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Mesophilic Cellulolytic Clostridia from Freshwater Environments

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Eight strains of obligately anaerobic, mesophilic, cellulolytic bacteria were isolated from mud of freshwater environments. The isolates (C strains) were rod-shaped, gram negative, and formed terminal spherical to oval spores that swelled the sporangium. The guanine plus cytosine content of the DNA of the C strains ranged from 30.7 to 33.2 mol% (midpoint of thermal denaturation). The C strains fermented cellulose with formation primarily of acetate, ethanol, CO_2, and H_2. Reducing sugars accumulated in the supernatant fluid of cultures which initially contained ≥0.4% (wt/vol) cellulose. The C strains resembled *Clostridium cellobioparum* in some phenotypic characteristics and *Clostridium papyrosolvens* in others, but they were not identical to either of these species. The C strains differed from thermophilic cellulolytic clostridia (e.g., *Clostridium thermocellum*) not only in growth temperature range but also because they fermented xylan and five-carbon products of plant polysaccharide hydrolysis such as D-xylose and L-arabinose. At 40°C, cellulose was degraded by cellulolytic mesophilic cells (strain C7) at a rate comparable to that at which *C. thermocellum* degrades cellulose at 60°C. Substrate utilization and growth temperature data indicated that the C strains contribute to the anaerobic breakdown of plant polymers in the environments they inhabit.

The fermentation of cellulose is of considerable interest as a potential source of liquid fuels such as ethanol. Most of the recent studies on production of ethanol from cellulose by microorganisms have dealt with thermophilic bacteria. One of the advantages often cited for the potential use of thermophiles in industrial cellulose fermentations is their high catabolic activity at temperatures optimal for their growth (30). Indeed, anaerobic digestion of solid wastes, a process presumably limited by the rate of cellulose breakdown, has been reported to occur about 50% more rapidly at thermophilic (65°C) than at mesophilic (37°C) temperatures (1). Other studies, however, have yielded somewhat different results. For example, Pfeffer (22) found two temperature optima for the digestion of solid waste, one at 42°C and the other at 60°C. Thus, studies of complex systems such as anaerobic digesters have been of limited value in understanding the effect of temperature on the rate of cellulose breakdown. Furthermore, there is a paucity of experimental data comparing rates of cellulose fermentation by pure cultures of mesophilic and thermophilic bacteria.

The results of studies by Hungate (8) indicate that free-living anaerobic mesophilic cellulose-fermenting bacteria are common in natural environments, and yet few have been described in detail. Ethanol-producing cellulolytic mesophiles have been isolated from soil and sewage sludge (8), from estuarine sediments (12), and from decayed grass (4). However, detailed studies of cellulose fermentation by these isolates have not been reported. Temperatures at which mesophilic bacteria grow and ferment cellulose occur commonly in nature. In contrast, temperatures that allow thermophiles to grow and metabolize at significant rates are limited to a few types of natural environments. It may be surmised that, in nature, the quantitative contribution of mesophilic bacteria to the anaerobic degradation of cellulosic materials is much greater than that of thermophilic bacteria. Because of the common occurrence of anaerobic mesophilic cellulolytic bacteria in various natural environments, it is possible that a wide diversity of these microorganisms exists in nature.

The objectives of the present investigation were to isolate anaerobic mesophilic cellulose-degrading bacteria from freshwater environments, to study their morphological and physiological characteristics, and to compare some of their metabolic properties with those of anaerobic thermophilic cellulolytic bacteria.

(A preliminary report of part of this work was presented at the Annual Meeting of the Ameri-

can Society for Microbiology, Atlanta, Ga., 7 through 12 March 1982 [abstr. no. I17].)

MATERIALS AND METHODS

Isolation of cellulolytic bacteria. Eight strains of mesophilic anaerobic cellulolytic bacteria, referred to as C strains, were isolated. Two of the C strains (strains C1 and C3) were isolated from mud collected from the bottom of Beaver's pond, a freshwater pond in Shutesbury, Mass. The other C strains (strains C5, C6, C7, C8, C9, and C10) were isolated from mud collected at different locations several centimeters below the surface of Cedar Swamp, a freshwater swamp in Woods Hole, Mass. The isolation procedure used was similar to procedures described by Stanton and Canale-Parola (28) and by Hungate (8) for the isolation of anaerobic cellulolytic bacteria from bovine rumen, sewage sludge, and soil. Enrichment cultures were prepared by serially diluting mud samples into Neoprene-stoppered anaerobic culture tubes (18 by 142 mm; Bellco Glass, Inc., Vineland, N.J.), each containing 5 ml of prereduced RFT-C broth (see below) under N_2 atmosphere. This medium contained ball-milled filter paper as the source of cellulose. After 1 to 3 weeks of incubation at 30°C, liquid and sediment from enrichment cultures showing cellulose disappearance were diluted serially into rubber-stoppered anaerobic culture tubes containing 5 ml of melted (45°C) prereduced RFT-C agar medium (see below) under N_2 atmosphere. After inoculation, the medium was allowed to solidify in a slanted position. Initially the agar medium slants were opaque because of the presence of cellulose fibers. However, during incubation (30°C, 1 to 3 weeks), clear zones appeared around colonies of cellulose-fermenting bacteria. Agar plug samples of colonies within clear zones were taken by means of Pasteur pipettes, diluted serially into melted prereduced RFT-C agar medium in tubes, and incubated as described above. To obtain pure cultures, this serial dilution procedure was carried out three or four additional times for each strain with RFT-C agar medium slants and at least once with a cellobiose-containing agar medium (RFT-CB medium slants, see below) before finally transferring the cells into tubes of RFT-C agar medium.

Culture conditions and media. Unless otherwise indicated, the cellulolytic bacteria were cultured anaerobically in prereduced media at 30°C under an atmosphere of N_2 (9). RFT-C agar medium, used in the isolation of the organisms, contained: distilled water, 68 ml; Bacto-Tryptone (Difco Laboratories, Detroit, Mich.), 0.1 g; yeast extract (Difco), 0.05 g; L-cysteine hydrochloride, 0.1 g; KH_2PO_4, 0.12 g; Na_2HPO_4, 0.2 g; resazurin solution (0.1% [wt/vol]), 0.1 ml; clarified rumen fluid (28), 10 ml; cellulose slurry, 20 ml; agar (Difco), 1.5 g. The cellulose slurry was prepared by wet-ball milling Whatman no. 1 filter paper (5 g/100 ml of distilled water) for 48 h, as described previously (28). The final cellulose concentration in the medium was 1% (dry wt/vol). RFT-C broth used in the isolation procedure was identical to RFT-C agar medium except that the agar was omitted. RFT-C broth, used for routine cultivation and maintenance of the isolates, contained a more finely dispersed cellulose slurry obtained by ball milling the filter paper for 72 h and then diluting the slurry to 3 g (dry weight) per 100 ml of distilled water before addition to the medium (final cellulose concentration in RFT-C broth, 0.6% [wt/vol]). The pH of RFT-C media was adjusted to 6.9. The media were heated under an N_2 atmosphere until the resazurin indicator became colorless and then distributed into culture tubes (4.9 ml of medium per anaerobic culture tube [18 by 142 mm]; Bellco Glass) that were being flushed with N_2. The tubes were sealed with Neoprene stoppers and autoclaved. After cooling either to room temperature (broth) or to 45 to 50°C (agar medium), the sterile media were supplemented with a filter-sterilized 5% (wt/vol) solution of sodium bicarbonate (0.1 ml/4.9 ml of medium). The final pH of RFT-C broth or agar medium was approximately 7.1.

RFT-C soft agar medium was identical to RFT-C agar medium except that it contained (per 100 ml) 0.75 g of agar (Difco) and 0.6 g of cellulose ball milled for 168 h. RFT broth and agar media, which lacked cellulose and other fermentable substrates, were prepared in the same way as RFT-C broth and RFT-C agar media except that distilled water replaced the cellulose slurry. RFT-cellobiose (RFT-CB) broth and agar media were prepared in the same way as RFT agar and broth media except that cellobiose was dissolved in the 5% solution of sodium bicarbonate (10 g of cellobiose per 100 ml of solution) before the solution was filter sterilized and added as a supplement to the cooled sterile medium. RFT-arabinose, RFT-glucose, and RFT-xylose broth media were prepared in the same way as RFT-CB broth except that L-arabinose, D-glucose, or D-xylose, respectively, was added to the 5% sodium bicarbonate solution (10 g of sugar per 100 ml of solution) instead of cellobiose. The final concentration of cellobiose, arabinose, glucose, or xylose in these media was 0.2% (wt/vol).

The isolates were grown on RFT agar plates overlaid with RFT-C soft agar medium to observe cellulose utilization and colony formation. The plates were incubated at 28°C for 6 to 12 days under an atmosphere of 5% CO_2, 10% H_2, and 85% N_2 in an anaerobic chamber (Coy Laboratory Products, Inc., Ann Arbor, Mich.).

Nutritional characteristics. The ability of the isolates to utilize various soluble compounds as fermentable substrates for growth was determined by estimating visually the turbidity of cultures after three transfers in liquid medium containing the potential substrate. Six-day-old RFT-C broth cultures were used to inoculate RFT broth medium (0.1 ml of inoculum added to 5 ml of medium) containing the potential substrate (final concentration, 0.2% [wt/vol]). Two additional successive transfers in the same medium were performed with 0.1 ml of culture as inoculum. The media (except broth containing polygalacturonic acid, carboxymethylcellulose, or xylan) were prepared in the same way as RFT broth except that the potential fermentable substrate was dissolved in a 5% solution of sodium bicarbonate (10 g of substrate per 100 ml of solution) before the solution was filter sterilized and added as a supplement to the cooled sterile medium (0.1 ml of solution per 4.9 ml of medium). Polygalacturonic acid was dissolved directly in RFT broth before autoclaving, and solutions (5% [wt/vol]) of carboxymethylcellulose or xylan were autoclaved separately and then added aseptically to sterile RFT broth. Xylan was washed three times in 70% ethanol before it was used

for medium preparation. No growth was observed in RFT broth that was not supplemented with a fermentable substrate.

The ability of the isolates to ferment various sources of cellulose was determined by inoculating 0.5 ml of a 6-day-old RFT-C broth culture into tubes containing 20 mg of a cellulosic material in 10 ml of RFT broth. Cellulose sources included microcrystalline cellulose (Avicel, a purified, particulate form of α-cellulose derived from fibrous plants), native cellulose (absorbent and nonabsorbent cotton), and purified wood cellulose (α-cellulose, filter paper, tissue paper, and Solka-Floc). After 2 weeks of incubation, the cultures were centrifuged at 27,000 × *g* for 15 min, and the ethanol content of the supernatant fluid was determined enzymatically by using alcohol dehydrogenase (Ethyl Alcohol Reagent Set; Worthington Diagnostics, Freehold, N.J.). Formation of ethanol was taken as an indication that an isolate was able to ferment the cellulose source being tested inasmuch as ethanol was a product of cellulose fermentation by all of the isolates (see below). In addition, cellulose utilization usually was accompanied by visible gas formation, appearance of turbidity in the culture, and an observable decrease in the amount of cellulosic material present in the test tubes.

Measurement of growth and cellulose utilization. RFT-C broth cultures (5 ml per tube) were each inoculated with 0.1 ml of an actively growing 3-day RFT-C broth culture. At various times during incubation, growth was measured by means of direct cell counts (Petroff-Hausser counting chamber), and residual cellulose in the cultures was determined by means of the filtration method of Weimer and Zeikus (29).

Growth of the isolates in RFT-arabinose, RFT-cellobiose, RFT-glucose, and RFT-xylose broth media (10 ml of medium inoculated with 0.5 ml of an 18-h culture grown in the same medium) was determined by measuring the optical density of cultures at 660 nm, using a Bausch & Lomb Spectronic 21 spectrophotometer.

Analysis of fermentation products. The effect of the initial cellulose concentration in the medium on the accumulation of fermentation end products was determined by using RFT broth cultures prepared with known amounts of lyophilized cellulose (filter paper ball milled for 72 h and then lyophilized). Cultures (10 ml) were inoculated with 0.5 ml of a 6-day-old culture in RFT-C broth and then incubated at 30°C for 28 days. Fermentation products and reducing sugars formed in these cultures were quantitated as described below.

Other analyses of fermentation products were performed to obtain data for calculating fermentation balances. For these analyses, cells were grown in 50 ml of either RFT broth containing 0.2% (wt/vol) lyophilized cellulose, RFT-glucose broth, or RFT-xylose broth. These media were prepared as described above except that the media were dispensed into serum bottles (125 ml, 54 by 107 mm; Wheaton Instruments, Millville, N.J.) according to the procedure of Miller and Wolin (16). Incubation was at 30°C for 24 h except for RFT-C cultures which were incubated for 6 days to allow complete utilization of cellulose in the medium.

Gaseous fermentation products were determined quantitatively by gas-liquid chromatography with a Varian 4600 gas chromatograph (equipped with a thermal conductivity detector) and accompanying Vista 44 data station (Varian Instrument Division, Palo Alto, Calif.). Fermentation gases were separated on a stainless steel column (12 ft by 1/8 in. [ca. 3.6 m by 0.3 cm] outer diameter) packed with Chromosorb 102, 80/100 mesh (Supelco, Inc., Bellefonte, Pa.). Operating parameters for sample separation and detection were: carrier gas (N_2) flow rate, 30 ml/min; injector temperature, 75°C; thermal conductivity detector temperature, 70°C; current through detector, 140 mA. Separation was carried out isothermally at 40°C.

Methods used for quantitative analysis of volatile and nonvolatile fatty acid fermentation products by gas-liquid chromatography have been described previously (5). Ethanol was assayed as described above. Glucose was assayed enzymatically with glucose oxidase (STATZYME glucose reagent; Worthington Diagnostics). Acetoin, diacetyl, and 2,3-butanediol were determined by conventional methods (17), formate was determined by the method of Lang and Lang (11), and reducing sugars were determined with dinitrosalicylic acid reagent (23).

Microscopy. Cells examined by phase-contrast and electron microscopy were grown in RFT-C broth or in RFT-arabinose broth. Methods and equipment used for phase-contrast photomicroscopy (27) and for electron microscopy (21) were described previously. Thin sections of chemically fixed cells were processed with ruthenium red (2, 31).

Determination of guanine plus cytosine content of DNA. DNA from each of the cellulolytic isolates was purified by the method of Marmur (14). Guanine plus cytosine composition of purified DNA was determined from its thermal denaturation temperature by the method of Mandel and Marmur (13). *Escherichia coli* K-12 DNA was used as a standard.

Chemicals and cellulosic substrates. All chemicals used were reagent grade. Avicel microcrystalline cellulose type PG-105 was a gift of FMC Corp., Philadelphia, Pa. Absorbent cotton (USP grade) was purchased from Absorbent Cotton Co., Valley Park, Mich. Nonabsorbent cotton coil (for 18- to 20-mm tubes) was from American Scientific Products, McGaw Park, Ill., and Solka-Floc was from Brown Co., Berlin, N.H.

RESULTS

Cell and colony morphology. The eight cellulolytic isolates (C strains) resembled one another in morphology (e.g., Fig. 1). All were curved rods, measuring 0.6 to 0.7 μm in diameter and 2 to 7 μm in length. The eight C strains formed terminal, spherical to oval endospores that swelled the sporangium (Fig. 1B). Cells grown in RFT-C broth were morphologically indistinguishable from cells grown in media containing soluble sugars except that spores were rarely observed when a soluble sugar (0.2%, final concentration) served as the growth substrate.

Cells were motile by means of peritrichous flagella, as determined by means of electron microscopy of cells negatively stained with phosphotungstic acid. All C strains consistently stained gram negative at different stages of growth. Electron micrographs of thin sections of

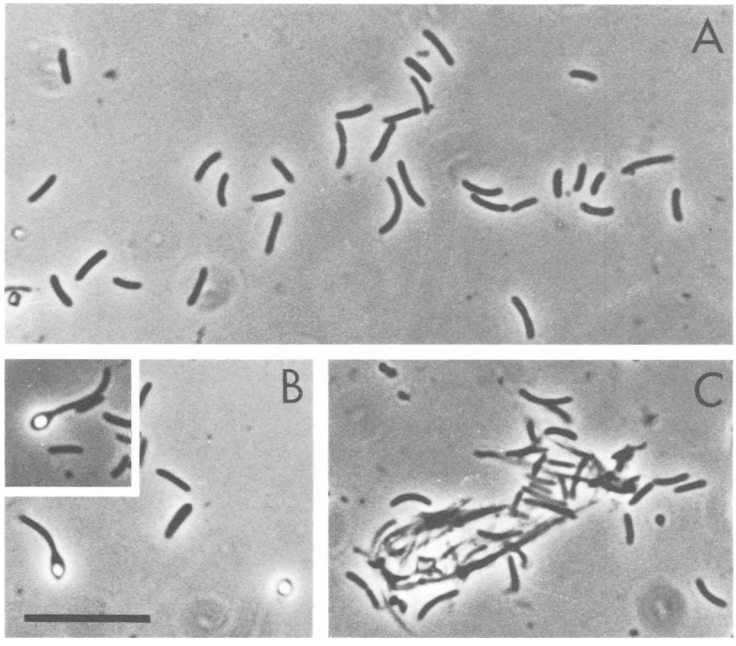

FIG. 1. Phase-contrast photomicrographs of cellulolytic isolates (wet-mount preparations). (A) Late-exponential-phase cells (24 h) of strain C5 grown in RFT-CB broth. (B) Late-exponential-phase culture (4 day) of strain C5 grown in RFT-C broth. Note the free spore and refractile spores within cells. (C) Exponential-phase culture (2 days) of strain C9 grown in RFT-C broth; cells are entangled in cellulose fibers. All cultures were incubated at 30°C. All micrographs are at the same magnification. Bar = 10 μm.

the bacteria (Fig. 2) showed that the cytoplasmic membrane was surrounded by a two-layered cell wall composed of an inner, densely stained layer 5 to 16 nm thick and an outer, less densely stained layer 8 to 13 nm thick. The cell envelope structure of the isolates resembled that of certain gram-negative and gram-variable clostridia which also have a two-layered cell wall (25). In these bacteria, the thin, densely staining layer is a lysozyme-sensitive murein layer, whereas the outer layer comprises a regular array of subunits (25).

Cellulose utilization and colony morphology were apparent when cells were grown on plates of RFT agar overlaid with RFT-C soft agar (Fig. 3). Clear areas resulting from cellulose fermentation surrounded the colonies. The size of the clear areas and the morphology of the colonies (star shaped, pinpoint, or diffuse) varied from strain to strain (Fig. 3).

Cells were present only within the colonies and not at the edge of the clear zones as determined by light microscopy. The appearance of cell-free clear zones around the colonies of the isolates indicated that active cellulolytic enzymes were released into the medium.

Growth characteristics. The C strains were obligately anaerobic. All C strains grew and fermented cellulose at 22 and 40°C, but not at 15 and 45°C. Other fermentable sugars supported growth at the above temperatures. In addition, slow growth of the C strains was observed at 15°C when a soluble sugar (e.g., L-arabinose) was the fermentable substrate. This observation indicates that the cellulase system of the C strains may be cold sensitive and that, in natural environments, growth at low temperatures (e.g., 15°C) depends on the availability of other fermentable substrates. The temperature range in which the most rapid rate of growth was observed was between 30 and 40°C.

As mentioned above, the C strains were isolated from mud of a freshwater pond and a freshwater swamp. Daytime temperatures were measured at numerous locations within the pond and swamp during the month of August. At 5 to 12 cm below the mud surface, the temperatures ranged from 20 to 27°C. These measurements indicated that, in the natural environments from which they were isolated, the C strains found temperatures that allowed them to grow and ferment cellulose.

In actively fermenting RFT-C broth cultures, gas bubbles rose from cellulose fibers present on the bottom of the tubes, and eventually the entire mass of cellulose often floated to the surface of the broth. Although the cellulose darkened slightly as it was fermented, a yellow

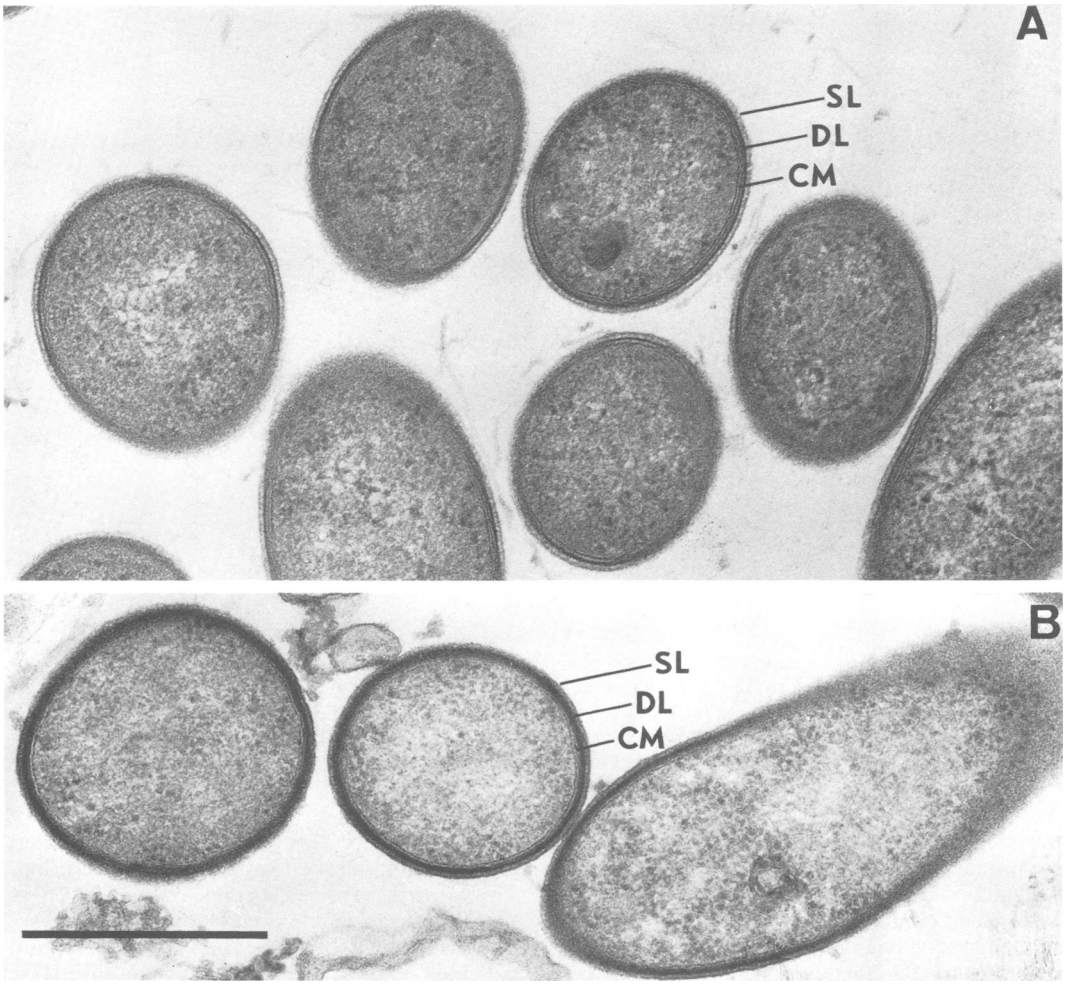

FIG. 2. Thin sections of cells of strains C5 (A) and C7 (B) fixed and stained with ruthenium red. The cytoplasmic membrane (CM) is surrounded by a cell wall consisting of an inner dense layer (DL) and a less dense surface layer (SL). The thickness of the dense layer of the cell wall varies from strain to strain; e.g., strain C7 has a relatively thick dense layer (B). Both micrographs are at the same magnification. Bar = 0.5 μm.

pigment tightly bound to the cellulose was not formed, in contrast to what occurs during cellulose fermentation by *Clostridium thermocellum* (29). However, the cell pellet remaining at the bottom of the culture tube after the cellulose was completely fermented had a yellow tinge. Light microscopic observation of samples from broth cultures revealed motile cells swimming in the liquid as well as cells intertwined within the cellulose fibers (Fig. 1C) and cells adhering end-on to cellulose fibers.

When cells from a 4-day-old RFT-C broth culture were inoculated into RFT-C broth, fermentation, as indicated by gas production and by cellulose utilization (Fig. 4), was apparent after a lag of about 12 to 24 h. The number of cells increased exponentially for 2 to 3 days (Fig. 4). After this time the number of cells containing refractile spores increased significantly. After 4 days of incubation, the number of vegetative cells and sporulating cells declined, and free spores were seen in the culture medium. The total number of refractile spores (within cells and free) was approximately 10% of the maximum number of vegetative cells. Cellulose degradation continued at an undiminished rate for 2 to 3 days after the number of cells began to decline.

Cellulose was completely utilized in 4 days when its initial concentration in media was 0.2 to 0.3% (wt/vol). This was determined by observing cellulose disappearance from RFT broth cultures that had been prepared with known amounts of lyophilized ball-milled filter paper.

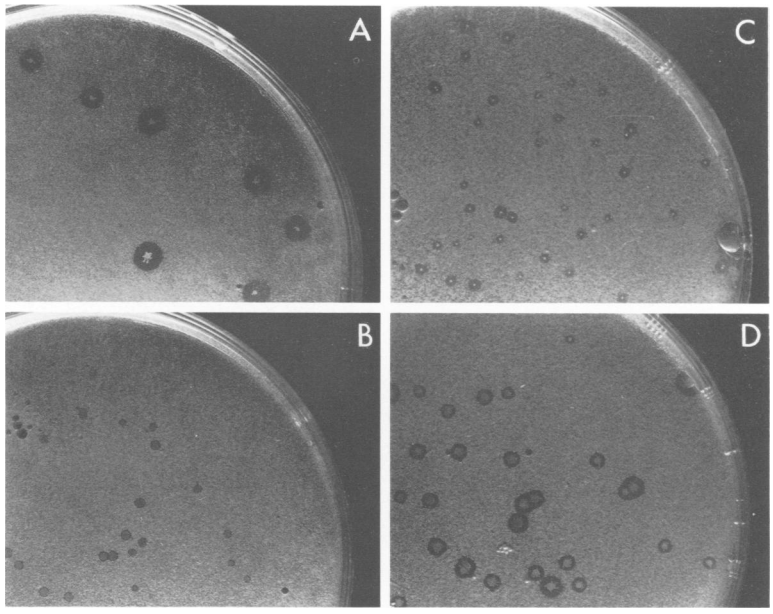

FIG. 3. Cellulose breakdown and colony formation on plates of agar medium. (A) Strain C3, star-shaped colonies. (B) Strain C5, diffuse colonies. (C) Strain C7, pinpoint colonies. (D) Strain C9, diffuse colonies with higher cell density than those of strain C5. The circular clear areas denote cellulose breakdown. Plates were incubated anaerobically for 12 days at 28°C.

During prolonged incubation (2 to 3 weeks), cellulose initially present at a concentration of 0.6% (wt/vol) was completely degraded. The pH of cultures in RFT-C broth ranged from 5.3 to 5.8 after cellulose was completely degraded.

Growth substrates. The C strains utilized carbohydrates, but not other compounds tested, as energy and carbon sources. Polysaccharides, hexoses, and pentoses that are commonly present in plant materials served as fermentable substrates. Thus, all C strains fermented cellulose, xylan, cellobiose, D-glucose, L-arabinose, and D-xylose. D-Fructose was fermented by three strains (C5, C8, and C10) and D-mannose by two (C5 and C10). Some of the strains (C7, C8, and C10) grew in RFT broth containing D-galactose as the fermentable substrate, but only after a lag of 2 to 5 days when transferred from RFT-C broth. None of the C strains utilized carboxymethylcellulose, polygalacturonate, starch, lactose, maltose, sucrose, D-galacturonate, sodium pyruvate, or amino acids as fermentable substrates.

It was not possible to determine accurately by means of turbidimetric measurements the cell density of cellulose-containing cultures because of the presence of cellulose particles in the medium. Thus, growth resulting from the utilization of different cellulosic substrates (0.2% [wt/vol] final concentration) was determined by measuring the amount of ethanol formed as a fermentation end product by the C strains. These determinations showed that ball-milled filter paper and microcrystalline cellulose (Avicel) were utilized as fermentable substrates by all C strains. However, the amount of ethanol produced by various strains varied considerably (e.g., strain C7 produced 5.9 mmol of ethanol per liter of culture fluid when fermenting ball-milled filter paper, whereas strain C8 produced 2.2 mmol/liter). Ethanol was produced in significant amounts (1.3 to 6.5 mmol/liter) by most strains in media containing tissue paper, Solka-Floc, or α-cellulose as fermentable substrates, indicating that these materials were utilized for growth. Insignificant amounts of ethanol (less than 1.3 mmol/liter) were produced from absorbent or nonabsorbent cotton by three strains (C3, C6, and C8), and low amounts (1.3 to 2.8 mmol/liter) were produced by the other strains.

Fermentation products. All C strains produced H_2, CO_2, acetate, and ethanol as major fermentation products from cellulose or glucose (Table 1) and from xylose (data not shown). Lactate and trace amounts of pyruvate and succinate were also detected (Table 1). The molar ratio of ethanol to acetate produced varied depending on the substrate (Table 1). This observation indicates that culture conditions affected the ratio of fermentation products of the isolates.

As mentioned above, when the C strains were grown in broth containing 0.6% (wt/vol) cellu-

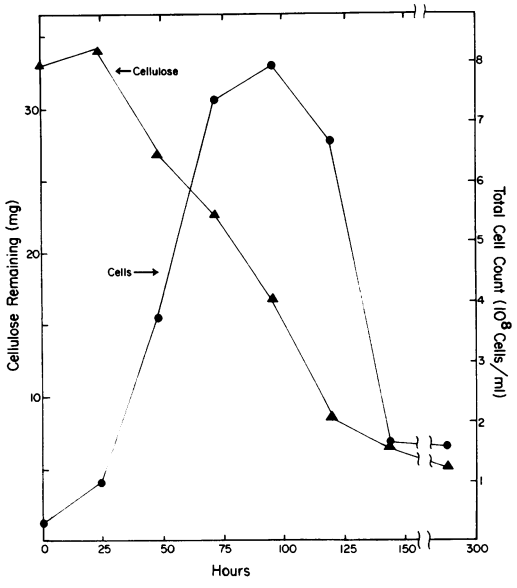

FIG. 4. Cellulose utilization during growth of strain C7. Cultures were grown anaerobically at 30°C in 5 ml of RFT-C broth. Cell counts include vegetative and sporulating cells but not free spores. Residual cellulose is expressed in milligrams of cellulose per 5-ml culture.

lose, the cellulose was completely degraded after prolonged incubation. Cellulose degradation continued even after the cell number declined drastically (Fig. 4). Reducing sugars accumulated in the supernatant fluid of cultures containing ≥0.4% (wt/vol) cellulose (Fig. 5). Approximately 55% (mol%) of the accumulated reducing sugars was glucose. The concentration of ethanol and acetate in culture supernatant fluid at the end of fermentation increased as the initial amount of cellulose added to the medium was increased up to 0.3% (wt/vol). In cultures initially containing ≥0.3% cellulose, the final concentration of acetate and ethanol remained constant (Fig. 5). These results indicate that the cellulase system of the C strains continues to hydrolyze cellulose to soluble saccharides after fermentation ceases.

In addition to the initial cellulose concentration in the growth medium, other culture conditions affected the amount of ethanol produced by the C strains. For example, cultures in RFT-C broth incubated at 40°C formed 33% more ethanol than cultures incubated at 30°C. As described in the previous section, ethanol yields were also affected by the source of cellulose in the culture medium. Generally, the highest ethanol yields (14 to 19 mmol/liter) were produced in media containing 0.6% (wt/vol) pebble-milled filter paper that had not been lyophilized. These concentrations are comparable to ethanol concentrations produced during cellulose fermentation by pure cultures of *C. thermocellum* (29).

DNA base composition. The guanine plus cytosine contents (mol%) of the DNAs of the isolates were: strain C1, 30.7; strain C3, 33.2; strain C5, 32.0; strain C6, 30.8; strain C7, 32.7; strain C8, 32.1; strain C9, 30.8; strain C10, 31.6.

Rates of growth and cellulose utilization. Strain C7 growing in cellobiose-containing broth (RFT-CB) had a doubling time of 1.8 h at 40°C and of 3.5 h at 30°C (Table 2). Thus, the rate of growth in RFT-CB at 40°C was approximately twice the rate of growth at 30°C. The growth rate of strain C7 at 40°C was comparable to the optimum growth rate of the thermophile *C. thermocellum* in cellobiose broth at 60°C (29) (Table 2). According to these results, strain C7 and *C. thermocellum* growing with a soluble sugar as fermentable substrate have similar maximum rates of growth, but strain C7 achieves this maximum rate at a temperature 20°C lower than that for *C. thermocellum*.

Both strain C7 and *C. thermocellum* grew more slowly on cellulose than on cellobiose (Table 2). The rates of growth and cellulose utilization by *C. thermocellum* at 60°C and by strain C7 at 40°C are comparable (Table 2). Also, strain C7 fermented cellulose only slightly more

TABLE 1. Products of cellulose and glucose fermentation by strain C10[a]

Product[b]	Amt of product[c]	
	Cellulose fermentation	Glucose fermentation
H_2	156.6	135.7
CO_2	131.1	101.1
Acetate	113.5	140.6
Ethanol	66.1	47.1
Lactate	23.6	17.5
Pyruvate	<1	3.3
Succinate	1.2	<1
Carbon recovery	94%	90%
Oxidation-reduction balance	0.91	0.89

[a] Cells were grown in RFT-C broth with ball-milled filter paper as the source of cellulose or in RFT-glucose broth.

[b] Assayed for but not detected: 2,3-butanediol, butyrate, diacetyl, formate, fumarate, malate, propionate, and valerate. Trace amounts (<1 μmol/100 μmol of substrate utilized) of acetoin and oxaloacetate were detected in cellulose- and glucose-containing cultures.

[c] Expressed as micromoles of product per 100 micromoles of glucose equivalents or glucose fermented.

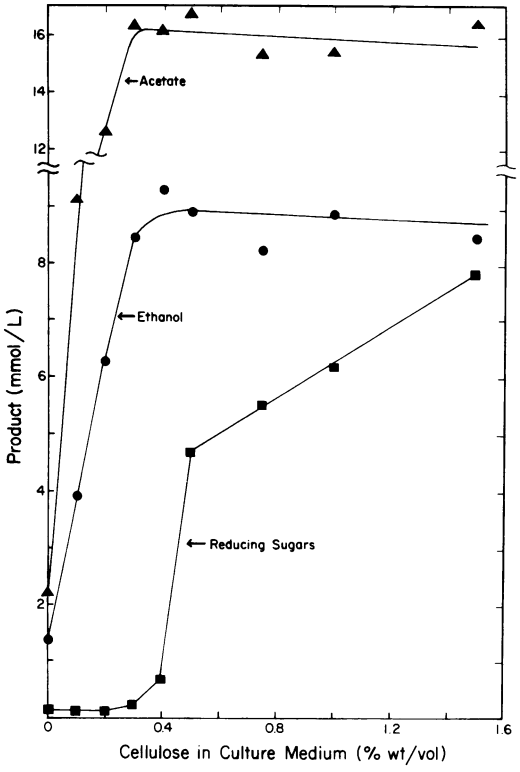

FIG. 5. Effect of initial cellulose concentration in the growth medium on the production of acetate, ethanol, and reducing sugars by strain C10. Cultures were incubated at 30°C for 28 days in RFT broth prepared with known amounts of cellulose. Products are expressed in millimoles per liter of culture supernatant fluid. Reducing sugars are expressed as glucose equivalents.

rapidly at 40°C than at 30°C (Table 2). These results suggest that a factor(s) other than temperature limits the rate of cellulose fermentation. Furthermore, the results imply that thermophilic bacteria do not necessarily degrade cellulose at a faster rate than mesophilic bacteria.

DISCUSSION

The eight strains of anaerobic cellulolytic bacteria described in this paper (C strains) are very similar to each other with regard to morphology, general physiological characteristics, substrate fermentation pattern, fermentation end products formed, growth temperature range, antibiotic sensitivity (data not shown), and guanine plus cytosine content of the DNA. The strains differ slightly in ultrastructure inasmuch as the dense layer of their cell walls (Fig. 2) is thicker in some strains than in others. These differences in thickness of the dense layer do not affect the Gram stain reaction of the strains, which was consistently negative. In addition, the C strains form different colony types on plates of cellulose-containing agar medium (Fig. 3). In spite of these few strain differences, the numerous similarities mentioned above indicate that the C strains are representatives of a single bacterial species.

All C strains are obligately anaerobic, gram-negative, spore-forming rods. Their substrate fermentation pattern and the relatively low guanine plus cytosine content of their DNA indicate that they are not strains of *Desulfotomaculum*, but rather that they represent a species of *Clostridium*.

The C strains differ from thermophilic cellulolytic clostridia (e.g., *C. thermocellum*) in several important respects. For example, the C strains behave as typical mesophiles in that they grow between 15 and 40°C but not at 45°C, whereas *C. thermocellum* grows optimally between 55 and 65°C (15, 19). Furthermore, the C strains differ from *C. thermocellum* because they ferment xylan and pentoses (e.g., D-xylose and L-arabinose). Although extracellular xylanase is produced by strains of *C. thermocellum* (3, 20), this organism is unable to utilize xylan, D-xylose, or L-arabinose as growth substrate (19, 24). The ability of the C strains to ferment xylan and pentoses, in addition to cellulose, cellobiose, and glucose, indicates that monocultures of these mesophilic clostridia have the potential of converting to ethanol both the cellulosic and hemicellulosic components of biomass. In contrast, thermophilic clostridia catabolize both of

TABLE 2. Comparison of rates of growth and cellulose utilization by strain C7 and *C. thermocellum*[a]

Organism	Incubation temp (°C)	Doubling time (h) when fermenting[b]:		Rate of cellulose utilization[c] (mg/liter per h)
		Cellobiose	Cellulose	
Strain C7	30	3.5	12.5	53[d]
Strain C7	40	1.8	10.1	59[e]
C. thermocellum	60	2.1	11.0	58[d]

[a] Strain C7 was grown in RFT-C (cellulose) broth or in RFT-cellobiose broth. Data for rates of growth and cellulose utilization by *C. thermocellum* are from Weimer and Zeikus (29).

[b] Growth was monitored turbidimetrically except for the growth of strain C7 in cellulose broth which was monitored by total cell counts.

[c] Determined by the method of Weimer and Zeikus (29).

[d] Cellulose utilization was preceded by a lag of 12 to 24 h.

[e] Cellulose utilization was preceded by a lag of approximately 120 h.

these biomass components only in mixed cultures consisting of a cellulolytic species (e.g., *C. thermocellum*) and a non-cellulolytic species (18).

Some of the morphological and physiological characteristics of the C strains are similar to those of *Clostridium cellobioparum*, a mesophilic cellulolytic bacterium isolated from the rumen (7). For example, both the C strains and *C. cellobioparum* have curved rod-shaped vegetative cells, as well as oval to spherical spores that swell the sporangium (6, 7, 26). Both ferment cellulose, forming similar products, except that *C. cellobioparum* produces formate and the C strains do not. However, the guanine plus cytosine content of the DNA of *C. cellobioparum* (28 mol%) (10) is slightly lower than that of the C strains (31 to 33 mol%). In addition, the C strains and *C. cellobioparum* differ in growth temperature range inasmuch as the former organisms grow at 15°C, whereas the latter grow slowly or not at all at 25°C and do not grow at 18°C (7, 26). Further differences are found in the substrate fermentation pattern of *C. cellobioparum* (7) and that of the C strains. On the other hand, the substrate fermentation pattern and the guanine plus cytosine content of the DNA of the C strains are similar to those of *Clostridium papyrosolvens*, a mesophilic cellulolytic bacterium recently isolated from intertidal mud banks at the estuary of the river Don in Scotland (12). This clostridium was reported to form acetate, ethanol, lactate, CO_2, and H_2 from cellulose (12). However, a significant comparison of its cellulose fermentation end products with those of C strains cannot be made because a quantitative fermentation balance for *C. papyrosolvens* has not been reported. The C strains and *C. papyrosolvens* differ significantly in morphology. We found that the vegetative cells of *C. papyrosolvens* (NCIB 11394; National Collection of Industrial Bacteria, Aberdeen Scotland), when growing in RFT-C medium and in other media tested, are straight rods that form spherical spores (data not shown). As previously reported by Madden et al. (12), oval spores are never seen in cultures of *C. papyrosolvens*. In contrast, oval spores are always present when the C strains are grown in the same media used for *C. papyrosolvens*, and the C strains vegetative cells are curved rods. In conclusion, the C strains resemble *C. cellobioparum* in some phenotypic characteristics and *C. papyrosolvens* in others, but apparently they are not identical to either of these species.

When cellobiose was the fermentable substrate, strain C7 grew approximately twice as rapidly at 40°C as it did at 30°C. However, when cellulose was the fermentable substrate, temperature had little effect on the rate of growth of strain C7 (Table 2). Furthermore, strain C7 degraded cellulose only slightly more rapidly at 40°C than it did at 30°C. The rate of cellulose degradation by this mesophile at 30 to 40°C was similar to the rate of cellulose degradation by *C. thermocellum* at 60°C (29). These results indicate that (i) temperatures in the range favorable for optimum growth of mesophiles do not necessarily limit the rate of cellulose fermentation, and (ii) anaerobic mesophilic bacteria apparently degrade cellulose at a rate comparable to the rate of cellulose degradation by anaerobic thermophilic bacteria.

The eight C strains described in this paper were isolated from two widely separated geographical locations. Thus, it seems likely that these bacteria are widespread in nature. The C strains ferment plant polysaccharides such as cellulose and xylan, as well as hydrolysis products of the polysaccharides. Furthermore, they grow at temperatures that are prevalent in the natural environments in which they are present. It may be concluded that the metabolic activities of these bacteria contribute to the anaerobic degradation of plant polymers in aquatic environments.

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