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Regulation and Distribution of *Fibrobacter succinogenes* subsp. *succinogenes* S85 Endoglucanases

MARTIN McGAVIN,† JOE LAM, AND CECIL W. FORSBERG*

Department of Microbiology, University of Guelph, Guelph, Ontario N1G 2W1, Canada

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The distribution of endoglucanase activities in cultures of *Fibrobacter succinogenes* subsp. *succinogenes* S85 grown on different carbon sources was examined by a variety of biochemical and immunological techniques. Total culture endoglucanase activity was primarily cell associated and was expressed constitutively, although synthesis of endoglucanase 1 (EG1) was repressed by cellobiose. Western immunoblotting showed that EG1 and EG3 were released into the culture fluid during growth, while EG2 remained largely associated with the cell. Subcellular localization showed low endoglucanase activity in the periplasmic fraction and similar, high levels in the cytoplasmic and membrane fractions. Western immunoblotting showed that EG2 was absent from the periplasmic fraction. Data from immunoelectron microscopy with either polyclonal or monoclonal antibody to EG2 revealed a high density of gold labeling at sites where there was a disruption in the regular features of the cell surface, such as in blebbing or physical tearing of the membrane. When cells were grown on cellulose, there was a high density of labeling on the cellulose but not on the cells, indicating that EG2 has limited exposure at the cell surface. On the basis of these data, export of enzymes from their intracellular locations appears to occur via three different mechanisms: a specific secretory pathway independent of cellulose, a secretory mechanism which is mediated by contact with cellulose, and a generalized blebbing process that occurs irrespective of the carbon source.

Fibrobacter succinogenes subsp. *succinogenes* S85, formerly *Bacteroides succinogenes* (39), is recognized as the most active of the rumen bacteria in degrading recalcitrant types of cellulosic materials (19, 51). However, the physiological mechanism of cellulose hydrolysis by this bacterium in obscure. Hungate (27) concluded that *F. succinogenes* possess a firmly bound cellulase because the organism did not produce a large zone of clearing in cellulose-containing agar. Cell-free rumen fluid contains very little cellulase activity, suggesting that most of the enzymes are either cell associated or tightly adsorbed to solid substrates (18). Attachment assays (14, 38) and electron microscopy (1, 8, 15) have demonstrated that *F. succinogenes* adheres tightly to plant materials, which is further indicative of a cell-bound cellulase.

Enzymological studies revealed that the bacterium possessed both extracellular and cell-bound endoglucanase activities. In stationary-phase cellulose-grown cultures, up to 80% of the total activity was found in the extracellular fluid. Most of the cell-free cellulase was associated with sedimentable membrane fragments, while the rest was divided between nonsedimentable protein aggregates of $>4 \times 10^6$ daltons, and a low-molecular-mass fraction of approximately 45 kilodaltons (15, 17, 46). More recently, three separate endoglucanases, designated EG1, EG2 (35), and EG3 (37); an extracellular Cl-stimulated cellobiosidase (25); and a periplasmic cellodextrinase (22, 23) have been purified and characterized. The cellobiosidases were shown to be cell associated in actively growing cultures (24).

In this study, we determined the distribution and expression of total endoglucanase activities in cultures grown on glucose, cellobiose, or crystalline cellulose. Antibodies specific for each of the endoglucanases were used as probes to test for the presence of the enzymes in the cell-associated and extracellular proteins of cultures grown on the three carbon sources, and immunoelectron microscopy was used to examine the distribution of EG2 on the surfaces of cells and cellulose in cultures growing on crystalline cellulose. This experimental approach allowed us to address such specific issues as whether the cellulase of *F. succinogenes* is primarily cell associated or extracellular, whether synthesis of endoglucanase activity is subject to carbon source regulation, and whether specialized cell surface structures which function in cellulose hydrolysis are produced, as has been observed in *Clostridium thermocellum* (3, 4, 30–32).

MATERIALS AND METHODS

Organism and growth conditions. *F. succinogenes* subsp. *succinogenes* S85 (previously obtained from M. P. Bryant, University of Illinois, Urbana) was maintained as described by Groleau and Forsberg (15). The growth medium was the chemically defined medium of Scott and Dehority (47) containing as a carbon source either 0.4% (wt/vol) Avicel PH105 crystalline cellulose (FMC Corp., Marine Colloids Div., Rockland, Maine) or either glucose or cellobiose at 0.5% (wt/vol). To study endoglucanase activities in cultures grown on glucose or cellobiose, an inoculum from a stock culture on Avicel cellulose was subcultured three times on the respective medium before introduction of a 10% inoculum into a 500-ml round-bottom flask containing 300 ml of medium. Incubation was at 37°C with constant gyrotary shaking at 150 rpm. The cultures were harvested in the early stationary phase. For growth of cultures on Avicel cellulose, the inoculum level was 10% and incubation was at 37°C for 60 h.

Harvesting of cultures and fractionation of total endoglucanase activity. Two 10-ml volumes were removed from the culture. One represented total culture protein and enzyme and was sonicated with nine 30-s bursts of 200 W with a Braun-Sonic 2000 sonicator equipped with a 3/8-in. (1 in. =

* Corresponding author.
† Present address: Department of Biochemistry, Schools of Medicine/Biochemistry, University of Alabama, Birmingham, AL 35294.

2.54 cm) probe. The second volume was centrifuged at 12,000 × *g* for 10 min in a Sorvall SS34 rotor. The supernatant was saved for quantification of extracellular protein and endoglucanase activity, while the cell pellet was washed once in 10 ml of 50 mM potassium phosphate buffer (pH 6.7), suspended in an equal volume of buffer, and sonicated as described above. This fraction represented cell-associated protein and endoglucanase activity. The rest of the culture was centrifuged at 13,200 × *g* for 10 min. The supernatant was concentrated and dialyzed against 50 mM potassium phosphate buffer (pH 6.5) by ultrafiltration through a PM-10 membrane (Amicon Corp., Lexington, Mass.) to a final volume of 15 ml. A 10-ml volume was ultracentrifuged at 100,000 × *g* for 2 h. The nonsedimentable supernatant fraction was poured off, and the sedimented membranous pellet was suspended in 10 ml of buffer. For further subcellular fractionation, late-exponential-phase glucose-grown cells were subjected to osmotic shock essentially as described by Neu and Heppel (40). The cells were washed twice in half of the previous sample volume of cold 0.05 M sodium phosphate buffer (pH 6.5) supplemented with 0.8% (wt/vol) NaCl (PBS) and suspended in the same volume of a solution containing 25% (wt/vol) sucrose and 1 mM EDTA. The suspension was shaken for 10 min at 22°C. After centrifugation at 20,000 × *g* for 20 min at 22°C, the cells were quickly and vigorously suspended in the same volume of ice-cold water. At this point, a 1 ml volume was removed for later use in Western blotting (immunoblotting). The suspension was shaken gently for 10 min at 4°C and then centrifuged at 30,000 × *g* for 20 min at 4°C. The supernatant (periplasmic fraction) was removed, and the pellet was suspended in the same volume of PBS. The shocked cell suspension was sonicated for nine 20-s bursts with 1-min intervals on ice. A portion of the sonic extract was ultracentrifuged at 100,000 × *g* for 1 h at 4°C to obtain the supernatant (cytoplasmic fraction) and the pellet (membrane fragments), which was suspended in the same volume of PBS.

Enzyme assays. Endoglucanase activity was assayed by incubating the appropriately diluted enzyme with 1.0% (wt/vol) carboxymethylcellulose (low viscosity; Sigma Chemical Co., St. Louis, Mo.) in 0.05 M sodium phosphate buffer (pH 6.5) at 39°C and quantifying the reducing sugars produced with the Nelson-Somogyi reagent (2) as described by Schellhorn and Forsberg (46). To remove residual reducing sugars from cellobiose- and glucose-grown cultures before enzyme assays, 1-ml volumes of whole sonicated culture, sonicated cells, and culture supernatants were dialyzed against 4 liters of 20 mM potassium phosphate buffer (pH 6.5). Cellobiosidase activity was measured by using the chromophore *p*-nitrophenyl-β-D-cellobioside (Sigma) as the substrate as described by Huang and Forsberg (22). Phosphoglucose isomerase was used as the periplasmic marker and assayed as described by Bergmeyer (5). Glutamic dehydrogenase was chosen as a cytoplasmic marker (34) and assayed under conditions identical to those used for phosphoglucose isomerase, except that glucose 6-phosphate dehydrogenase was omitted from the assay mixture and an equimolar amount of glutamic acid was added in place of fructose 6-phosphate. In both cases, reduction of NADP to NADPH was monitored at 340 nm on a Varian 2290 recording spectrophotometer and rates were calculated as A_{340} units per minute.

Protein assay. Protein was determined by the method of Bradford (6), with bovine serum albumin as the standard. For the sedimentable fractions, membrane-associated proteins were solubilized by being heated at 100°C for 10 min in 0.5 N NaOH. The protein was then diluted such that the final sodium hydroxide concentration in the assay mixture was less than 5 mM and consequently unable to cause interference by neutralization of the Bradford reagent.

Preparation and affinity purification of antibodies. Preparation of monoclonal antibody (MAb) N5A and an affinity-purified polyclonal antibody to EG2 was as described by McGavin and Forsberg (36). Preparation of a monospecific polyclonal antibody to EG3 was described by McGavin et al. (37). EG1 was not purified in sufficient quantity to facilitate the preparation of specific antiserum. However, monospecific antibodies were isolated from antiserum to nonsedimentable culture fluid proteins of cellulose-grown cells.

To ensure preservation of tertiary antigenic structures, concentrated nonsedimentable culture fluid protein (420 μg/ml) was mixed with an equal volume of 0.6 (wt/vol) Formalin in PBS and allowed to stand at 25°C for 30 min. The mixture was then diluted 10-fold in PBS and concentrated by ultrafiltration through an Amicon PM-10 membrane. The mixture was again diluted 10-fold and concentrated to a final volume of 3.5 ml. Each of two male New Zealand White rabbits was injected subcutaneously with 100 μg of this antigen emulsified in Freund complete adjuvant. Subsequent injections of 100 μg emulsified in Freund incomplete adjuvant were administered 19 and 27 days later. A final booster of 50 μg was given intravenously two weeks after the third injection. Serum was collected 1 week later. Monospecific antibody to EG1 was isolated from this antiserum by using a modification of previously published methods (42). Briefly, 14 μg of purified EG1 was electrophoresed on a preparative sodium dodecyl sulfate (SDS)-polyacrylamide gel and electrophoretically transferred to 0.45 μm-pore-size nitrocellulose (Schleicher & Schuell, Inc., Keene, N.H.). The transfer conditions were as described in the section on SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting. A narrow vertical strip was cut from the middle of the sheet and stained for protein with 0.1% (wt/vol) amido black in 45% (vol/vol) methanol–10% (vol/vol) acetic acid. The position of the antigen band was located, and the corresponding horizontal strip was cut from the unstained nitrocellulose. The strip was incubated in a blocking solution containing 3% (wt/vol) gelatin in Tris-buffered saline (20 mM Tris hydrochloride, 0.5 M NaCl [pH 7.5]) and then overnight with 50 ml of 100-fold-diluted antiserum in Tris-buffered saline containing 1% gelatin and 0.05% Tween 20. After three 5-min washes in Tris-buffered saline, the bound antibodies were eluted from the blot by incubation for 5 min in 2 ml of a solution consisting of 0.2 M glycine hydrochloride and 0.5 M NaCl (pH 2.7). This step was repeated once, and the eluates were neutralized immediately with 5% (by volume) 1.5 M Tris hydrochloride (pH 8.8).

SDS-PAGE and Western blotting. SDS-PAGE and Western blotting were performed by using the Bio-Rad Mini Protean II electrophoresis cell and Trans-blot apparatus. For SDS-PAGE, the gel formulation and buffering system were those of Laemmli (28), and resolving gels containing 10% (wt/vol) acrylamide and 5% stacking gels were used. Samples were dissociated in Laemmli sample buffer by being heated at 100°C for 10 min before electrophoresis. For Western blotting, the transfer buffer was that of Towbin et al. (55). Transfer was typically at 30 V overnight at 4°C.

Transmission electron microscopy. (i) Immunolabeling with colloidal gold markers. The ages of the cellulose-grown cultures used for colloidal gold labeling experiments ranged between 24 and 48 h, as indicated. When cells were to be labeled with a polyclonal antibody, the cultures were pre-

fixed with glutaraldehyde. A 1-ml volume of culture was added to 0.5 ml of 1% (vol/vol) glutaraldehyde made up in HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered saline (HBS; 20 mM HEPES buffer, 0.8% NaCl [pH 6.7]) and incubated at room temperature for 30 min. The fixed cells were centrifuged in an Eppendorf benchtop centrifuge at 480 × *g* for 3 min, and the pellet was suspended in 1 ml of 0.2 M NH_4Cl made up in HBS as a negative control. After incubation for an additional 30 min, the cells were washed three times in one-ml volumes of HBS containing 0.2% bovine serum albumin. After the third wash, the cells were split into two 50-μl volumes to which was added either 20 μl of an affinity-purified polyclonal antibody to EG2 (36) or an additional 20 μl of HBS–0.2% bovine serum albumin as a negative control. After incubation for 30 min, the samples were washed three times in HBS–0.2% bovine serum albumin and suspended in 40 μl of HBS–0.2% bovine serum albumin. To this was added 10 μl of a goat anti-rabbit immunoglobulin G-colloidal gold conjugate (10-nm-diameter particles; Sigma). After incubation for 30 min, the samples were washed twice in HBS and suspended in 100 μl of 0.5% glutaraldehyde made up in HBS.

For labeling with MAb N5A specific for EG2 (36), a 1-ml volume of a 24-h culture was centrifuged at 480 × *g* for 10 min and suspended in 1 ml of HBS. To this was added either 50 μl of the MAb or 50 μl of HBS as a negative control. After incubation for 30 min at room temperature, the sample was washed twice in 1 ml of HBS and then suspended in 50 μl of HBS–20 μl of a goat anti-mouse immunoglobulin G-colloidal gold conjugate (15-nm-diameter particles; Janssen Pharmaceuticals). After incubation for 30 min, the centrifugation and washing procedures were repeated as before, followed by suspension in 1 ml of 0.5% glutaraldehyde made up in HBS.

In addition to the control sample in which either the MAb or the polyclonal antibody was omitted, a second control was included to further ensure that labeling was due to specific antigen-antibody interaction. This involved treatment of a sample of sterile cellulose-containing medium with both first and second antibodies to test for entrapment of antibodies on or within the cellulose.

(ii) Negative staining of labeled cells. Carbon-Formvar-coated 200-mesh copper grids were floated on a drop of sample for 5 min. Excess sample was drawn off by touching the edge of the grid to a disk of filter paper. The grids were then floated briefly on a drop of 0.5% (wt/vol) ammonium molybdate (pH 7.0), and excess stain was removed with filter paper as before. The grids were then examined in a Philips 300 transmission electron microscope operating at an accelerating voltage of 60 kV.

(iii) Thin-section preparation. Labeled samples were enrobed in 2% (wt/vol) Noble agar and washed three times for 10 min each time with 50 mM HEPES (pH 6.7). The agar cores were dehydrated through a stepwise alcohol series and infiltrated with propylene oxide. The samples were then embedded in Epon resin (Epon 812) and cured for 2 days at 60°C. Ultrathin sections were obtained by cutting the embedded samples with an ultramicrotome (Reichert Jung Ultracut E). The thin sections were transferred onto carbon-Formvar-coated 200-mesh copper grids and double stained with 2% (wt/vol) uranyl acetate and 2% (wt/vol) lead citrate as described by Reynolds (45). The sections were examined in a Philips 300 transmission electron microscope operating at an accelerating voltage of 60 kV.

TABLE 1. Distribution of protein in cultures grown on different carbon sources[a]

Carbon source	Protein concn (μg/ml) in:		% of supernatant protein which was:	
	Culture	Supernatant[b]	Sedimentable	Nonsedimentable
Glucose	761	31 (4.1)	53.1	46.9
Cellobiose	521	19.5 (3.7)	55.1	44.9
Avicel	546	78.6 (14.4)	34.1	65.9

[a] Glucose- and cellobiose-grown cultures were harvested after 12 h of growth, while cellulose-grown cells were harvested after 60 h of growth.
[b] The values in parentheses represent the supernatant protein as a percentage of the total culture protein.

TABLE 2. Distribution of endoglucanase activity in cultures grown on different carbon sources

Carbon source	U/ml (nmol/min per ml)			% of extracellular activity which was:	
	Total culture	Cell associated[a]	Extracellular[a]	Sedimentable	Nonsedimentable
Glucose	117.5	94.4 (80.3)	19.6 (16.7)	64.2	35.8
Cellobiose	84.8	70.3 (82.9)	14.4 (17.0)	64.6	35.4
Avicel	187.0	92.8 (49.6)	86.1 (46.0)	41.1	58.9

[a] The values in parentheses represent percentages of total culture activity.

RESULTS

Distribution of protein and endoglucanase activity. Greater than 95% of total culture protein (Table 1) and 80% of total endoglucanase activity (Table 2) were found to be cell associated in glucose- and cellobiose-grown cultures, while in the Avicel cellulose-grown culture, 85% of the protein and 50% of the endoglucanase activity was cell associated. Of the extracellular protein and endoglucanase activity, the major proportion was associated with sedimentable membrane fragments in the glucose- and cellobiose-grown cultures but was predominantly nonsedimentable in the Avicel-grown culture supernatant. All three cultures produced similar levels of cell-associated endoglucanase, whereas the Avicel-grown culture supernatant had four- to sixfold greater activity than the glucose- or cellobiose-grown culture supernatant (Table 2). The specific activity of endoglucanase was almost identical in the glucose- or cellobiose-grown whole-culture or cell-associated samples (Table 3). The endoglucanase specific activity of the Avicel whole culture was about twofold higher than that of either the glucose- or cellobiose-grown cultures, and the specific activity of each of the culture supernatants was about fivefold greater than the corresponding cell-associated activity.

To examine the relationship between cell growth and endoglucanase synthesis, growth was monitored in a glucose-grown culture by measuring both the optical density of the culture at 675 nm and the total protein content at defined time intervals. With a 10% (vol/vol) inoculum from a late-

TABLE 3. Specific endoglucanase activities of culture fractions

Carbon source	Sp act of culture fraction (μmol/mg per min)		
	Total culture	Cell associated	Extracellular
Glucose	0.15	0.13	0.63
Cellobiose	0.16	0.14	0.74
Avicel	0.34	0.20	1.10

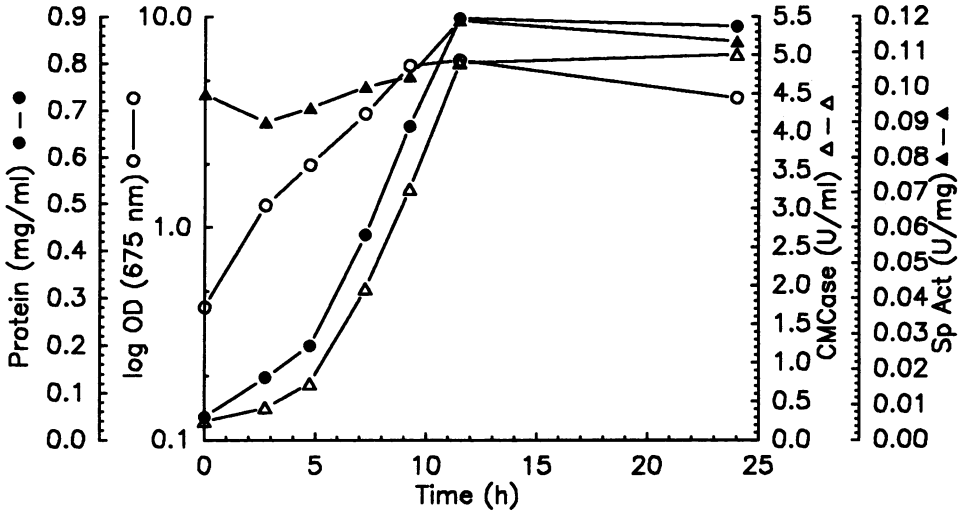

FIG. 1. Time course analysis of endoglucanase production in a batch culture with 0.5% (wt/vol) glucose as the carbon source. OD, Optical density; CMCase, carboxymethylcellulase.

exponential-phase culture and 0.5% (wt/vol) glucose as the carbon source, the optical density of the culture increased in an exponential fashion for 9.25 h (Fig. 1). During this time, the specific activity of endoglucanase slowly increased in a linear relationship, and after a initial lag of 4.25 h, total protein increased at a linear rate. Growth decreased rapidly between 9.25 and 11.5 h, whereas protein synthesis was maintained at the same constant rate. A rapid increase in total endoglucanase and a modest increase in the specific activity of endoglucanase were observed. Beyond 11.5 h, protein synthesis stopped, optical density decreased, and there was a slow reduction in the specific activity of endoglucanase.

Distribution of individual endoglucanases. Before the use of antibodies for these experiments, it has been established by Western immunoblotting that there was no cross-reactivity of the specific antibody with the other purified endoglucanases. Antibodies monospecific to EG1 did not interact with any cell-associated proteins. However, an immunoreactive band equal in size to purified EG1 was observed in the supernatants of the glucose and Avicel cultures (Fig. 2). This band was not observed in the cellobiose culture supernatant. MAb N5A, which is specific for EG2, showed the presence of EG2 in the cell-associated proteins from all three cultures but not in the culture supernatants. Using monospecific antibody affinity purified from antiserum to EG3 in immunoblots, a unique distribution for this enzyme was observed. The anti-EG3 antibody interacted with both cell-associated and supernatant proteins of the Avicel-grown culture, but the predominant antigen-antibody interactions were seen in the supernatant of the glucose- and cellobiose-grown cultures. At equal protein loading, the immunoreactive band could also be detected in the cell-associated fraction from the glucose-grown culture but was barely discernible in the cell-associated fraction from the cellobiose-grown culture.

Localization of EG2 by immunoelectron microscopy. Although Western blotting clearly showed that EG2 was cell associated, immunoelectron microscopy with MAb N5A showed very little interaction of the antibody with the cell surface. In Fig. 3a, a cellulose particle was very heavily labeled. At a higher magnification (Fig. 3b), the fibrous nature of the cellulose and the clearly defined boundary between the surface of the cell and the cellulose became apparent. Similar results were obtained in thin sections of a cellulose-grown culture incubated with MAb N5A in which colloidal gold labels were found to be associated with cellulose particles (Fig. 3c).

The antigenic determinant of the 118-kilodalton EG2, which interacts with MAb N5A, has previously been localized to a 43-kilodalton substrate-binding domain (36). It is possible that localization of EG2 with MAb N5A was limited by the availability and orientation of this epitope. Thus, to confirm the aforementioned observations, immunolabeling

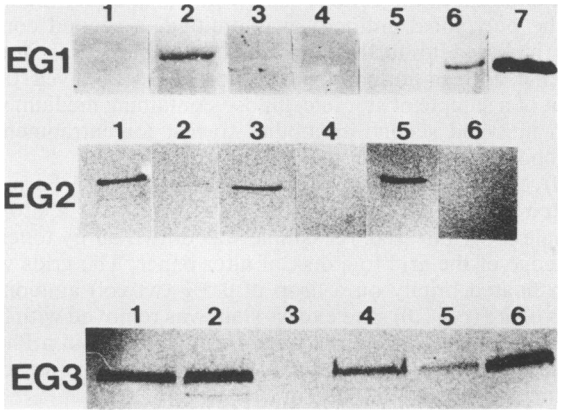

FIG. 2. Western blotting to examine the expression and distribution of EG1, EG2, and EG3 in cultures grown on glucose, cellobiose, or Avicel. For EG1, 30 μg of cell-associated proteins or 15 μg of supernatant proteins was applied to the gel, while for EG2 and EG3, the corresponding values were 7 and 3.3 μg, respectively. Lanes 1, 3, and 5 contained Avicel cellulose-, cellobiose-, and glucose-grown culture cellular proteins, respectively, while lanes 2, 4, and 6 contained the respective culture supernatant proteins. For EG1, lane 7 contained 0.3 μg of pure EG1. After SDS-PAGE, proteins were transferred to nitrocellulose and incubated for 16 h with a monospecific antibody to EG1 (a), 2 h with MAb N5A to EG2 plus tissue culture supernatant diluted 500-fold or (b), 2 h with a monospecific antibody to EG3 (c). Incubation with a second antibody was for 1 h in a 3,000-fold-diluted Bio-Rad goat anti-rabbit immunoglobulin G-immunoglobulin M-alkaline phosphatase conjugate (EG1 and EG3) or a 5,000-fold-diluted goat anti-mouse F(ab')2-alkaline phosphatase conjugate (EG2).

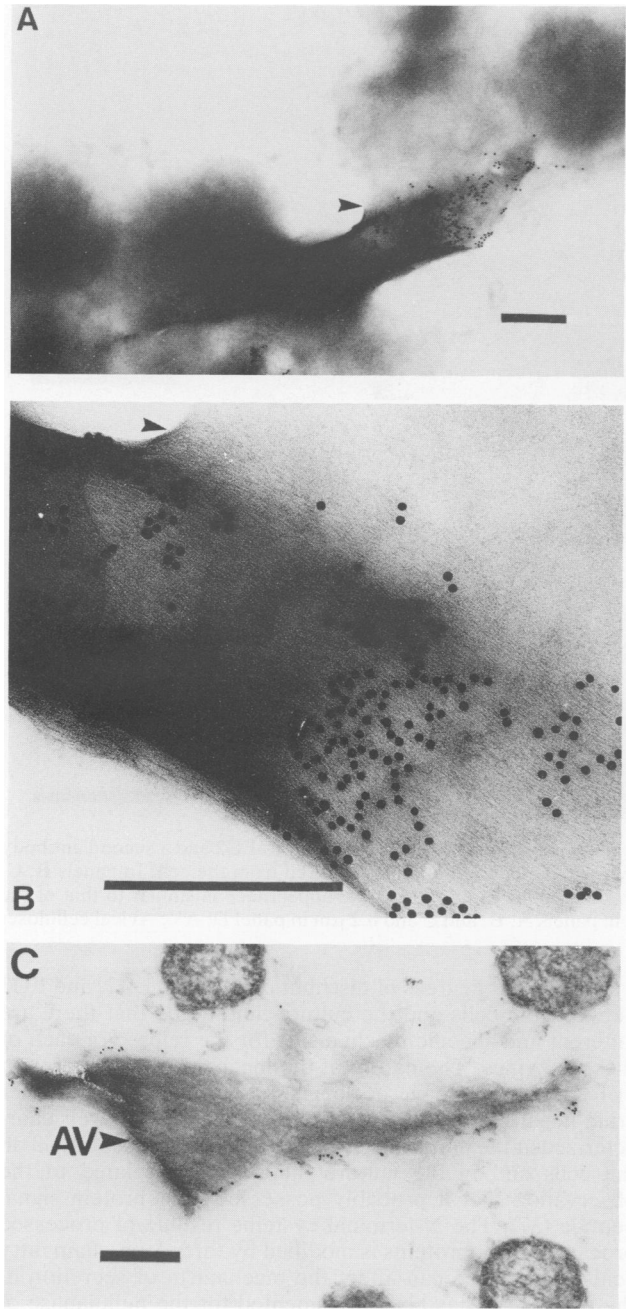

FIG. 3. Cells grown for 24 h on Avicel cellulose were treated with MAb N5A and a second antibody conjugated to 15-nm-diameter colloidal gold particles. (A) Negative staining of a whole mount. The Avicel cellulose (AV) was heavily labeled, whereas the cells bound to it were not. The arrowhead points to the boundary between the cell surface and cellulose. (B) Enlargement of panel A. The arrowhead points to the same position as in panel A. The fibrous nature of the cellulose is clearly visible, and the boundary where cellulose fibers merge with the cell can be distinguished. (C) Thin section through cells growing on cellulose. The surface of the cellulose was labeled with gold. Bars, 0.4 μm.

of cellulose-grown cultures was also performed by using an affinity-purified polyclonal antibody to EG2. Again, in thin sections, cell-associated labels were not commonly observed. However, the pattern of cellulose-bound labeling was different. There was a frequent association with discrete particulate structures, possibly protein aggregates or membrane vesicles bound to cellulose (Fig. 4b, c, and d). In negatively stained preparations which had been treated with either a polyclonal antibody (Fig. 5a and b) or MAb N5A (Fig. 5c), cell-associated labels were found to be localized on what appeared to be either protrusions from or disturbances of the cell surface (Fig. 5a and b). Similar observations were also made when labeling was performed on a glucose-grown culture in which the gold markers were found to be associated with a membrane bleb which was about to be released from the cell (Fig. 5c).

The different patterns of cellulose-bound labeled observed when using either MAb N5A or a polyclonal antibody may reflect a difference in the conformation of EG2 and consequently the epitopes which are exposed, depending on whether the protein is bound to cellulose or in association with membranes and other proteins. The polyclonal antiserum was raised against EG2 purified by SDS-PAGE, and specific antibodies were subsequently purified by affinity chromatography by using native antigen coupled to cyanogen bromide-activated Sepharose (36). The MAb was prepared from mice immunized with native antigen and is specific for an epitope on the substrate-binding domain.

The paucity of cell-associated labels observed when both the Mab and an affinity-purified polyclonal antibody were used to localize EG2 suggested that the enzyme was not predominantly cell surface associated. In a preliminary experiment in which cells were grown on glucose and lysed by passage through a French press, a high level of endoglucanase activity was found in both the soluble and particulate (membrane) fractions (data not shown). This prompted further localization of the endoglucanase activity by osmotic shock (Table 4). The distribution of glutamate dehydrogenase, the cytoplasmic marker, indicates that the osmotic shock procedure did not cause the cells to lyse. The cytoplasmic fraction contained a high level of endoglucanase activity (46%), as well as phosphoglucose isomerase, the periplasmic marker, and cellobiosidase, which was previously determined to be periplasmic (22). Similarly, the periplasmic fraction also contained high levels of the latter two enzymes, as found in previous studies (22, 24). In comparison with these enzymes, there was a low level of endoglucanase activity in the periplasm. There was a high level of membrane-associated endoglucanase activity (35% of the total), in contrast to both the cytoplasmic and periplasmic markers. EG2 was present in each of the cellular fractions which were examined by Western immunoblotting with MAb N5A, except for the periplasmic fraction (Fig. 6), and the abundance of EG2 in each fraction appeared to correlate with the percentage of total endoglucanase contained.

DISCUSSION

In confirmation of previous studies, we found that the endoglucanase activity of *F. succinogenes* was primarily cell associated in cultures grown on glucose or cellobiose. Furthermore, of the activity which was extracellular, almost two-thirds was associated with sedimentable membrane fragments. This indicates that the primary means by which endoglucanase activity appears in the culture supernatant is probably due to its release in the form of membrane vesicles. The substantially higher levels of protein and both total and nonsedimentable endoglucanases in the Avicel culture supernatant suggest that cellulose has a role in triggering the specific secretion of endoglucanases.

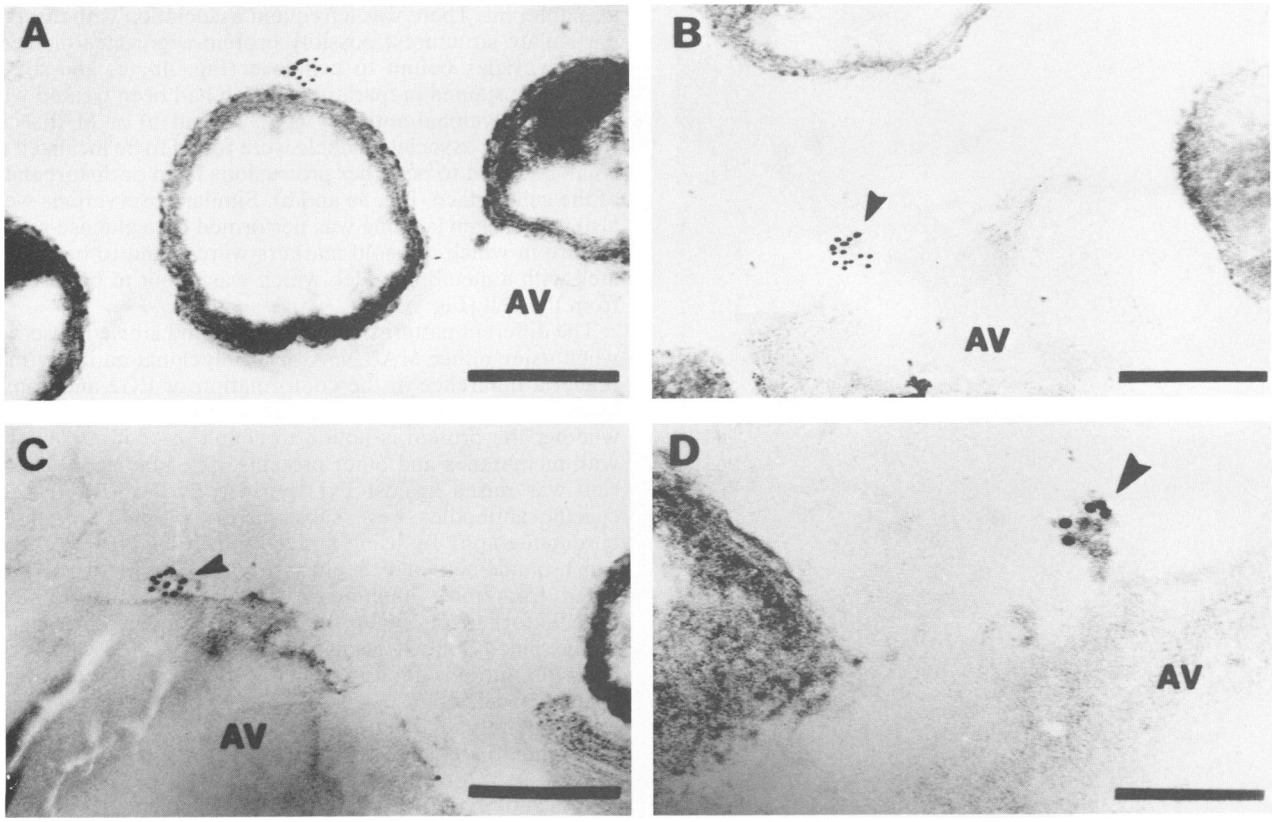

FIG. 4. Thin sections through a cellulose-grown culture treated with an affinity-purified polyclonal antibody to EG2 and a second antibody conjugated to 10-nm-diameter colloidal gold particles. In panel A, the enzyme in the process of being released from the cell. In panels B, C, and D, the arrowheads point to heavily labeled cellulose-bound particles 75 to 100 nm in diameter. The appearance is similar to that of the membrane vesicles observed on cellulose in previous studies. Bars, 0.4 μm in panels A, B, and C and 0.2 μm in panel D. AV, Avicel cellulose.

EG1 was not detected in the cellobiose-grown culture, whereas EG3 was present but visibly less abundant than in the glucose- and cellulose-grown cultures. All three enzymes are synthesized with either glucose or cellulose as the carbon source. The end product of the action of EG1 on amorphous cellulose is cellobiose, while EG2 and EG3 produce cellotetraose and cellotriose (35, 37). This may explain the selective repression of EG1 synthesis by cellobiose. However, the specific activity of the cellulose-grown culture was only 2.1- to 2.2-fold greater than that of the cellobiose- and glucose-grown cultures. This change is very small compared with that observed in other cellulolytic bacteria, in which endoglucanase synthesis is known to be regulated by a carbon source-dependent induction-and-repression mechanism (7, 33, 41, 49, 52, 56). In contrast, the cellulases of rumen bacteria are believed to be constitutive in nature (20, 43), although there have been reports to the contrary (11, 48).

Our results indicate that with the exception of EG1, endoglucanase synthesis in *F. succinogenes* is of a constitutive nature. The approximately twofold higher specific activity of endoglucanase in the cellulose-grown culture may be due to growth-rate-dependent control of endoglucanase synthesis, as has been implicated in other cellulolytic bacteria (33, 43, 53). Similarly, in this study, we observed that a glucose-grown culture in transition between exponential growth and the stationary phase experienced a more rapid increase in specific and total endoglucanase activities than was observed over the previous part of the growth curve (Fig. 1).

The various degrees of distribution of EG1, EG2, and EG3 between the cells and the culture fluid argue that there are different and specific mechanisms for the release of each of these enzymes. The nature of the mechanisms for secretion of EG1 and EG2 have not been studied; however, in other gram-negative bacteria, secretion of enzymes is well characterized at the molecular level (21). The presence of EG3 on the cells and in the culture fluid may be related to the observation that it probably possesses a lipoprotein signal peptide (37). The N-terminal cysteine residue of processed procaryotic lipoproteins is modified by three long-chain fatty acids (57), which can affect the mechanism of secretion of the protein, as has been documented for the pullulanase of *Klebsiella pneumoniae* (9, 44). The enzyme is localized to the outer face of the outer membrane during exponential growth and then progressively released into the growth medium. The abundance of EG3 in both the cell and supernatant fractions of the cellulose-grown culture may reflect a similar mechanism of secretion.

EG2 is the only truly cell-associated endoglucanase of *F. succinogenes* of the three thus far characterized. In previous work, this enzyme was purified from the culture fluid of cells grown in a chemostat with 1% Avicel cellulose as the substrate (35). Under these conditions, there was a generation time of 20 h and the culture fluid had a sixfold higher protein content than could be obtained in a batch culture. It was evident that proteolysis had occurred, as a 94-kilodalton degradation product of the 118-kilodalton EG2 was also purified. Hence, the appearance of EG2 in the culture

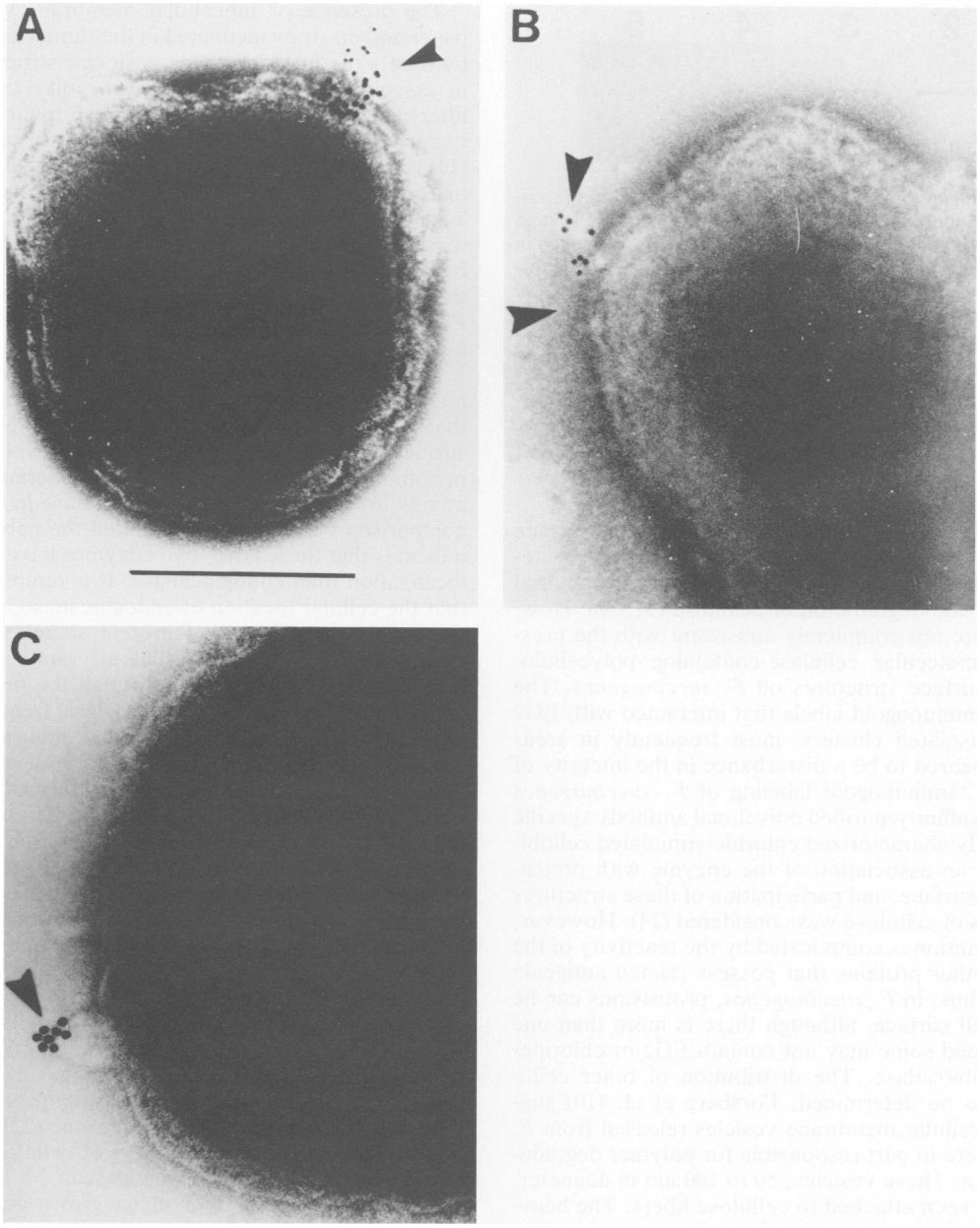

FIG. 5. Negative staining of cells treated with an affinity-purified polyclonal antibody (A and B) or MAb N5A (C). (A) A 48-h cellulose-grown culture. The label is associated with particulate material extending about 75 nm outward from the cell surface. (B) A 24-h cellulose culture. The lower arrowhead points to a disruption in the integrity of the cell surface. Adjacent to this, labeled membrane appears to be peeling away from the cell surface. (C) A 12-h glucose culture. A labeled, membranous bleb is in the process of being released from the cell surface. Markers, 0.4 μm.

supernatant can be correlated with conditions characterized by proteolysis, slow growth, and a high concentration of extracellular protein.

It was our intention to further elucidate the mechanism of cellulose hydrolysis by studying the localization of this enzyme on the cell surface. Studies conducted with *C. thermocellum* have shown that the cellulose-binding activity of cells and their ability to hydrolyze cellulose are associated with a compact multienzyme complex on the cell surface, termed a cellulosome (4, 30, 32), which upon interaction with cellulose, undergoes extensive structural transformation, protracting rapidly to form fibrous "contact corridors"

TABLE 4. Cellular distribution of endoglucanase and cellobiosidase in late-exponential-phase glucose-grown cells[a]

Fraction	% of total activity			
	Glutamate dehydrogenase	Phosphoglucose isomerase	Cello-biosidase	Endo-glucanase
Cytoplasm	95.2	64.3	53.6	46.5
Membranes	4.8	3.6	4.3	34.0
Periplasm	0	32.1	42.1	19.5

[a] Cells were harvested after 12 h of growth (A_{675}, 5.5) and subjected to osmotic shock as described in Materials and Methods. The total activity of each enzyme was taken as the sum of activities from the cytoplasmic, periplasmic, and membranous fractions.

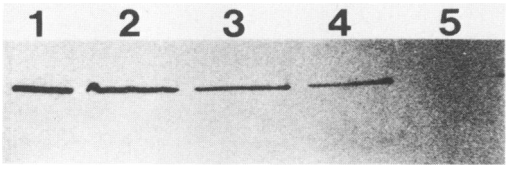

FIG. 6. Subcellular localization of EG2. As described in Materials and Methods, glucose-grown cells were washed twice in PBS and once in 25% sucrose containing 1 mM EDTA and then suspended in deionized water (lane 1). The shocked cell suspension was centrifuged to obtain the cell pellet, which was suspended in an equal volume of PBS and sonicated (lane 2), and the supernatant (periplasm) fraction (lane 5). The sonic extract was ultracentrifuged to obtain the supernatant (cytoplasm) fraction (lane 3) and the membrane pellet (lane 4), which was suspended in an equal volume of PBS. A 20-μl volume of each fraction was subjected to SDS-PAGE and Western immunoblotting with MAb N5A as described for Fig. 2b.

between the cells and the cellulose (29). Similar cell surface structures were recently found in several cellulolytic bacterial species, suggesting a common mechanism for bacterial interaction with and degradation of cellulose (31, 38). However, our data are not completely consistent with the presence of macromolecular cellulase-containing polycellulosome-like cell surface structures on *F. succinogenes*. The cell-associated immunogold labels that interacted with EG2 were found in isolated clusters, most frequently in areas where there appeared to be a disturbance in the integrity of the cell surface. Immunogold labeling of *F. succinogenes* cells by using an affinity-purified polyclonal antibody specific for the previously characterized chloride-stimulated cellobiosidase showed an association of the enzyme with protrusions of the cell surface, and participation of these structures in the hydrolysis of cellulose was considered (24). However, such an interpretation is complicated by the reactivity of the antibody with other proteins that possess related antigenic epitopes (26). Thus, in *F. succinogenes*, protrusions can be found on the cell surface, although there is more than one structural type and some may not contain EG2 or chloride-stimulated cellobiosidase. The distribution of other cellulases remains to be determined. Forsberg et al. (10) suggested that subcellular membrane vesicles released from *F. succinogenes* were in part responsible for polymer degradation in the rumen. These vesicles, 50 to 100 nm in diameter, were frequently seen attached to cellulose fibers. The heavily labeled particulate structures, 75 to 100 nm in diameter, seen in this study both on the cell surface and adhering to cellulose confirm the proposal of Forsberg et al. (10) that subcellular membrane vesicles released by *F. succinogenes* contain hydrolytic enzymes which may contribute to cellulose hydrolysis. In a separate study, Gaudet and Gaillard (12) concluded that these vesicles were primarily a result of culture aging and the absence of vesicles in young cellulose cultures was taken as an indication that they play no role in the adherence of bacteria or in their nutrition. Indeed, vesicle formation may not be a prerequisite for efficient hydrolysis of crystalline cellulose by *F. succinogenes*. However, *F. succinogenes* cells in the rumen do contribute to an extracellular population of subcellular vesicles that possess both cellulolytic and hemicellulolytic activities. This undoubtedly enhances polymer digestion and provides a source of sugars for microbes that lack polymer-degrading activity, thereby contributing to a stable, heterogeneous microbial population.

The presence of subcellular membrane vesicles on filter paper and on straw incubated in the rumen has been reported by others (8, 50). Numerous vesicular structures were seen in association with *F. succinogenes*-like cells attached to filter paper-cellulose which had been incubated in a nylon bag within the rumen (10). In a separate study, Gawthorne (13) isolated from a rumen sample of partially digested plant material a membrane-like fraction which had cellulase, xylanase, endoglucanase, and cellobiose activities. This vesicular mechanism of enzyme release is not restricted to rumen bacteria. Thompson et al. (54) demonstrated by immunoelectron microscopy that blebs that appeared on the surface of *Pseudomonas fragi* contained high concentrations of protease.

Although EG2 can be released by bleb formation, the best interpretation of the osmotic-shock experiment would be that the endoglucanase activity may not be primarily cell surface associated, in view of the high levels of the enzyme in both the cytoplasm and membrane fractions. The comparatively low percentage of endoglucanase in the periplasm in comparison with cellobiosidase and the periplasmic marker indicates that these latter two enzymes have a more external localization than endoglucanase. It is tempting to speculate that the cellular location of endoglucanase is a direct reflection of the mechanism of protein secretion. A review of current literature suggests that in gram-negative bacteria, most secreted proteins pass through the periplasm as they traverse the cell envelope and, less frequently, may be extruded through zones of inner and outer membrane adhesion (21). In either case, accumulation of endoglucanase in the cytoplasmic membrane as an intermediate step would not be unexpected, and the periplasmic activity would be low in comparison with that of the cytoplasmic and membrane fractions.

Further evidence in dispute of cell surface localization of endoglucanase comes from the observations of Groleau and Forsberg (15, 16). Trypsin was able to solubilize endoglucanase activity from the extracellular sedimentable membrane fraction but not from whole cells, and 70% of the endoglucanase activity from cellobiose-grown cells was in soluble form. Finally, in agreement with our present results, the distribution of endoglucanase activity in cells grown on cellulose in a chemostat was 23% periplasmic, 44% membrane associated, and 33% cytoplasmic (22).

With respect to the physiology of cellulose hydrolysis, a number of important conclusions can be drawn from this study. In glucose- and cellobiose-grown cultures, endoglucanase activity is primarily cell associated. Nevertheless, EG1 and EG3 are specifically secreted from the cells during growth on these substrates. In the presence of cellulose, the distribution of endoglucanase activity shifts and there are approximately equal amounts of cell-associated and extracellular activities. Therefore, the presence of cellulose appears to trigger endoglucanase secretion. EG2 was not secreted in glucose- or cellobiose-grown cultures. It did not appear in the cellulose culture supernatant, yet it was abundant on the cellulose surface. Therefore, this enzyme appears to be secreted only by cells which are in contact with cellulose. The documented affinity of this enzyme for cellulose (35, 36) ensures that once separated by cells, it will bind to cellulose and not be released into the culture fluid. Therefore, of the three endoglucanases characterized, EG2 is the only one which is truly cell associated. A substantial body of the data indicated that the enzyme is not a cell surface protein. Insofar as EG2 is representative of total cell-associated endoglucanase, it is difficult to conceive how

cellulose hydrolysis by *F. succinogenes* may be mediated by macromolecular cell surface structures, as has been proven for *C. thermocellum* and implicated in several other cellulolytic bacteria (31). To develop a more complete understanding of the mechanism of cellulose hydrolysis by this bacterium, it will be necessary to more thoroughly study the cellular distribution and mechanism of secretion of both endoglucanase and cellobiosidase.

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