Clostridium herbivorans sp. nov., a Cellulolytic Anaerobe from the Pig Intestine

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A new cellulolytic anaerobic clostridium was isolated from the intestinal tract of pigs. The single isolate was a gram-positive, motile rod, formed terminal to subterminal swollen sporangia, and required a fermentable carbohydrate for growth. Cellulose, cellobiose, maltose, starch, and glycogen supported growth, but glucose and fructose did not. The major end products from the fermentation of cellobiose were butyrate and formate; minor amounts of hydrogen and ethanol were also formed. Ruminant fluid (15%) or yeast extract (1%) was required for good growth. The optimum temperature for growth was 39 to 42°C, and the optimum pH was 6.8 to 7.2. Cell lysis occurred rapidly once stationary growth was reached. A 16S rRNA sequence analysis showed that the strain was related to a group of gram-positive anaerobes that includes *Clostridium oroticum* and the cellulolytic species *Clostridium polysaccharolyticum* and *Clostridium populeti*. The DNA base composition of the isolate is 38 mol% G+C. We propose the name *Clostridium herbivorans* for this organism; strain 54408 (= ATCC 49925) is the type strain.

The most probable numbers for the cellulolytic bacterial population in the large intestines of pigs were estimated to be 4 and 6% of the viable bacterial counts when the animals were fed low- and high-fiber diets, respectively (33). Thus, significant quantities of cellulose may be degraded in the large intestines of pigs. The volatile fatty acids produced from this degradation are absorbed by the animals and influence the energy requirements of the animals. Our understanding of the cellulolytic bacterial species that make up this population is incomplete.

The anaerobic cellulolytic bacteria that have been isolated from pig intestinal tracts include *Fibrobacter intestinalis* (*Fibrobacter succinogenes*) (18, 33), *Ruminococcus flavefaciens* (33), and an unidentified clostridial species (34). The *Clostridium* species was isolated initially from pig intestinal tracts when the ruminant cellulolytic species "*Clostridium longisporum*" (32) was fed to pigs in an effort to ascertain whether this organism can colonize the pig intestinal tract (34). In this report we provide additional characteristics of the pig intestinal clostridium and propose the name *Clostridium herbivorans*.

MATERIALS AND METHODS

The cellulolytic clostridium described in this paper was isolated from pig intestinal contents by a procedure described previously (34). This isolate was named *C. herbivorans*, was designated strain 54408^T (T = type strain), and was deposited in the American Type Culture Collection as strain ATCC 49925^T.

Biochemical characterization. Phenotypic characteristics of strain 54408^T were determined previously (34) and in this study. The media used for growth and characterization experiments were prepared under CO₂ by the Hungate anaerobic culturing method described by Bryant (2) or by the methods described by Balch and Wolfe (1). Biochemical tests were performed by using the standard procedures described by Holdeman et al. (9) and Smibert and Krieg (27).

The medium used for routine growth of the culture contained (per 100 ml) 15 ml of clarified preincubated ruminal fluid, 0.3 g of cellobiose, 0.2 g of Trypticase (BBL Microbiology Systems, Cockeysville, Md.), 0.1 g of yeast extract, 0.04 g of NH₄Cl, 5 ml of mineral S2 (25), 0.5 ml of vitamins (32), 0.0001 g of resazurin, 0.4 g of Na₂CO₃, and 0.05 g of cysteine hydrochloride. Good growth could be expected if the ruminal fluid was deleted and the concentration of yeast extract was increased to 1% (wt/vol).

The optimum pH was determined with the medium described above, except

that the Na₂CO₃ and CO₂ atmosphere were replaced with phosphate buffer and an N₂ atmosphere. In a second set of experiments we used the buffers PIPES (piperazine-*N,N'*-bis-2-ethanesulfonic acid) and HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid) (Sigma Chemical Co., St. Louis, Mo.) in the medium described above, and the concentrations of Na₂CO₃ and CO₂ were reduced to 0.04 g/100 ml and 10%, respectively; an N₂ atmosphere was used in these experiments. The PIPES-HEPES buffer system allowed the culture to grow better; however, the optimum pH for growth was the same as the optimum pH obtained with the phosphate buffer. CO₂ appeared to stimulate growth.

Microscopy. A culture of strain 54408^T was cooled on ice and fixed for 15 min with glutaraldehyde at a final concentration of 0.5%. The fixed cells were allowed to settle onto carbon-coated Formvar films on copper grids and were stained with 1% phosphotungstate (pH 7). Cellular morphology was determined with a Zeiss model EM-10 transmission electron microscope.

Cellular fatty acid analysis. The cellular fatty acid compositions of strain 54408^T and *Clostridium polysaccharolyticum* ATCC 33142^T were determined with cells grown on a cellobiose-yeast extract medium and harvested while they were in exponential growth phase. A cellular fatty acid analysis was performed by Microcheck, Inc. (Northfield, Vt.). Whole-cell fatty acids were analyzed as fatty acid methyl esters with a MIDI microbial identification system (MIDI, Inc., Newark, Del.). Fatty acid methyl esters were synthesized and analyzed as described previously (17, 20, 26).

16S rRNA sequence analysis. Strain 54408^T was grown on a cellobiose-yeast extract medium and harvested in the exponential growth phase. *Clostridium oroticum* ATCC 13619^T and *Clostridium symbiosum* ATCC 14940^T were provided by John L. Johnson, and *Eubacterium eligens* ATCC 27750^T and *Eubacterium ventriosum* ATCC 27560^T were provided by Robert L. Gherna. The 16S rRNAs of these strains were sequenced by using previously described methods (15, 22). The sequences of the other *Clostridium* and *Eubacterium* species used in this study have been published previously (4, 16, 23, 24, 35). The sequences were analyzed by using a maximum-likelihood method (21).

Nucleotide sequence accession numbers. The 16S rRNA sequences of strain 54408^T (*C. herbivorans* ATCC 49925^T), *C. oroticum*, *C. symbiosum*, *E. eligens*, and *E. ventriosum* have been deposited in GenBank database under accession numbers L34418, M59109, M59112, L394420, and L34421, respectively.

RESULTS AND DISCUSSION

Colony and cellular morphology. Electron microscopy and phase-contrast microscopy showed that cells of strain 54408^T were motile, straight rods (0.7 to 0.9 by 3.5 to 4.0 µm) that occurred in pairs or as single cells (Fig. 1) (34). The cells were peritrichous, and cells from exponential-phase cultures frequently had 15 to 20 flagella per cell. Spores were rarely observed; however, when spores were present, they were subterminal to terminal and 1 µm wide by 1 to 2 µm long and caused swelling of the cells. When strain 54408^T was grown in cellulose roll tubes, it produced two zones of clearing (Fig. 2), depend-

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FIG. 1. Transmission electron micrograph of *C. herbivorans*. Cells were fixed with glutaraldehyde, spread on a carbon-coated Formvar grid, and stained with 1% phosphotungstic acid (pH 7). Bar = 1 μ m.

ing on the location of the organism in the agar. If cells were on the surface of the agar, no visible colonies were formed and the zone of clearing was larger than the zone of clearing produced when cells were embedded in the agar. When cells were imbedded in the agar distinct colonies were produced.

Biochemical reactions. Growth of strain 54408^T was supported by cellulose, cellobiose, maltose, starch, and glycogen. In the presence of cellobiose plus yeast extract the generation time was 2.3 h at 37°C. Strain 54408^T did not utilize substrates such as glucose, Casamino Acids, fructose, pectin, sucrose, xylose, and xylan. Other substrates that did not support growth were amygdalin, arabinose, erythritol, galactose, inositol, lactose, lactate, mannitol, mannose, melezitose, melibiose, pyruvate, raffinose, rhamnose, ribose, sorbitol, and trehalose. Strain 54408^T did not digest chopped meat or reduce sulfate or nitrate and was negative for catalase, oxidase, and urease activities. It produced formate (28 mM) and butyrate (12 mM) as major fermentation products when cells were cultured with cellobiose. Ethanol (1 mM) and hydrogen (5 mM) were also

produced. Anaerobic conditions were required for growth. The optimum temperature for growth was between 39 and 42°C; however, cultures could be adapted to grow at 45°C. Growth did not occur at 25 or 55°C. The optimum pH for growth was between 6.8 and 7.2. No growth occurred at pH 5.8 or 8.0. We have isolated other strains of this organism, including some strains isolated from Meishan pigs imported from the People's Republic of China. The characteristics of two strains which were obtained from Meishan pigs and which were characterized phenotypically were similar to the characteristics of type strain 54408 (34).

Cellular fatty acid composition. The cellular fatty acid composition of strain 54408^T was distinct and was not similar to the cellular fatty acid composition of any other bacterium in the gram-positive low-G+C-content phylogenetic cluster (clostridia and clostridium-like organisms) (4, 29) in the MIDI database. The major components identified in strain 54408^T were C_{14:0} (43.8% of the total fatty acids), iso-3-OH C_{13:0} (8.9%), C_{16:0} (5.3%), iso C_{14:0} (2.2%), *cis*-9 C_{18:1} (1.4%), C_{14:0} di-

FIG. 2. Zones of clearing produced by *C. herbivorans* after 48 h in an agar roll tube (18 by 150 mm) with ball-milled filter paper as the substrate. A large zone of clearing without a colony was formed when cells were on the surface of the agar. Cells embedded in the agar produced a smaller zone of clearing with a visible colony.

methyl acetal (22.1%) and $C_{14:0}$ aldehyde (10.2%). The presence of the two latter components indicated that plasmalogen was present in the cells (11). *C. polysaccharolyticum* was phylogenetically related to strain 54408^T (see below) and was analyzed to determine its fatty acid composition since it was not included in the MIDI database. The cellular fatty acid profile of this organism was distinct from that of strain 54408^T. The major components identified in *C. polysaccharolyticum* were iso-3-OH $C_{13:0}$ (24.9% of the total fatty acids), $C_{14:0}$ (22.1%), iso $C_{14:0}$ (11.9%), $C_{16:0}$ (5.9%), $C_{15:0}$ (5.8%), anteiso $C_{15:0}$ (2.9%), iso $C_{16:0}$ (2.2%), $C_{14:0}$ dimethyl acetal (8.8%), $C_{15:0}$ anteiso dimethyl acetal (5.1%), and $C_{14:0}$ aldehyde (3.4%). The presence of the three latter components also indicated that plasmalogen is present in *C. polysaccharolyticum*.

Phylogeny. Figure 3 shows the phylogenetic relationship of strain 54408^T to other clostridia and related microorganisms, including several mesophilic, cellulolytic species (Table 1). This phylogenetic tree, which was prepared by using a maximum-likelihood method (21), is essentially identical topographically to the tree prepared with a distance matrix method (5, 13, 22) previously used by us (data not shown).

Strain 54408^T was more closely related to *C. polysaccharolyticum* than to any of the other species whose sequences were deposited in the rRNA database (16). However, the 16S rRNA sequences of strain 54408^T and *C. polysaccharolyticum* differ by

FIG. 3. Phylogeny of *C. herbivorans* 54408^T as determined from a maximum-likelihood (21) 16S rRNA sequence analysis. The bar indicates the average number of base changes per position in the sequences compared. C., *Clostridium*; A., "*Acetitomaculum*"; and E., *Eubacterium*.

3.3%, indicating that these organisms belong to different species (28).

Strain 54408^T is a member of a group of clostridia, designated cluster XIVa by Collins et al. (4), which includes "*Acetitomaculum ruminis*" (6), *Clostridium celerecrescens*, *C. oroticum*, *Clostridium populeti*, *C. symbiosum*, *E. eligens* (10), and *E. ventriosum*. This group of clostridia is phylogenetically distinct from clostridial rRNA homology group I defined by Johnson and Francis (12), which was called cluster I by Collins et al. (4), was represented in this study by *Clostridium butyricum*, "*Clostridium chartatabidum*," and *Clostridium pasteurianum*, and also includes the cellulolytic species *Clostridium cellulovorans* and "*Clostridium longisporum*." Clostridial rRNA homology group II of Johnson and Francis (12), which was designated cluster cluster XI by Collins et al. (4), is represented in Fig. 3 by *Clostridium lituseburense* and *Clostridium mayombeii*. The phylogeny of the other cellulolytic clostridia has been discussed by Collins et al. (4) and was specifically examined by Rainey and Stackebrandt (24). Strain 54408^T is phylogenetically distinct from the other mesophilic, cellulolytic clostridia.

Distinguishing characteristics. Some of the characteristics which distinguish *C. herbivorans* from phylogenetically related species and the mesophilic cellulolytic clostridia are listed in Table 1. The closest relative, phylogenetically and phenotypically, of *C. herbivorans* is *C. polysaccharolyticum*. Both species utilize cellulose, cellobiose, maltose, and starch, although they do not utilize glucose. The distinguishing characteristics include G+C content (38 and 42 mol%) and the fermentation of

TABLE 1. Characteristics that differentiate *C. herbivorans* from phylogenetically related bacteria and the mesophilic cellulolytic clostridia

Organism ^a	G+C content (mol%)	End products ^b	Motility	Fermentation of ^c :									
				Arabinose	Glucose	Cellobiose	Cellulose	Fructose	Galactose	Maltose	Starch	Xylose	Xylan
<i>C. herbivorans</i>	38	F,B,e	+	—	—	+	+	—	—	+	+	—	—
<i>C. polysaccharolyticum</i>	42	F,B,e	+	+	—	+	+	+/-	—	+	+	+	+
<i>C. aldrichii</i>	40	A,P,B,L,S	+	—	—	+	+	—	NR	—	—	—	+
<i>C. celerecrescens</i>	38	A,E,F,B,L,S	+	+	+	+	+	+	+	+	—	+	NR
<i>C. cellobioparum</i>	28	A,E,l,f	+	+	+	+	+	+	+	+	—	+	NR
<i>C. celluloferrum</i>	34	A,E	+	—	+	+	+	+	+	+	—	+	NR
<i>C. cellulolyticum</i>	41	A,E,l,f	+	+	+	+	+	+	W	—	—	+	NR
<i>C. cellulovorans</i>	27	B,a,f	—	—	+	+	+	+	+	+	—	—	+
“ <i>C. chartatabidum</i> ”	31	A,B,E	+	NR	+	+	+	+	—	—	—	+	—
<i>C. josui</i>	40	A,E,p,b	—	+	+	+	+	—	—	+	—	+	+
<i>C. lentocellum</i>	36	A,E,l	+	+	+	+	+	+	+	+	+	+	+
“ <i>C. longisporum</i> ”	33	F,A,B,e	+	—	+	+	+	+	+	—	—	—	—
<i>C. papyrosolvans</i>	30	A,L,E	+	+	+	+	+	+	+	—	NR	+	NR
<i>C. populeti</i>	28	B,a,l	+	+	+	+	+	+	+	+	—	+	+
<i>C. termitidis</i>	39	A,E	+	—	+	+	+	+	+	+	—	+	NR
<i>C. oroticum</i> ^d	44	A,E,l,f	—	+/-	+	+/-	—	+	NR	+	—	+	NR
<i>C. symbiosum</i> ^e	46	A,B	—	+	+	—	—	+	+	—	—	+	NR
<i>E. ventriosum</i> ^f	NR	F,b,l,a	—	+/-	+	+/-	—	+	+	+	+	+/-	NR

^a See reference 7 and the references cited in reference 7 for other characteristics.

^b F and f, formate; A and a, acetate; P and p, propionate; B and b, butyrate; L and l, lactate; S, succinate; E and e, ethanol. Uppercase letters indicate major end products, and lowercase letters indicate minor end products.

^c +, positive; —, negative; +/-, weak or some strains are positive while others are negative; NR, not reported.

^d See references 3, 8, and 9.

^e See references 3 and 14.

^f See reference 19.

arabinose, xylan, and xylose by *C. polysaccharolyticum* but not by *C. herbivorans*. The ecosystems from which these organisms were isolated (pig intestinal tracts and sheep rumina for *C. herbivorans* and *C. polysaccharolyticum*, respectively) are also different. The cell morphologies and peritrichous flagella of these two species are similar, and both organisms are straight rods that occur singly or in pairs. *C. polysaccharolyticum* is typically 0.8 µm wide by 3 to 6 µm long, while *C. herbivorans* is 0.8 µm wide by 4.0 µm long. Long chains and aseptate filaments that are 50 µm or more long have been observed in *C. polysaccharolyticum* cultures (30, 31), but have not been seen in *C. herbivorans* cultures.

C. herbivorans can be distinguished from all of the remaining mesophilic cellulolytic and phylogenetically related organisms except *Clostridium aldrichii* by its lack of glucose fermentation. *C. herbivorans* and *C. aldrichii* can be clearly differentiated by their fermentation products and fermentation of glycogen, maltose, starch, and xylan. *C. herbivorans* is also distinct from all organisms listed in Table 1 except *C. polysaccharolyticum* in that it does not produce acetate.

Description of *Clostridium herbivorans* sp. nov. *Clostridium herbivorans* (her.bi.vo'rans. L. fem. n. *herba*, a green plant; L. v. *voro*, to devour; M. L. part. adj. *herbivorans*, devouring plants). Cells are gram-positive straight rods (0.7 to 0.9 by 3.5 to 4.0 µm) that occur in pairs or as single cells. Cells are motile, and peritrichous with 15 to 20 flagella per cell. Cells rarely sporulate; however, when spores are present, they are subterminal to terminal and 1 µm wide by 2 µm long and cause the sporangium to swell. Cultures grown with insoluble substrates such as cellulose or plant cell walls more readily produce spores than do those grown with soluble substrates.

Obligate anaerobe. Growth requires a fermentable carbohydrate such as cellobiose, cellulose, maltose, starch, or glycogen. The following compounds do not support growth: amygdalin, arabinose, Casamino Acids, erythritol, fructose, glucose, inositol, lactate, lactose, mannitol, mannose, melezitose, melibiose,

pectin, pyruvate, raffinose, rhamnose, ribose, salicin, sorbitol, sucrose, trehalose, xylose, and xylan. Nitrate and sulfate are not reduced. Catalase, oxidase, and urease negative. Esculin, lecithin, and gelatin are not hydrolyzed. Meat is not digested. Indole is not produced.

The optimum temperature for growth is 39 to 42°C, and the optimum pH for growth is 6.8 to 7.2. Rapid lysis of cells occurs in broth media once the stationary growth phase is reached; this is slightly less true with agar media. Ruminal fluid (15%, vol/vol) and yeast extract (1%, wt/vol) stimulate growth.

The major end products of cellobiose fermentation are formate and butyrate; minor amounts of ethanol and hydrogen are also produced.

The G+C content of the DNA is 38 mol% (as determined by the buoyant density method).

The type strain, strain 54408, was isolated from intestinal contents of a pig and has been deposited in the American Type Culture Collection as strain ATCC 49925.

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