

Clostridium phytofermentans sp. nov., a cellulolytic mesophile from forest soil

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An obligately anaerobic, mesophilic, cellulolytic bacterium, strain ISDg^T, was isolated from forest soil. Cells of this isolate stained Gram-negative, despite possessing a Gram-positive cell-wall ultrastructure, and were motile, straight rods that formed spherical terminal spores that swelled the sporangium. Cellulose, pectin, polygalacturonic acid, starch, xylan, arabinose, cellobiose, fructose, galactose, gentiobiose, glucose, lactose, maltose, mannose, ribose and xylose supported growth. The major end products of fermentation were ethanol, acetate, CO₂ and H₂; formate and lactate were minor products. The optimum temperature for growth was 35–37 °C. Phylogenetic analyses based on 16S rRNA sequence comparisons showed that strain ISDg^T was related to a group of anaerobes that included *Clostridium herbivorans*, *Clostridium polysaccharolyticum* and *Clostridium populeti*. The G+C content of this strain was 35.9 mol%. On the basis of numerous genotypic and phenotypic differences between strain ISDg^T and its close relatives, strain ISDg^T is proposed as a novel species in the genus *Clostridium*, for which the name *Clostridium phytofermentans* sp. nov. is proposed. The type strain is ISDg^T (= ATCC 700394^T).

Keywords: *Clostridium phytofermentans*, cellulolytic, cellulose fermentation, ethanol production, forest soil

INTRODUCTION

Since cellulose is the most abundant organic material on Earth, the microbes involved in its breakdown are of interest both in ecological terms, for their importance in the global carbon cycle, and in economic terms, for their role in processes involving the conversion of cellulosic wastes to valuable products such as ethanol and organic acids (Ljungdahl & Eriksson, 1985; Leschine, 1995). Although most of the cellulose produced globally each year is broken down aerobically, there are many anaerobic environments in which cellulose decomposition occurs. Anaerobic cellulolytic bacteria have been isolated from such diverse habitats as soils, sediments and estuarine muds (Madden *et al.*, 1982; Murray *et al.*, 1986; He *et al.*, 1991; Monserrate *et al.*, 2001), anaerobic digestors and the rumens or intestines of various mammals. As part of a study of the diversity of cellulolytic microbes from soils and sediments, we isolated several anaerobic

strains from a wide range of locations. One isolate was sufficiently different from previously described species to warrant a detailed characterization. In this report, the isolation and characterization of a novel cellulolytic species of *Clostridium* from forest soil is described. The name *Clostridium phytofermentans* sp. nov. is proposed for this isolate, with strain ISDg^T as the type strain.

METHODS

Media and culture conditions. The anaerobic techniques of Hungate (1969) were used, unless specified otherwise. Medium GS-2C, used for enrichment, isolation and routine cultivation of strain ISDg^T, was derived from GS-2 of Johnson *et al.* (1981) and contained the following (g l⁻¹): ball-milled cellulose (Leschine & Canale-Parola, 1983), 6.0; yeast extract, 6.0; urea, 2.1; K₂HPO₄, 2.9; KH₂PO₄, 1.5; MOPS, 10.0; trisodium citrate dihydrate, 3.0; cysteine hydrochloride, 2.0; resazurin, 0.001; and the pH was adjusted to 7.0. In medium GS-2CB, cellulose was replaced with 3.0 g cellobiose l⁻¹, added as a filter-sterilized solution to the sterile medium. GS-2 agar media were supplemented with 15 g agar l⁻¹ and soft-agar media contained 7.5 g agar l⁻¹.

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain ISDg^T is AF020431.

Medium MI, which was used in growth and characterization studies, was a modified version of GS-2 that contained no yeast extract or urea but was supplemented with the following (g l^{-1}) as growth factors: tryptone (Difco), 2.0; adenine, 0.02; cytosine, 0.05; guanosine, 0.02; thymine, 0.05; uracil, 0.04. After autoclaving, 10 ml sterile vitamin solution (Wolin *et al.*, 1964) was added per litre medium.

Broth cultures were incubated in an atmosphere of O_2 -free N_2 at 30 °C. Cultures on plates of agar media were incubated at room temperature in an atmosphere of $\text{N}_2/\text{CO}_2/\text{H}_2$ (83:10:7) in an anaerobic chamber (Coy Laboratory Products).

Isolation procedure. Strain ISDg^T was isolated from damp silt in the bed of an intermittent stream in a forested site near Quabbin Reservoir in Massachusetts (USA). A soil sample was inoculated into GS-2C medium and incubated at 30 °C for 2 weeks. Following two transfers into fresh medium, the culture was diluted and inoculated into tubes containing 4 ml melted GS-2C soft-agar medium, which was poured onto plates of GS-2 basal agar medium. Strain ISDg^T was isolated by following the procedure of Warshaw *et al.* (1985). Colonies that produced clear zones in cellulose overlays were streaked and restreaked several times on plates of GS-2CB agar medium and finally transferred back into tubes of GS-2C broth to determine whether the isolate was cellulolytic.

Substrate utilization and temperature- and pH-optima studies. The ability of strain ISDg^T to utilize various soluble compounds as fermentable substrates was determined by measuring the turbidity of cultures in medium MI containing the potential substrate, as described previously (Leschine & Canale-Parola, 1983; Monserrate *et al.*, 2001). Soluble substrates were added to tubes of MI medium as filter-sterilized solutions, to a final concentration of 2 g l^{-1} , and pebble-milled cellulose (Leschine & Canale-Parola, 1983) was added, before autoclaving, to a final concentration of 6 g l^{-1} .

The optimum temperature and pH for growth were determined in MI medium. For determination of the optimum pH, MOPS in MI was replaced with an equimolar quantity of MES, TAPS or CHES, and the proportions of K_2HPO_4 and KH_2PO_4 were altered to obtain a range of pH values outside the range of MOPS buffer.

Biochemical characterization and antibiotic sensitivity. Biochemical tests were performed by following the procedures outlined by Smibert & Krieg (1994). Antibiotic sensitivity was tested using antibiotic discs on plates of GS-2CB agar medium, as described previously (Monserrate *et al.*, 2001). The antibiotics tested included ampicillin, chloramphenicol, erythromycin, kanamycin, neomycin, penicillin, rifampicin, streptomycin, tetracycline and vancomycin. Liquid fermentation products were determined by HPLC using an Aminex HPX 87H column at 4 °C with 0.0025 M H_2SO_4 as the running buffer, an ISCO model 2300 HPLC pump and a Spectra Physics SP8430 refractive index detector. Ethanol was also determined by means of an enzymic assay (Sigma kit no. 332-A). Production of CO_2 and H_2 was measured by GC with a Shimadzu GC-8A apparatus fitted with a silica gel 60/80 column (Alltech) and a thermal conductivity detector. The carrier gas was helium at a flow rate of 12 ml s^{-1} and the column temperature was 60 °C. H_2 production was also analysed using an RGD2 reduction gas analyser (Trace Analytical) equipped with a Carbosieve S-II column (Supelco) and a type 50 rotary sampling valve (Rheodyne) with a 0.1 ml sampling loop. N_2 was the carrier gas, at a flow rate of 20–30 ml min^{-1} .

Morphology. Cells were examined by phase-contrast and electron microscopy. Cells were prepared for electron microscopy using methods similar to those described previously (Paster & Canale-Parola, 1982; Monserrate *et al.*, 2001). Cells from liquid cultures were negatively stained with 1% uranyl acetate. Cells from plates of GS-2CB agar medium were used for preparation of thin sections. Negatively stained preparations and thin sections were examined using a Philips CM10 transmission electron microscope.

Comparative analyses of 16S rRNA sequences. The 16S rRNA from strain ISDg^T was isolated and sequenced using methods essentially as described by Paster & Dewhirst (1988). The RNA was extracted and partially purified by the method of Pace *et al.* (1982). Nucleotide sequences of the rRNA were determined by using the dideoxynucleotide technique (Lane *et al.*, 1985), as modified by Paster & Dewhirst (1988). A nearly complete sequence was determined using DNA primers (primers 3–9; Dewhirst *et al.*, 1992) that were complementary to conserved regions of the rRNA molecule. A putative secondary structure for the strain ISDg^T sequence was examined for anomalies by comparison with a previously deduced secondary structure for a 16S rRNA sequence from the related Gram-positive bacterium *Clostridium innocuum*. The percentage similarity of the strain ISDg^T sequence to each of its closest neighbours was calculated manually.

Closely related sequences of 16S rRNA were identified in the GenBank database using BLAST (Altschul *et al.*, 1997; Benson *et al.*, 1993) and in the Ribosomal Database Project, version 8.0, using SEQUENCE_MATCH and SEQUENCE_ALIGN (Maidak *et al.*, 2000). The most closely related sequences derived from type strains of recognized species, as well as representative sequences from less closely related organisms, were obtained from GenBank and aligned with the PILEUP algorithm available in the Wisconsin Package (version 10) with graphical user interface (SEQLAB) (Genetics Computer Group). The alignment was verified and further refined manually. Only those nucleotide positions that could be aligned unambiguously (1298 nt) were used for further analysis. Phylogenetic trees, including rate-corrected versions, were constructed using distance, parsimony and maximum-likelihood methods available in version 4.0b2 of PAUP* (Swofford, 1998). Bootstrap analyses were used to evaluate the robustness of the inferred tree topologies recovered.

DNA base composition. DNA was isolated and purified by the method of Marmur (1961), as modified by Johnson (1994), from cells grown for 48 h in GS-2CB. The G+C content was determined by HPLC using the method of Mesbah *et al.* (1989).

RESULTS AND DISCUSSION

Colony and cellular morphology

Strain ISDg^T, cultured for 1 week on plates of GS-2CB agar medium, formed round, glossy, translucent colonies (2–5 mm in diameter) with slightly raised centres. The colony margins became somewhat undulate in older colonies.

Under phase-contrast microscopy, cells of strain ISDg^T appeared as long, thin, straight, motile rods (0.5–0.8 × 3.0–15.0 μm , occasional cells reaching 33 μm in length) and formed round, terminal spores (0.9–

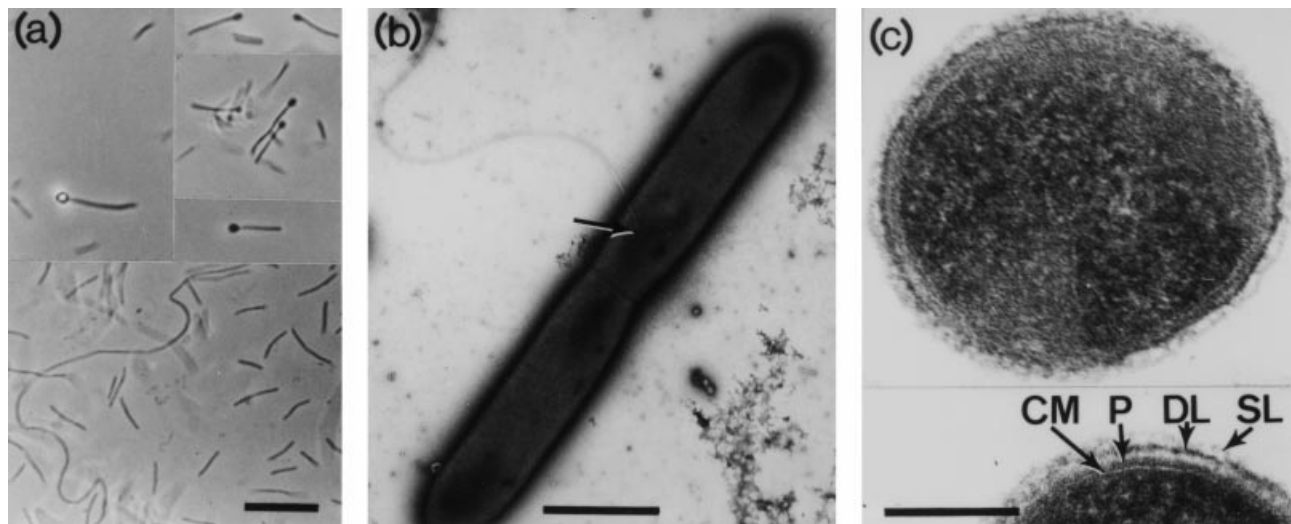


Fig. 1. Morphology of strain ISDg^T. (a) Phase-contrast micrographs of cells from a 1-day-old culture in GS-2CB broth, showing a sporulating cell, several cells in which the sporangium has begun to swell but spores have not yet formed and an exceptionally long cell. Bar, 10 µm. (b) Transmission electron micrograph of a negatively stained cell from a 2-day-old culture in MICB medium (MI medium plus cellobiose), showing attachment of single flagellum (arrow). Bar, 1 µm. (c) Transmission electron micrograph of thin sections of cells cultured for 1 week on plates of GS-2CB agar medium. The cell membrane (CM) is surrounded by a relatively thin, densely staining layer, presumably peptidoglycan (P), which, in turn, is surrounded by additional layers, including a densely staining layer (DL) and a more diffuse surface layer (SL). Bar, 0.1 µm.

1.5 µm in diameter) that caused swelling of the cells (Fig. 1a). Cells were usually single and only rarely occurred in pairs. Electron micrographs of negatively stained cells revealed that cells had one or two usually subterminal flagella. A flagellated cell is shown in Fig. 1(b). Electron micrographs of thin sections of cells showed that the cytoplasmic membrane was surrounded by a multilayered cell envelope composed of a relatively thin electron-dense (peptidoglycan) layer and at least two additional exterior layers (Fig. 1c). Cells from logarithmic- and stationary-phase cultures of strain ISDg^T stained Gram-negative, although cell walls were not surrounded by outer membranes typical of Gram-negative cells (Fig. 1c).

Physiological and metabolic characteristics

Growth of strain ISDg^T was supported by arabinose, cellobiose, cellulose, fructose, galactose, gentiobiose, glucose, lactose, maltose, mannose, pectin, polygalacturonic acid, ribose, starch, xylan and xylose. Strain ISDg^T did not grow with glycerol, pyruvate, sucrose, trehalose or tryptone as substrate. Very poor growth occurred in MI medium without purines and pyrimidines, without vitamins or without tryptone. Apparently, strain ISDg^T required amino acids and/or peptides as a source of nitrogen, inasmuch as addition of either NH₄Cl or urea did not stimulate growth. Strain ISDg^T was negative in tests for urease, aesculin hydrolysis, nitrate reduction and H₂S production.

When cultured with either cellulose or cellobiose as substrate, strain ISDg^T produced acetate, ethanol,

CO₂ and H₂ as major products and formate and lactate as minor products. Growth in MI medium containing 0.3% cellobiose resulted in the production of the following (mmol product per 100 mmol cellobiose, mean of nine determinations): ethanol (265), acetate (109), lactate (49), formate (15).

Anaerobic conditions were required for growth of strain ISDg^T. The optimum temperature for growth was 37 °C. Cells grew slowly but to a relatively high density at 15 °C. Growth was poor at 42 °C and no growth occurred at 5 or 45 °C. Growth was observed at pH 6.0–9.0 but was very poor at pH 9.5. Cells did not grow at pH 5.5. The maximum growth rate occurred when the initial pH was 8.0 and the maximum growth yield was achieved when the initial pH was 8.5. Strain ISDg^T was resistant to kanamycin and streptomycin.

Phylogeny

Phylogenetic analysis using distance, parsimony and maximum-likelihood methods produced highly congruent inferred tree topologies. Each analysis clearly placed strain ISDg^T in cluster XIVa of the genus *Clostridium* (Collins *et al.*, 1994), with *Eubacterium xylanophilum* and *Clostridium populeti* as the nearest neighbours (Fig. 2). The sequence similarities between strain ISDg^T and *E. xylanophilum* and *C. populeti* were respectively 93 and 92% over 1392 nt. However, the terminal branching between strain ISDg^T and *E. xylanophilum* was not well resolved, as revealed by the low bootstrap support at this node (Fig. 2), indicating that the order of speciation events cannot be de-

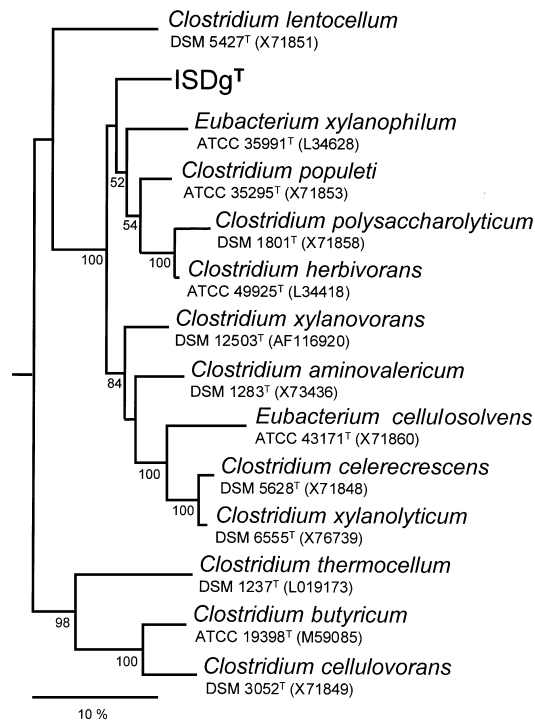


Fig. 2. Phylogeny of strain ISDg^T and related species within cluster XIVa of the low-G+C-content Gram-positive bacteria, as determined by a maximum-likelihood analysis of 16S rRNA sequences. *Clostridium butyricum* is included as the type species and *Clostridium cellulovorans*, *Clostridium thermocellum* and *Clostridium lentocellum* are included as cellulolytic representatives of clusters I, III and XIVb, respectively, of the genus *Clostridium* (Collins *et al.*, 1994). The tree was rooted using *Escherichia coli* as the outgroup. Bootstrap values greater than 50%, expressed as percentages of 100 replications, are shown at the branching points. Bar, 10 substitutions per 100 sequence positions.

terminated using the 16S rRNA gene as a phylogenetic marker.

G+C content of the DNA

The G+C content of the DNA of strain ISDg^T was 35.9 ± 0.2 mol%, based on five determinations.

Distinguishing characteristics

Some of the characteristics that distinguish strain ISDg^T from other cellulolytic members of *Clostridium* clusters XIVa and XIVb (Collins *et al.*, 1994) are presented in Table 1. Strain ISDg^T may be distinguished from two of its close cellulolytic relatives, *Clostridium herbivorans* and *Clostridium polysaccharolyticum* (van Gylswyk, 1980; van Gylswyk *et al.*, 1980), by its ability to utilize glucose, galactose, lactose and mannose as well as by its end products. Both *C. herbivorans* and *C. polysaccharolyticum* produce formate and butyrate as major products, whereas strain ISDg^T produced acetate and ethanol. Strain ISDg^T is

also relatively closely related to *C. populeti* (Sleat & Mah, 1985), but can be readily distinguished from it by the fact that *C. populeti* has a much lower G+C content (28 mol%) and produces butyrate and lactate as major fermentation products. According to 16S rRNA phylogenetic analyses, strain ISDg^T is less closely related to the other cellulolytic members of *Clostridium* cluster XIV but, in terms of substrate utilization and end products, strain ISDg^T appears to be more similar to *Clostridium celerecrescens* (Palop *et al.*, 1989) and *Clostridium lentocellum* (Murray *et al.*, 1986). Strain ISDg^T can be distinguished from *C. celerecrescens* most easily by the much wider range of fermentation end products produced by *C. celerecrescens* and by the fact that *C. celerecrescens* does not ferment starch. Strain ISDg^T differs from *C. lentocellum* in that the former did not ferment pyruvate, sucrose or trehalose, whereas the latter does, and the former fermented mannose and ribose, whereas the latter does not. Strain ISDg^T and *C. lentocellum* also differ in cell morphology. Cells of strain ISDg^T were long, straight rods (typically 0.6–0.7 µm in diameter and 3–15 µm long), while cells of *C. lentocellum* are smaller, shorter, slightly curved rods (typically 0.3–0.5 µm in diameter and 2.5–4 µm long). *Eubacterium cellulosolvens* (van Gylswyk & van der Toorn, 1986) has a much higher G+C content than strain ISDg^T and also does not produce either acetate or ethanol. Three other close relatives of ISDg^T, *Clostridium aminovalericum* (Hardman & Stadtman, 1960), *Clostridium xylanovorans* (Mechichi *et al.*, 1999) and *E. xylanophilum* (van Gylswyk & van der Toorn, 1985), can be distinguished from strain ISDg^T by their inability to use cellulose as a growth substrate. Strain ISDg^T may be distinguished from most of the other mesophilic, cellulolytic clostridia by its ability to utilize starch.

Strain ISDg^T was obligately anaerobic, formed endospores, did not carry out dissimilatory sulfate reduction and, although cells stained Gram-negative, they possessed a Gram-positive-type cell envelope structure. These results indicate that strain ISDg^T represents a species of *Clostridium* as this genus is currently defined. Phenotypic and phylogenetic characteristics of strain ISDg^T readily distinguish it from other cellulolytic clostridia. Therefore, it is concluded that strain ISDg^T represents a novel species of the genus *Clostridium*, for which the name *Clostridium phytofermentans* sp. nov. is proposed. Presumably, following a proposed taxonomic revision of the genus *Clostridium* (Collins *et al.*, 1994), members of cluster XIVa will receive a new genus epithet, assuming that *Clostridium* is retained for members of cluster I.

Description of *Clostridium phytofermentans* sp. nov.

Clostridium phytofermentans (phy.to.fer.men'tans. Gr. n. *phyton* plant; L. part. adj. *fermentans* fermenting; N.L. part. adj. *phytofermentans* plant-fermenting, re-

Table 1. Characteristics that differentiate strain ISDg^T from phylogenetically related cellulolytic bacteria

Strains: 1, strain ISDg^T (= ATCC 700394^T); 2, *C. celerecrescens* CECT 954^T; 3, *C. herbivorans* ATCC 49925^T; 4, *C. lentocellum* NCIB 11756^T; 5, *C. polysaccharolyticum* ATCC 33142^T; 6, *C. populeti* ATCC 35295^T; 7, *E. cellulosolvans* ATCC 43171^T. All species are members of *Clostridium* clusters XIVa and XIVb as defined by Collins *et al.* (1994). All are motile and all ferment cellulose, cellobiose and maltose. Abbreviations: A, acetate; B, butyrate; C, caproate; E, ethanol; F, formate; I, isobutyrate; L, lactate; M, 3-methylbutanoate; S, succinate; v, *n*-valerate; +, positive; -, negative; +/-, weak, or some strains are positive while others are negative; NR, not reported. Data for species other than ISDg^T are from Palop *et al.* (1989), Varel *et al.* (1995), Murray *et al.* (1986), van Gylswyk (1980), van Gylswyk *et al.* (1980), Sleat & Mah (1985) and van Gylswyk & van der Toorn (1986).

Characteristic	1	2	3	4	5	6	7
Gram stain	-	+	+	-	-	-	+ to -
G + C content (mol%)	36	38	38	36	42	28	50
End products	A, E, F, L	A, B, C, E, F, I, L, M, S	B, E, F	A, E, L	B, E, F	A, B, L	B, F, L, V
Optimum temperature	37	30-37	39-42	40	30-38	35	37-39
Fermentation of:							
Arabinose	+/-	+	-	+	+	+	-
Fructose	+/-	+	-	+	+/-	+	+/-
Galactose	+	+	-	+	-	+	+
Glucose	+	+	-	+	-	+	+
Lactose	+	+/-	-	+	-	-	+
Mannose	+	+	-	-	-	-	-
Pectin	+/-	NR	-	NR	+/-	+	+
Ribose	+	+	-	-	NR	NR	NR
Starch	+	-	+	+	-	-	-
Sucrose	-	+/-	-	+	-	+	+
Trehalose	-	+	-	+	-	-	-
Xylan	+	NR	-	+	+	+	-
Xylose	+	+	-	+	+	+	-

ferring to the wide range of plant polysaccharides that this organism is capable of utilizing as growth substrate).

Cells are straight rods (0.5-0.8 × 3-15 µm), usually single or in pairs. Cells are motile, with one or two (usually subterminal) flagella per cell. Round, terminal spores (0.9-1.5 µm in diameter) are produced that make the sporangium swell. Cells stain Gram-negative and have a multilayered cell wall. Surface colonies (in GS-2CB agar medium) are round, glossy and translucent with slightly raised centres measuring 2-5 mm in diameter; margins become somewhat undulate in older colonies. Obligate anaerobe. Fermentable compounds include cellulose, pectin, polygalacturonic acid, starch, xylan, arabinose, cellobiose, fructose, galactose, gentiobiose, glucose, lactose, maltose, mannose, ribose and xylose. The following compounds do not support growth: glycerol, pyruvate, sucrose, trehalose and tryptone. Nitrate and sulfate are not reduced. Aesculin is not hydrolysed. Urease-negative or very weakly positive. The optimum temperature for growth is 37 °C; growth occurs at pH values ranging from 6.0 to 9.0. The major end products of cellulose fermentation are ethanol, acetate, CO₂ and H₂; the minor end products are formate and lactate. Phylogenetically, a member of cluster XIVa of the low-G+C-content Gram-positive bacteria. The G+C content of the DNA is 35.9 ± 0.2 mol%. Isolated

from forest soil. The type strain is strain ISDg^T (= ATCC 700394^T).

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