Clostridium hungatei sp. nov., a mesophilic, N₂-fixing cellulolytic bacterium isolated from soil

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Two strains of obligately anaerobic, mesophilic, cellulolytic, N₂-fixing, spore-forming bacteria were isolated from soil samples collected at two different locations near Amherst, MA, USA. Single cells of both strains were slightly curved rods that measured between 2 and 6 µm in length and approximately 0.5 µm in diameter. The spores were spherical, terminally located, distended the sporangium and measured 0.8–1.0 µm in diameter. The cells of both isolates (designated strain AD[†] and strain B3B) stained Gram-negative, but did not have a typical Gram-negative cell wall structure as demonstrated by transmission electron microscope analysis. The cells of both strains were motile with subpolarly inserted flagella and exhibited chemotactic behaviour towards cellobiose and D-glucose. Both strains fermented cellulose, xylan, cellobiose, cellodextrins, D-glucose, D-xylose, D-fructose, D-mannose and gentiobiose. In addition, strain B3B fermented L-arabinose. For both strains, fermentation products from cellulose were acetate, ethanol, H₂ and CO₂, as well as small amounts of lactate and formate. The G+C content of strain AD was 40 mol% and that of strain B3B was 42 mol%. Based on their morphological, physiological and phylogenetic characteristics, it was concluded that the two isolates are representatives of a novel species of *Clostridium*. The name *Clostridium hungatei* is proposed for the new species. The type strain of *Clostridium hungatei* sp. nov. is strain AD[†] (= ATCC 700212[†]).

Keywords: *Clostridium hungatei*, mesophilic, N₂-fixing, cellulolytic anaerobic bacterium, cellulase/xylanase activity

INTRODUCTION

Approximately 7.5×10^{10} tons of cellulose, the most abundant biopolymer on Earth, are produced by photosynthesis every year (Ljungdahl & Eriksson, 1985). Although it is commonly believed that the bulk of this material is degraded in aerobic environments, up to 5–10% is degraded under anaerobic conditions by physiologically diverse bacteria (Coughlan & Mayer, 1991; Fenchel & Jorgensen, 1977; Leschine, 1995; Ljungdahl & Eriksson, 1985; McInerney & Beaty, 1988; Wolin & Miller, 1983; and references therein). In nature, cellulose is primarily found in plant

cell walls where it is embedded in a matrix consisting of xylan, other hemicelluloses and lignin. Many anaerobic cellulolytic bacteria from soils and sediments secrete cellulases and xylanases that associate into high molecular mass complexes (Cavedon *et al.*, 1990a, b, Gal *et al.*, 1997; Lamed & Bayer, 1988; Wolin & Miller, 1983). Because of the relative abundance of cellulose in nature, the micro-organisms involved in its degradation play an important role in the carbon cycle. In addition, interest in research on anaerobic cellulose degradation has increased because of the potential for converting large amounts of photo-synthetically produced cellulosic material into industrial substrates.

Many of the anaerobic environments in which cellulose is degraded (i.e. peat soils, water-logged sediments, municipal and agricultural wastes) are deficient in combined nitrogen (Leschine, 1995; Postgate, 1982). Anaerobic cellulolytic bacteria that fix N₂ have been

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Abbreviation: CMCCase, carboxymethylcellulase.

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reported to be widespread in nature (Leschine *et al.*, 1988). Several previously described obligately anaerobic cellulolytic species [i.e. *Clostridium papyrosolvens* (Madden *et al.*, 1982), *Clostridium papyrosolvens* C7 (Leschine & Canale-Parola, 1983), strain JW-2 (Warshaw *et al.*, 1985)] exhibited ammonium-repressible nitrogenase activity (Leschine *et al.*, 1988). In this report, the isolation and characterization of two strains of a new N_2 -fixing cellulolytic species of *Clostridium*, isolated from samples of soil rich in decaying plant material, are described. The name *Clostridium hungatei* is proposed for these isolates, with strain AD^T as the type strain.

METHODS

Culture media. The basal medium used in this study contained (g l⁻¹): KH_2PO_4 , 2.39; Na_2HPO_4 , 0.98; $MgSO_4 \cdot 7H_2O$, 0.25; $CaCl_2 \cdot 2H_2O$, 0.15; $FeSO_4$, 0.003; $NaMoO_4$, 0.01; L-ascorbic acid, 0.3; sodium thioglycolate, 0.3; and 1 ml resazurin solution (0.1%, w/v). The pH of the medium was adjusted to 7.2. In addition, 10 ml sterile vitamin solution (Wolin *et al.*, 1963) was added to the medium after autoclaving. The medium containing cellulose in a growth-limiting concentration (DNF) was prepared by adding a cellulose slurry to a final concentration of 0.2% (w/v). The medium containing cellulose in excess (DNF_x), used for enrichment cultures and for agar overlays, was prepared by adding the cellulose slurry to a final concentration of 0.8% (w/v). The source of cellulose was a slurry prepared by wet-ball milling Whatman no. 1 filter paper (3 g per 100 ml distilled water) (Leschine & Canale-Parola, 1983). Media containing a soluble sugar as the carbon and energy source (e.g. medium DNF_{cb}, containing cellobiose) were prepared by adding a sterile solution of the desired sugar to the basal medium to a final concentration of 0.2% (w/v). All media were sterilized by autoclaving. Some of the cultures were grown in a medium containing 1.0 g NH_4Cl l⁻¹ (DNF + N). Solid media and overlays contained 10.0 g Noble agar l⁻¹ (Difco).

Anaerobic methods, measurement of growth and cellulose utilization. Anaerobic techniques of Hungate (1950) were used throughout this study, unless specified otherwise. Cultivation of strains AD^T or B3B was in pre-reduced medium DNF contained in 18 × 150 mm test tubes sealed with rubber stoppers crimped into place with aluminium seals (Balch *et al.*, 1979) in an N_2 atmosphere. Growth was measured by direct cell counts using a Petroff-Hausser counting chamber. Residual cellulose was measured by means of the gravimetric method of Weimer & Zeikus (1977). Growth in media containing soluble sugars was determined by measuring the OD of the cultures at 660 nm using a spectrophotometer (Spectronic 20; Milton Roy).

Sources of organisms. Medium DNF_x, lacking a source of combined nitrogen and containing cellulose as the carbon and energy source, was used for the enrichment of strain AD^T. Test tubes (18 × 150 mm), each containing 10 ml medium DNF_x, were inoculated with a small amount of soil collected from under a pile of rotting wood chips. The cultures were incubated without shaking at 30 °C in air. After approximately 15 d, the amount of cellulose in some of the cultures had decreased considerably, as determined by visual observation. Sediment (0.1 ml) from cellulose-utilizing cultures was inoculated into test tubes containing medium

DNF_x. The cellulose-utilizing cultures obtained after incubation were transferred repeatedly in the same manner at intervals of 1–2 weeks. These cultures consisted of a heterogeneous microbial population which converted cellulose to methane and other products in the absence of combined nitrogen.

Strain AD^T was isolated from one of these cellulose-utilizing mixed cultures. Serial dilutions of samples from a mixed culture were prepared in melted DNF_x agar medium and used to inoculate overlay basal medium agar plates in an anaerobic chamber (Coy Laboratory Products) containing an atmosphere of 80% nitrogen, 13% carbon dioxide and 7% hydrogen (by vol.). After incubating the plates for approximately 2 weeks at 30 °C in the anaerobic chamber, zones of clearing appeared on the opaque cellulose-containing overlay medium. A small amount of material from these zones was transferred to 5 ml basal solution and then heated at 80 °C for 15 min before streaking for cell isolation on cellobiose-containing agar medium DNF_{cb}. The inoculated DNF_{cb} plates were incubated at 30 °C in the anaerobic chamber until isolated colonies became visible (approx. 15 d).

Strain B3B was isolated from forest soil as described by Leschine *et al.* (1988). *Clostridium cellulolyticum* ATCC 35319^T was obtained from the ATCC.

Morphology. Cells examined by phase-contrast or electron microscopy were grown in a medium containing NH_4Cl . Cells were negatively stained for electron microscopy by a method similar to that described by Paster & Canale-Parola (1982) except that a uranyl acetate solution (1%, w/v; pH of 4.5) was used. Thin sections of cells were prepared as previously described (Paster & Canale-Parola, 1982). Negatively stained preparations and thin sections were examined using a Phillips CM10 transmission electron microscope operating at 80 kV.

Biochemical reactions and antibiotic sensitivity. Biochemical assays for catalase production, nitrate reduction, sulfate reduction, urease production, gelatin or casein hydrolysis, blood haemolysis and lipase production were performed by procedures described by Holdeman *et al.* (1977). Cells grown to mid-exponential phase in medium DNF_{cb} were used for the tests. Antibiotic sensitivity was tested by placing an antibiotic disk (Difco) on a plate of medium DNF_{cb} which had been inoculated with one of our isolates and allowing them to grow in the anaerobic chamber. The antibiotics tested were: rifampin, streptomycin, penicillin, erythromycin, vancomycin, tetracycline, ampicillin, kanamycin, neomycin and chloramphenicol. A ring of no growth around the bacterial lawn determined the sensitivity to the antibiotic.

Temperature and pH optima studies. The optimum temperature and pH for growth were determined in both DNF_{cb} and DNF_{cb} + N media cultures. For determination of the optimum growth temperature, cultures were incubated at an initial pH of 7.2. For determination of the optimum pH, cultures were grown at 30 °C.

Enzyme and protein assays. Avicelase activity was determined using the method of Johnson *et al.* (1982) by measuring the decrease in turbidity of a suspension of Avicel (microcrystalline cellulose, 20 µm particles; FMC) incubated at 42 °C in N_2 . Reaction mixtures were as described previously by Cavedon *et al.* (1990a). A unit of Avicelase activity was defined as the amount of enzyme that hydrolysed 10.0 µg Avicel per h.

Carboxymethylcellulase (CMCase) and xylanase activities were assayed by determining the production of reducing sugars (Miller *et al.*, 1960; Pohlschröder *et al.*, 1994) from either carboxymethylcellulose or soluble xylan after incubation of the reaction mixtures for 20 min at 42 °C. A unit of CMCase or xylanase activity was defined as the amount of enzyme that released 1.0 µmol reducing sugar (expressed in glucose or xylose equivalents, respectively) per min.

Ammonium-repressible nitrogenase activity was demonstrated by means of the acetylene reduction assay (Postgate, 1972). Cells of strain AD^T, B3B and *C. cellulolyticum* were grown and assayed as described by Leschine *et al.* (1988), except that medium DNF was used to grow our strains.

Protein concentration was measured by the method of Bradford (1976) with the Bio-Rad protein assay kit; BSA was used as the protein standard.

DNA base composition analysis. DNA was purified by the method of Marmur (1961) and its G+C content was determined by thermal denaturation using the method described by Mandel & Marmur (1968). Thermal denaturation was carried out with a Perkin-Elmer model Lambda 4B UV/VIS spectrophotometer equipped with a temperature program controller. *Escherichia coli* K-12 DNA was used as standard.

Analytical methods. The production of acetate, ethanol and lactate by strains AD^T and B3B cells grown in DNF or DNF+N media was determined by GLC. A Shimadzu Mini-GC fitted with a 10% SP 1200, 1% phosphoric acid, 80/100 Chromosorb WAW column (Supelco) was used to separate fermentation products present in culture supernatant samples. The steel injection block and a flame-ionization detector were operated at 190 °C and the column was operated at 120 °C. A 1 ml supernatant sample was pre-treated with 2 M phosphoric acid (100 µl) and periodic acid (365 µl) in order to oxidize lactate to acetaldehyde (Brotz & Schaefer, 1987; Teunissen *et al.*, 1989). Formate was detected by the colorimetric method of Sleat & Mah (1984). Soluble sugars were measured by HPLC (Spectra-Physics) fitted with an Aminex HPX-87P (Bio-Rad) column and a refractive index detector. Reducing sugars were determined by the dinitrosalicylic acid reducing sugar assay (Miller *et al.*, 1960). Gaseous products were measured by GC with a Shimadzu model GC-8A GC fitted with a thermal conductivity detector. H₂ and CO₂ gas standards were obtained from Supelco.

Isolation and SDS-PAGE analysis of cellulase systems. Cultures of strains AD^T, B3B and *C. cellulolyticum* (Petitdemange *et al.*, 1984) were grown in medium DNF+N. Cells were pelleted by centrifugation (20 min, 5000 g, 4 °C) after most of the cellulose was utilized (approx. 6 d incubation for all cultures). Following methods described previously (Pohlschröder *et al.*, 1994), Avicelase-active enzyme systems were isolated from culture supernatants by means of stirred-cell ultrafiltration and gel-filtration chromatography, and enzyme samples were analysed by SDS-PAGE using a PhastSystem (Pharmacia). Prior to PAGE analyses, enzyme samples were boiled for 5 min in sample buffer (Laemmli, 1970). Samples containing approximately 0.1 µg protein were applied to 4–15% linear gradient polyacrylamide gels (PhastGels; Pharmacia). Molecular mass standards were obtained from Bio-Rad.

Phylogenetic analysis based on 16S rRNA. Procedures used to sequence 16S rRNA from strains AD^T and B3B were

essentially the same as those described by Paster & Dewhirst (1988). RNA was isolated and partially purified by the method of Pace *et al.* (1982) and nucleotide sequences of rRNA were determined by the reverse transcriptase dideoxynucleotide method (Lane *et al.*, 1985) following the modifications of Paster & Dewhirst (1988). Nearly complete sequences were obtained using seven DNA primers complementary to conserved regions of the rRNA molecule [primers 3–9 (Dewhirst *et al.*, 1992)]. Nearest relatives of strains AD^T and B3B were identified by submitting the 16S rRNA sequences to the SUGGEST_TREE program of the Ribosomal Database Project (Maidak *et al.*, 1997). Reference sequences most related to the newly generated sequences were extracted from the GenBank database, with *E. coli* included as an outgroup. Sequences were aligned using the program PILEUP of the GCG package (Devereux *et al.*, 1984), alignment was verified manually and positions of uncertain alignment were omitted from analyses. Evolutionary distances, based on 1384 nt positions, were computed by the method of Jin & Nei (1990) and neighbour-joining phylogenetic trees were inferred using TREECON (Van de Peer & De Wachter, 1994). Maximum-likelihood and parsimony trees were constructed using version 4.0b2 of PAUP* (Swofford, 1998). Phylogenetic trees generated using different methods for dendrogram construction were essentially identical topologically.

RESULTS

Cell and colony morphology

Cells of the two isolates were similar in morphology (Fig. 1a, b). The cells were slightly curved rods measuring mostly between 2 and 6 µm in length and approximately 0.5 µm in diameter. When grown on agar medium DNFcb, cells of both isolates formed spherical terminal spores measuring 0.8–1.0 µm in diameter (Fig. 1c). The isolates stained Gram-negative; however, they did not exhibit the ultrastructure typical of Gram-negative cell walls. Electron micrographs (Fig. 2a) showed that the cytoplasmic mem-

Fig. 1. Phase-contrast photomicrographs of strain AD^T (a), strain B3B (b), and of strain AD^T sporulating cells (c). Bar, 10 µm (all photomicrographs at same magnification). Vegetative cells (a, b) were grown in liquid medium DNFcb and the sporulating cells (c) were grown on plates of agar medium DNFcb supplemented with 1.0% (w/v) Noble agar.

Fig. 2. Transmission electron micrographs of thin sections of strain AD^T (a) and *Clostridium cellulolyticum* (b) cells stained with uranyl acetate. The cell envelope of AD^T (A) appears to consist of a cell membrane (cm) surrounded by a peptidoglycan layer (p), a middle layer (ml), a dense outer layer (dol), and a less dense outer layer (lol). The cell membrane in *Clostridium cellulolyticum* (b) is surrounded by a peptidoglycan layer and an outer layer (ol). Bar, 0.1 μ m (both micrographs at same magnification).

brane of strain AD^T was surrounded by a multi-layered cell envelope composed of an electron-dense (peptidoglycan) layer, a thick layer of low electron density (presumably a periplasmic space), an electron-dense layer and a thick electron-dense layer. In contrast, electron microscopy of the cell wall of *C. cellulolyticum* showed only one layer surrounding the peptidoglycan layer (Fig. 2b).

On cellulose agar medium overlays (medium DNFx), zones of clearing appeared 7–10 d after inoculation. These zones of clearing continued expanding until the entire plate was cleared. Distinctive colonies were not formed within the zones of clearing. However, when wet mount preparations of material from the clear zones were viewed by phase-contrast microscopy, rod-shaped bacteria were observed in large numbers. Apparently, the bacteria were able to migrate through the agar overlays and, therefore, did not form discrete colonies. On cellobiose agar medium DNFcb + N, smooth, circular, slightly raised, unpigmented, butyrous textured colonies appeared after 5–6 d incubation at 30 °C.

Motility

Cells of the isolates accumulated near or attached to cellulose fibres during growth on medium DNF (Fig. 3), possibly as a result of a chemotactic response to hydrolysis products of cellulose (Hsing & Canale-Parola, 1992). Swarm plate assays revealed that both strains are chemotactic towards cellobiose and D-

glucose (data not shown). Cells of the isolates were motile by means of one or two subpolar flagella (Fig. 4a). *C. cellulolyticum* cells had 8–10 peritrichous flagella (Fig. 4b). *C. papyrosolvans* (Madden *et al.*, 1982), *Clostridium cellobioparum* (Hungate, 1944), *Clostridium termitidis* (Hethener *et al.*, 1992) and other cellulolytic clostridia are peritrichously flagellated (Table 1).

Physiological and metabolic characteristics

Both strains AD^T and B3B were strict anaerobes as demonstrated by their inability to grow in shake cultures of liquid media. In addition, the strains only grew in solid media when incubated in the anaerobic chamber. Strain AD^T had an optimum growth temperature between 30 and 40 °C both under N₂-fixing conditions and in the presence of NH₄Cl. Maximum growth temperature was 45 °C and no growth was observed at either 50 or 15 °C. The optimum initial pH for growth in medium DNFcb was 7.2.

Both strains had ammonium-repressible nitrogenase activity. Cells reduced acetylene to ethylene when growing in the cellulose-containing medium DNF or cellobiose-containing medium DNFcb in an atmosphere of 100% N₂, but not when growing in the same medium supplemented with 0.1% (w/v) NH₄Cl. Cultures of strain AD^T and B3B in medium DNF exhibited nitrogenase specific activities of 500 and 630 nmol acetylene reduced h⁻¹ (mg cell protein)⁻¹. The strains did not grow in medium DNF or medium DNFcb in an argon atmosphere. *C. cellulolyticum* did not grow in medium DNF in the absence of combined nitrogen.

Various sources of cellulose (ball-milled filter paper, Avicel, tissue paper and Solka-Floc) were hydrolysed by strain AD^T at different rates, with Avicel being hydrolysed at the slowest rate and ball-milled filter paper at the fastest rate. Other substrates fermented by strains AD^T and B3B included cellobiose, cellotriose, cellotetraose, cellopentaose, D-glucose, D-fructose, D-mannose, D-xylose, xylan and gentiobiose. Strain B3B also fermented L-arabinose. The following substrates were not fermented: D-ribose, sucrose, maltose, lactose, glycerol, starch, pectin, polygalacturonic acids and Bacto-tryptone (Difco) (Table 1). Cells of both strains hydrolysed aesculin, but did not hydrolyse gelatin or casein and did not reduce nitrate or sulfate. In addition, they did not produce lipase or urease, and did not lyse red blood cells. Exogenous vitamins or fatty acids were not required for growth.

Products of fermentation of cellulose by strain AD^T were acetate, ethanol, H₂ and CO₂, as well as small amounts of lactate and formate (Table 2). The ratio of ethanol to acetate produced did not change significantly when the cells were growing under N₂-fixing conditions (Table 2). However, this ratio increased when the amount of initial substrate was in excess or when the concentration of H₂ accumulated in the headspace.

Fig. 3. Phase-contrast photomicrograph of strain AD^T cells entangled in cellulose fibres. Free-floating cells found in the same culture are shown in the inset. Bar, 10 µm.

Fig. 4. Transmission electron micrograph of negatively stained cells of strain AD^T (a) showing the subpolar flagella as compared to peritrichously flagellated *Clostridium cellulolyticum* (b). Bar, 1 µm (both micrographs at same magnification).

Strains AD^T and B3B grew in the presence of rifampin, streptomycin, penicillin, erythromycin and vancomycin. Growth was inhibited in the presence of tetracycline, ampicillin, kanamycin, neomycin or chloramphenicol.

In medium DNFx, which contained cellulose in excess, growth ceased when acetate accumulated and the pH dropped below 5.5. Cellulose degradation continued and reducing sugars accumulated after growth

stopped. Cellobiose constituted approximately 95 mol% of the accumulated reducing sugars, as demonstrated by HPLC analysis of culture supernatant fluids.

G+C content of the DNA

The G+C contents of the DNA of strain AD^T and strain B3B were 40 and 42 mol%, respectively.

Table 1. Differential characteristics of species of mesophilic cellulolytic clostridia

+, Ferments readily; —, does not ferment; Weak, ferments less readily; NR, not reported. Data from this study and Hethener *et al.* (1992), Hungate (1944), Madden *et al.* (1982) and Petitdemange *et al.* (1984). Strain B3B ferments L-arabinose, otherwise substrate utilization pattern was identical to strain AD^T. All species were positive for utilization of cellulose, cellobiose, D-glucose, D-xylose. All species were negative for utilization of sucrose.

Character	Strain AD ^T	<i>Clostridium cellulolyticum</i>	<i>Clostridium papyrosolvens</i>	<i>Clostridium cellobioparum</i>	<i>Clostridium termitidis</i>
Substrate utilization:					
D-Ribose	—	+	+	+	+
L-Arabinose	—	+	+	+	—
D-Mannose	+	+	—	+	+
Maltose	—	—	—	+	+
Lactose	—	—	—	+	+
Glycerol	—	—	+	Weak	Weak
Starch	—	—	—	—	NR
Pectin	—	—	—	NR	NR
Xylan	+	+	+	NR	NR
Polygalacturonate	—	—	—	NR	NR
Bacto-tryptone	—	—	—	—	NR
Gram reaction	Negative	Positive	Negative	Negative	Positive
Flagella	Subpolar	Peritrichous	Peritrichous	Peritrichous	Peritrichous
G + C (mol %)	40	41	30	28	39

Table 2. Products of cellulose fermentation by strain AD^T growing under N₂-fixing conditions or in the presence of combined nitrogen

Cells were grown in medium DNF supplemented with 0.1 % (w/v) NH₄Cl (DNF + N) or medium DNF under N₂-fixing conditions. Amount of product is expressed as μ mol product per 100 μ mol glucose equivalents. Data are means of three replicates.

Product	Amount of product	
	DNF + N	DNF
H ₂	165	192
CO ₂	131	138
Acetate	122	119
Ethanol	49	52
Lactate	18	23
Formate	14	17
Carbon recovery (%)	90	94
Oxidation-reduction balance	1.05	0.99

Analysis of 16S rRNA sequences

Nearly complete sequences of the 16S rRNA of strains AD^T and B3B were determined and compared to sequences of other cellulolytic and non-cellulolytic clostridia (Fig. 5). The 16S rRNA sequences of strains AD^T and B3B were nearly identical (similarity value of 99.5 %). Of all the species for which rRNA sequences are available in databases, the 16S rRNA sequence of strain AD^T was most similar to those of *C. cello-*

Fig. 5. Phylogenetic tree based on 16S rRNA gene sequence similarities and constructed using the neighbour-joining method, indicating the position of strain AD^T relative to representative members of cluster III of low-G + C-containing Gram-positive bacteria (Collins *et al.*, 1994; denoted by a bracket), including cellulolytic strains (with GenBank accession numbers in parentheses) *Clostridium termitidis* (X71854), *Clostridium cellobioparum* (X71856), *Clostridium papyrosolvens* (X71852), *Clostridium cellulolyticum* (X71847), *Clostridium josui* (AB011057), *Bacteroides cellulosoelvens* (L35517), *Clostridium aldrichii* (X71846), *Acetivibrio cellulolyticus* (L35516) and *Clostridium thermocellum* (L09173). The type species, *Clostridium butyricum* (M59085), and representative cellulolytic members of cluster I, *Clostridium cellulovorans* (X71849) and *Clostridium chartatabidum* (X71850), are also shown. Bootstrap values > 50 (based on 100 replications) are given at branch nodes.

Fig. 6. SDS-PAGE of the partially purified cellulase/xylanase systems of strain AD^T (a), strain B3B (b) and *C. cellulolyticum* (c). The numbers on the left represent $M_r (\times 10^3)$.

bioparum and *C. termitidis*. However, sequence similarity values for 16S rRNA from strain AD^T and from both of these species were less than 97% indicating that it is unlikely that these organism would have more than 70% DNA sequence similarity (Stackebrandt & Goebel, 1994), a result that is consistent with the conclusion that strain AD^T represents a separate species of *Clostridium*.

The cellulase system

The extracellular enzyme systems in culture supernatants of strain AD^T, strain B3B and *C. cellulolyticum* were fractionated by gel-filtration chromatography. A 650 000 M_r protein peak (A_{280}), containing Avicelase, CMCase and xylanase activities was produced by each strain (data not shown). This protein peak fraction isolated from strain AD^T was found to exhibit high Avicelase activity [163 U (mg protein)⁻¹], as well as CMCase [12.7 U (mg protein)⁻¹] and xylanase [14.0 U (mg protein)⁻¹] activities.

Samples of the enzyme systems of strain AD^T, strain B3B and *C. cellulolyticum* were analysed by SDS-PAGE. In silver-stained gels, the protein banding patterns in lanes containing samples from strain AD^T or strain B3B (Fig. 6, lanes a and b, respectively) were very similar, but they differed from the protein banding pattern in the lane which contained the sample from *C. cellulolyticum* (Fig. 6, lane c).

Cellulose degradation and growth kinetic studies

Strain AD^T cells grew at a much faster rate and to much higher cell densities in medium DNF + N (which contained NH₄Cl final concentration of 0.1%, w/v) (Fig. 7b) than under N₂-fixing conditions (Fig. 7a). However, the rate of cellulose degradation in both cultures was virtually the same (4.1 µg cellulose ml⁻¹ h⁻¹ in the N₂-fixing culture vs 4.4 µg cellulose ml⁻¹ h⁻¹ in the culture growing in the presence of NH₄Cl). Because the number of cells present in the N₂-fixing

culture was consistently smaller than that of the NH₄Cl-containing culture, it appeared that the rate of cellulose degradation *per cell* in the N₂-fixing culture (Fig. 7a) was greater than that in the culture containing combined nitrogen.

Reducing sugars were not detected in supernatant fluids of cultures growing in medium DNF + N or under N₂-fixing conditions. The amount of fermentation end products formed *per cell mass* was greater in cultures growing under N₂-fixing conditions (data not shown), when compared to cultures growing with NH₄Cl. These observations indicated that cells growing under N₂-fixing conditions were fermenting cellulose at a faster rate *per cell* than cells growing in the presence of combined nitrogen.

DISCUSSION

Strains AD^T and B3B, cellulolytic mesophiles isolated from soil rich in decaying plant material, were very similar to each other with respect to morphology, physiological characteristics, substrate fermentation pattern, fermentation end products formed, growth temperature range, antibiotic sensitivity and DNA G + C content. The numerous similarities indicate that strains AD^T and B3B are representatives of a single bacterial species. Consistent with this conclusion, analysis of 16S rRNA sequences of strains AD^T and B3B showed a 99.5% sequence similarity. Both strains were obligately anaerobic, formed endospores, did not carry out dissimilatory sulfate reduction and, although cells stained Gram-negative, they possessed a cell wall structure more similar to a Gram-positive type structure. These results indicate that strains AD^T and B3B represent a species of *Clostridium*. Similar to most cellulolytic clostridia previously described, strains AD^T and B3B did not utilize compounds other than carbohydrates as carbon and energy sources.

Comparison with other cellulolytic mesophiles indicated that, morphologically and physiologically, strains AD^T and B3B resembled *C. papyrosolvens*, *C. cellobioparum*, *C. cellulolyticum* and *C. termitidis*. In addition, a close phylogenetic relationship between strain AD^T and these other cellulolytic bacteria was indicated by analysis of 16S rRNA sequences (Fig. 5). On the basis of DNA G + C content, strains AD^T and B3B, with G + C contents of 40 and 42 mol%, respectively, can be readily distinguished from either *C. cellobioparum* or *C. papyrosolvens*, which have G + C contents of 28 and 30 mol%, respectively (Johnson & Francis, 1975; Madden *et al.*, 1982). Strains AD^T and B3B can be distinguished from *C. cellulolyticum* and *C. termitidis* based on their Gram staining reaction, the former staining Gram-negative whereas the other two species staining Gram-positive. Cell wall differences were confirmed by transmission electron microscopy, which indicated that strain AD^T has a multilayered cell wall, whereas *C. cellulolyticum* has a Gram-positive-like cell wall surrounded by an outer layer (Fig. 2) similar in structure to the cell walls of *C. termitidis*.

Fig. 7. Cellulose utilization during growth of strain AD^T at 30 °C in medium DNF under N_2 -fixing conditions (a), and growing in the presence of NH_4Cl (b). Residual cellulose was determined gravimetrically and cells were counted using a Petroff–Hausser counting chamber. Spores were not included in the counts. Sporulation did not begin until all cellulose was utilized and spore numbers were less than 5% of total counts.

(Hethener *et al.*, 1992). The species also differ in the arrangement and number of flagella, which are peritrichous in *C. cellulolyticum* (Fig. 4) and *C. termitidis* (Hethener *et al.*, 1992) and subpolar in strain AD^T. In addition, strains AD^T and B3B have ammonium-repressible nitrogenase activity and fix N_2 in medium DNF containing either cellulose or cellobiose as carbon and energy source, whereas neither *C. cellulolyticum* nor *C. termitidis* has been reported to fix N_2 .

Comparison of the protein composition of the cellulase systems of strains AD^T and B3B (Fig. 6) revealed a very similar protein banding pattern which, however, was strikingly different from that of *C. cellulolyticum* (Fig. 6).

The phenotypic and phylogenetic characteristics of the cellulolytic isolates readily distinguish them from *C. papyrosolvens*, *C. cellobioparum*, *C. termitidis* and *C. cellulolyticum*. Therefore, it is concluded that strains AD^T and B3B represent a new species of the genus *Clostridium*, for which the name *Clostridium hungatei* is proposed.

As previously discussed (Leschine *et al.*, 1988), cellulolytic bacteria are widespread in environments deficient in combined nitrogen. Cellulolytic bacteria able to fulfil their nitrogen requirements by fixing N_2 may have a selective advantage over bacteria that require a source of combined nitrogen. Furthermore, the ability to fix N_2 may allow these mesophilic bacteria to develop physiological interactions with other bacteria present in these environments (Cavedon & Canale-Parola, 1992). Our studies indicate that under conditions of combined nitrogen deprivation, the cellulose activity of N_2 -fixing bacteria *per cell mass* is significantly enhanced.

Description of *Clostridium hungatei* sp. nov.

Clostridium hungatei (hun.gat'e.i. M.L. gen. n. *hungatei* of Hungate, named after R. E. Hungate who pioneered the study of the ecology of cellulolytic bacteria).

Cells are motile, subpolarly flagellated, slightly curved rod ($0.5 \times 2.0\text{--}6.0 \mu\text{m}$) which form round terminal spores ($0.8\text{--}1.0 \mu\text{m}$) when grown in agar medium (medium DNFcb). Cells stain Gram-negative and have a multilayered cell wall. Surface colonies (in medium DNFcb + N containing 1.5 g agar per 100 ml medium) are smooth, slightly raised, circular, unpigmented, with butyrous texture and measure 1–2 mm in diameter. Optimum growth temperature is between 30 and 40 °C, both under N_2 -fixing conditions and in the presence of NH_4Cl . Maximum growth temperature is 45 °C; no growth is observed at either 50 or 15 °C. The optimum initial pH for growth in medium DNFcb is 7.2. Obligately anaerobic, cellulolytic, N_2 -fixing, utilizes carbohydrates as carbon and energy sources. Fermentable compounds include cellulose, cellobiose, cellotriose, cellotetraose, cellopentaose, D-glucose, D-fructose, D-mannose, D-xylose, xylan and gentiobiose. Strain B3B also ferments L-arabinose. The following substrates are not fermented: D-ribose, sucrose, maltose, lactose, glycerol, starch, pectin, polygalacturonic acids and Bacto-tryptone (Difco). Exogenous vitamins or fatty acids are not required for growth. Products of cellulose fermentation are H_2 , CO_2 , acetate, ethanol, lactate and formate. Cells produce an extracellular cellulase system that yields a large 650 000 M_r protein peak (A_{280}) upon fractionation by Sephacryl S-300 gel filtration. Grows in the presence of rifampin, streptomycin, penicillin, erythromycin and vancomycin. Growth is inhibited by tetracycline, ampicillin, kanamycin, neomycin or chloramphenicol. Isolated from

moist soil rich in decaying plant material. Strains AD^T and B3B are phylogenetically closely related, based on 16S rRNA sequence analysis (99.5% sequence similarity). The DNA G + C content of is 40 mol% (strain AD^T). Type strain is strain AD^T (= ATCC 700212^T).

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