

Antigenic Nature of the Chloride-Stimulated Cellobiosidase and Other Cellulases of *Fibrobacter succinogenes* subsp. *succinogenes* S85 and Related Fresh Isolates

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Polyclonal and monoclonal antibodies to the Cl-stimulated cellobiosidase of *Fibrobacter succinogenes* subsp. *succinogenes* S85 reacted with numerous proteins of both higher and lower molecular weights from *F. succinogenes* subsp. *succinogenes* S85, but not with *Escherichia coli* proteins, and only one protein each from *Butyrivibrio fibrisolvens* and *Ruminococcus albus*. Different profiles were observed for Western blots (immunoblots) of peptide digests of both the purified enzyme from *F. succinogenes* and immunoreactive proteins of higher and lower molecular weights, demonstrating that they were different proteins. Therefore, *F. succinogenes* appeared to produce numerous proteins with one or more common antigenic determinants. However, with the exception of Cl-stimulated cellobiosidase, none were cellulases that have been characterized. An affinity-purified polyclonal antibody to Cl-stimulated cellobiosidase reacted with numerous proteins in cells of each of three fresh isolates of *F. succinogenes* subsp. *succinogenes* and one of *F. succinogenes* subsp. *elongata* when analyzed by Western blotting. Antibodies to periplasmic cellodextrinase, endoglucanase 2 (EG2), and EG3, when reacted in Western blots with the various cellulases, including Cl-stimulated cellobiosidase, revealed limited antigenic similarity among the different proteins and none with either *B. fibrisolvens* or *R. albus* proteins. The periplasmic cellodextrinase antibody reacted with an antigen with a size corresponding to cellodextrinase in each of the three *F. succinogenes* subsp. *succinogenes* isolates but not with any antigens from the *F. succinogenes* subsp. *elongata* isolate. The anti-EG2 antibody reacted with single antigens in each of the four isolates, while the anti-EG3 antibody reacted with only one of the four isolates. The apparent absence of cellodextrinase from the *F. succinogenes* subsp. *elongata* isolate may indicate a subspecies difference, whereas the absence of EG3 from several isolates of both subspecies may be related to the stage of growth. Therefore, it appears that the cellulase enzymes of strain S85 are generally present in other strains of *F. succinogenes*.

Fibrobacter succinogenes subsp. *succinogenes* S85, formerly *Bacteroides succinogenes* (22), is one of the major fibrolytic bacteria in the rumina of cattle and sheep (3, 25). Studies of the cellulase system of *F. succinogenes* subsp. *succinogenes* S85 have revealed that it is extremely complex, consisting of as many as six different genes that code for endoglucanases (5). Three endoglucanases have been purified and characterized in detail (18, 21). *F. succinogenes* also possesses a Cl-stimulated cellobiosidase (13), a periplasmic cellodextrinase (10, 11), and a cellobiase (8).

Antibody probes specific for endoglucanase 1 (EG1), EG2, and EG3 (20), periplasmic cellodextrinase (11), and Cl-stimulated cellobiosidase (this study) have been prepared and used to study the regulation and cellular distribution of endoglucanases (20) and cellobiosidases (12).

S85, the type strain of *F. succinogenes* subsp. *succinogenes*, was isolated from the bovine rumen by Bryant and Doetsch in 1954 (2) and has been maintained in pure culture ever since. Cultivation of the bacterium in laboratory media for more than 30 years has offered growth conditions different from those in the rumen and a period of time sufficient to allow genotypic change in the organism to occur. Indeed,

there is evidence to suggest that variants with altered physiological properties do arise when *F. succinogenes* isolates are cultured under laboratory conditions (27).

In this study, we explored the antigenic nature of the Cl-stimulated cellobiosidase of *F. succinogenes*. The structural relatedness of the cellulase enzymes of this bacterium was examined by using specific antibodies. The antibodies were used as probes to determine the presence of cellulase enzymes of strain S85 in four fresh *Fibrobacter* isolates from cattle.

MATERIALS AND METHODS

Organisms and growth conditions. *F. succinogenes* subsp. *succinogenes* S85 (ATCC 19169), *Butyrivibrio fibrisolvens* D1, and *Ruminococcus albus* B199 had previously been obtained from M. P. Bryant (6). Fresh *Fibrobacter* sp. strains LRS088, LRS107, LRS110, and LRS111 were isolated from fistulated cattle fed wheat straw at Lethbridge, Alberta, Canada, by using the method described by Stewart et al. (26). The medium used for the isolation procedure was that described by Scott and Dehority (24) with 10% (vol/vol) added strained rumen fluid. All cultures were grown for the experiments in L-10 medium which contained (per liter) the following components in addition to those in the medium described by Scott and Dehority (24): tryptone, 2 g; yeast extract, 0.5 g; glucose, 2 g; maltose, 1 g; cellobiose, 1 g; hemin, 1 mg; and menidione, 2 mg. Isobutyric acid and valeric acid were the only fatty acids included. The *Escherichia coli* RR1(PRE3) culture used harbors plasmid PRE3, a

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pUC8-derived construct which contains the *cel-3* gene that encodes endoglucanase 3 of *F. succinogenes* (21).

Enzyme preparation. Purified preparations of EG1, EG2 (18), EG3 (21), periplasmic cellodextrinase (11), and CI-stimulated cellobiosidase (13) were made previously. Similarly, chemostat culture fluid from strain S85 (13) and periplasmic proteins from *E. coli* RE3 (21) were also prepared previously.

Enzyme assays and analytical techniques. Enzyme assays were conducted as previously described (10). Fermentation products in the culture supernatant were determined by high-pressure liquid chromatography. Samples of the cell-free culture supernatant were freed from cations by mixing with Amberlite IR-120 cation-exchange resin (hydrogen form), filtered through a 0.45- μ m-pore-size Metrical filter, and analyzed by using a high-pressure liquid chromatography system (Waters Associates, Inc., Milford, Mass.) equipped with an ERC-7510 refractive-index detector (Erma Optical Works, Ltd., Tokyo, Japan). The fermentation products were separated on an Aminex HPX-87H column (Bio-Rad Laboratories, Richmond, Calif.) at 35°C with 0.013 N sulfuric acid as the eluent at a flow rate of 0.4 ml/min.

Soluble proteins were determined by the method of Bradford (1) by using bovine serum albumin as the standard. For quantification of cell-associated proteins, samples were solubilized by being heated at 100°C for 10 min in 0.5 N NaOH. Following centrifugation in a benchtop microcentrifuge to remove extraneous particulate material, the protein content of the supernatant samples was measured by using a standard curve constructed by dissolving bovine serum albumin in 0.5 N NaOH.

Preparation and purification of antibodies. Polyclonal antisera to the periplasmic cellodextrinase, EG2, and EG3 had been prepared previously in rabbits (11, 19, 21). Polyclonal antiserum to purified CI-stimulated cellobiosidase was prepared in a fashion identical to that described for EG2 (19). Monospecific polyclonal antibodies were prepared by affinity purification from the respective antisera by using modifications of the method of Olmsted (23) as previously described (11).

Mouse monoclonal antibody (MAb) H4A to purified CI-stimulated cellobiosidase was prepared as described by McGavin and Forsberg (19). The antibody was found to belong to the immunoglobulin M class by using the Bio-Rad Mouse-Typer Sub-Isotyping Kit. It had a titer of 10, where the titer was defined as the \log_{10} of the last serial 10-fold dilution of the antibody that gave an optical density at 405 nm of ≥ 0.1 in an enzyme-linked immunosorbent assay with 0.1 μ g of CI-stimulated cellobiosidase per ml as the antigen (16).

Western blotting. Western immunoblotting was conducted as described by Huang and Forsberg (11). Samples subjected to electrophoresis included purified proteins, peptide digests, or whole cells lysed in sample buffer. The specific antigens bound to nitrocellulose were detected by using the procedure described in the Bio-Rad immunoblot manual. The first antibody was appropriately diluted polyclonal antiserum, affinity-purified polyclonal antibodies, or undiluted MAb H4A. For immunoblotting with polyclonal antibodies, the second antibody was either 3,000-fold-diluted protein A conjugated to alkaline phosphatase (Sigma Chemical Co., St. Louis, Mo.) or a 3,000-fold-diluted goat anti-rabbit immunoglobulin G antibody conjugated to alkaline phosphatase (Bio-Rad). For Western blotting with MAb H4A, the second antibody was a 5,000-fold-diluted affinity-purified goat anti-mouse immunoglobulin G F(ab')₂ alkaline phosphatase-con-

jugated antibody (Jackson Immunoresearch Laboratories, Inc., West Grove, Pa.).

Peptide mapping of *F. succinogenes* proteins recognized by antibody to CI-stimulated cellobiosidase. Samples of purified CI-stimulated cellobiosidase (4 μ g of protein) and the concentrated chemostat culture supernatant (100 μ g of protein) were electrophoresed on a 1.5-mm-thick sodium dodecyl sulfate-polyacrylamide gel (10% acrylamide). The gel was stained for 2 min with Coomassie brilliant blue and then destained briefly. Three protein bands known to be recognized by antibodies to CI-stimulated cellobiosidase were cut out. One band contained the CI-stimulated cellobiosidase derived from the purified enzyme sample. The other two bands were from the lane containing the culture supernatant sample and contained a protein which was either approximately 10 kilodaltons larger than CI-stimulated cellobiosidase or approximately 10 kilodaltons smaller than the enzyme. The gel slices containing these protein bands were placed at the bottom of the separate wells of a second sodium dodecyl sulfate-15% polyacrylamide gel, treated with *Staphylococcus aureus* V8 protease (5 μ g per well) as described by Cleveland et al. (4). Following separation of proteolytic fragments by electrophoresis, the specific antigens were analyzed by immunoblotting with 500-fold-diluted antiserum to CI-stimulated cellobiosidase.

Proteinase K digestion of proteins recognized by rabbit antibody to CI-stimulated cellobiosidase. Proteinase K digestion of *F. succinogenes* proteins was done by the method of Hitchcock and Brown (9). *F. succinogenes* cells grown on Avicel cellulose were washed in 0.05 M sodium phosphate buffer (pH 6.5) supplemented with 0.8% (wt/vol) NaCl and suspended in the same buffer. Samples of the cell suspension (20 μ l; 68 μ g of protein) and purified CI-stimulated cellobiosidase (0.6 μ g of protein) were mixed with lysis buffer (30 μ l) containing 2% (wt/vol) sodium dodecyl sulfate, 4% (wt/vol) 2-mercaptoethanol, 10% (vol/vol) glycerol, 1 M Tris hydrochloride (pH 6.8), and 0.01% (wt/vol) bromophenol blue and heated at 100°C for 10 min. Proteinase K (4 mg/ml in lysis buffer; Sigma) was added to each sample to give a final concentration of 0.7 mg/ml. In the controls, lysis buffer replaced the proteinase K solution. Samples (20 μ l) were electrophoresed and blotted as described above.

RESULTS

Immunogenic nature of CI-stimulated cellobiosidase. In Western blots, antisera to CI-stimulated cellobiosidase exhibited cross-reactivity with numerous proteins in the extracellular culture fluid of *F. succinogenes* which were nonsedimentable at 100,000 $\times g$ for 2 h, but not with *E. coli* cell proteins. After affinity purification of the antibodies, the same banding pattern as with the untreated antiserum was observed (Fig. 1). Peptide analysis of two selected immunogenic proteins with higher and lower molecular weights than the enzyme showed no similarity, either with one another or with purified cellobiosidase (Fig. 2), thereby precluding the possibility that the multiplicity of bands was caused by either aggregation or proteolysis. When the extracellular culture fluid sample was pretreated with proteinase K before electrophoresis, no bands were detected by the antibodies, suggesting that the antigens were proteins or perhaps covalently modified proteins rather than lipopolysaccharide, which is unaffected by protease action.

Immunological relatedness of purified cellobiosidases and endoglucanases. Purified samples of periplasmic cellodextrinase, CI-stimulated cellobiosidase, EG1, EG2, EG3, and

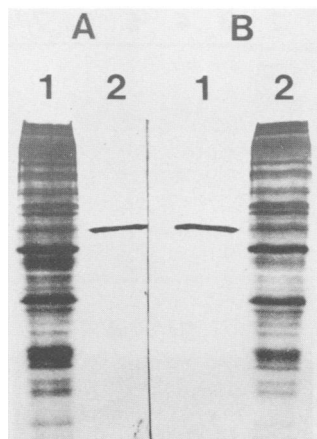


FIG. 1. Western blotting of nonsedimentable cell-free culture fluid of *F. succinogenes* with 500-fold-diluted rabbit antiserum to Cl-stimulated cellobiosidase (A) and affinity-purified antibodies to Cl-stimulated cellobiosidase (B). Lanes: 1 in panel A and 2 in panel B, nonsedimentable culture fluid (38.5 μ g of protein); 2 in panel A and 1 in panel B, 0.2 μ g of purified Cl-stimulated cellobiosidase. The second antibody was 3,000-fold-diluted protein A conjugated to alkaline phosphatase.

other samples were subjected to Western blotting by using antibodies specific for each of the enzymes, with the exception of EG1. Cellodextrinase-specific antibodies reacted with EG1 and EG2, in addition to cellodextrinase; however, they did not react with either Cl-stimulated cellobiosidase or EG3 (Fig. 3A). No cellulase other than Cl-stimulated cellobiosidase was recognized by affinity-purified polyclonal antibodies to Cl-stimulated cellobiosidase (Fig. 3B). The antibody to EG2 reacted weakly with Cl-stimulated cellobiosidase and with both EG2 and its previously described (18) lower-molecular-weight proteolytic degradation product (Fig. 3C).

Culture characteristics and cellobiosidase activities of *Fibrobacter* isolates. The new *Fibrobacter* isolates grew with Avicel cellulose as the source of carbon. Three of these (LRS088, LRS107, and LRS111) were pleomorphic, coccoid cells characteristic of *F. succinogenes* subsp. *succinogenes*,

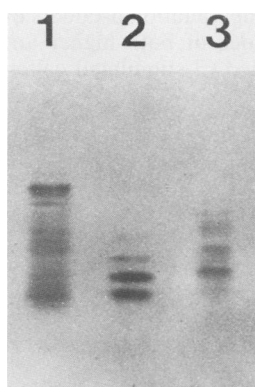


FIG. 2. Western blotting of *S. aureus* V8 protease peptide digests of Cl-stimulated cellobiosidase and two immunologically related proteins from the nonsedimentable fraction of the culture supernatant of *F. succinogenes* by using affinity-purified polyclonal antibodies to Cl-stimulated cellobiosidase. Lanes: 1, protein larger than the enzyme; 2, Cl-stimulated cellobiosidase; 3, protein smaller than the enzyme.

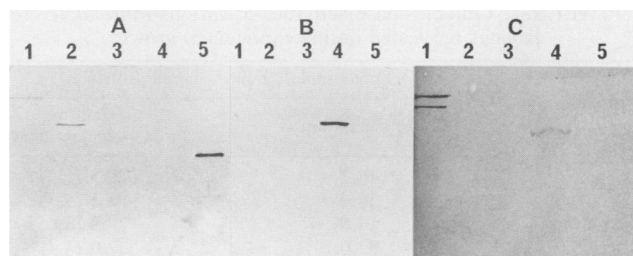


FIG. 3. Western blotting to examine relatedness of purified endoglucanases and cellobiosidases by using an affinity-purified polyclonal antibody to each cellulase. (A) Periplasmic cellodextrinase antibody. Lanes: 1, 0.4 μ g of EG2; 2, 0.4 μ g of EG1; 3, 0.4 μ g of EG3; 4, 0.4 μ g of Cl-stimulated cellobiosidase; 5, 0.4 μ g of periplasmic cellodextrinase. (B) Cl-stimulated cellobiosidase antibody. Lanes: 1 to 5, same as in panel A. (C) EG2 antibody. Lanes: 1, 0.8 μ g of partially purified EG2; 2, 0.4 μ g of EG1; 3, 0.8 μ g of Cl-stimulated cellobiosidase; 4, 0.5 μ g of EG3; 5, 0.4 μ g of periplasmic cellodextrinase.

while one, LRS110, was a thin rod characteristic of *F. succinogenes* subsp. *elongata*. After three serial subcultures of S85 and the four isolates in rumen fluid-free medium, the fermentation products and total culture protein were determined (Table 1). All cultures exhibited identical fermentation profiles, with acetate and succinate as the major products. The cellobiosidase activities of whole cultures and cell-free culture fluid were also determined for late-exponential-phase cultures (Table 2). All cultures exhibited similar cellobiosidase specific activities, with the exception of LRS110, which had a lower specific activity. Release of cellobiosidase into the culture medium differed among the cultures, with the release from S85 being the least. These data represent an approximation of the release of cellobiosidase, since a single time point was analyzed for each culture.

Western blotting to detect cellulase enzymes in fresh *Fibrobacter* isolates. Cellular proteins of strain S85 and the various isolates were examined by Western blotting. By using an affinity-purified polyclonal antibody to cellodextrinase, a single protein band corresponding in molecular weight to periplasmic cellodextrinase was detected in *F. succinogenes* subsp. *succinogenes* LRS088, LRS107, LRS111, and S85, but not in *F. succinogenes* subsp. *elongata* LRS110 (Fig. 4). Since the protein contents of all samples were identical, the similarity in intensity of the bands indicates that the three positive isolates had comparable levels of cellodextrinase.

The Cl-stimulated cellobiosidase-related antigens in whole-cell lysates of the isolates were probed by using both affinity-purified polyclonal antibodies (Fig. 5A) and MABs (Fig. 5B). Analysis with polyclonal antibodies revealed similar banding patterns for samples from strains S85, LRS107, LRS111, and LRS088, although the antibodies reacted more strongly with S85 antigens (Fig. 5A). Strain

TABLE 1. Fermentation products of *Fibrobacter* isolates

Strain	Succinate (mM)	Acetate (mM)	Protein (mg/ml)
S85	21.4	6.2	0.56
LRS107	14.7	ND ^a	0.36
LRS110	19.3	6.1	0.47
LRS111	16.9	5.0	0.45
LRS088	15.3	6.5	0.36

^a ND, Not determined.

TABLE 2. Cellobiosidase activities of various *Fibrobacter* isolates harvested during exponential growth

Strain	Sp act (nmol · min ⁻¹ · mg of protein ⁻¹) in:	
	Whole culture	Cell-free supernatant fluid ^a
S85	46.2	20.5 (1.7)
LRS107	38.9	28.5 (5.3)
LRS110	14.0	24.9 (39.5)
LRS111	32.0	17.0 (4.4)
LRS088	38.5	48.0 (22.5)

^a The data in parentheses represent percentages of the total cellobiosidase activity found in the cell-free supernatant fluid.

LRS110 exhibited a few differences in the antigen pattern. Only a single cross-reacting band each was detected in *B. fibrisolvens* and *R. albus*, and their sizes were similar to that of the Cl-stimulated cellobiosidase. MAb H4A reacted more uniformly with Cl-stimulated cellobiosidase from the different strains than did the polyclonal antibodies. Furthermore, it reacted more intensely with a protein antigen which migrated to the same position as purified Cl-stimulated cellobiosidase in each of the samples (Fig. 5B) than did the polyclonal antibodies (Fig. 5A). With the exception of strain LRS110, all isolates exhibited banding patterns practically identical to that of strain S85. EG2 was detected in all four fresh isolates but not in either *B. fibrisolvens* or *R. albus* (Fig. 6).

The data obtained with antibodies to EG3 should be interpreted with caution, since the antibodies were raised against a 43-kilodalton C-terminal fragment of the enzyme purified from the periplasm of *E. coli* as indicated previously (21). The protein in the extracellular culture fluid of *F. succinogenes* subsp. *succinogenes* S85 had a molecular weight of 118,000. With the anti-EG3 antibody, the enzyme was detected in strains S85, LRS088, and LRS110 (stained very lightly) but not in LRS107 or LRS111 (Fig. 7).

DISCUSSION

Cl-stimulated cellobiosidase apparently shares one or more antigenic epitopes with numerous other proteins of *F. succinogenes*. Evidence obtained in this study which sup-

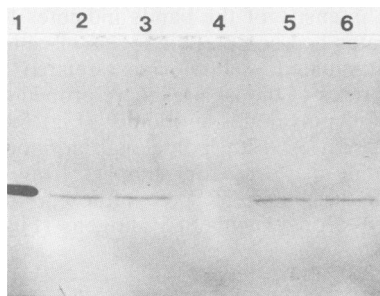


FIG. 4. Immunological detection of cellodextrinase in various *F. succinogenes* isolates by Western blotting. An affinity-purified polyclonal anti-periplasmic-cellodextrinase antibody was used as the first antibody. Lanes: 1, 0.4 μ g of purified periplasmic cellodextrinase; 2, strain S85; 3, strain LRS107; 4, strain LRS110; 5, strain LRS111; 6, strain LRS088. Late-exponential-phase cells washed once in 0.05 M sodium phosphate buffer (pH 6.5) were solubilized in disruption buffer, and 27 μ g of cellular protein was applied per lane for electrophoresis.

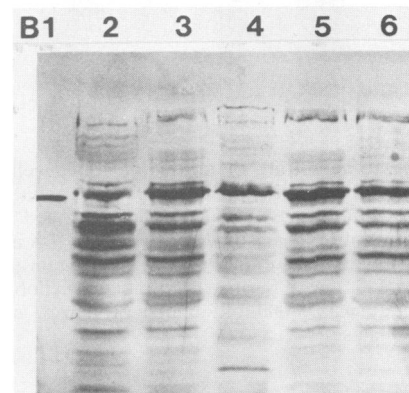
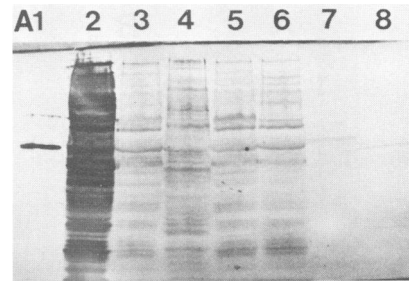


FIG. 5. (A) Immunological detection of Cl-stimulated cellobiosidase in various *F. succinogenes* isolates and two other rumen cellulolytic bacteria. An affinity-purified polyclonal anti-Cl-stimulated cellobiosidase antibody was used as the first antibody. Lanes: 1, 0.2 μ g of purified Cl-stimulated cellobiosidase; 2, strain S85; 3, strain LRS107; 4, strain LRS110; 5, strain LRS111; 6, strain LRS088; 7, *B. fibrisolvens*; 8, *R. albus*. (B) Immunological detection of Cl-stimulated cellobiosidase in various *F. succinogenes* isolates by Western blotting. MAb H4A to Cl-stimulated cellobiosidase was used as the first antibody. Lanes: 1, 0.2 μ g of purified Cl-stimulated cellobiosidase; 2, strain S85; 3, strain LRS107; 4, strain LRS110; 5, strain LRS111; strain 6, LRS088.

ports this conclusion includes the following. Both affinity-purified polyclonal antibodies and MAbs reacted with a large number of proteins from cells. The antigen is either a protein or attached to protein, because it was completely destroyed by proteinase K. The individual proteins recognized by the antibodies are not degradation products of one large protein, because polypeptides of both higher and lower molecular weights than purified Cl-stimulated cellobiosidase exhibited

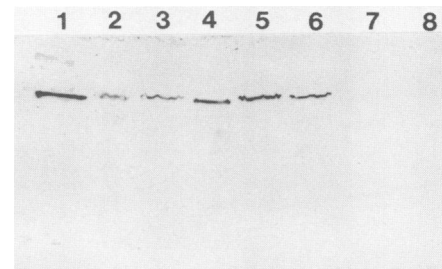


FIG. 6. Immunological detection of EG2 in various *F. succinogenes* isolates and two other rumen bacteria by Western blotting. Affinity-purified polyclonal anti-EG2 antibody was used as the first antibody. Lanes: 1, 0.5 μ g of EG2; 2, strain S85; 3, strain LRS107; 4, strain LRS110; 5, strain LRS111; 6, strain LRS088; 7, *B. fibrisolvens*; 8, *R. albus*.

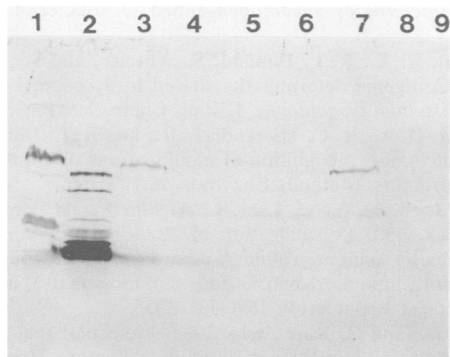


FIG. 7. Immunological detection of EG3 in various *F. succinogenes* isolates and two other rumen bacteria. An affinity-purified polyclonal anti-EG3 antibody was used as the first antibody. Lanes: 1, 34 μ g of chemostat culture fluid protein; 2, 15 μ g of periplasmic proteins from RE3; 3, strain S85; 4, strain LRS107; 5, strain LRS110; 6, strain LRS111; 7, strain LRS088; 8, *B. fibrisolvans*; 9, *R. albus*.

peptide profiles different from that of cellobiosidase. The cross-reactivity of antibodies to Cl-stimulated cellobiosidase with other proteins apparently is not due to a nonspecific reaction, since the antibodies reacted with neither the other known cellulases of *F. succinogenes* nor *E. coli* proteins and they recognized only one protein each from *B. fibrisolvans* and *R. albus*. Thus, on the basis of the criteria outlined by Knecht et al. (15), it appears reasonable to conclude that Cl-stimulated cellobiosidase contains one or more antigenic determinants common to a wide range of other cellular proteins of this species. Since the enzyme is a glycoprotein (13), it is possible that the carbohydrate moieties are antigenic determinants. The fact that the antigen was purified by denaturing polyacrylamide gel electrophoresis could have caused extensive unfolding of the polypeptide, exposing the carbohydrate moieties, thereby increasing their contribution to the immunogenicity of the molecule (15).

The observation in this study of the shared antigenic determinants of Cl-stimulated cellobiosidase resembles a parallel situation with lysosomal enzymes from the cellular slime mold *Dictyostelium discoideum* (14). The lysosomal hydrolases of the slime mold possess a common posttranslational modification which is extremely antigenic in rabbits and mice. Rabbit antisera and mouse MAbs that recognize this determinant cross-react with a group of at least 40 to 50 highly negatively charged proteins, which include most or all of the lysosomal enzymes. The common antigenic determinant from one protein was shown to be an N-linked oligosaccharide containing an esterified sulfate (7). Whether the common epitope(s) shared among the *F. succinogenes* proteins is an oligosaccharide remains to be determined. The similarity of function of the lysosomal proteins that share the same epitope raises the possibility that the antigenically related proteins in *F. succinogenes* also exhibit common structure-function relationships. In a recent immunolabeling study, the Cl-stimulated cellobiosidase-common antigen(s) was detected in patches on the cell surface which were usually present on protuberant structures (12).

The cross-reactivity of polyclonal antibodies to periplasmic cellobiosidase, EG2, and EG3 was minimal. When these antibodies were used in Western blots of *F. succinogenes* cell extracts or supernatant proteins, they were highly specific for their respective antigens and there was no indication of cross-reactivity with other proteins (Fig. 4, 6,

and 7). However, when the individual purified enzymes were subjected to Western blotting, antibodies to periplasmic cellobiosidase were cross-reactive with EG1 and EG2, while antibodies to EG2 were cross-reactive only with Cl-stimulated cellobiosidase. This may be due to a localized concentration of epitopes from the purified proteins on the nitrocellulose, a situation which would not be encountered in Western blots of cell extracts or culture supernatants. The weak cross-reactivity observed suggests that there are unique conserved recognition sites in each of two groups of enzymes, periplasmic cellobiosidase, EG1, and EG2 (Fig. 3A) in the first group and Cl-stimulated cellobiosidase and EG2 (Fig. 3C) in the second (17). In the latter group (Fig. 3C), it is interesting that both of these enzymes bind to crystalline cellulose (13, 18), whereas the other enzymes do not. Hence, it may be speculated that the cross-reactivity observed is due to conservation of structure in the cellulose-binding domains of the respective proteins. In *Thermomonospora fusca* YX, cross-reactivity of endoglucanases has also been observed (28). Five endoglucanases have been purified from this bacterium. Of these, EG1, EG2, and EG5 are distinct antigenically, as antiserum to each enzyme reacted only with that enzyme, while EG3 and EG4 reacted with antisera to each other but were antigenically distinct from the other enzymes and differed from one another in molecular weight, N-terminal sequence, and glycosylation.

The antibody to EG3 did not react with any of the other cellulase enzymes (data not shown). However, data concerning the immunological relatedness of EG3 to the other purified enzymes must be tempered by the consideration that the 43-kilodalton enzyme purified from the periplasm of *E. coli* RR1(PRE3) is actually the carboxyl-terminal catalytic portion of the protein and that the N-terminal 265 amino acids have been removed by proteolytic cleavage (21). Therefore, antibodies specific for potential cross-reactive epitopes at the N terminus of the protein would not be present in the antiserum, and in addition, the N-terminal 265 amino acids are not available for interaction with antiserum specific for the other purified enzymes.

The culture characteristics of all four fresh isolates corresponded to those of *F. succinogenes*. Three (LRS088, LRS107, and LRS111) exhibited an oval morphology, indicating that they belonged to *F. succinogenes* subsp. *succinogenes*, while the fourth was rod shaped, which is a characteristic of *F. succinogenes* subsp. *elongata* (22). Cellobiosidase activity was lower in the *F. succinogenes* subsp. *elongata* isolate.

Western blotting of fresh *Fibrobacter* isolates to detect the presence of Cl-stimulated cellobiosidase, cellobiosidase, EG2, and EG3 demonstrated that all were present in some or all of the fresh isolates. The exceptions were cellobiosidase, which was absent from *F. succinogenes* subsp. *elongata* (LRS110), and EG3, which was not detected in cells of two *F. succinogenes* subsp. *succinogenes* isolates (LRS107 and LRS111). It was observed by Huang and Forsberg (12) that cellobiosidase is produced constitutively in *F. succinogenes* subsp. *succinogenes* S85. Failure to detect it in *F. succinogenes* subsp. *elongata* may indicate that the enzyme is absent in this subspecies. EG3 was also observed to be produced constitutively in *F. succinogenes* subsp. *succinogenes* S85; however, it is secreted from cells during growth (20). Consequently, its absence from cells is not necessarily due to lack of synthesis by the bacteria. Therefore, it is reasonable to conclude that the cellulases of *F. succinogenes* subsp. *succinogenes* S85 that have been studied in detail are broadly distributed among members of the genus.

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