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Adhesion and Growth Rate of *Clostridium cellulolyticum* ATCC 35319 on Crystalline Cellulose

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The rate of tritiated-thymidine incorporation into DNA was used to estimate *Clostridium cellulolyticum* H10 growth rates on Avicel cellulose, taking into consideration both the unattached cells and the cells adhered to the substrate. The generation time on cellobiose calculated from the data on cell density (4.5 h) agreed well with the generation time calculated by tritiated-thymidine incorporation (3.8 h). Growth on Avicel cellulose occurred when bacteria were adhered to their substrate; 80% of the biomass was detected on the cellulose. Taking into consideration attached and free bacteria, the generation time as determined by thymidine incorporation was about 8 h, whereas by bacterial-protein estimation it was about 13 h. In addition to the growth rate of the bacteria on the cellulose, the release of adhered cells constituted an important factor in the efficiency of the cellulolysis. The stage of growth influenced adhesion of *C. cellulolyticum*; maximum adhesion was found during the exponential phase. Under the conditions used, the end of growth was characterized by an acute release of biomass and cellulase activity from the cellulose. An exhaustion of the accessible cellulose could be responsible for this release.

The degradation of biopolymers such as cellulose and hemicellulose is of considerable interest in terms of both microbial ecology and industrial microbiology. Fundamental studies of cellulolytic bacteria developed about 20 years ago and were devoted essentially to the cellulase system (2, 5, 16, 17, 27, 31, 34, 36) and to adhesion of bacteria to cellulose (1, 4, 8, 11, 15, 18, 28).

Until now, however, no study on colonization of cellulose that takes into consideration unattached cells and cells adhered to the substrate has been published. The reason for this has been the absence of reliable quantitative techniques for studying insoluble-substrate colonization. Therefore, we have adapted a technique to measure growth rates of bacteria both adhered to crystalline cellulose and free-living in the bulk liquid phase during colonization. This technique is incorporation of [^{3}H]thymidine into DNA, defined as the cold-trichloroacetic acid (cold-TCA)-precipitable fraction, which has been commonly used as a measure of bacterial growth in water and for cells attached to particles (7, 12–14, 22, 32).

In this study, we investigated some of the properties of adhesion and colonization of *Clostridium cellulolyticum* H10 (ATCC 35319) on crystalline cellulose. *C. cellulolyticum* is able to degrade crystalline cellulose completely (30), and its physiology has been studied previously (9, 10). *C. cellulolyticum* possesses cellulosome-like structures with a complex polypeptide distribution, very much resembling those described for *Clostridium cellulovorans* (24); in addition, three genes coding for endo-1,4-β-glucanase activities have been cloned (6, 29, 33).

MATERIALS AND METHODS

Organisms, substrates, and culture conditions. *Clostridium acetobutylicum* ATCC 824 was grown anaerobically at 34°C in reinforced clostridial medium.

C. cellulolyticum ATCC 35319 was grown anaerobically at 34°C in Hungate tubes containing the following (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. The desired carbon source (Avicel cellulose [7.5 g/liter] or cellobiose [0.6 or 2 g/liter]) was included in this basal medium.

Materials. Reinforced clostridial medium was purchased from Oxoid Ltd., Basingstoke, Hampshire, England. Whatman no. 1 filter paper and filters of cellulose nitrate were purchased from Whatman International Ltd., Maidstone, England. Carboxymethyl cellulose and Avicel cellulose were purchased from Fluka, Buchs, Switzerland. [*methyl*-^{3}H]thymidine (specific activity, 75 to 80 Ci/mmol) and L-[^{35}S]methionine (specific activity, 500 mCi/mmol) were purchased from Amersham, Les Ulis, France. The scintillation fluid was "Ready safe" purchased from Beckman Instruments, Inc., Fullerton, Calif. Unless otherwise specified, all other chemicals were reagent grade and purchased from Sigma Chemical Co., St. Louis, Mo.

Thymidine incorporation rate measurement. Batch cultures (9 ml) with or without agitation (reciprocating roller; Bioblock Scientific no. 95212) were grown in Hungate tubes. In the course of fermentation, filter-sterilized thymidine (labeled and unlabeled) was added anaerobically, so that the final concentration was 42 μM and the radioactivity was 2.1 μCi/ml. The cultures were incubated for 2 h at 34°C after being mixed. At the end of the time course, cellulose had settled and two samples of 2 ml of the upper phase containing unattached (free) cells were carefully collected (fraction 1). The remaining culture (fraction 2) was mixed, and two samples of 2 ml containing attached and free cells were collected. For cultures grown on 2 g of cellobiose per liter, the same treatment was applied. After 2 h, the culture was mixed and two 2-ml samples were collected.

The collected samples were immersed immediately in ice water for 1 min, and an equal volume of ice-cold 10% TCA was added to precipitate the macromolecules as described by Fuhrman and Azam (7). RNA, macromolecular proteins,

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and DNA were included in this insoluble fraction. After 1 h of extraction on ice, the cold-TCA-insoluble material was collected by filtration through 25-mm-diameter cellulose nitrate filters (pore size, 0.22 μm). The filters were washed five times with 2.5 ml of ice-cold 5% TCA. With five washings, cellulose did not interfere with the radioactivity detection. Filters were placed in scintillation vials. Both ethyl acetate (1 ml, to dissolve the filter) and scintillation fluid (9 ml) were added, and the radioactivity was assayed by liquid scintillation spectrometry; efficiency and quenching were determined by comparing actual and theoretical values, using standards.

Incorporation into free cells was calculated directly with the upper-phase samples (fraction 1). Incorporation into attached cells was the difference between radioactivity in fraction 2 and that in fraction 1, since the same free population was found in both fractions. Values obtained were converted to disintegrations per minute per milliliter.

Isotope dilution analysis. Isotope dilution analysis measured the dilution of incorporated radioactivity (32). *C. cellulolyticum* was grown on cellobiose in Hungate tubes. At a cell concentration of 0.12 mg/ml, tritiated thymidine was added (0.4 or 2.1 μCi/ml) together with increased amounts of unlabeled thymidine (1 to 50 μM). After 2 h, samples were immediately filtered and washed with cold 5% TCA.

Exhaustion of the carbon source. *C. cellulolyticum* was grown on 0.6 g of cellobiose per liter. At this concentration, exhaustion of the carbon source occurred when the cells were in the exponential phase (10). To monitor the rapid changes in the thymidine incorporation rate, thymidine was added so that the final concentration was 28 μM and the radioactivity was 3.8 μCi/ml. The cultures were incubated for 15 min. After incubation, 2 samples (2 ml each) were collected and the samples were filtered and washed with cold 5% TCA.

Bacterial-protein estimation. The determination of biomass in the cellulose-grown cultures was based on bacterial-protein estimation as described by Bensadoun and Weinstein (3). An aliquot (2 ml) of fraction 2 or of fraction 1 was centrifuged. The pellet was washed twice with 2 ml of NaCl (0.9%) and incubated with 0.025 ml of sodium desoxycholate (2%) for 15 min. A 1-ml volume of 24% TCA was added to the medium, and the assay was centrifuged at 6,000 × *g* for 15 min. Under these conditions, the cells burst and intracellular proteins were found in the pellet. The protein concentration was determined by the method of Lowry et al. (23). Unattached biomass was measured directly with the upper-phase samples (fraction 1). Attached biomass was the difference between the biomass in fraction 2 and the free biomass found in the upper phase (fraction 1). The values obtained were converted to milligrams of cells per milliliter. Calibrations were performed with cells grown on cellobiose to estimate the relationship between optical density, dry weight, and protein content of the cells.

CMCase activity. Endoglucanase activity was determined in vitro by the method of Miller et al. with carboxymethyl cellulose as the substrate (26). The incubation was performed for 30 min at 34°C. One unit of carboxymethyl cellulase (CMCase) activity was defined as the amount of enzyme which releases 1 μmol of reducing sugar (with glucose as the standard) per min under the conditions indicated. Unattached-cellulose CMCase activity was measured directly with the upper-phase samples (fraction 1). Attached-cellulose CMCase activity was the difference between the activity found in fraction 2 and the free activity (fraction 1).

Reducing sugars. The amount of reducing sugar was determined by the method of Miller (25).

Influence of stage of growth. Cellobiose-grown cultures (2 g/liter) were specifically labeled with L-[^{35}S]methionine (500 μCi/mmol) at a concentration of 1 μCi/ml. The methionine was added at the start of the fermentation. The cells were harvested by centrifugation (10,000 × *g* for 30 min) in the exponential and stationary phases, and the pellets were washed and suspended in 50 mM Tris-HCl buffer (pH 7.5). These labeled cells were used in adhesion experiments (see below).

Heterogeneous population. Cellulose-grown cultures (7.5 g/liter) were specifically labeled with L-[^{35}S]methionine (500 μCi/mmol) at a concentration of 1 μCi/ml. The methionine was added at the start of the fermentation. After 50 to 60 h of fermentation, cultures (50 ml) were harvested and mixed, and the cellulose was allowed to settle for 2 h at 4°C. The upper phase was separated carefully from the settled cellulose pellet. The upper phase was centrifuged at 10,000 × *g* for 30 min, and the resulting pellet was mixed with 10 ml of 50 mM Tris-HCl buffer (pH 7.5). This cell suspension constitutes population A.

The remaining settled cellulose pellet was mixed with 50 ml of 50 mM Tris-HCl buffer (pH 7.5) and shaken (1 min), and the cellulose was allowed to settle for 2 h at 4°C. The upper phase was collected and the treatment described above was applied again to the settled cellulose pellet. Both upper phases were pooled and centrifuged at 10,000 × *g*. The pellet was mixed with 10 ml of 50 mM Tris-HCl buffer (pH 7.5). This cell suspension constitutes population B.

Adhesion measurement. Labeled cells were used in the following adherence assay. Calibrations were performed to determine the relationship between the detected radioactivity and the dry weight of the cells. Adhesion was allowed to occur by adding 5 ml of bacterial suspension to 0.1 g of Whatman filter paper (1 by 10 cm) in a Hungate tube. This tube was stoppered and placed horizontally on a reciprocating roller (Bioblock Scientific no. 95212) at 34°C. This arrangement afforded gentle agitation and adequate opportunity for adhesion (8). After 30 min, the filter paper was removed from the sample and the unadhered cells were collected by filtration of the bacterial suspension (5 ml) through 25-mm-diameter cellulose nitrate filters (pore size, 0.45 μm). The filters were washed three times with 2.5 ml of 50 mM Tris-HCl buffer (pH 7.7) and placed into scintillation vials. Ethyl acetate (1 ml) was added, and the radioactivity was assayed by liquid scintillation spectrometry. Total adhered cells were determined by using the following formula: % adhered cells = {[total dpm added − total dpm in supernatant (unadhered cells)]/(total dpm added)} × 100. All samples were analyzed in triplicate, and each experiment was repeated several times.

Cellulose degradation. At the end of fermentation (200 h), the percentage of degraded cellulose was estimated by filtering the culture medium. Cells were lysed with 8% (vol/vol) formic acid, filtered, and dried at 70°C to a constant weight. The quantity of degraded cellulose was calculated as the difference between the initial and final weights.

RESULTS

Validity of the method. (i) *C. acetobutylicum* ATCC 824 cannot grow on crystalline cellulose (19–21). The same percentages of radioactively labeled thymidine added to a *C. acetobutylicum* batch culture were removed from the extracellular medium in the presence and absence of cellulose.

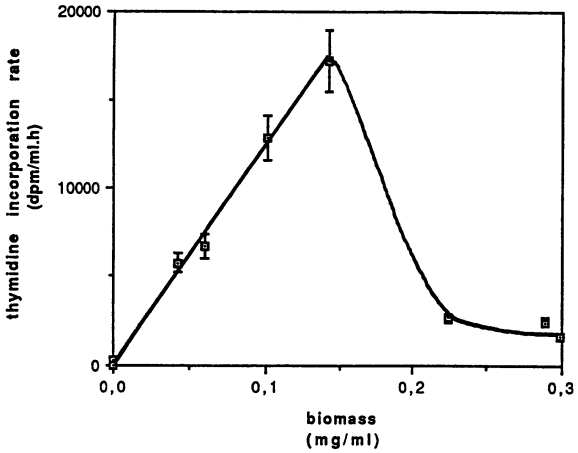


FIG. 1. Relation between biomass production and rate of thymidine incorporation during *C. cellulolyticum* growth on cellobiose (2 g/liter). Each point is the mean ± SD of four determinations for two different cultures.

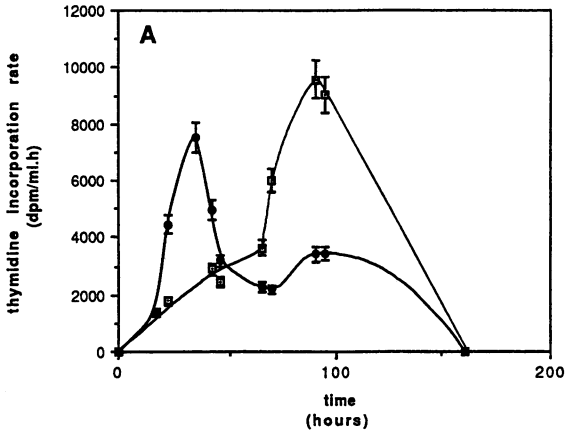
Thus, nonspecific adsorption of the thymidine was not observed in the presence of cellulose. Furthermore, experiments performed with and without cellulose showed that cellulose had no effect on the cold-TCA-insoluble material extraction.

(ii) To assess the significant concentration of extracellular thymidine needed to suppress cellular synthesis by *C. cellulolyticum*, isotope dilution experiments were done as described in Materials and Methods. At thymidine concentrations less than 5 μM, the amount of incorporated radioactivity increased with that of added unlabeled thymidine, showing a limitation in thymidine incorporation. At concentrations greater than 25 μM, isotope dilution was not observed. Consequently, at the thymidine concentration used (42 μM), the thymidine incorporation was not limited.

(iii) During experiments with *C. cellulolyticum* growth on cellobiose (2 g/liter), incorporation of tritiated thymidine was measured. The rate of thymidine incorporation was maximal when cells were in the exponential phase. In this phase, biomass formation was proportional to thymidine incorporation up to about 0.14 mg/ml (Fig. 1). The generation times (means ± standard deviations [SD] of four experiments) calculated by the optical density and thymidine methods were similar: 4.5 ± 0.3 and 3.8 ± 0.7 h, respectively. These results indicated that an acceptable estimate of the rate of growth of *C. cellulolyticum* on cellobiose was obtained by the thymidine method.

Cellulose growth characteristics. *C. cellulolyticum* was grown on Avicel cellulose in Hungate tubes without shaking except during thymidine addition. Solubilization of Avicel cellulose by *C. cellulolyticum* can be divided into two stages, the first corresponding to the colonization of cellulose from 0 to 50 h and the second occurring after 50 h with an acute release of cells from their substrate.

In the first stage (0 to 50 h), measurement of the thymidine incorporation rate (Fig. 2A) and biomass (Fig. 2B) revealed that growth occurred essentially on cellulose. The rate of thymidine incorporation into attached cells increased strongly and was maximal after about 35 h (about 8,000 dpm/ml). This increase was correlated with the amount of attached biomass. After 50 h of fermentation, about 80% of the total biomass had adhered to the cellulose (0.5 mg/ml). At the same time, a slight increase in the rate of thymidine



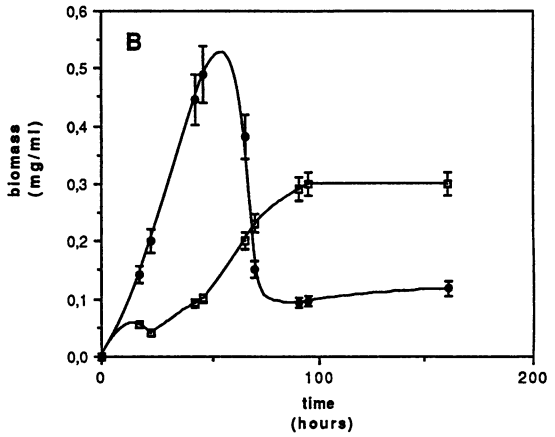


FIG. 2. Growth and thymidine incorporation rate kinetics of *C. cellulolyticum* on Avicel cellulose. (A) Rate of thymidine incorporation into attached and free cells; (B) biomass of attached (measured by bacterial-protein estimation) and free (measured by optical density) cells. Each point is the mean ± SD of four determinations for two different cultures. Symbols: ●, attached; ⊡, free.

incorporation into free cells (0 to 3,000 dpm/ml) was correlated with the amount of unattached biomass (0 to 0.100 mg/ml). Sugars resulting from cellulose degradation were almost entirely consumed (Fig. 3A), and the cellulase production was found to be essentially related to the growth of the attached bacteria (Fig. 3B).

In the second stage, after 50 h of fermentation, CMCase activity progressively began to appear in the supernatant (Fig. 3B). In this stage, the total biomass remained constant (Fig. 2B) and an accumulation of reducing sugars occurred (Fig. 3A), confirming the end of growth. Consequently, the release of cells from cellulose was responsible for the increase in nonadhering biomass (Fig. 2B).

The generation time of attached cells calculated from cellular-protein estimation was about 13 h, and the time calculated from the thymidine incorporation rate was about 8 h. The rate of specific thymidine incorporation into attached cells was maximal in the exponential phase: about 50,600 dpm/mg/h. The difference between calculations by

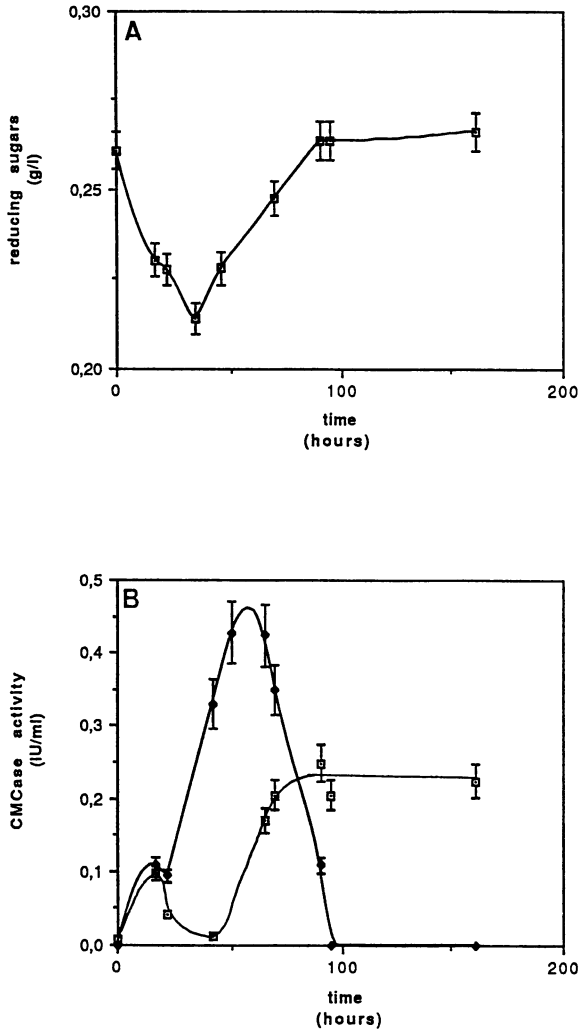


FIG. 3. Production of soluble sugar and attached- and free-cellulose CMCase activities by *C. cellulolyticum* cultivated on Avicel cellulose (7.5 g/liter). (A) Soluble-sugar production; (B) CMCase activity production. Symbols: ●, attached; ⊡, free. Each point is the mean ± SD of four determinations for two different cultures.

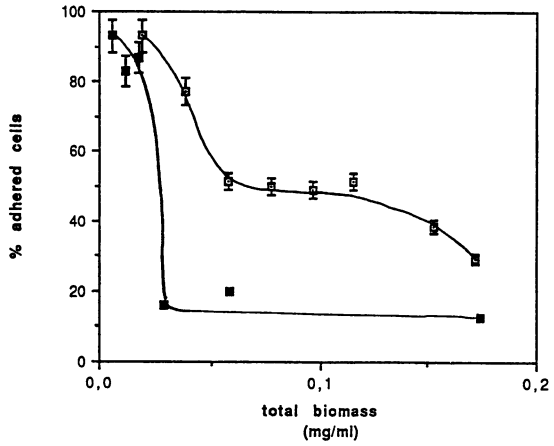


FIG. 4. Influence of stage of growth on adhesion to filter paper of *C. cellulolyticum* cultivated on cellobiose (2 g/liter). Each point is the mean ± SD of four determinations for two different cultures. Symbols: ⊡, cells harvested in exponential phase; ■, cells harvested in stationary phase.

bacterial-protein estimation and the thymidine method could result from consumption of the culture medium proteins, which might interfere with the estimation.

Examination of factors involved in release of cells from cellulose. (i) Adhesion experiments with originally attached labeled cells (population B) were conducted. Cells were suspended, at a concentration of 0.1 mg/ml, in fresh medium and in a previous culture (Avicel, 7.5 g/liter) supernatant (200 h of fermentation). Only a slight reduction of cell adhesion was observed in the presence of the supernatant (0.35 mg of adhered cells per g of cellulose) compared with cell adhesion observed in the presence of the fresh medium (0.45 mg/g of cellulose). Cellobiose, glucose (0 to 5 g/liter), and pH (between 7.0 and 5.0) had no effect on *C. cellulolyticum* adhesion. Acetate, the major metabolite produced by *C. cellulolyticum* (10), at concentrations between 0 and 4 g/liter reduced the cell adhesion only slightly (about 0.38 mg/g of cellulose). These results suggested that the accumulation of end products had little effect on *C. cellulolyticum* adhesion.

(ii) *C. cellulolyticum* was grown on cellobiose (2 g/liter). Cells in the late exponential and stationary phases were harvested, and their adhesion to filter paper was measured (Fig. 4). At low cell densities (0.03 mg/ml or lower) in the test system, the same amount of adhered cells was found (about 0.23 mg/g of cellulose) with cells harvested in the exponential and the stationary phases. At higher biomass concentrations (0.03 mg/ml or higher), adhesion of cells harvested in the stationary phase was greatly reduced compared with that of cells harvested in the exponential phase. Thus, the stage of growth strongly influenced the percentage of adhesion at high cell densities.

(iii) With the treatment described in Materials and Methods ("Heterogeneous population"), two bacterial populations (populations A and B) were separated after 50 h of fermentation. Adhesion of these populations to filter paper was examined. A cell density of 0.2 mg/ml was used for these experiments. Only 0.04 mg of cells of population A adhered under these conditions, whereas 0.08 mg of cells of population B adhered under the same conditions. Thus, at this stage of growth, a heterogeneous population was found in regard to the affinity for cellulose.

(iv) During the release of bacteria from cellulose, a sharp increase in thymidine incorporation into free cells was detected (Fig. 2A), whereas total biomass remained constant (Fig. 2B). The changes in incorporation rate as a function of free-cell density are represented in Fig. 5 and can be divided into three stages. In the first stage, corresponding to the phase of growth on cellulose (Fig. 2A), the rate of incorporation into free cells was proportional to the cell density between 0 and 0.100 mg/ml. From this plot, an attempt to determine the specific rate of thymidine incorporation (disintegrations per minute incorporated per milligram of bacteria per hour) was made. In this stage, the specific incorporation rate was about 31,500 dpm/mg/h. At the same time, cells attached to the cellulose were in the exponential phase and the specific incorporation rate was about 50,600 dpm/mg/h. The second stage was characterized by a decrease in specific incorporation for cell densities between 0.100 and 0.200 mg/ml, corresponding to the end of growth on cellulose. The specific incorporation rate obtained was about 6,700 dpm/mg/h. The third stage began with a sharp increase

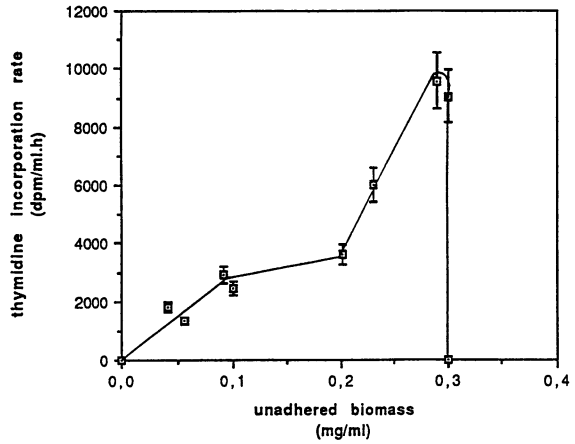


FIG. 5. Relation between free biomass production and the rate of incorporation of thymidine into unattached cells during *C. cellulolyticum* growth on Avicel cellulose (7.5 g/liter). Each point is the mean ± SD of four determinations for two different cultures.

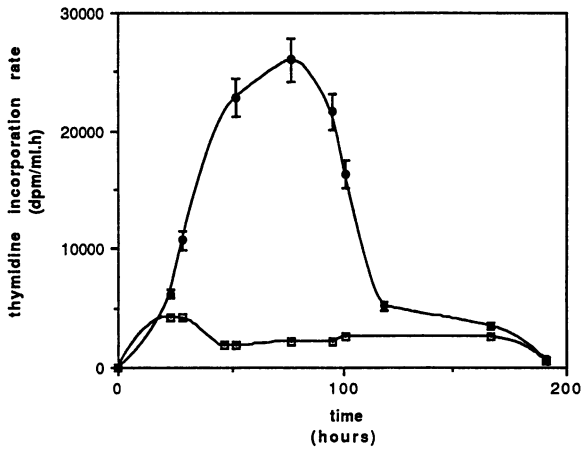


FIG. 7. Rate of incorporation of thymidine into attached and free cells in shaken culture of *C. cellulolyticum* on Avicel cellulose. Each point is the mean ± SD of four determinations for two different cultures. Symbols: ●, attached; ⊡, free.

in thymidine incorporation rate (specific rate, about 68,000 dpm/mg/h) followed by a rapid decrease.

(v) *C. cellulolyticum* was cultivated on 0.6 g of cellobiose per liter and the thymidine incorporation rate was measured as described in Materials and Methods. At this cellobiose concentration, exhaustion of the carbon source occurred when cells were in the exponential phase. The kinetics of growth and thymidine incorporation rate are shown in Fig. 6. In the first stage of fermentation (0 to 0.175 mg/ml), corresponding to the exponential phase, the thymidine incorporation rate was proportional to the biomass formation. Following this phase, a lag of the thymidine incorporation rate was observed (from 0.175 to 0.200 mg/ml) followed by a sharp increase, whereas biomass remained constant. The biomass decrease at the end of fermentation probably corresponded to the cellular lysis. Consequently, a high level of homology was observed in the changes in the thymidine incorporation rate in the population depleted of cellobiose (Fig. 6) and in free bacteria in the last growing stage of cellulose culture (Fig. 5).

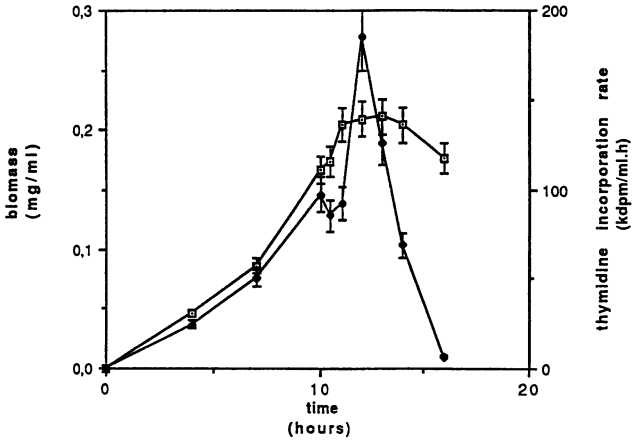


FIG. 6. Growth and thymidine incorporation rate kinetics of *C. cellulolyticum* on cellobiose (0.6 g/liter). Each point is the mean ± SD of four determinations for two different cultures. Symbols: ●, thymidine incorporation rate; ⊡, biomass.

(vi) Kinetics of thymidine incorporation were studied by using mildly shaken cellulose cultures (7.5 g/liter) (Fig. 7). Under these conditions, growth occurred essentially on cellulose. Neither a release of bacteria (about 0.110 mg found free per ml after 200 h of fermentation) nor a burst of thymidine incorporation into unattached cells occurred, contrary to what was observed in static cultures. Shaking altered considerably the percentage of cellulose degraded after 200 h of fermentation: i.e., about 18% (12 mg in the test system) of cellulose was degraded in static cultures and about 40% (28 mg in the test system) was degraded in the shaken cultures. These results suggested that in static cultures the substrate was probably less accessible.

DISCUSSION

This paper describes the colonization and fermentation of Avicel cellulose by the cellulolytic, mesophilic bacterium *C. cellulolyticum* H10 (ATCC 35319), taking into consideration both unattached bacteria and bacteria attached to the substrate, using incorporation of tritiated thymidine.

C. acetobutylicum ATCC 824 was chosen for validating the developed method, since this strain can neither adhere to cellulose (8) nor degrade crystalline cellulose (19–21). With this strain, nonspecific adsorption of the thymidine in the presence of cellulose was not found. Furthermore, cellulose did not interfere with TCA extraction.

Growth of *C. cellulolyticum* occurred essentially on the insoluble substrate, with generation times of about 8 h as determined by the thymidine method and 13 h as determined by bacterial-protein estimation. The difference obtained could be the result of the presence of extracellular proteins which interfered with the estimation. Under the conditions used, a significant overestimation could not occur, since this technique has been demonstrated to be useful for measuring the rate of growth of *C. cellulolyticum* on cellobiose. In both cases, the generation time for *C. cellulolyticum* on Avicel cellulose was comparable to that on glucose (10 h) and superior to that on cellobiose, about 4 h (10). Taking into consideration only the total biomass, Giallo et al. reported for *C. cellulolyticum* on cellulose a generation time of about 24 h (9).

In static cultures, an acute release of attached bacteria

from cellulose was observed at the end of fermentation, followed by a burst of thymidine incorporation. This phenomenon was not detected in shaken cultures when particles of cellulose were accessible in suspension. It could be interpreted as an exhaustion of the carbon source because the total biomass remained constant in this stage. The transition from exponential to stationary phase due to exhaustion of the supplied carbon source is always observed to be abrupt, and the decelerating phase is virtually absent (35). Although total biomass remains constant during the first hours of the stationary phase, the number of cells usually increases. This results from the fact that already-initiated rounds of DNA replication and cell division have to be completed. All cells in cultures depleted of the primary carbon and energy source should complete DNA synthesis; RNA can be expected to serve as a supply of building blocks and energy after exhaustion of the carbon source (35).

Since the accumulation of end products was not responsible for the release of attached cells, a possible explanation for the observations of this study might be that, under static growth conditions (i.e., in Hungate tubes without agitation), accessible cellulose was limited, leading to a release of bacteria in several stages of growth. This could explain the presence of a heterogeneous population with regard to the affinity for cellulose, since adhesion of *C. cellulolyticum* was dependent on the stage of growth. Bacteria depleted of primary carbon sources should complete the initiated rounds of DNA replication, explaining the change in thymidine incorporation rate detected at the end of growth.

This study points out an important aspect of the cellulolysis, namely, the release of cellulolytic bacteria from cellulose, and suggests that exhaustion of usable carbon sources could be important in this phenomenon.

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REFERENCES

1. **Bayer, E. A., R. Kenig, and R. Lamed.** 1983. Adherence of *Clostridium thermocellum* to cellulose. J. Bacteriol. **156:**818–827.
2. **Bayer, E. A., E. Setter, and R. Lamed.** 1985. Organization and distribution of the cellulosome in *Clostridium thermocellum*. J. Bacteriol. **163:**552–559.
3. **Bensadoun, A., and D. Weinstein.** 1976. Assay of proteins in the presence of interfering materials. Anal. Biochem. **70:**241–250.
4. **Bhat, S., R. J. Wallace, and E. R. Ørskov.** 1990. Adhesion of cellulolytic ruminal bacteria to barley straw. Appl. Environ. Microbiol. **56:**2698–2703.
5. **Cavedon, K., S. B. Leschine, and E. Canale-Parola.** 1990. Cellulase system of a free-living, mesophilic Clostridium (strain C7). J. Bacteriol. **172:**4222–4230.
6. **Faure, E., A. Belaich, C. Bagnara, C. Gaudin, and J. P. Belaich.** 1989. Sequence analysis of the *Clostridium cellulolyticum* endoglucanase A-encoding gene cel-CCA. Gene **84:**39–46.
7. **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.
8. **Gelhaye, E., B. Claude, C. Cailliez, S. Burle, and H. Petitdemange.** 1992. Multilayer adhesion to filter paper of two mesophilic, cellulolytic clostridia. Curr. Microbiol. **25:**307–311.
9. **Giallo, J., C. Gaudin, and J. P. Belaich.** 1985. Metabolism and solubilization of cellulose by *Clostridium cellulolyticum* H10. Appl. Environ. Microbiol. **49:**1216–1221.
10. **Giallo, J., C. Gaudin, J. P. Belaich, E. Petitdemange, and F. Caillet-Mangin.** 1983. Metabolism of glucose and cellobiose by cellulolytic mesophilic *Clostridium* sp. strain H10. Appl. Environ. Microbiol. **45:**843–849.
11. **Imam, S. H., R. V. Greene, and H. L. Griffin.** 1990. Adhesive properties of a symbiotic bacterium from a wood-boring marine shipworm. Appl. Environ. Microbiol. **56:**1317–1322.
12. **Jeffrey, W. H., and J. H. Paul.** 1986. Activity of an attached and free-living *Vibrio* sp. as measured by thymidine incorporation, *p*-iodonitrotetrazolium reduction, and ATP/DNA ratios. Appl. Environ. Microbiol. **51:**150–156.
13. **Karl, D. M.** 1982. Selected nucleic acid precursors in studies of aquatic microbial ecology. Appl. Environ. Microbiol. **44:**891–902.
14. **Kirchman, D.** 1983. The production of bacteria attached to particles suspended in a freshwater pond. Limnol. Oceanogr. **28:**858–872.
15. **Kudo, H., K. J. Cheng, and J. W. Costerton.** 1987. Electron microscopy study of the methylcellulose-mediated detachment of cellulolytic rumen bacteria from cellulose fibers. Can. J. Microbiol. **33:**267–272.
16. **Lamed, R., J. Naimark, E. Morgenstern, and E. A. Bayer.** 1987. Specialized cell surface structures in cellulolytic bacteria. J. Bacteriol. **169:**3792–3800.
17. **Lamed, R., E. Setter, and E. A. Bayer.** 1983. Characterization of a cellulose-binding complex in *Clostridium thermocellum*. J. Bacteriol. **156:**828–836.
18. **Latham, M. J., B. E. Brooker, G. L. Pettipher, and P. J. Harris.** 1978. *Ruminococcus flavefaciens* cell coat and adhesion to cotton cellulose and to cell walls in leaves of perennial ryegrass (*Lolium perenne*). Appl. Environ. Microbiol. **35:**156–165.
19. **Lee, S. F., and C. W. Forsberg.** 1987. Isolation and some properties of a β-D-xylosidase from *Clostridium acetobutylicum* ATCC 824. Appl. Environ. Microbiol. **53:**651–654.
20. **Lee, S. F., C. W. Forsberg, and L. N. Gibbins.** 1985. Cellulolytic activity of *Clostridium acetobutylicum*. Appl. Environ. Microbiol. **50:**1068–1076.
21. **Lee, S. F., C. W. Forsberg, and J. B. Rattray.** 1987. Purification and characterization of two endoxylanases from *Clostridium acetobutylicum* ATCC 824. Appl. Environ. Microbiol. **50:**644–650.
22. **Lovell, C. R., and A. Konopka.** 1985. Thymidine incorporation by free-living and particle-bound bacteria in a eutrophic dimictic lake. Appl. Environ. Microbiol. **49:**501–504.
23. **Lowry, O. H., N. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
24. **Madarro, A., J. L. Pena, J. L. Lequerica, S. Valles, R. Gay, and A. Flors.** 1991. Partial purification and characterization of the cellulases from *Clostridium cellulolyticum* H10. J. Chem. Technol. Biotechnol. **52:**393–406.
25. **Miller, G. L.** 1959. Use of dinitrosalicylic reagent for determination of reducing sugars. Anal. Chem. **31:**426–428.
26. **Miller, G. L. R., W. E. Blum, and A. L. Burton.** 1960. Measurements of carboxymethylcellulase activity. Anal. Biochem. **2:**127–132.
27. **Morag, E., I. Halevy, E. A. Bayer, and R. Lamed.** 1991. Isolation and properties of a major cellobiohydrolase from the cellulosome of *Clostridium thermocellum*. J. Bacteriol. **173:**4155–4162.
28. **Morris, E. J.** 1988. Characteristics of the adhesion of *Ruminococcus albus* to cellulose. FEMS Microbiol. Lett. **51:**113–118.
29. **Perez-Martinez, G., L. Gonzales-Candelas, J. Polaina, and A. Flors.** 1988. Expression of an endoglucanase gene from *Clostridium cellulolyticum* in *E. coli*. J. Ind. Microbiol. **3:**365–371.
30. **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.
31. **Pettipher, G. L., and M. J. Latham.** 1979. Characteristics of enzymes produced by *Ruminococcus albus* which degrade plant cell walls. J. Gen. Microbiol. **122:**278–287.
32. **Pollard, P. C., and D. J. W. Moriarty.** 1984. Validity of the tritiated thymidine method for estimating bacterial growth rates: measurement of isotope dilution during DNA synthesis. Appl. Environ. Microbiol. **48:**1076–1083.

33. **Shima, S., Y. Igarashi, and T. Kodama.** 1991. Molecular cloning of a new endoglucanase from *Clostridium cellulolyticum* and its expression in *Escherichia coli*. Appl. Microbiol. Biotechnol. **35:**233–236.
34. **Shoseyov, O., and R. H. Doi.** 1990. Essential 170 kDa subunit for degradation of crystalline cellulose by *Clostridium cellulovorans* cellulase. Proc. Natl. Acad. Sci. USA **87:**2192–2195.
35. **Wanner, U., and T. Egli.** 1990. Dynamics of microbial growth and cell composition in batch culture. FEMS Microbiol. Rev. **75:**19–44.
36. **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.