).

Cells are strictly anaerobic, peritrichously flagellated, slightly curved rods with oval ends, 0·5-0·6 μm in diameter and 2·0-5·0 μm in length, occurring 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 yellow pigment. Cells stain Gram-negative, but react Gram-positively with the KOH test. Although spore formation is not observed for cells grown in PYG liquid and PY4S agar slant cultures and the cells do not grow after treatment at 80˚C for 10 min, thermotolerant cells occur when grown on filter paper fragments. Grows at 20-33˚C (optimum 33˚C), pH 5·9-8·2 (optimum pH 7·4). NaCl tolerance is very low; does not grow in PYG liquid medium containing 5 g $l^{-1}$ of NaCl. Oxidase, catalase and nitrate-reducing activities are negative. Utilizes xylose, fructose, glucose, cellobiose, xylooligosaccharide, cellulose (filter paper fragments and ball-milled filter paper) and xylan as growth substrates. Ribose, CMC and sorbitol are slightly utilized. Does not use arabinose, galactose, mannose, rhamnose, lactose, maltose, melibiose, sucrose, trehalose, melezitose, raffinose, glycogen, inulin, pectin, inositol, mannitol, amygdalin and salicin. Acetate, ethanol, $H_2$ and $CO_2$ are produced as major fermentation products from saccharides used. Aesculin and starch are not hydrolyzed. Indole, hydrogen sulfide, lecithinase and lipase are not produced. Milk is unchanged and no growth occurs in chopped meat broth. The genomic DNA G+C content is 40·7 mol%. Cell wall contains *meso*-DAP in amino acids of peptidoglycan. The major cellular fatty acids are iso-$C_{15:0}$, iso-$C_{14:0}$, $C_{16:0}$ DMA. Does not have any respiratory quinones.

The type strain, CDT-$1^T$ (=JCM $14807^T$=DSM $19573^T$), was isolated from rice-straw residue in a methanogenic reactor treating waste from cattle farms in Japan.

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**Legends for figures**

**Fig. 1.** Phase-contrast photomicrograph of cells of strain CDT-1$^T$ on an agar slant of PY4S medium (a). Photomicrograph of flagella-stained cells of strain CDT-1$^T$ (b). Bar, 10 μm.

**Fig. 2.** Neighbour-joining tree, based on 16S rRNA gene sequences showing the phylogenetic relationship of strain CDT-1$^T$ and related species in the genus *Clostridium* (group III). Bootstrap values (based on analysis of 1000 replicates) are shown at branch nodes. Bar, estimated difference of 2% in nucleotide sequence positions. The sequence of *Bacillus subtilis* ATCC 6051$^T$, which belongs to the class *Bacilli*, was used as the outgroup.

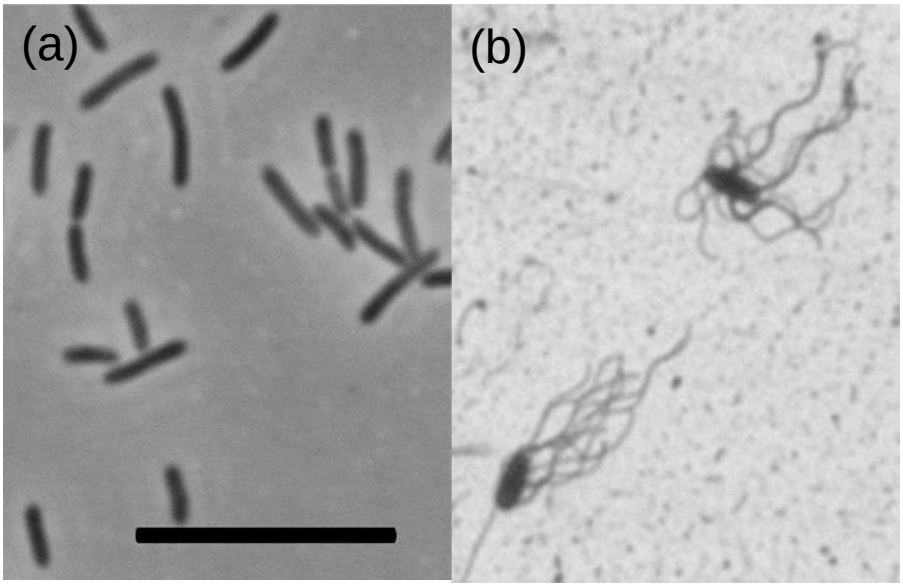
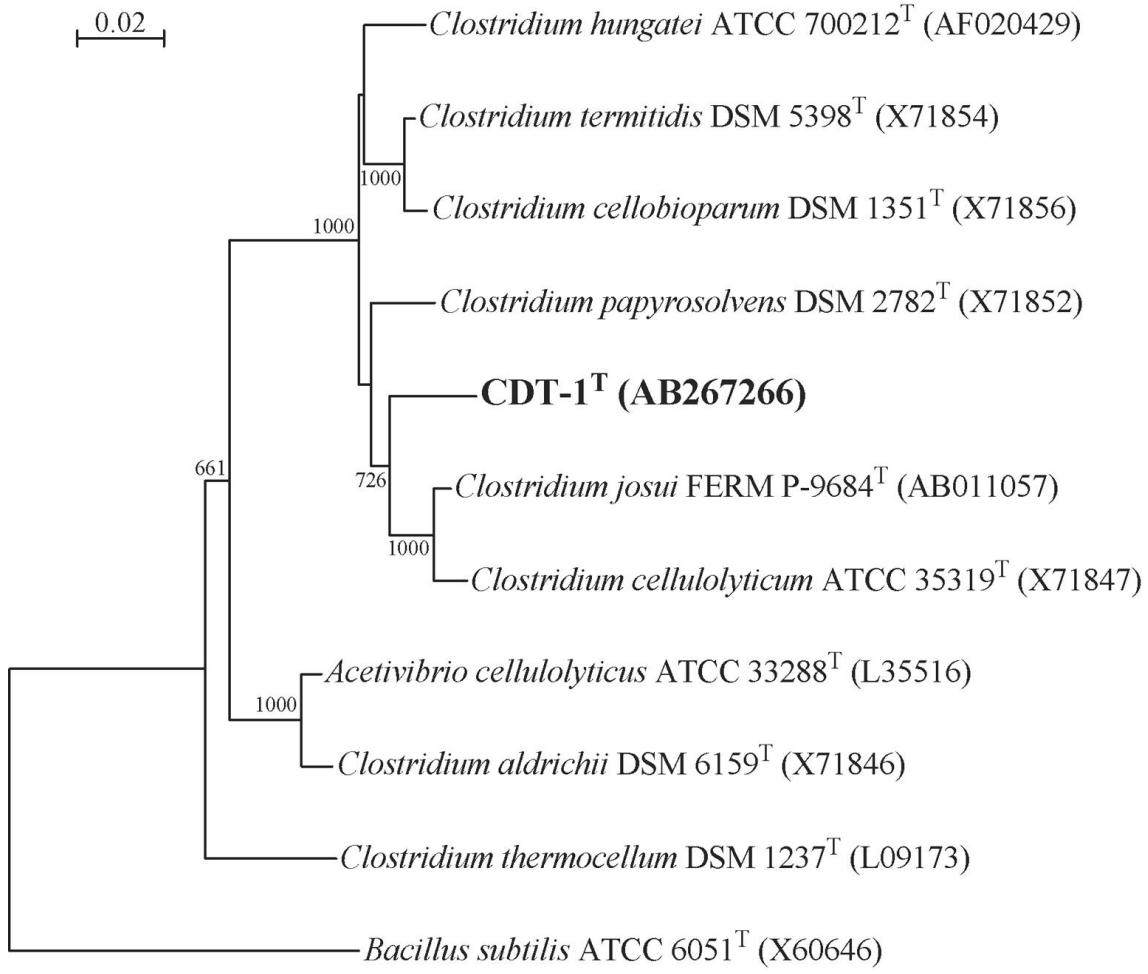



Fig. 1.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Clostridium termitidis DSM 5398T (X71854)
1000
Clostridium cellobioparum DSM 1351T (X71856)
1000
Clostridium papyrosolvens DSM 2782T (X71852)
CDT-1T (AB267266)
661
726
Clostridium josui FERM P-9684T (AB011057)
1000
Clostridium cellulolyticum ATCC 35319T (X71847)
Acetivibrio cellulolyticus ATCC 33288T (L35516)
1000
Clostridium aldrichii DSM 6159T (X71846)
Clostridium thermocellum DSM 1237T (L09173)
Bacillus subtilis ATCC 6051T (X60646)

Fig. 2.

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

Strains: 1, CDT-1$^T$; 2, *Clostridium hungatei* ATCC 700212$^T$ (data from Monserrate *et al*., 2001); 3, *Clostridium termitidis* DSM 5396$^T$ (Hethener *et al*., 1992); 4, *Clostridium papyrosolvens* 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 | Moist soil rich in decaying plant material | Gut of the wood-feeding termite | Estuarine sediments |
| Cell shape | Slightly curved rods | Slightly curved rods | Slightly curved rods | Straight rods |
| Flagellation | Peritrichous | Subpolar | Peritrichous | Peritrichous |
| Temperature (°C) (optimum/range) | 33/20-33 | 30-40/20-45 | 37/20-48 | 25-30/15-37 |
| Gram-staining | - | - | + | - |
| Aesculin hydrolysis | - | + | + | + |
| Substrate utilization : | | | | |
| Arabinose | - | - | - | + |
| Ribose | w | - | + | + |
| Mannose | - | + | + | - |
| Lactose | - | - | + | - |
| Maltose | - | - | + | - |
| DNA G+C (mol%) | 40.7 | 40.0 | 39.2 | 30.0 |