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Clostridium cellulolyticum Viability and Sporulation under Cellobiose Starvation Conditions

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Depending on the moment of cellobiose starvation, *Clostridium cellulolyticum* cells behave in different ways. Cells starved during the exponential phase of growth sporulate at 30%, whereas exhaustion of the carbon substrate at the beginning of growth does not provoke cell sporulation. Growth in the presence of excess cellobiose generates 3% spores. The response of *C. cellulolyticum* to carbon starvation involves changes in proteolytic activities; higher activities (20% protein degradation) corresponded to a higher level of sporulation; lower proteolysis (5%) was observed in cells starved during the beginning of exponential growth, when sporulation was not observed; with an excess of cellobiose, an intermediate value (10%), accompanied by a low level of sporulation, was observed in cells taken at the end of the exponential growth phase. The basal percentage of the protein breakdown in nonstarved culture was 4%. Cells lacking proteolytic activities failed to induce sporulation. High concentrations of cellobiose repressed proteolytic activities and sporulation. The onset of carbon starvation during the growth phase affected the survival response of *C. cellulolyticum* via the sporulation process and also via cell-cellulose interaction. Cells from the exponential growth phase were more adhesive to filter paper than cells from the stationary growth phase but less than cells from the late stationary growth phase.

In nature, most bacteria are challenged by conditions of widely changing nutrient availability as well as by exposure to various forms of physical stress. A remarkable feature of bacterial species is their capacity for rapid growth when nutrients are available. Perhaps even more remarkable is their ability to remain viable under conditions not propitious for growth.

In both soil and marine environments, nutrient supplies are often scarce (17). Many bacteria possess highly sophisticated mechanisms that allow them to maintain cell viability during starvation for essential nutrients and resume growth when nutrients again become available. Some species form dormant spores, whereas others form multicellular aggregates and fruiting bodies in response to starvation conditions (10, 12). However, even without the formation of such elaborately differentiated cells, many bacteria enter a starvation-induced program that results in a metabolically less active and more resistant state.

It is generally believed that the capability of cells to survive starvation and to tolerate treatments that are lethal during growth is based on the synthesis of a set of proteins commonly referred to as starvation and stress proteins (14). During starvation conditions, one role of protein degradation in bacteria is to provide a source of amino acids for cells (8, 9, 15).

Clostridium cellulolyticum ATCC 35319, a gram-positive, anaerobic, mesophilic bacterium, is able to degrade cellulose efficiently (19). *C. cellulolyticum* possesses cellulosome-like structures with a complex peptide distribution (13), and its cellulolytic system has been studied by molecular genetic approaches (3, 4, 18, 21). In a previous paper, we showed that the colonization of cellulose by *C. cellulolyticum* occurs by the process of adhesion, colonization, release, and readhesion (6). It seemed possible to consider the release stage to be a starvation period. In this paper, we report on the behavior of *C. cellulolyticum* cells under different cellobiose growth conditions, focusing our investigation on cell viability, adhesion of cells, and sporulation capabilities.

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MATERIALS AND METHODS

Organisms, substrates, and culture conditions. *C. cellulolyticum* ATCC 35319 was grown anaerobically at 34°C in CM_3 medium (per liter): KH_2PO_4, 1.5 g; $K_2HPO_4 \cdot 3H_2O$, 2.9 g; $(NH_4)_2SO_4$, 1.3 g; $MgCl_2 \cdot 6H_2O$, 0.1 g; $CaCl_2$, 0.02 g; yeast extract, 5 g; 5% $FeSO_4$, 25 μl; 0.2% resazurin, 1.0 ml; and L-cysteine hydrochloride, 0.5 g. Cellobiose (0.5, 1, or 7 g/liter; OSI, Paris, France) was included in this basal medium. The Hungate roll tube contained 4.5 ml of basal medium with cellobiose at 3 g/liter and agar at 20 g/liter.

C. cellulolyticum was grown anaerobically at 34°C in a 2-liter fermentor (LSL Biolafitte SA) with 1.5 liters of basal medium. The medium was reduced during cooling and bubbled with nitrogen 3 h before inoculation. A 1-day-old culture on 3 g of cellobiose per liter was used as the inoculum (10%, vol/vol). Gentle stirring (50 rpm) was carried out after 24 h of culture. The pH was maintained at 7.2 by automatic addition of 6 N NH_4OH. During fermentation, samples were taken daily and different experiments were performed.

Materials. Whatman no. 1 filter paper and cellulose nitrate filters were purchased from Whatman International Ltd., Maidstone, England. L-[4,5-^{3}H]leucine (specific activity, 68 Ci/mmol) was purchased from Amersham, Les Ulis, France. The scintillation fluid was Ready Safe (Beckman Instruments, Inc., Fullerton, Calif.). Unless otherwise specified, all other chemicals were reagent grade and were purchased from Sigma Chemical Co., St. Louis, Mo.

Growth measurements and sugar consumption. Growth was measured by reading the optical density at 600 nm with a Beckman model 25 spectrophotometer. An optical density of 1 at 600 nm corresponded to 0.5 g (dry weight) per liter. Reducing sugars were determined by the method of Miller (16) with glucose as the standard.

Viable cells, spores, and epifluorescence. Culture samples were diluted stepwise in CM_3 medium. The number of viable *C. cellulolyticum* cells during the time course of fermentation was calculated from the number of CFU on solid CM_3 (cellobiose, 3 g/liter; agar, 20 g/liter) roll tubes incubated for 3 days at 34°C. The assay for the number of spores was performed in the same way, but the roll tubes were heated for 10 min at 80°C. Viable-cell and spore counts were determined in duplicate with three different fermentations from roll tubes containing 20 to 300 colonies.

Epifluorescence counts were made by a standard acridine orange staining method for which 10 μl of culture was deposited on a 1-cm^2 slide and counted, after staining with acridine orange (0.025% in 0.1 M sodium citrate buffer [pH 6.0]), with a Nikon epifluorescence microscope (model HB 10101 AF). The cellular viability and sporulation percentage were expressed as a percentage of the total cells counted by microscopy.

Adhesion measurements. Adhesion was allowed to occur by addition of 15 ml

of bacterial or spore suspension (the cells were suspended in 50 mM Tris HCl buffer [pH 7.0]) to a 240-cm^2 Whatman no. 1 filter paper (a single piece measuring 6 by 20 cm) in a glass tube (25 by 2.5 cm) by the method of Gelhaye et al. (7). The tube was stoppered and placed horizontally on a reciprocating roller (Bioblock Scientific no. 95212) for 30 min at 34°C. This arrangement afforded gentle agitation, and the incubation time allowed maximal adhesion of cellulolytic bacteria to cellulose (1, 20). After incubation, the optical density of the supernatant at 600 nm was determined. These optical density values were converted to dry weight per milliliter by harvesting cells by centrifugation at 10,000 $\times$ *g* for 30 min and drying the pellet to constant weight at 70°C. The initial concentration of the biomass was 0.25 mg (dry weight)/ml.

Protein degradation studies. The methods used to measure protein degradation were an adaptation of those used by Damereau and St-John (2). For studies examining the degradation of proteins, cells growing in CM_3 medium with 3 g of cellobiose per liter were labeled with L-[4,5-^{3}H]leucine (final concentration, 5.0 μCi/ml) for 5 h at the beginning, middle, and end of the exponential growth phase. The labeled cultures were divided and then harvested by centrifugation in an anaerobic atmosphere, washed twice, and resuspended in CM_3 medium without cellobiose (starvation conditions) or with 3 g of cellobiose per liter (growth conditions). Duplicate 1-ml aliquots were taken immediately upon resuspension of the cells (zero time) and at various time points thereafter. Proteins were precipitated with 1 ml of 10% trichloroacetic acid as described by Fuhrman and Azam (5). After 1 h of extraction on ice, the cold trichloroacetic acid-insoluble material was collected by filtration through 25-mm-diameter cellulose nitrate filters (pore size, 0.22 μm). The filters were placed in scintillation vials. Both ethyl acetate (1 ml, to dissolve filter) and scintillation fluid (9 ml) were added, and the radioactivity was assayed by liquid scintillation spectrometry.

RESULTS

Study of *C. cellulolyticum* growth under different cellobiose conditions. Three cellobiose concentrations were used for cell growth: 7, 1, and 0.5 g/liter. The pH was monitored during the cultures, because unregulated conditions lead to weak viability. Figure 1 shows growth, cellobiose consumption, and cell viability for *C. cellulolyticum* with the different cellobiose concentrations. When cellobiose was used at 7 g/liter, cells entered the stationary phase when the cellobiose concentration was still at 3 g/liter. This growth curve corresponded to the expected growth of *C. cellulolyticum* with a soluble substrate. When cellobiose was used at 1 g/liter, carbon starvation occurred when the cells were in their exponential phase of growth. When cellobiose was used at 0.5 g/liter, the cells were at the beginning of their fermentation phase when the substrate was totally consumed.

The maximal optical densities at 600 nm were 1.05, 0.6, and 0.25 for the cultures with 7, 1, and 0.5 g of cellobiose per liter, respectively. The generation times were 5 $\pm$ 0.4 h for the different fermentations. Cells grown on cellobiose at 7 g/liter entered a long stationary phase when the biomass reached 0.5 g/liter, whereas they showed only between 60 and 30% viability during 10 h after the exponential growth, and then the viability fell to 3%. In Fig. 1B, the behavior of *C. cellulolyticum* grown on cellobiose at 1 g/liter is compared with that of the other two cultures (Fig. 1A and C). Cell division took place as long as the substrate was available, and then the cells entered a stationary phase of growth and cellular lysis. Cells from the 1-g/liter culture were viable during a very long period, since 30% of them could form colonies on an adequate solid medium. At the opposite extreme, cells from the 0.5-g/liter culture were viable for only 6 h after the growth phase, when an important lysis occurred (i.e., half of the cells were lysed in 6 h). As shown in Fig. 1B, the optical density of the culture increased after 125 h of fermentation.

Spore formation by *C. cellulolyticum*. Spore formation in each culture was observed as described above. The more interesting behavior was the culture of *C. cellulolyticum* on 1 g of cellobiose per liter. Indeed, Fig. 2 shows viability and sporulation percentages during the course of fermentation on 1 g of cellobiose per liter. Sporulation of *C. cellulolyticum* began after 150 h of fermentation, when 30% of the cells were viable

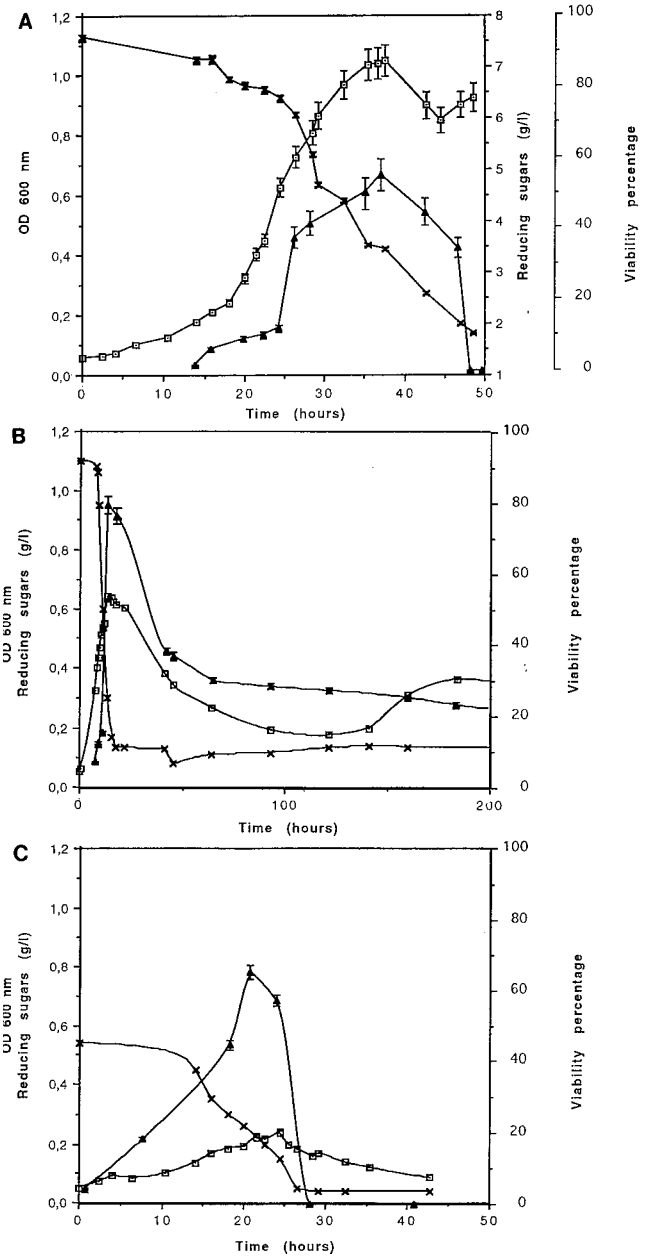

FIG. 1. Growth curves, reducing-sugar consumption, and viability curves of *C. cellulolyticum* in a fermentor at 34°C with pH regulation at 7.2 on cellobiose at 7 g/liter (A), 1 g/liter (B), and 0.5 g/liter (C). Each point is the mean $\pm$ standard deviation of three determinations for two different cultures. Symbols: ⊡, growth; ×, reducing sugars; ▲, viability. OD, optical density.

although growth had ceased 120 h previously. After 180 h of culture, cells had sporulated significantly. Starvation of cells during the exponential growth phase induced a state which allowed the cells to survive. The fermentation with 7 g of cellobiose per liter gave only a low sporulation percentage, since 3% of the total cells were viable and sporulated. With 0.5 g of substrate per liter, spores were not detected. These results indicate that a particular cellular state is necessary to initiate the sporulation process.

Thus, depending on the growth conditions used, *C. cellulolyticum* cells were able to behave in different ways. Onset of

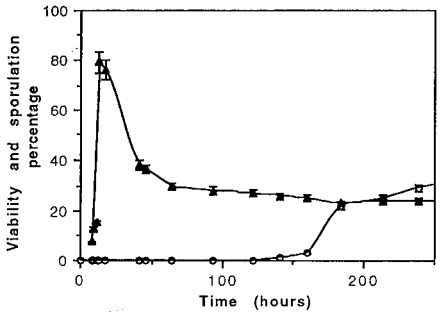

FIG. 2. Percentages of viability and sporulation of *C. cellulolyticum* cells grown on cellobiose at 1 g/liter in a fermentor. Each point is the mean ± standard deviation of three determinations for two different cultures. Symbols: ▲, viability; ○, sporulation.

carbon starvation during exponential growth induced sporulation. A long period occurred during which cells were viable without sporulating.

Rate of protein degradation during carbon starvation. To further analyze the effects of carbon starvation on the levels of proteolytic activities, we withdrew cells grown from medium with 3 g of cellobiose per liter and with L-[4,5-^{3}H]leucine when cells reached optical densities of 0.25, 0.6, and 1.05. These values correspond to the optimal optical densities obtained on 0.5, 1, and 7 g of cellobiose per liter (Fig. 1). Thus, they correspond to starvation occurring at the beginning, middle, and end of growth, respectively. Figure 3 presents the proteolysis rates obtained for experiments under starvation and growth conditions. The proteolysis was greater (20%) when cells were starved during the mid-exponential phase. A lower activity (5%) was observed in cells starved at the beginning of the exponential growth, and an intermediate value (10%) was observed in cells starved at the end of the growth. When exponential growth was allowed by resuspending the cells in a complete medium, there was a basal rate of proteolysis (around 4%).

Cell adhesion. Beside sporulation, adhesion and colonization of new sites on cellulose allow *C. cellulolyticum* to escape carbon starvation. Figure 4 presents the relationship between the percentage of *C. cellulolyticum* adhesion and the fermentation time. Adhesion represents a main step in the colonization of cellulose. This is why it seemed important to study the capability of cells to adhere according to the age of the bacteria. Cells harvested in the exponential phase were more adherent to the filter paper than were cells harvested in the early stationary phase of growth. At a later time, adhesion of cells from the late stationary phase was increased and reached 80%. At this time, spores composed about 30% of the total suspension used for the adhesion experiment. By microscopic observation of the suspension before and after the adhesion on the filter paper, the percentage of adherent spores was 90% of the initial spores, suggesting that the increased adhesion of the "old cells" could result from the high level of spore adhesion. Therefore, the stage of growth strongly influenced the percentage of adhesion at the cell density of 0.25 mg (dry weight)/ml, used in this experiment.

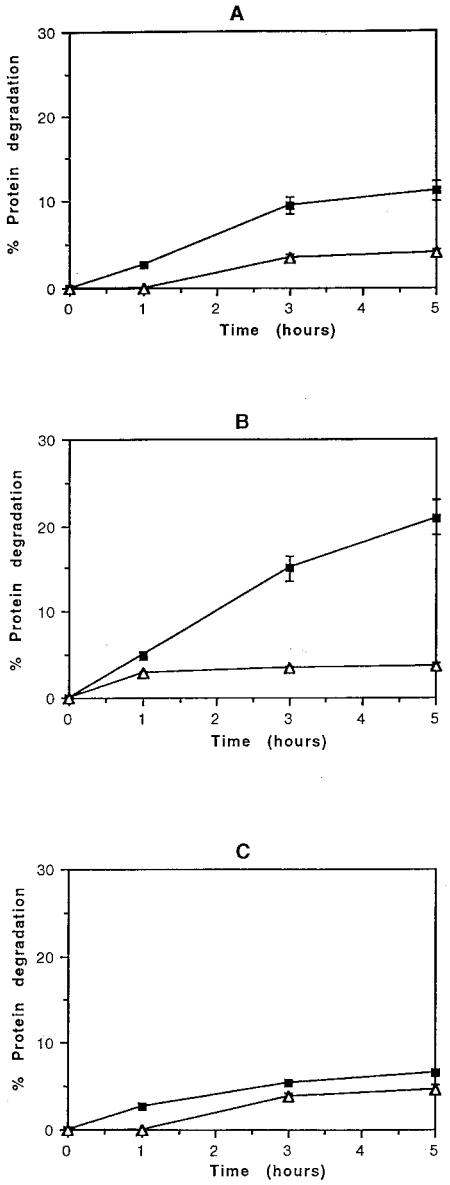

FIG. 3. Effect of starvation and readdition of cellobiose on the degradation of proteins labeled during *C. cellulolyticum* exponential growth. Proteolysis of proteins synthesized at the end (A), the middle (B), and the beginning (C) of the exponential growth phase when cells were resuspended in a cellobiose complete medium (growth conditions with 3 g of cellobiose per liter) and in a cellobiose-free medium (starvation conditions) as described in Materials and Methods. Each point is the mean ± standard deviation of three determinations for two different cultures. Symbols: △, cellobiose complete medium; ■, cellobiose-free medium.

DISCUSSION

This paper describes cell viability, cell sporulation, proteolysis, and cell adhesion of the cellulolytic, mesophilic *C. cellulolyticum* ATCC 35319. Depending on the concentration of cellobiose used for the growth of the microorganism, carbon starvation started at the beginning or in the middle of the exponential phase when cellobiose was present at 0.5 and 1 g/liter, respectively. The control growth was the culture with an excess of cellobiose (7 g/liter).

As shown in experiments dealing with viability related to growth (Fig. 1), the onset of carbon starvation during the growth phase affects the survival response of *C. cellulolyticum*. Cells starved during the mid-exponential phase could possess the intracellular information needed to remain viable, by means of a combination of factors which allow cells to survive in environments with scarce nutrients. Moreover, there was a

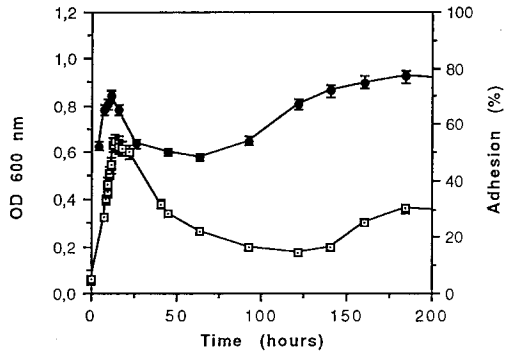

FIG. 4. Growth curve of *C. cellulolyticum* grown on 1 g of cellobiose per liter and cell adhesion percentage. The adhesion experiment was performed with a cell suspension of 0.25 mg/ml for 30 min at 34°C. Each point is the mean ± standard deviation of three determinations for two different cultures. Symbols: ⊡, growth; ●, adhesion. OD, optical density.

long period between the end of growth on 1 g of cellobiose per liter and the beginning of the sporulation. This time could be an adaptative state preceding a carbon starvation, during which there is an induction of starvation-specific proteins. Cells deprived of cellobiose in early exponential growth were not able to fight against the exhaustion of the substrate and died. Therefore, the induction of sporulation by starvation conditions allows *C. cellulolyticum* cells to survive when the cells have been released from their substrate.

In Fig. 1B, the optical density of the culture increased at around 125 h of fermentation. This increase could be due to changes in the optical properties of the sporulating cells, to elongation of individual cells without appreciable cell division, or to an increase in cell size as previously demonstrated (11).

One role of protein degradation in bacteria is to provide a source of amino acids for cells during starvation (8, 9, 15). The breakdown of proteins synthesized during the exponential phase of growth was great when cells were starved during the mid-exponential phase. This means that carbon-starved cells in the mid-exponential growth phase generated an endogenous protein degradation to survive the carbon deprivation. In this starved culture, many new starvation-specific polypeptides could be induced. When growth was completed by resuspending cells in a cellobiose basal medium, the rate of proteolysis reached 4% at 5 h and corresponded to the basic turnover of proteins in growing cells. Indeed, in starved *C. cellulolyticum* cells, the increased proteolysis was correlated to the need for amino acids required to synthesize new proteins. At this stage of the exponential growth, cells probably possessed the signals which allowed viability when they were challenged by a stress such as starvation.

Therefore, we demonstrated in this study that carbon starvation during the growth of *C. cellulolyticum* provoked various types of cell behavior. In *C. cellulolyticum*, a strong correlation exits between the ability to survive and the aptitude for sporulation. This is particularly true in experiments with high cellobiose concentrations, which repressed sporulation and hence the survival response, as well as in experiments with low cellobiose concentrations, which did not allow the induction of proteolytic activities required for the sporulation. The life cycle of cellulolytic bacteria in natural environments should therefore depend on the stage of growth when the release of cellulolytic bacteria from the substrate occurs as a result of either exhaustion of the lignocellulosic substrate or saturation of the adhesion sites. We demonstrated that the physiological state of a cell upon cellulose release is a determining factor for the future of this released cell and hence that in the colonization process of the cellulose (6), a starved *C. cellulolyticum* cell should readhere to another site, die, or sporulate and readhere.

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REFERENCES

1. **Bhat, S., R. J. Wallace, and E. R. Orskov.** 1990. Adhesion of cellulolytic ruminal bacteria to barley straw. Appl. Environ. Microbiol. **56:**2693–2703.
2. **Damereau, K., and A. C. St-John.** 1993. Role of Clp protease subunits in degradation of carbon starvation proteins in *Escherichia coli*. J. Bacteriol. **175:**53–63.
3. **Faure, E., C. Bagnara, A. Belaich, and J.-P. Belaich.** 1988. Cloning and expression of two cellulase genes of *Clostridium cellulolyticum* in *Escherichia coli*. Gene **65:**51–58.
4. **Fierobe, H. P., C. Gaudin, A. Belaich, M. Loutfi, E. Faure, C. Bagnara, D. Baty, and J.-P. Belaich.** 1991. Characterization of endoglucanase A from *Clostridium cellulolyticum*. J. Bacteriol. **173:**7956–7962.
5. **Fuhrman, J. A., and F. Azam.** 1982. Thymidine incorporation as a measure of heterotrophic bacterioplankton production in marine surface waters: evaluation and field results. Mar. Biol. **66:**109–120.
6. **Gelhaye, E., A. Gehin, and H. Petitdemange.** 1993. Colonization of crystalline cellulose by *Clostridium cellulolyticum* ATCC 35319. Appl. Environ. Microbiol. **59:**3154–3156.
7. **Gelhaye, E., H. Petitdemange, and R. Gay.** 1993. Adhesion and growth rate of *Clostridium cellulolyticum* ATCC 35319 on crystalline cellulose. J. Bacteriol. **175:**3452–3458.
8. **Goldberg, A. L., and A. C. St John.** 1976. Intracellular protein degradation in mammalian and bacterial cells. Part 2. Annu. Rev. Biochem. **45:**747–803.
9. **Gottesman, S.** 1989. Genetics of proteolysis in *Escherichia coli*. Annu. Rev. Genet. **23:**163–198.
10. **Kaiser, D.** 1984. Regulation of multicellular development in *Myxobacteria*, p. 197–218. *In* R. Losick and L. Shapiro (ed.), Microbial development. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
11. **Landuyt, S. L., and E. J. Hsu.** 1992. Preparation of refractile spores of *Clostridium thermosaccharolyticum* involves a solventogenic phase. Appl. Environ. Microbiol. **58:**1797–1800.
12. **Losick, R., and P. Youngman.** 1984. Endospore formation in *Bacillus*, p. 63–88. *In* R. Losick and L. Shapiro (ed.), Microbial development. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. **Madarro, A., J. L. Pena, J. L. Lequerica, S. Valles, R. Gay, and A. Flors.** 1991. Partial purification of the cellulases from *Clostridium cellulolyticum* H10. J. Chem. Technol. Biotechnol. **52:**393–406.
14. **Matin, A.** 1991. The molecular basis of carbon starvation induced general resistance in *Escherichia coli*. Mol. Microbiol. **5:**3–10.
15. **Miller, C. G.** 1987. Protein degradation and proteolytic modification, p. 680–691. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
16. **Miller, G. L.** 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugars. Anal. Chem. **31:**426–428.
17. **Morita, R. Y.** 1988. Bioavailability of energy and its relationship to growth and starvation survival in nature. Can. J. Microbiol. **34:**346–441.
18. **Perez-Martinez, G., L. Gonzales-Candelas, J. Polaina, and A. Flors.** 1988. Expression of endoglucanase gene from *Clostridium cellulolyticum* in *E. coli*. J. Ind. Microbiol. **3:**365–371.
19. **Petitdemange, E., F. Caillet, J. Giallo, and C. Gaudin.** 1984. *Clostridium cellulolyticum* sp. nov., a cellulolytic mesophilic species from decayed grass. Int J. Syst. Bacteriol. **34:**155–159.
20. **Rasmussen, M. A., B. A. White, and R. B. Hespell.** 1989. Improved assay for determining adherence of ruminal bacteria to cellulose. Appl. Environ. Microbiol. **55:**2089–2091.
21. **Shima, S., Y. Ygarashi, and T. Kodama.** 1991. Molecular cloning of a new endoglucanase gene from *Clostridium cellulolyticum* and its expression in *Escherichia coli*. Appl. Microbiol. Biotechnol. **35:**233–236.