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Susan Leschine, University of Massachusetts - Amherst
T. A Warnick

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

Thomas A. Warnick, Barbara A. Methé and Susan B. Leschine

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.
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), 20; adenine, 0.02; cysteine, 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 N\(_2\)/CO\(_2\)/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 KH\(_2\)PO\(_4\) in MI was replaced with an equimolar quantity of KH\(_2\)PO\(_4\) in an anaerobic chamber (Coy Laboratory Products).

**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 (Alschul 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 MUSCLE 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.

**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 x 3.0–15.0 µm), occasional cells reaching 33 µm in length) and formed round, terminal spores (0.9–
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 ISDgT 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 ISDgT was supported by arabinose, cellobiose, cellulose, fructose, galactose, gentiobiose, glucose, lactose, maltose, mannose, pectin, polygalacturonic acid, ribose, starch, xylan and xylose. Strain ISDgT 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 ISDgT required amino acids and/or peptides as a source of nitrogen, inasmuch as addition of either NH₄Cl or urea did not stimulate growth. Strain ISDgT was negative in tests for urease, aesculin hydrolysis, nitrate reduction and H₂S production.

When cultured with either cellulose or cellobiose as substrate, strain ISDgT 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 ISDgT. 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 ISDgT 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 ISDgT 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 ISDgT and E. xylanophilum and C. populeti were respectively 93 and 92% over 1392 nt. However, the terminal branching between strain ISDgT 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-
Clostridium lentocellum

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 ISDgT is less closely related to the other cellulolytic members of Clostridium cluster XIV but, in terms of substrate utilization and end products, strain ISDgT appears to be more similar to Clostridium celerecrescens (Palop et al., 1989) and Clostridium lentocellum (Murray et al., 1986). Strain ISDgT 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 ISDgT 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 ISDgT and C. lentocellum also differ in cell morphology. Cells of strain ISDgT 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 ISDgT and also does not produce either acetate or ethanol. Three other close relatives of ISDgT, Clostridium aminovalericum (Hardman & Stadtman, 1960), Clostridium xylanovorans (Mecchini et al., 1999) and E. xylanophilum (van Gylswyk & van der Toorn, 1985), can be distinguished from strain ISDgT by their inability to use cellulose as a growth substrate. Strain ISDgT may be distinguished from most of the other mesophilic, cellulolytic clostridia by its ability to utilize starch.

Strain ISDgT 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 ISDgT represents a species of Clostridium as this genus is currently defined. Phenotypic and phylogenetic characteristics of strain ISDgT readily distinguish it from other cellulolytic clostridia. Therefore, it is concluded that strain ISDgT 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.menˈtans. Gr. n. phyton plant; L. part. adj. fermentans fermenting; N.L. part. adj. phytofermentans plant-fermenting, re-
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 x 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 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 the 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 ISDgT (= ATCC 700394T).

ACKNOWLEDGEMENTS

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REFERENCES


Table 1. Characteristics that differentiate strain ISDgT from phylogenetically related cellulolytic bacteria

<table>
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Clostridium phytofermentans sp. nov.


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sp. nov. and Fusobacterium polysaccharolyticum sp. nov. and S. B. Leschine & S. B. Leschine (1983).


