

Colonization of Crystalline Cellulose by *Clostridium cellulolyticum* ATCC 35319

E. GELHAYE, A. GEHIN, AND H. PETITDEMANGE*

Laboratory of Biological Chemistry I, University of Nancy I, Boite Postale 239,
54506 Vandoeuvre-lès-Nancy, France

Received 5 May 1993/Accepted 12 July 1993

Cellulose colonization by *Clostridium cellulolyticum* was studied by using [methyl-³H]thymidine incorporation. The colonization process indicated that a part of the bacterial population was released from cellulose to the liquid phase before binding and colonizing another adhesion site of the cellulose. We postulate that cellulose colonization occurs according to the following process: adhesion, colonization, release, and readhesion.

Clostridium cellulolyticum ATCC 35319, a gram-positive, anaerobic, mesophilic bacterium, is able to degrade cellulose efficiently (10). *C. cellulolyticum* possesses cellulosome-like structures with a complex peptide distribution (8), and its cellulolytic system has been studied by molecular genetic approaches (2, 3, 9, 12). However, interactions between *C. cellulolyticum* and cellulose have not been well characterized. In a previous paper, we reported the adhesive properties of *C. cellulolyticum* (5). Analysis of the adhesion process indicated a multilayer adhesion with cell-cellulose interaction in the first step and cell-cell interaction due to an aggregation of cells from the surface in the second step. Furthermore, growth of *C. cellulolyticum* on crystalline cellulose occurs when bacteria are attached to their substrate (7). The purpose of this study was to examine the colonization of cellulose by *C. cellulolyticum* by using [methyl-³H]thymidine incorporation into DNA defined as the cold-trichloroacetic acid (TCA)-precipitable fraction (4). This method has been reported to be valid for measuring bacterial growth rates (6, 11), in particular the *C. cellulolyticum* growth rate (7).

Medium. *C. cellulolyticum* was grown anaerobically at 34°C in a previously described medium, CM₃ medium (1). The desired carbon source (Avicel cellulose or filter paper) was included in this basal medium.

Colonization measurement. At the start of the experiments, the N₂-flushed Hungate tubes contained only 0.120 mg of Whatman no. 1 filter paper (a single piece, 12 by 1 cm). This filter paper band was inoculated with 50 µl of bacterial suspension from an Avicel cellulose (7.5-g/liter) culture harvested in the exponential phase and was spotted on the extremity of the filter paper band. After 5 min at 34°C, the filter paper was washed three times with 2 ml of CM₃ medium to remove unattached cells. Finally, 8 ml of CM₃ medium was added and the cultures were incubated with gentle agitation (with a Bioblock Scientific no. 95212 reciprocating roller) at 34°C. In the course of fermentation (times are indicated in the figure legends), [methyl-³H]thymidine (specific activity, 82 Ci/mmol) (Amersham) diluted with unlabeled thymidine (final concentration, 15 µM, 2.1 µCi/ml) was added anaerobically. The cultures were incubated for 1 h at 34°C. At the end of this time, the filter paper band was removed, washed with 8 ml of CM₃ medium, and cut into six sections (2 by 1 cm each). Each section was mixed with 6 ml

of ice-cold TCA to precipitate the macromolecules, as described by Fuhrman and Azam (4). RNA, macromolecular protein, and DNA were included in this insoluble fraction (4). After 1 h of extraction on ice, the cold-TCA-insoluble material (filter paper sections were washed five times with 2.5 ml of ice-cold 5% TCA) was collected by filtration through 25-mm cellulose nitrate filters (pore size, 0.22 µm) (Whatman). Filters were washed five times with 2.5 ml of ice-cold 5% TCA and placed in scintillation vials. Both ethyl acetate (1 ml) and scintillation fluid (9 ml) (Ready Safe; Beckman) were added, and the radioactivity was monitored with a scintillation counter (LS 5000 TD; Beckman). The counting efficiency and quenching were estimated by comparing actual and theoretical values with standards.

Colonization experiments. To determine the inoculum position on the filter paper, Avicel cellulose-grown cells were labeled with [methyl-³H]thymidine (final concentration, 42 µM, 2.1 µCi/ml) added in the exponential phase. These labeled cells were used as the inoculum (see above). Radioactivity was detected only on the first and second sections (0 to 4 cm). No radioactivity was found in the liquid phase, demonstrating that washings had removed the unattached cells.

The colonization of filter paper by *C. cellulolyticum* was performed as described above (Fig. 1). At 1 h, thymidine incorporation was found only on the first section (Fig. 1A); at 4 h, it was detected only on the inoculated sections (0 to 4 cm) (Fig. 1B). At 7 h (Fig. 1C), radioactivity was found on the entire filter paper band. However, the thymidine incorporation was greater on the first sections (0 to 6 cm) than on the last (6 to 12 cm). After 12 h (Fig. 1D), the entire filter paper was colonized, and a high incorporated radioactivity was detected on each section of filter paper. At each point, the proportion of total incorporated thymidine detected in the liquid phase was measured. This amount always included between 5 and 15% of the total detected radioactivity without significant development, and the concentration of unattached biomass (detected by optical density measurement) remained less than 0.03 mg/ml. These results suggested that (i) *C. cellulolyticum* colonized a large area of the substrate starting from attached cells and (ii) the colonization advanced and thymidine incorporation was always detected in previously colonized sections.

To determine whether the colonization process occurred with a release of bacteria from cellulose, a part (the third section [4 to 6 cm]) of the filter paper was covered with an adhesive plastic (848408; Plasto S.A., Chenove, France)

* Corresponding author.

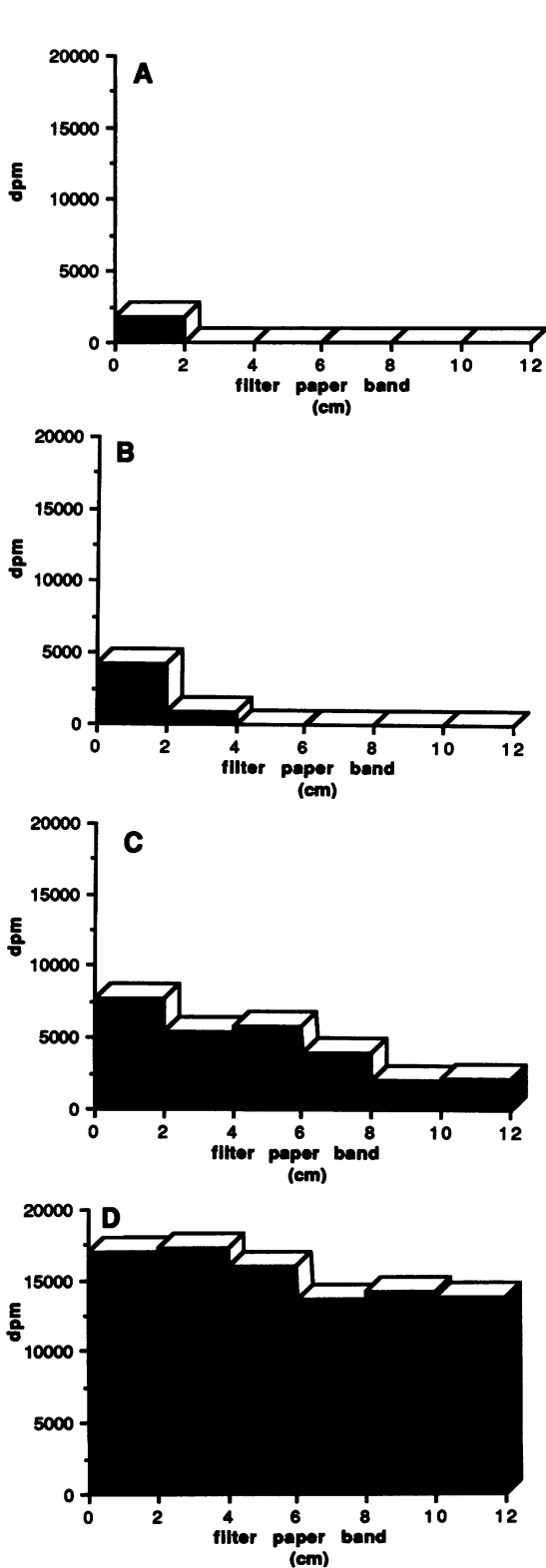


FIG. 1. Colonization of filter paper by *C. cellulolyticum*. Detection of the incorporated thymidine after 1 h (A), 4 h (B), 7 h (C), and 12 h (D) is shown. Each value is the mean of three experiments, with a coefficient of variation of about 12%.

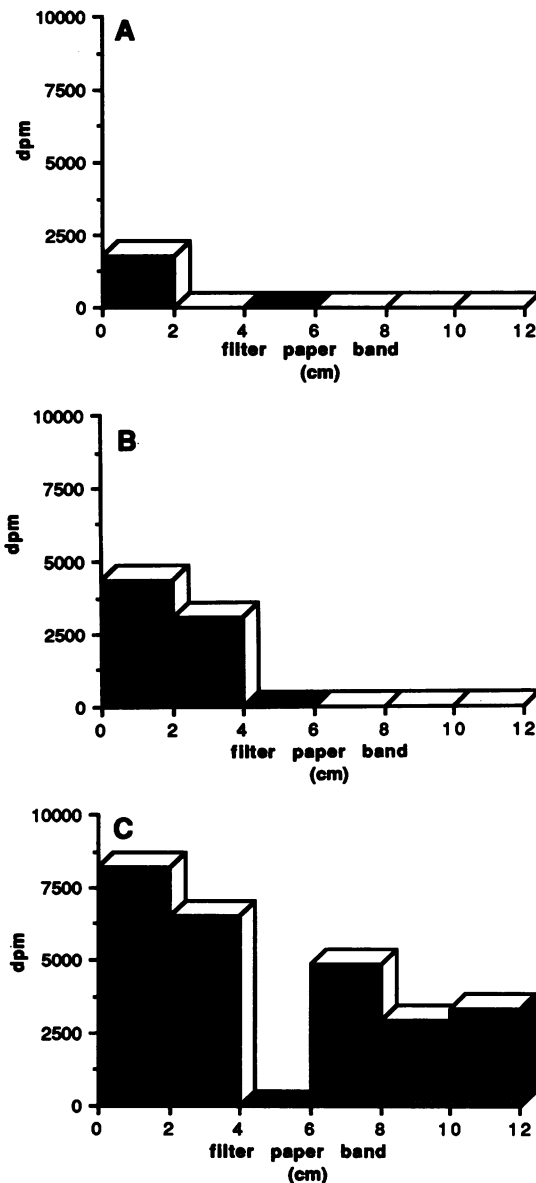


FIG. 2. Colonization of filter paper by *C. cellulolyticum* in the presence of a nonusable piece (4 to 6 cm). Detection of the incorporated thymidine after 1 h (A), 4 h (B), and 7 h (C) is shown. Each value is the mean of three experiments, with a coefficient of variation of about 12%.

which cannot be colonized by *C. cellulolyticum*. The same procedure as described above was performed. As shown in Fig. 2, thymidine incorporation was detected on the last section of filter paper (6 to 12 cm) after 7 h, in spite of the presence of the nonusable section. At the same time, the first (0 to 4 cm) and last (6 to 12 cm) sections were colonized. During this experiment, the amount of radioactivity detected in the liquid phase included between 5 and 15% of the total detected thymidine without significant development. These results suggested that some of the bacteria are released and adhere again to cellulose.

Since growth of *C. cellulolyticum* on cellulose occurs when bacteria are attached to their substrate (7), we postu-

late that cellulose colonization by *C. cellulolyticum* occurs according to the following process: adhesion, colonization, release, and readhesion. Adhesion of *C. cellulolyticum* to filter paper occurs on specific sites (6). A possible explanation for the observed bacterial release might be that, in the course of fermentation, some adhesion sites are saturated (or hydrolyzed), leading to a transfer of bacteria from cellulose to the liquid phase. Our data demonstrated that these released bacteria could, at least in part, adhere again and colonize another site. Further investigations are needed to determine the effects of this bacterial release on *C. cellulolyticum* metabolism.

This work was supported by a fellowship to E.G. from the Agence de l'Environnement et de la Maîtrise de l'Energie (ADEME) and from Gaz de France.

REFERENCES

1. Benoit, L., C. Cailliez, E. Petitdemange, and J. Gitton. 1992. Isolation of cellulolytic mesophilic clostridia from a municipal solid waste digester. *Microb. Ecol.* **23**:117-125.
2. 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.
3. 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.
4. 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.
5. 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.
6. Gelhaye, E., H. Petitdemange, and R. Gay. 1992. Characteristics of cellulose colonization by a mesophilic cellulolytic *Clostridium* (strain C401). *Res. Microbiol.* **143**:891-895.
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. 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.
9. Perez-Martinez, G., L. Gonzalez-Candelas, J. Polaina, and A. Flors. 1988. Expression of an endoglucanase gene from *Clostridium cellulolyticum* in *Escherichia coli*. *J. Ind. Microbiol.* **3**:365-371.
10. 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.
11. 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.
12. 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.