JOURNAL OF BACTERIOLOGY, Aug. 1990, p. 4222–4230
0021-9193/90/084222-09$02.00/0

Vol. 172, No. 8

Cellulase System of a Free-Living, Mesophilic Clostridium (Strain C7)

KATHERINE CAVEDON,† SUSAN B. LESCHINE, AND E. CANALE-PAROLA*
Department of Microbiology, University of Massachusetts, Amherst, Massachusetts 01003

Received 4 December 1989/Accepted 8 May 1990

The enzymatic activity responsible for crystalline cellulose degradation (Avicelase activity) by a mesophilic clostridium (strain C7) was present in culture supernatant fluid but was not detected in significant amounts in association with whole cells or in disrupted cells. Cells of the mesophilic clostridium lacked cellulosome clusters on their surface and did not adhere to cellulose fibers. The extracellular cellulase system of the mesophilic clostridium was fractionated by Sephacryl S-300 gel filtration, and the fractions were assayed for Avicelase and carboxymethylcellulase activities. The Avicelase activity coincided with an A_{280} peak that eluted in the 700,000-M_r region. Nondenaturing polyacrylamide gel electrophoresis and sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of the 700,000-M_r fractions showed that Avicelase was present as a multiprotein aggregate that lost the ability to hydrolyze crystalline cellulose when partially dissociated by sodium dodecyl sulfate treatment. Proteins resulting from the partial dissociation of the aggregate retained carboxymethylcellulase activity. An Avicelase-deficient mutant of strain C7 (strain LS), which was not capable of degrading crystalline cellulose, lacked the Avicelase-active 700,000-M_r peak. The results indicated that an extracellular 700,000-M_r multiprotein complex, consisting of at least 15 proteins, is utilized by the mesophilic clostridium for the hydrolysis of crystalline cellulose. At least six different endo-1,4-β-glucanases may be part of the cellulase system of strain C7. Sephacryl S-300 column fractions, corresponding to an A_{280} peak in the 130,000-M_r region, contained carboxymethylcellulase-active proteins that may serve as precursors for the assembly of the Avicelase-active complex by the mesophilic clostridium.

Much of what is known about anaerobic cellulose biodegradation has been learned from studies on *Clostridium thermocellum*, a thermophilic bacterium that produces a multiprotein enzyme system capable of hydrolyzing crystalline cellulose (8). *C. thermocellum* grows optimally between 60 and 64°C and does not grow at temperatures that occur commonly in natural environments (e.g., at or below 37°C) (6). Thus, it may be inferred that its contribution to cellulose degradation in nature is limited to cellulose-containing environments in which relatively high temperatures prevail. Most likely, mesophilic microorganisms, rather than thermophiles such as *C. thermocellum*, are primarily responsible for the degradation of the vast amounts of cellulose produced by photosynthesis.

In the course of a project aimed at understanding the complex process of anaerobic cellulose biodegradation as it takes place in nature, we have isolated various strains of mesophilic, obligately anaerobic, cellulolytic bacteria from different environments in which plant material was undergoing decomposition (18, 20, 23). Some of the isolates formed endospores and were identified as strains of undetermined species of *Clostridium*, whereas others are presently taxonomically unassigned. Six isolates that were tested fixed N_2, a property expected to be advantageous to them in their habitats inasmuch as natural environments rich in cellulose are frequently deficient in nitrogen (20). In this paper we describe the isolation and some of the properties of the extracellular cellulase system produced by one of these N_2-fixing mesophiles, a *Clostridium* strain (strain C7), which we isolated from mud collected several centimeters below the surface of a freshwater swamp (18). As our results indicate, the extracellular cellulase system of *Clostridium* sp. strain C7 is significantly different from the extracellular cellulase system produced by *C. thermocellum*.

(A preliminary report of part of this work was presented previously [Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, K127, p. 223].)

MATERIALS AND METHODS

Bacterial strains and culture conditions. The cellulolytic bacterium used in this investigation is a mesophilic *Clostridium* strain that is referred to as strain C7 (18). Strain C7 was isolated from mud as previously described (18). This strain utilizes crystalline cellulose (Avicel, type PH 105, 20-μm particles; FMC Corp., Marcus Hook, Pa.) as fermentable substrate for growth, and has been routinely grown in media containing ball-milled filter paper (see below) as the source of cellulose.

A strain C7 mutant (strain LS), which does not utilize cellulose as fermentable substrate, was isolated as described below.

C. thermocellum YS, isolated from soil collected from a hot spring in Yellowstone National Park, was a gift from R. Lamed and E. Morgenstern.

All media were prereduced (12). Liquid cultures were incubated in an N_2 atmosphere, whereas agar medium plate cultures were incubated in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.; 10% CO_2–7% H_2–83% N_2, vol/vol/vol). The incubation temperature was 30°C.

Chemically defined medium MJ-CB was identical to medium MJ of Johnson and co-workers (13), except that the only vitamins added were *d*-biotin (2×10^{-5} g/100 ml, final concentration) and *p*-aminobenzoic acid (4×10^{-5} g/100 ml, final concentration). Unless otherwise indicated, cellobiose at a concentration of 0.2 g/100 ml was the fermentable

* Corresponding author.
† Present address: Laboratory of Microbial Ecology, National Institute of Dental Research, Bethesda, MD 20892.

substrate in medium MJ-CB. In some experiments 0.6 g of yeast extract (Difco Laboratories, Detroit, Mich.) was added per 100 ml of medium MJ-CB instead of the vitamins. This yeast extract-containing medium is referred to as GS2-CB broth. In some of the experiments aimed at determining induction of Avicelase activity by various growth substrates, cellobiose in medium GS2-CB was replaced by another fermentable sugar (0.2% [wt/vol], final concentration). Avicelase activity in the culture supernatant was assayed after the fermentable sugar had been almost completely utilized by the growing cells.

Medium MJ-C was identical to medium MJ-CB, except that cellulose (0.1 g [dry weight] of ball-milled Whatman no. 1 filter paper per 100 ml of medium) (18) was the fermentable substrate instead of cellobiose. GS2-C broth was identical to medium GS2-CB, except that it contained cellulose (0.6 g [dry weight] of ball-milled filter paper per 100 ml of medium) instead of cellobiose. Agar media were prepared by adding 1.5 g of agar (Difco) per 100 ml of the media described above. However, GS2-CB agar medium contained 0.5 g (instead of 0.2 g) of cellobiose per 100 ml.

Mutant strain LS was cultured on GS2-CB agar plates and in GS2-CB and MJ-CB broths. *C. thermocellum* YS was grown in GS2-CB broth.

Isolation of mutant strain LS. All steps for isolation of strain LS were carried out in an N_2 atmosphere or, where indicated, in the anaerobic chamber. An 18-h GS2-CB-grown culture of strain C7 (5 ml) was added to a mixture consisting of 4 ml of GS2 WM (sterile GS2 wash medium, identical to GS2-CB broth except that the cellobiose was omitted) and 1 ml of a solution of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (500 μg/ml) (1). After the cells were added, the mixture was incubated in the anaerobic chamber at 30°C for 20 min; after dilution with 40 ml of GS2 WM, the cells were pelleted by centrifugation (10 min, 6,800 × *g*, room temperature), washed by suspending them in 10 ml of GS2 WM and then centrifuging, and finally suspended in 20 ml of GS2-CB broth. The cell suspension was incubated for 24 h at 30°C; then the cells were pelleted by centrifugation and suspended in 20 ml of GS2-C medium containing 10,000 U of penicillin G per ml (9, 17). After incubation for 18 h at 30°C, the cells were pelleted, washed in 20 ml of GS2 WM, suspended in 25 ml of GS2-CB broth, and incubated for 24 h at 30°C. The cells were pelleted, washed in 10 ml of GS2 WM, diluted, and plated on a selective agar medium (GS2-C agar medium containing 0.01 g of cellobiose per 100 ml). The plates were incubated in the anaerobic chamber. The selective medium was opaque due to the presence of cellulose. Mutants unable to utilize cellulose (cellulose utilization mutants) grew at the expense of the small amount of cellobiose present in the medium, forming very small colonies that were not surrounded by a clear area. Cells from these colonies were transferred to GS2-CB and GS2-C agar medium plates to verify their inability to utilize cellulose as a fermentable substrate. Strain LS was one of the mutants isolated by means of this procedure.

Enzyme assays. The Avicelase assay measures the ability of cellulase preparations to hydrolyze crystalline cellulose (Avicel). In the present study Avicelase activity was determined by the method of Johnson et al. (14) by measuring the decrease in turbidity of a suspension of Avicel (see above). Reaction mixtures were incubated at 42°C in an N_2 atmosphere in 18- by 142-mm neoprene-stoppered anaerobic culture tubes (Bellco Glass Inc., Vineland, N.J.). Each reaction mixture contained the following (in a total volume of 5 ml): succinate-NaOH buffer (pH 6.0), 0.3 mmol; Avicel, 1.33 mg; dithiothreitol, 50 μmol; $CaCl_2$, 25 μmol; enzyme preparation; and distilled water. Reactions were initiated by the addition of enzyme. At various times after the initiation of reactions, the mixtures were gently mixed by inversion, and turbidities at 660 nm were determined. A unit of Avicelase activity was defined as the amount of enzyme resulting in the hydrolysis of 0.5 μg of Avicel per h, as determined by correlating the decrease in turbidity with the decrease in weight of cellulose. Measurements were carried out only during the linear decrease in turbidity (0 to 72 h). In addition, Avicelase activity was determined by incubating the reaction mixture described above for 72 h at 42°C, removing insoluble material by centrifugation, and determining reducing sugars by the dinitrosalicylic acid reducing sugar method of Miller et al. (22). In some of the Avicelase assays by the latter method, dithiothreitol was omitted from the reaction mixture.

The carboxymethylcellulase (CMCase) assay has been widely used to measure endo-1,4-β-glucanase activity (25). CMCase activity was determined by incubating the enzyme with carboxymethylcellulose (CMC; sodium salt, medium viscosity; Sigma Chemical Co., St. Louis, Mo.) and measuring reducing sugars as mentioned above. Each reaction mixture (in air, in 16- by 150-mm test tubes) contained succinate-NaOH buffer (pH 6.0, 0.065 mmol), CMC (13.0 mg), and enzyme preparation in a total volume of 2.3 ml. Reactions were initiated by the addition of enzyme. Incubation was at 42°C for 15 min. A unit of CMCase activity was defined as the amount of enzyme that released 1.0 μmol of reducing sugar (expressed as glucose) per min under the assay conditions.

Protein was measured by the method of Bradford (4) with the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, Calif.) and bovine serum albumin as the protein standard, unless specified otherwise.

Determination of extracellular and cell-associated cellulase activities. Enzymatic assays of culture supernatant fluids, disrupted cells, and cell extracts were used to determine whether the cellulase activity was secreted by strain C7 cells into the external environment or whether it remained associated with the cells or both. Supernatant fluid from a 200-ml culture (grown in an N_2 atmosphere for 72 h in MJ-CB broth) was obtained by pelleting the cells by centrifugation (10 min, 4,000 × *g*, 4°C). The pelleted cells were washed twice by centrifugation in phosphate-buffered saline (0.15 M NaCl [final concentration] in 10 mM potassium phosphate buffer [pH 7]). The washed cells were suspended in 10 ml of 20 mM Tris hydrochloride buffer (pH 7.5). Then 1 ml of a 1% (wt/vol) lysozyme (Sigma) solution was added to 9 ml of the cell suspension, and the mixture was incubated at room temperature (23°C) or at 37°C for 30 min. The above-mentioned washing and incubation steps were not carried out under stringent anaerobic conditions because we had determined that the Avicelase activity in culture fluid was not appreciably affected even by prolonged exposure to air (7).

In some cases, lysozyme-treated cells were disrupted by passing the mixture through a French pressure cell at 10,000 lb/in^2 (7×10^6 kg/m^2). In some experiments, cells were disrupted by passing the mixture through the French pressure cell three times without previous treatment with lysozyme; in other experiments, cells were disrupted by adding lysozyme and Triton X-100 (0.5 g/100 ml; final concentration) without passing the mixture through the French pressure cell. Cell debris was separated by centrifugation (10 min, 27,000 × *g*, 4°C). Avicelase and CMCase activities in

culture fluids, in supernatants resulting from centrifugation of disrupted cells (cell extracts), and in the particulate fraction (cell debris) were assayed as described above.

To determine whether Avicelase activity was present in association with whole cells of strain C7, cell suspensions (prepared by carrying out all procedural steps in an N_2 atmosphere) were incubated with Avicel under anaerobic conditions, and assays for products were performed. Mid- or late-log-phase cells used in these experiments were washed once and suspended in succinate-NaOH buffer (0.1 M [pH 6]; containing 0.002 M dithiothreitol, final concentration). One milliliter of cell suspension (5.5×10^9 cells) was added to the Avicelase assay reaction mixture (see above) instead of the enzyme preparation. After 48 and 72 h the reaction mixture supernatant was assayed for soluble sugars (e.g., glucose, cellobiose) and fermentation end products (e.g., acetate, ethanol) by high-pressure liquid chromatography with an Aminex HPX-87H column (Bio-Rad) by the method of Ehrlich et al. (10).

Separation of the extracellular cellulase system complex. Strain C7 cells were grown in an N_2 atmosphere either in MJ-CB broth containing 0.25 g of cellobiose per 100 ml or in MJ-C broth containing a growth-limiting amount of cellulose (0.1 g [dry weight] of ball-milled filter paper per 100 ml). Late-log or early-stationary-phase cells were pelleted by centrifugation (15 min, 4,000 × *g*, 4°C) from 4 liters of MJ-CB broth. Cells grown in MJ-C broth (1 liter) were pelleted after the cellulose in the culture was depleted. The pellet was discarded, and the supernatant fluid was subjected to ultrafiltration with an Amicon CH2 Ultrafiltration Unit (Amicon Corp., Danvers, Mass.) equipped with a 100,000-M_r-cutoff Diaflo hollow fiber cartridge (Amicon). Ultrafiltration was continued until the volume of the material retained by the cartridge (concentrate) was 90 ml. When the protein level in the concentrate was not sufficiently high (i.e., less than 1.5 mg/ml), the concentrate was further reduced in volume by placing it in dialysis tubing and blowing cool air over it (pervaporation), or by dialysis against 30% polyethylene glycol (15,000 to 20,000 M_r; Sigma). In both of these concentration methods a 12,000- to 14,000-M_r-cutoff dialysis membrane (Spectra/Por; Fisher Scientific Co., Pittsburg, Pa.) was used.

Ultrafiltration concentrate (2.7 ml, 1.5 to 2.5 mg of protein per ml) was applied to a Sephacryl S-300 column (91 by 1.7 cm, 40- to 105-μm wet bead size, Superfine; Pharmacia, Uppsala, Sweden) equilibrated with succinate-NaOH buffer (0.1 M, pH 6). Gel chromatography was carried out at 6°C. Protein was eluted with the succinate-NaOH buffer (flow rate, 12 ml/h), and fractions (2 ml each) were collected. The protein concentration (A_{280}) and Avicelase and CMCase activities of individual fractions were measured (see above). For each absorption peak the three fractions with the highest A_{280} (corresponding to the highest enzyme activities) were pooled, and the pooled material was concentrated by pervaporation or by dialysis against 30% polyethylene glycol. The pooled and concentrated fractions were used for electrophoretic analyses and other experiments (see below). The following molecular weight standards were used to calibrate the Sephacryl S-300 column: blue dextran, thyroglobulin, ferritin, phycocyanin, and vitamin B_{12}.

In some cases, the following adsorption-desorption step was used in conjunction with the separation procedure. The concentrate from the ultrafiltration step (>100,000 M_r, 100 ml) was incubated with a mixture of Avicel (0.5 g) and ashless cellulose powder (1 g; J. T. Baker Chemical Co., Phillipsburg, N.J.) for 1 h at 30°C. Then 1.7 ml of ball-milled cellulose slurry (3 g of Whatman no. 1 filter paper per 100 ml of distilled water) was added, and the mixture was incubated at 30°C for 1 h with occasional stirring. After the supernatant fluid was decanted, the mixture was washed briefly with 10 ml of succinate-NaOH buffer (0.1 M, pH 6.0), suspended in 10 ml of the same buffer, and poured into a glass column (2.7 by 9.5 cm), and protein was eluted with 130 ml of distilled water at room temperature. Fractions (3.5 ml each) were collected and assayed for CMCase activity. Fractions containing activity were pooled, concentrated by dialysis against 30% polyethylene glycol, and applied to a Sephacryl S-300 column (see above). Protein was eluted from the column with succinate-NaOH buffer, as described above.

Electrophoretic analysis of proteins. Proteins were separated by polyacrylamide gel electrophoresis (PAGE) in the presence and absence of sodium dodecyl sulfate (SDS). Two mini-slab vertical electrophoresis systems (Mini-Protein II, Bio-Rad; and Mini-Slab, Idea Scientific Co., Corvallis, Oreg.) were used. Electrophoresis in polyacrylamide slab gels (stacking gel, 5% acrylamide; running gel, 7.5% acrylamide; 8 by 10 by 0.75 cm) was performed in the absence of SDS at 6°C or in the presence of SDS (0.1%, wt/vol) with the gel system of Laemmli (15).

Glycerol to a final concentration of 10% (vol/vol) was added to samples from the Sephacryl S-300 column before they were applied to nondenaturing (native) polyacrylamide gels. The following additions were made to Sephacryl S-300 column samples before analysis by SDS-PAGE (final concentrations): SDS, 1.5% (wt/vol); glycerol, 6% (vol/vol); 2-β-mercaptoethanol, 6.3% (vol/vol). Before SDS-PAGE, the mixtures were boiled for 3 min for extensive denaturation (i.e., complete loss of Avicelase and CMCase activities) or kept at room temperature for 1 h for mild denaturation. For determination of molecular weight, the standards (Bio-Rad) used were myosin, β-galactosidase, phosphorylase B, bovine serum albumin, and ovalbumin. Bromophenol blue in 10% glycerol (0.08 g/100 ml, final dye concentration) was used as reference dye. Gels were stained with Coomassie brilliant blue R-250 (Bio-Rad). CMCase activity in polyacrylamide gels of nondenatured and mildly denatured preparations was determined as described below.

Polyacrylamide gel overlays (zymograms) for CMCase activity. CMCase activity in gels subjected to electrophoresis was detected by using polyacrylamide gel zymograms that contained CMC as a substrate. The zymogram solution contained the following, in a total volume of 100 ml: succinate-NaOH buffer (pH 6), 5.4 mmol; polyacrylamide, 4.75 g; bisacrylamide, 0.15 g; CMC, 0.5 g; ammonium persulfate, 0.03 g; *N,N,N',N'*-tetramethylethylenediamine, 0.05 ml; riboflavin, 0.0005 g; and distilled water. The zymogram solution was cast on a polyacrylamide gel support film (FMC Corp.) to a thickness of 0.4 mm. Then the zymogram was incubated in a humidity chamber within the anaerobic chamber for 75 min at 30°C in contact with the gel that had been subjected to electrophoresis. After incubation, the CMC-containing zymogram was stained for residual carbohydrate with an aqueous solution of Congo red (1 mg/ml), destained with 1 M NaCl, and fixed with 1.0% (wt/vol) NaOH. Clear areas in the zymogram indicated CMCase activity.

Ferritin-stained cells. Cells grown in GS2-CB broth (0.25 g of cellobiose per 100 ml) were harvested in the mid- and late-log phases, prepared for staining, and then stained with cationized ferritin (Sigma) by the procedure of Bayer et al. (3). Stained cells were processed for electron microscopy as described by Bayer et al. (3), sectioned with an MT2

ultramicrotome, and viewed with a JEOL 100S electron microscope.

RESULTS

Production of cellulolytic enzymes by mesophilic clostridia. When cellulose was the fermentable substrate, the supernatant fluid of strain C7 broth cultures remained clear, inasmuch as the cells grew in association with the sedimented cellulose material (ball-milled filter paper). However, when the sediment in the cultures was examined by light microscopy of wet-mount preparations, the cells of strain C7 did not appear to adhere to the cellulose fibers, in contrast to cells of *C. thermocellum* strains, which show strong adherence to the cellulosic substrate when examined in a similar manner (24).

Avicelase activity was present in the supernatant fluid of cultures of strain C7 grown in media containing cellulose as the fermentable substrate (19). The Avicelase activity was inducible, inasmuch as it was found in culture supernatant fluids when some but not all of the fermentable sugars were individually present in the growth medium as the carbon and energy source. For example, 5.5 to 6.0 U of Avicelase activity per ml was detected in early-stationary-phase cultures of strain C7 grown in a medium (MJ-CB) containing cellobiose as the fermentable substrate. Growth yields in these cultures ranged from 7.8×10^8 to 1×10^9 cells per ml, as determined by light microscopy enumeration. Xylan and, to a smaller extent, L-arabinose also induced Avicelase activity, whereas D-xylose did not serve as an inducer, nor did it repress Avicelase in cultures containing both cellobiose and D-xylose. D-Glucose did not induce Avicelase production under the growth conditions used.

Extracellular Avicelase activity in MJ-CB medium cultures of strain C7 increased during the log phase of growth and reached a maximum (e.g., 6 U/ml) during the late-log or early stationary phase. During growth significant cell lysis did not occur in the cultures, as determined by light microscopy examination and by measuring the amount of DNA present in culture fluids (5, 11). This indicated that Avicelase was released into the culture fluid via processes that did not involve cell lysis.

To determine whether cell-associated Avicelase activity was present in strain C7, cells in the mid- or late-log growth phase were disrupted by a number of different procedures (see Materials and Methods). In some of the experiments, less than 0.06% of the total Avicelase activity in the culture was detected in the particulate fraction (cell debris) from log-phase C7 cells. In other experiments, cell debris-associated Avicelase activity was completely absent. Likewise, Avicelase activity was either not detected or present at very low levels in the liquid fraction (cell extract) resulting from cell disruption. The treatments used to disrupt the cells did not affect Avicelase activity, inasmuch as this activity in culture fluid did not decrease when the fluid was passed three times through a French pressure cell (13,000 lb/in^2), treated with lysozyme, or treated with lysozyme and Triton X-100 (see Materials and Methods). Furthermore, nongrowing, log-phase washed cells of strain C7 did not hydrolyze crystalline cellulose, as determined by measuring formation of reducing sugars and end products (e.g., acetate, ethanol) from Avicel. These observations indicated that Avicelase activity in strain C7 cultures is extracellular and that only an extremely low level of activity, if any, is cell associated.

Approximately 91% (0.16 U/ml) of the CMCase activity was present in the fluid of cultures of strain C7 (late-log or early-stationary phase, medium MJ-CB), with the remainder being cell associated. β-Glucosidase activity in late-log-phase strain C7 cultures in GS2-CB medium was mostly (72%) cell associated (J. J. Kane and E. Canale-Parola, unpublished data).

TABLE 1. Growth substrates for strain C7 (wild-type) and LS (mutant)

Substrate	Growth on substrate	
	C7	LS
D-Glucose	+	+
Cellobiose	+	+
Cellotriose	+	+
Cellotetraose	+	+
Cellopentaose	+	+
Cellohexaose	+	+
CMC	−	−
Ball-milled filter paper	+	−
Avicel	+	−
D-Xylose	+	+
Xylan	+	+
L-Arabinose	+	+

The cellulose utilization mutant (strain LS) had the same substrate utilization pattern as wild-type strain C7, except that it did not ferment Avicel and ball-milled filter paper (Table 1). Avicelase activity was not detected either in disrupted cells or in culture supernatants of mutant strain LS, whereas CMCase activity was present both in culture fluids and in disrupted cells of the mutant.

Absence of polycellulosomes from the surface of strain C7 cells. *C. thermocellum* synthesizes high-molecular-weight, multiprotein complexes, named cellulosomes, that hydrolyze crystalline cellulose (2, 16, 21). Clusters of cellulosomes (polycellulosomes) are present on the *C. thermocellum* cell surface and are responsible for the strong adherence of the cells to cellulose (2). Bayer and co-workers (2, 3) demonstrated by electron microscopy the presence of polycellulosomal organelles appearing as protuberances on the surface of *C. thermocellum* cells treated with cationized ferritin.

We examined cationized ferritin-treated cells of strain C7 and of *C. thermocellum* by transmission electron microscopy, using methods similar to those of Bayer and coworkers (3). Structures similar to the polycellulosomal protuberances described by Bayer et al. (3) were observed on the surface of *C. thermocellum* (Fig. 1B). However, the protuberances were never present on the surface of strain C7 cells (Fig. 1A) examined in the early, middle, and late logarithmic phases of growth. These results are consistent with the observations that strain C7 cells do not have cell-associated Avicelase activity and do not adhere to cellulose.

Isolation of cellulolytic enzymes. Preliminary experiments involving gel filtration chromatography of culture supernatant fluids indicated that Avicelase activity was associated only with high-molecular-weight protein fractions (results not shown). Therefore, ultrafiltration through a hollow-fiber cartridge was used as the first step in the isolation of strain C7 extracellular cellulolytic enzymes, to eliminate low-molecular-weight (<100,000) proteins from culture supernatant fluids. This step served to increase the concentration of protein and resulted in a 1.5-fold purification of Avicelase activity (Table 2). After treatment of the concentrate by gel filtration chromatography (Sephacryl S-300 column), a 4.7-fold total increase in Avicelase specific activity was obtained (Table 2). When the concentrate obtained by ultrafiltration of

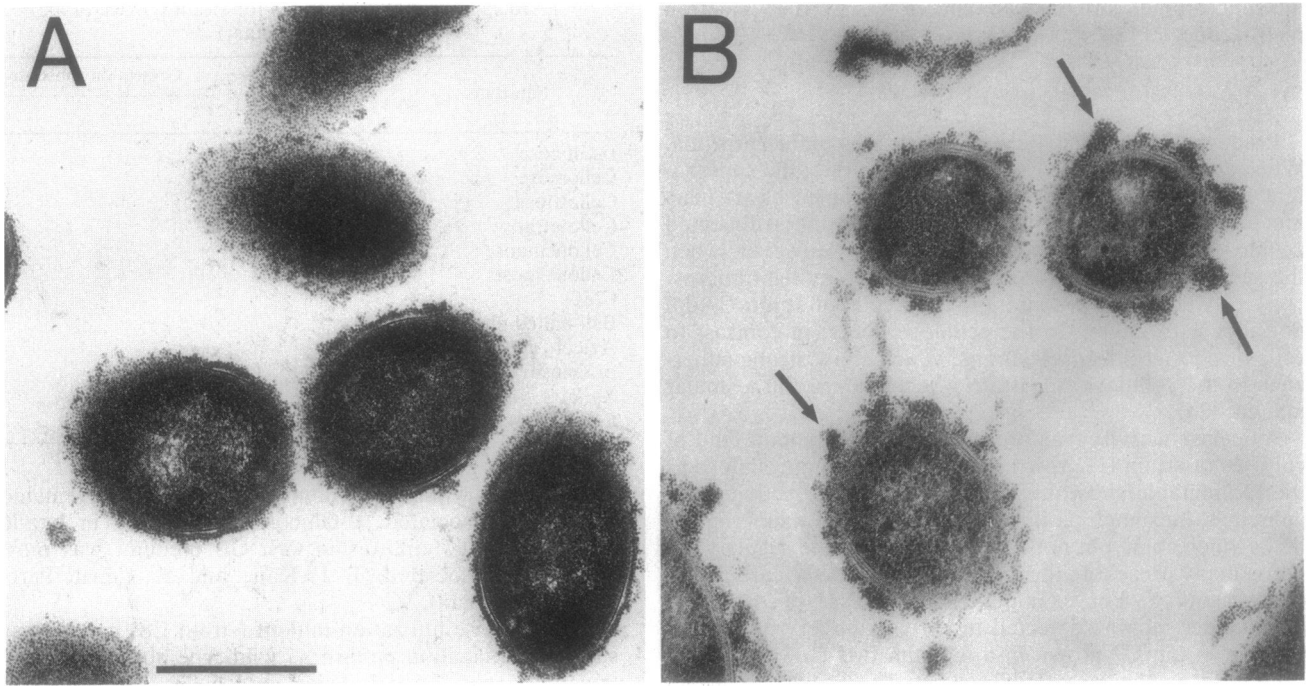

FIG. 1. Comparison of the cell surface ultrastructure of cellobiose-grown strain C7 (A) and *C. thermocellum* YS (B) cells. Shown are transmission electron micrographs of thin sections of cells stained with cationized ferritin. Note that protuberant structures (cellulosomes) indicated by arrows are present on the cell surface of *C. thermocellum* (B) but are absent from the surface of strain C7 cells (A). Bar, 0.5 μm.

strain C7 culture supernatant was fractionated on a Sephacryl S-300 gel filtration column, we consistently observed a major absorbance peak (A_{280}) that eluted in the 700,000-M_r region and a minor absorbance peak that eluted in the 130,000-M_r region (Fig. 2 and 3). Gel filtration protein and activity profiles of ultrafiltration concentrates from strain C7 grown either in cellulose-containing medium (Fig. 2) or in cellobiose-containing medium (Fig. 3) were very similar. Both Avicelase and CMCase activities coincided with the 700,000-M_r peak (Table 2, Fig. 2), whereas only CMCase activity was present in the fractions corresponding to the 130,000-M_r peak (Fig. 2). Avicelase activity in the pooled 700,000 M_r fractions ranged from 24.6 to 32 U/ml, depending on the experiment. Low levels of Avicelase activity were present in the void-volume fractions (e.g., 5.5 U of activity per ml in fractions with the highest A_{280}), but a peak(s) of Avicelase activity was not detected in the void volume. In fractions other than those corresponding to the void volume and to the 700,000-M_r peak, significant Avicelase activity was not present. CMCase activity was always detected in fractions that had Avicelase activity. Thus, fractions lacking CMCase activity also lacked Avicelase activity.

The ultrafiltration concentrate from the culture superna-

TABLE 2. Isolation of extracellular Avicelase from strain C7 cultures

Prepn	Avicelase activity (U/ml)	Protein (mg/ml)	Sp act (U/mg of protein)	Purification (fold)	Yield (%)
Culture supernatant	5.8	0.095	61.1	1.0	100
After ultrafiltration	134.0	1.430	93.7	1.5	52
After gel filtration[a]	24.6	0.085	289.4	4.7	28

[a] Data for the pooled 700,000-M_r fractions. Cells were grown in MJ-CB broth.

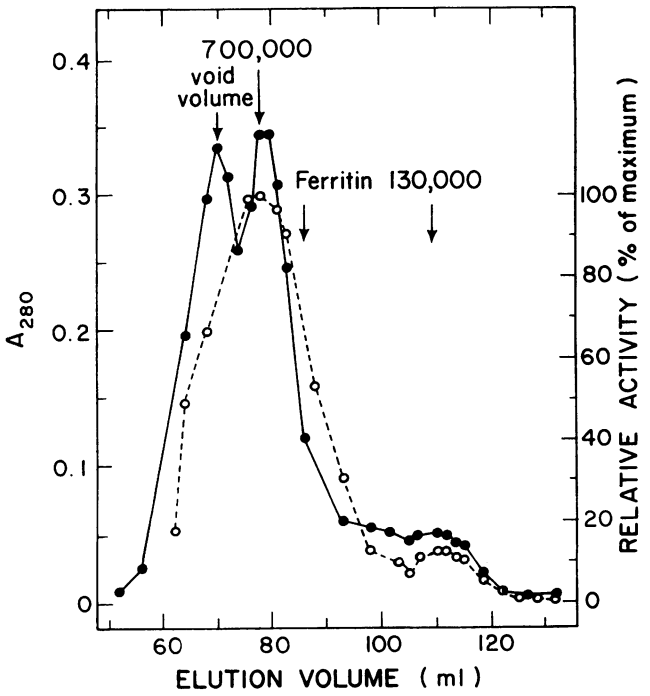

FIG. 2. Sephacryl S-300 chromatography of ultrafiltration-concentrated cellulase preparation from culture fluid of cellulose-grown strain C7. Cells were grown in MJ-C broth. Protein as A_{280} (●) and CMCase activity (○) were determined as described in Materials and Methods. The elution positions of ferritin (M_r 443,000), the 700,000- and 130,000-M_r fractions, and the void volume are indicated by arrows. The fraction with the highest CMCase activity contained 0.32 U of CMCase activity per ml.

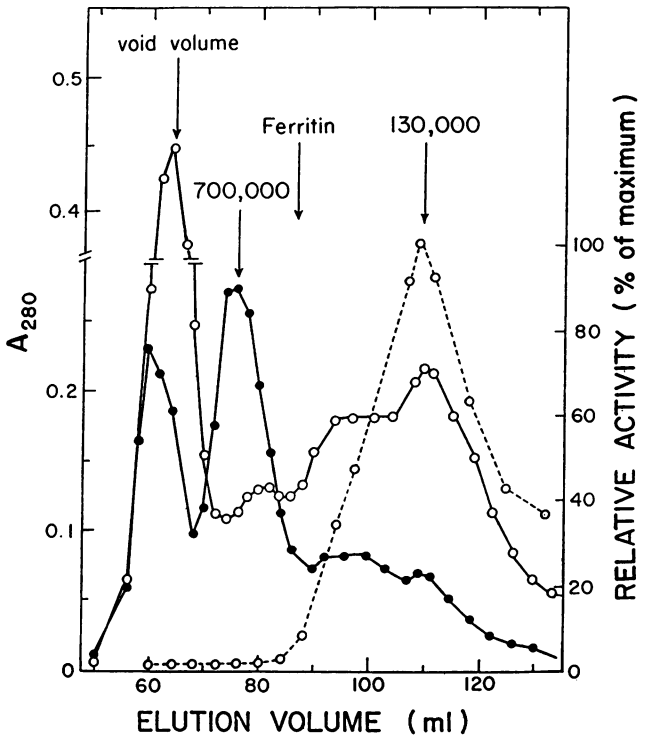

FIG. 3. Sephacryl S-300 chromatography of ultrafiltration-concentrated cellulase preparation from culture fluids of cellobiose-grown strain C7 and mutant strain LS. Cells were grown in MJ-CB broth. Protein (A_{280}) in strain C7 (●——●) and strain LS (○——○) fractions and CMCase activity in strain LS (○----○) fractions were determined as described in Materials and Methods. The elution positions of ferritin, the 700,000- and 130,000-M_r fractions, and the void volume are designated by arrows. The fraction with the highest CMCase activity contained 0.15 U of CMCase activity per ml. The CMCase activity profile for cellobiose-grown strain C7 (data not shown) was similar to that for cellulose-grown strain C7 (Fig. 2).

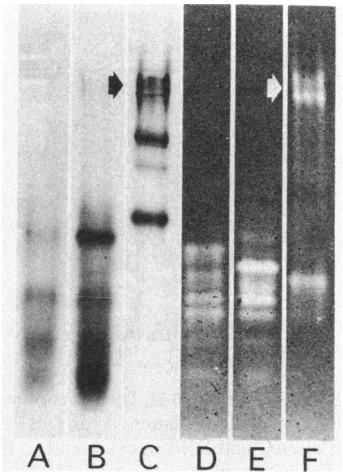

FIG. 4. Nondenaturing (native) PAGE of enzyme-active Sephacryl S-300 column fractions. Gels were stained for protein with Coomassie brilliant blue R (lanes A, B, and C) or analyzed for cellulolytic activity by means of a CMC-polyacrylamide zymogram (lanes D, E, and F) as described in Materials and Methods. All samples contained 15 μg of protein. Lanes: A and D, 130,000-M_r fraction of wild-type strain C7; B and E, 130,000-M_r fraction of mutant strain LS; C and F, 700,000-M_r fraction of wild-type strain C7. Arrows point to the broad, slowly migrating, multiple-protein band that contained much of the CMCase activity (lanes C and F).

tant of the cellulose utilization mutant (strain LS) lacking both extracellular and cell-associated Avicelase activity was subjected to Sephacryl S-300 gel filtration. Concentrate from strain LS lacked the Avicelase-active 700,000-M_r absorption peak but contained a CMCase-active 130,000-M_r peak (Fig. 3).

Strain C7 ultrafiltration concentrate was treated by means of an adsorption-desorption procedure involving binding of the enzymes to cellulose, followed by elution (see Materials and Methods). Sephacryl S-300 gel filtration of the eluate from cellulose yielded the same protein and enzymatic activity profiles observed when the concentrate was not treated by the adsorption-desorption procedure.

The pooled Avicelase-containing fractions (M_r 700,000) from strain C7, when analyzed by nondenaturing PAGE, showed a broad, slowly migrating band that contained much of the CMCase activity (Fig. 4, lanes C and F). In addition, several lower-molecular-weight protein bands were observed (lane C), as well as a high-specific-activity band that showed in the CMC gel overlay (lane F) but not in the gel stained with Coomassie brilliant blue (lane C).

Mild denaturation of the pooled 700,000-M_r fractions was accomplished by treating them with SDS at room temperature (see Materials and Methods) before SDS-PAGE. After this treatment, Avicelase activity was no longer present in the fractions, whereas CMCase activity was still detected. When the pooled, mildly denatured, Avicelase-lacking fractions were analyzed by SDS-PAGE, approximately 15 protein bands were present (Fig. 5, lane B), six of which exhibited CMCase activity (lane D). Apparently, some of these bands were derived from proteins that formed the broad, slowly migrating, CMCase-active band in nondenaturing polyacrylamide gels. These results supported the idea that a multiprotein aggregate was responsible for the formation of the broad, slowly migrating band. Nearly identical

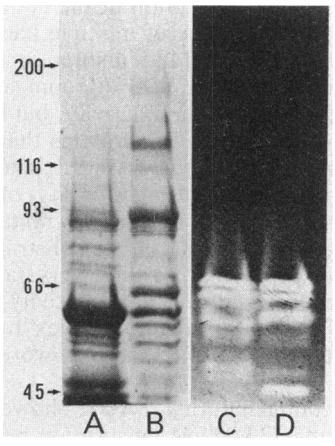

FIG. 5. SDS-PAGE of mildly denatured CMCase-active Sephacryl S-300 column fractions. Gels were stained for protein (lanes A and B) or analyzed for CMCase activity (lanes C and D). All samples contained 15 μg of protein. Lanes: A and C, 130,000-M_r fraction of mutant strain LS; B and D, 700,000-M_r fraction of wild-type strain C7. Note protein bands with similar migration rates in the 130,000-M_r fraction of mutant strain LS (lane A) and the 700,000-M_r fraction of wild-type strain C7 (lane B). CMCase-active protein bands with similar migration rates are present in lanes C and D. The numbers indicate M_rs in thousands.

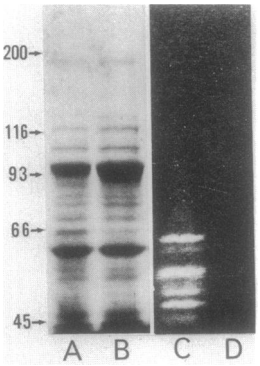

FIG. 6. SDS-PAGE of 130,000-M_r fractions of mutant strain LS. Gels were stained for protein (lanes A and B) or analyzed for CMCase activity (lanes C and D). Lanes: A and C, mildly denatured fraction; B and D, extensively denatured fraction. All samples contained 20 μg of protein. Note the similarity between the protein profiles of the mildly and extensively denatured samples. CMCase activity was absent in the extensively denatured sample (lane D). The numbers indicate M_rs in thousands.

protein band patterns were yielded by mildly denatured 700,000-M_r preparations (Fig. 5, lane B) and extensively denatured 700,000-M_r preparations (data not shown).

The results described above indicated that the Avicelase activity resided in a large protein complex, with a molecular weight of approximately 700,000, that apparently consisted of at least 15 proteins. This complex had both Avicelase and CMCase activities and appeared to be eluted as a single entity from Sephacryl S-300 chromatography columns (Fig. 3). Mutant strain LS, which lacked Avicelase activity, lacked the complex as well. The Avicelase-active complex was relatively stable, inasmuch as reapplication of the pooled 700,000-M_r fractions to Sephacryl S-300 columns again yielded protein that was eluted as a single peak (A_{280}, 700,000 M_r) and had both Avicelase and CMCase activities. However, during electrophoresis on nondenaturing polyacrylamide gels, some disruption of the complex occurred, as indicated by the presence of multiple bands on the gels (Fig. 4, lanes C and F). Mild disruption of the protein complex by treatment with SDS at room temperature resulted in total loss of Avicelase activity, but CMCase activity was retained by some of the proteins that resulted from the breaking apart of the complex (Fig. 5, lane D). Inasmuch as disruption of the structural integrity of the complex resulted in loss of the ability to degrade crystalline cellulose, it appears that the 700,000-M_r complex of strain C7 functions as a unit in the depolymerization of crystalline cellulose. Attempts to reconstitute Avicelase activity by combining components of the multiprotein complex by using procedures similar to those of Wu and co-workers (26) were unsuccessful.

Analysis by nondenaturing PAGE showed that pooled fractions from the 130,000-M_r absorption peak from either strain C7 or mutant strain LS lacked the slowly migrating, multiple-protein, CMCase-active complex observed in the 700,000-M_r fractions but contained various lower-molecular-weight protein bands that had CMCase activity (Fig. 4, lanes A, B, D, and E). Upon mild denaturation, the pooled 130,000-M_r fractions from strain C7 (data not shown) or mutant LS yielded similar protein band profiles consisting of three major protein bands and many other protein bands (Fig. 6, lane A). Six of the bands contained CMCase activity (lane C). Extensively denatured preparations yielded band profiles (lane B) similar to those of mildly denatured preparations (lane A), except that all CMCase activity was lost (lane D).

The 130,000-M_r CMCase activity in both strain C7 and the mutant strain was eluted as a single peak when the ultrafiltration concentrate was directly fractionated on a Sephacryl S-300 column (Fig. 2 and 3) and also when the ultrafiltration concentrate was treated by the adsorption-desorption procedure before fractionation. These observations suggested that the 130,000-M_r CMCase activity may reside in a multiprotein complex that retained its structural integrity during the purification procedures we used. As indicated by PAGE (Fig. 4, lanes A, B, D, and E), the CMCase activity is present in various protein bands, and it is possible that the CMCase-active proteins in these bands are components of the postulated complex. Furthermore, SDS-PAGE analysis indicated that five of the six CMCase-active protein bands from the pooled 130,000-M_r fractions had migration rates similar to those of CMCase-active bands from the pooled 700,000-M_r fractions (Fig. 5, lanes C and D).

DISCUSSION

Clostridium sp. strain C7, like the thermophilic bacterium *C. thermocellum*, produces a multiprotein complex that is capable of degrading crystalline cellulose. The multiprotein complex (cellulosome) (16) of *C. thermocellum* forms ferritin-stainable clusters (polycellulosomes), which are located on the cell surface and are responsible for attachment of the cells to cellulose (8). As fermentation proceeds, the multiprotein complexes of *C. thermocellum* become separated from the cell surface and from the substrate and are released into the culture supernatant fluid, where they retain their enzymatic activity for a time and finally break down, forming polypeptides (8, 21). Thus, *C. thermocellum* cultures contain cell-associated and extracellular (i.e., free in the culture fluid) multiprotein complexes, both capable of degrading crystalline cellulose (8, 21).

The following observations indicated that, in strain C7, the enzymatic activity responsible for the degradation of crystalline cellulose (Avicelase activity) resided in a multiprotein complex.

(i) The Avicelase activity coincided with an A_{280} peak that eluted from Sephacryl S-300 columns in the 700,000-M_r region. When ultrafiltration concentrate was treated by an adsorption-desorption procedure before fractionation by gel filtration, the Avicelase-active peak was still present in the Sephacryl S-300 column eluate. Furthermore, the Avicelase-active peak persisted through repeated Sephacryl S-300 gel filtration of the 700,000-M_r fractions.

(ii) Nondenaturing PAGE analysis of the 700,000-M_r fractions showed a broad, slowly migrating band. SDS-PAGE analysis showed that, after mild denaturation, these fractions yielded a number of protein bands derived from the broad, slowly migrating band. This observation is in agreement with the idea that the slowly migrating band comprised a multiple-protein aggregate.

(iii) Fractions from the 700,000-M_r region, when subjected to mild SDS treatment, which caused the multiple-protein aggregate to break apart, lost Avicelase activity while retaining CMCase activity.

(iv) An Avicelase-deficient mutant (strain LS) that lacked the ability to utilize crystalline cellulose lacked the Avicelase-active 700,000-M_r peak as well.

(v) When wild-type strain C7 was cultured under condi-

tions that did not induce Avicelase synthesis (e.g., with D-xylose as the fermentable substrate), the Avicelase-active 700,000-M_r peak was not detected (data not shown).

Our studies indicated that the cellulolytic system of mesophilic *Clostridium* sp. strain C7 differs significantly from that of *C. thermocellum*. We found that the mesophilic *Clostridium* sp. lacked cell-associated cellulases capable of degrading crystalline cellulose. At all stages of growth tested, the enzyme activity responsible for crystalline cellulose degradation was present in the culture supernatant fluid but was not detected in significant amounts in disrupted cells or in association with whole cells. In addition, whereas we readily demonstrated ferritin-stainable structures (polycellulosomes) on the surfaces of cells of a *C. thermocellum* strain used as a control, we did not detect these structures on cells of *Clostridium* sp. strain C7. Furthermore, cells of *Clostridium* sp. strain C7 did not adhere to cellulose, in contrast with *C. thermocellum* cells, which possess polycellulosomes that mediate their attachment to the cellulosic substrate. Finally, the multiprotein complex utilized by strain C7 in the hydrolysis of crystalline cellulose was estimated to have a molecular weight of approximately 700,000 and, therefore, is much smaller than the *C. thermocellum* cellulosome, which has a molecular weight ranging from 2×10^6 to 4.5×10^6 (21).

Our observations that the mesophilic *Clostridium* (i) lacks significant cell-associated Avicelase activity, (ii) releases Avicelase activity into the culture fluid, and (iii) does not have cellulosome clusters on its cell surface are in agreement with the idea that the multiprotein complex used by this bacterium to degrade crystalline cellulose is entirely or almost entirely assembled extracellularly (e.g., neither within the cell nor in the form of structures adhering to the cell surface).

When protein in the pooled 700,000-M_r fractions from the Sephacryl S-300 column was mildly denatured by SDS treatment, the Avicelase activity was totally lost, whereas CMCase activity was still present. SDS-PAGE of the mildly denatured preparations showed that CMCase activity was retained in 6 of the 15 protein bands resulting from the dissociation of the 700,000-M_r multiple-protein complex. Examination by SDS-PAGE indicated that these CMCase-active proteins had migration rates similar to those of CMCase-active proteins present in the 130,000-M_r fractions of either strain C7 or mutant strain LS (Fig. 5). These observations suggest that, in strain C7 cultures, the 130,000-M_r fraction proteins may serve as precursors for the assembly of the large Avicelase-containing complex and/or may be derived from its partial dissociation. Mutant strain LS, which lacks the 700,000-M_r complex and does not have the ability to hydrolyze crystalline cellulose, may be deficient in protein(s) or other components necessary to assemble the Avicelase-containing complex.

The results indicate that at least six different endo-1,4-β-glucanases may be part of the cellulase system of strain C7. We have not determined whether proteins with exoglucanase (exocellobiohydrolase) activity are part of the 700,000-M_r strain C7 complex (7). The presence of exoglucanases in *C. thermocellum* cellulosomes has not been demonstrated (8). However, mechanisms of crystalline cellulose degradation that do not involve exoglucanases have been postulated for anaerobic bacteria (8).

ACKNOWLEDGMENTS

We thank E. Musante for expert assistance with electron microscopy and photography procedures. We are grateful to T. Warnick for high-pressure liquid chromatography determinations of sugars and fermentation end products.

This research was supported by Department of Energy grants DE-FG02-88ER13898 and DE-AC02-81ER10878 and by U.S. Department of Agriculture grant 87-CRCR-1-2398.

LITERATURE CITED

1. **Adelberg, E. A., M. Mandel, and G. C. C. Chen.** 1965. Optimal conditions for mutagenesis by *N*-methyl-*N'*-nitrosoguanidine in *Escherichia coli* K12. Biochem. Biophys. Res. Commun. **18:** 788–795.
2. **Bayer, E. A., and R. Lamed.** 1986. Ultrastructure of the cell surface cellulosome of *Clostridium thermocellum* and its interaction with cellulose. J. Bacteriol. **167:**828–836.
3. **Bayer, E. A., E. Setter, and R. Lamed.** 1985. Organization and distribution of the cellulosome in *Clostridium thermocellum*. J. Bacteriol. **163:**552–559.
4. **Bradford, M. M.** 1976. A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principles of protein dye binding. Anal. Biochem. **72:**248–254.
5. **Burton, K.** 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. **62:**315–323.
6. **Cato, E. P., W. L. George, and S. M. Finegold.** 1986. Genus *Clostridium* Prazmowski, p. 1141–1200. *In* P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 2. The Williams & Wilkins Co., Baltimore.
7. **Cavedon, K., S. B. Leschine, and E. Canale-Parola.** 1990. Characterization of the extracellular cellulase from a mesophilic clostridium (strain C7). J. Bacteriol. **172:**4231–4237.
8. **Coughlan, M. P., and L. G. Ljungdahl.** 1988. Comparative biochemistry of fungal and bacterial cellulolytic enzyme systems, p. 11–30. *In* J.-P. Aubert, P. Beguin, and J. Millet (ed.), Biochemistry and genetics of cellulose degradation, FEMS Symposium no. 43. Academic Press, Inc., New York.
9. **Davis, B. D.** 1948. Isolation of biochemically deficient mutants of bacteria by penicillin. J. Am. Chem. Soc. **70:**4267.
10. **Ehrlich, G. G., D. F. Goerlitz, J. H. Bourell, G. V. Eisen, and E. M. Godsy.** 1981. Liquid chromatographic procedure for fermentation product analysis in the identification of anaerobic bacteria. Appl. Environ. Microbiol. **42:**878–885.
11. **Giles, K. W., and A. Myers.** 1965. An improved diphenylamine method for the estimation of deoxyribonucleic acid. Nature (London) **206:**93.
12. **Hungate, R. E.** 1969. A roll tube method for cultivation of strict anaerobes. Methods Microbiol. **3B:**117–132.
13. **Johnson, E. A., A. Madia, and A. L. Demain.** 1981. Chemically defined minimal medium for growth of the anaerobic cellulolytic thermophile *Clostridium thermocellum*. Appl. Environ. Microbiol. **41:**1060–1062.
14. **Johnson, E. A., M. Sakajoh, G. Halliwell, A. Madia, and A. L. Demain.** 1982. Saccharification of complex cellulosic substrates by the cellulase system from *Clostridium thermocellum*. Appl. Environ. Microbiol. **43:**1125–1132.
15. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
16. **Lamed, R., E. Setter, R. Kenig, and E. A. Bayer.** 1983. The cellulosome—a discrete cell surface organelle of *Clostridium thermocellum* which exhibits separate antigenic, cellulose-binding and various cellulolytic activities. Biotechnol. Bioeng. Symp. **13:**163–181.
17. **Lederberg, J., and N. D. Zinder.** 1948. Concentration of biochemical mutants of bacteria with penicillin. J. Am. Chem. Soc. **70:**4267–4268.
18. **Leschine, S. B., and E. Canale-Parola.** 1983. Mesophilic cellulolytic clostridia from freshwater environments. Appl. Environ. Microbiol. **46:**728–737.
19. **Leschine, S. B., and E. Canale-Parola.** 1984. Ethanol production from cellulose by a coculture of *Zymomonas mobilis* and a clostridium. Curr. Microbiol. **11:**129–136.

20. **Leschine, S. B., K. Holwell, and E. Canale-Parola.** 1988. Nitrogen fixation by anaerobic cellulolytic bacteria. Science **242:** 1157–1159.
21. **Mayer, F., M. P. Coughlan, Y. Mori, and L. G. Ljungdahl.** 1987. Macromolecular organization of the cellulolytic enzyme complex of *Clostridium thermocellum* as revealed by electron microscopy. Appl. Environ. Microbiol. **53:**2785–2792.
22. **Miller, G. L., R. Blum, W. E. Glennon, and A. L. Burton.** 1960. Measurement of carboxymethylcellulase activity. Anal. Biochem. **2:**127–132.
23. **Warshaw, J. E., S. B. Leschine, and E. Canale-Parola.** 1985. Anaerobic cellulolytic bacteria from wetwood of living trees. Appl. Environ. Microbiol. **50:**807–811.
24. **Wiegel, J., and M. Dykstra.** 1984. *Clostridium thermocellum*: adhesion and sporulation while adhered to cellulose and hemicellulose. Appl. Microbiol. Biotechnol. **20:**59–65.
25. **Wood, T. M., and K. M. Bhat.** 1988. Methods for measuring cellulase activities. Methods Enzymol. **160:**87–112.
26. **Wu, J. H. D., W. H. Orme-Johnson, and A. L. Demain.** 1988. Two components of an extracellular protein aggregate of *Clostridium thermocellum* together degrade crystalline cellulose. Biochemistry **27:**1703–1709.