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Cellulosome-Like Entities in *Bacteroides cellulosolvens*

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Abstract. Cellulosome-like complexes were identified in the broth and sonic extracts of cellobiose- and cellulose-grown cells of *Bacteroides cellulosolvens*. The extracellular fractions contained three to four major polypeptides and several minor polypeptide bands that were localized in two major gel filtration peaks indicating average molecular weights of about 700 kDa and >10 MDa. A relatively large molecular weight component (M_r 230 kDa) was found to contain carbohydrate, but no apparent enzymatic activity of its own could be detected. The cell sonicate displayed a more complicated polypeptide profile, and glycosylated polypeptides were larger (ca. 310 and 290 kDa) than that of the extracellular fraction. The 230-kDa extracellular component interacted strongly with the GSI isolectin from *Griffonia simplicifolia*, exhibited immunochemical cross-reactivity with the S1 subunit of the cellulosome from *Clostridium thermocellum*, and displayed anomalous pH- and salt-dependent migratory behavior in SDS-PAGE. Taken together, this evidence strongly suggests a structural similarity between the glycoconjugates of these two distinct cellulolytic bacteria. A major 84-kDa polypeptide was identified as a xylanase, and a 50-kDa polypeptide displayed endoglucanase activity. Additional biochemical and cytochemical evidence indicated that cellulosome-like cellulolytic complexes are associated with the cell surface in this bacterium.

Aggregative forms of cellulases have been identified in several cellulolytic bacteria either as cell-bound complexes and/or in the cell-free form [10]. In the case of *Clostridium thermocellum*, the most extensively studied cellulolytic bacterium, adherence to cellulose is directly mediated by a multicellulase complex termed the cellulosome [1, 12]. The cellulosome of *C. thermocellum* was found to be exquisitely organized for highly efficient degradation of its insoluble cellulosic substrate. The cellulosome complex contains at least 14 different subunits, many of which exhibit endoglucanase, xylanase, and exoglucanase-like activities [17]. One of its most interesting components, the S1 subunit, has no known enzymatic role, but may contribute indirectly to the observed synergism among the other components of the cellulosome [11, 16]. The S1 is highly glycosylated and antigenic [4, 11].

We have recently reported that a variety of cellulolytic bacteria exhibit a range of structural properties that are remarkably similar to those of *C. thermocellum* [13]. These include the presence of cell surface protuberance-like structures, the interaction with an αGal-specific lectin that selectively recognizes the S1 subunit of the cellulsome, and the cross-reactivity with an anti-cellulosome-specific antibody preparation. One of these strains, *Bacteroides cellulosolvens*, a Gram-negative mesophilic bacterium, was previously found to contain a very active cell-associated cellulase [6, 21] and to exhibit the above-mentioned properties reminiscent of the cellulosome from *C. thermocellum* [5]. The purpose of this work was to determine whether such a multienzyme complex exists in *B. cellulosolvens* and to characterize its properties on the molecular level, with the cellulosome from *C. thermocellum* as a reference.

Materials and Methods

Organism and growth conditions. *Bacteroides cellulosolvens* ATCC 35603 was cultured anaerobically at 37°C according to Murray et al. [20], with either cellobiose (Sigma Chemical Co., St. Louis, Missouri) or microcrystalline cellulose (Avicel, E. Merck AG, Darmstadt, FRG) as substrate.

Electron microscopy. Cationized ferritin staining and processing for scanning electron microscopy was performed as reported earlier [14].

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Lectin-induced agglutination and labeling of cells. Cell suspensions were washed once by centrifugation (17,000 *g*, 10 min) with phosphate-buffered saline, pH 7.4 (PBS),[1] and the cells were suspended to one absorbance unit (400 nm). A 50-μl sample was combined with 10 μl of the GSI isolectin derivatized with fluorescein isothiocyanate (FITC-GSI; 0.4 mg/ml, Sigma). After 10 min at 25°C, the cells were washed once, suspended to the original volume with PBS, and mounted on a glass microscope slide for fluorescence analysis under an Olympus BH2 fluorescence microscope with a blue exciter filter.

Adherence assay. The assay was performed essentially according to Bayer et al. [2]. Briefly, a 1-ml aliquot of a washed cell suspension (A_{400} = 2.5) was brought to a total volume of 3 ml with 1 ml of 2% (wt/vol in PBS) microcrystalline cellulose and 1 ml of PBS (containing the various additives when indicated). The suspension was vortexed for 30 s, and the cellulose and bacterial cells were allowed to settle at room temperature for 60 min. The turbidity (A_{400}) of the supernatant fluids was measured and compared with control tubes wherein PBS was substituted for the cellulose suspension.

Determination of cellulolytic and xylanolytic activities. Cellulase and xylanase activities were performed by determining reducing sugars released from the respective polymer. CMCase activity was determined using carboxymethylcellulose (CMC) essentially according to Miller et al. [15]; briefly, a solution containing the enzyme sample, CMC (low viscosity, 1% wt/vol) and citrate–phosphate buffer (50 m*M* K_2HPO_4, 12.5 m*M* citric acid, pH 6.3), was brought to a total volume of 2 ml with distilled water. The samples were incubated for 2 h at 38°C. A 3-ml aliquot of dinitrosalicylic acid reagent was added, and the tubes were heated at 90°C for 10 min. The extent of reaction was measured spectrophotometrically at 640 nm. Xylanase activity was determined in a similar manner by substituting 0.5% (wt/vol) xylan for the CMC. A cellobiose standard was used for cellulase, and xylose for xylanase activity.

Gel chromatography. Samples (5–25 mg, 5 ml) were applied to a Sepharose 4B column (1.4 × 63 cm), and gel filtration was carried out at room temperature. The column was equilibrated and eluted with 50 m*M* Tris-HCl buffer (pH 7.5) containing 0.05% sodium azide. The flow rate was 0.25 ml/min, and fractions of 1.5 ml were collected.

Lectin labeling of cell-derived material. The desired extracellular or cell-associated sample was examined for lectin binding by a dot blot assay. Dot blots were dried and quenched with 2% bovine serum albumin in PBS. Blots were incubated for 1 h with GSI-peroxidase (3 μg/ml, Sigma). After incubation, the blots were rinsed several times with PBS, and a substrate solution [0.013% (wt/vol) H_2O_2 in a Tris-saline buffer (pH 7.4), containing 0.5 mg of chloro-1-naphthol per ml (initially prepared from a 3-mg/ml methanolic stock solution)] was added. Color development occurred within 5–10 min, and the reaction was terminated by washing the blots with tap water.

[1] Abbreviations used: CMC, carboxy methyl cellulose; FITC, fluorescein isothiocyanate; GSI, isolectin I from *Griffonia simplicifolia*; PBS, phosphate-buffered saline, pH 7.4; PAGE, polyacrylamide gel electrophoresis; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

Cellulose-based affinity chromatography. The cell-free supernatant fluids from a 1-liter culture were brought to pH 7.7 with 1 *N* NaOH, and 10 g of microcrystalline cellulose was added. The suspension was then stirred for 1 h at room temperature. The supernatant fluids were removed through a sintered glass funnel, and the residue was washed with 50 m*M* Tris-HCl buffer (pH 7.4). The adsorbed proteins were eluted from the cellulosic matrix with 100 ml of a 1% solution of triethylamine. The eluent solution was immediately neutralized with 10% acetic acid.

Lectin-based affinity chromatography. A 0.1-ml sample of GSI-agarose (EY Labs, Inc., San Mateo, CA) was washed with Tris-HCl buffer and added to a 1-ml solution containing 20-fold concentrated spent growth medium from *B. cellulosolvens*. The suspension was mixed for 60 min at room temperature, centrifuged, and the supernatant fluids were saved for subsequent studies. The adsorbed proteins were eluted from the affinity column by 1 *M* galactose.

Slab-gel electrophoresis. The conditions for SDS–PAGE (6% gels) were those described previously [7]. Native PAGE was carried out under similar conditions with the omission of SDS and mercaptoethanol. Gels were stained with Coomassie brilliant blue R250, destained, and photographed. In some cases, gels were stained for the presence of carbohydrates by the PAS method [23].

Blot transfer of separated proteins was carried out electrophoretically onto nitrocellulose sheets. The GSI lectin, conjugated to peroxidase, was employed to detect the relevant carbohydrate-containing polypeptide component. Staining with anticellulosomal antibodies was carried out as described previously [13].

Zymograms. In order to detect enzymatic activity associated with a given polypeptide band, the gels were stained by a modification of the Congo red procedure [3]. Gels were run as above with the addition of the desired substrate, i.e., xylan (0.1% soluble fraction) for xylanase activity or 0.1% CMC for endoglucanase activity. Following the electrophoresis step, the gels were washed twice for 30 min with citrate–phosphate buffer containing 25% (vol/vol) 2-propanol and twice again with the alcohol-free buffer alone. The washed gels were incubated for 16 h at 37°C and stained with 0.1% Congo red. Finally, the gels were rinsed with 1 *M* NaCl.

Results

Reisolation and characterization of *B. cellulosolvens*. In the early stages of this work, we encountered difficulties in maintaining the strain used (ATCC 35603). During growth in a simplified synthetic medium [18], which contained 0.5% cellobiose as carbon source, an unidentified mesophilic sporulating rod was consistently found to overtake the original nonsporulating rod. Since the bacterium was originally isolated from a stable coculture with *Clostridium saccharolyticum* [19, 20], we suspected that the latter was the contaminant strain. After reisolation of the bacterium by a roll bottle modification of

the roll tube technique, the nonsporulating strain (*B. cellulosolvens*) could be transferred repeatedly in cellobiose-containing medium and exhibited a doubling time of 13 h. In accordance with the original publication on this bacterium [20], the reisolate grew on cellulose and cellobiose as growth substrates, but not on starch, xylan, xylose, glycerol, glucose, fructose, galactose, or arabinose. The culture was insensitive to 25 μg/ml penicillin G, but sensitive to 2.5% yeast extract, results which are in line with the original publication.

Adherence of *B. cellulosolvens* to insoluble polymer. Cells of *B. cellulosolvens* have been reported earlier to adhere to cellulose [8]. This observation was confirmed here by the strong adherence of the cells to cellulose during growth, even upon vigorous shaking of the culture. In order to characterize the specificity of the adherence, washed cells were added to various charged and uncharged cellulosic and noncellulosic polymers, either in the presence or in the absence of potentially inhibitory sugar derivatives and salts. Adherence of *B. cellulosolvens* to microcrystalline cellulose was very similar to that of *C. thermocellum* in terms of the capacity of the polymer to adsorb the cells, and the lack of effect of various additives to interfere with the adsorption (including 5% concentrations of glucose, galactose or cellobiose, and 1 *M* concentrations of NaCl, CMC, hydroxyethyl cellulose, or polyethylene glycol). Methyl cellulose inhibited adherence in *B. cellulosolvens* but not in *C. thermocellum*. Like *C. thermocellum*, alfalfa and wheat straw adsorbed the bacterium. Significantly, in contrast to *C. thermocellum*, cells of *B. cellulosolvens* readily bound to the insoluble fraction of birch xylan. Starch and agarose particles adsorbed cells of *B. cellulosolvens* only nominally. Positively charged forms of cellulose such as DEAE- and PEI-cellulose adsorbed the bacterium, and the adsorption was partially inhibited by salt.

Scanning electron microscopic delineation of the cell surface. Protuberant structures on the cell surface were visualized with the cationized ferritin procedure [14] in line with our previously published observations, obtained before reisolation of the bacterium [13]. Extended protuberances linking the bacteria to cellulose particles could be observed in a cellulose-grown culture (not shown). Cellobiose-grown cells exhibited smaller and less abundant protuberances compared with cellulose-grown cells.

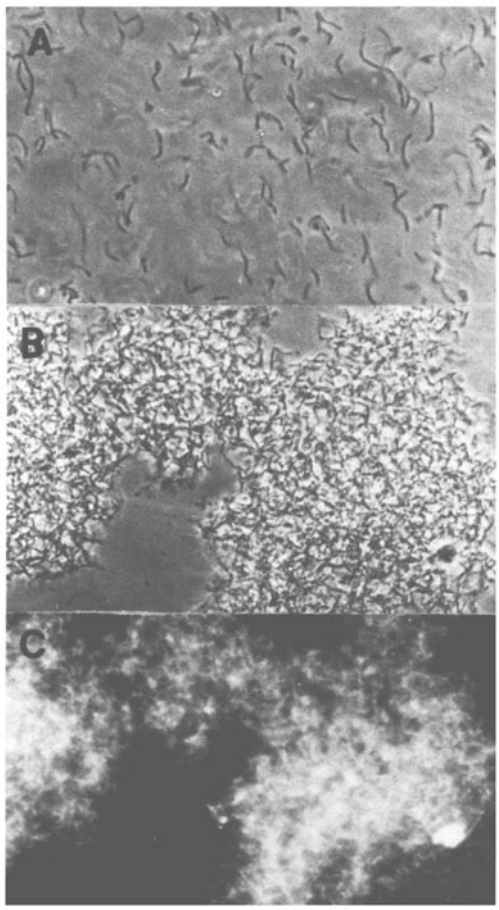

Fig. 1. Agglutination and cell surface labeling of *Bacteroides cellulosolvens* cells by fluorescein-derivatized lectin. Cells were grown on cellobiose-containing medium, washed twice with PBS, and labeled with FITC-GSI for 5 min at room temperature. Panel A, phase micrograph of untreated cells; panel B, phase micrograph of lectin-treated cells; panel C, fluorescence micrograph of lectin-treated cells.

Cell surface labeling and agglutination by GSI isolectin. Several different cellulolytic bacteria were previously reported to undergo labeling by an isolectin from *Griffonia simplicifolia* that is specific for terminally linked α-galactosyl moieties, suggesting the general occurrence of αGal on the surface of this bacterium. The reisolate was extensively labeled and agglutinated by an FITC derivative of this lectin (Fig. 1).

Fractionation and analysis of cell-derived material. The growth supernatant of cellobiose-grown *B. cellulosolvens* was concentrated and applied to 6% native PAGE gels (in the absence of SDS). The results

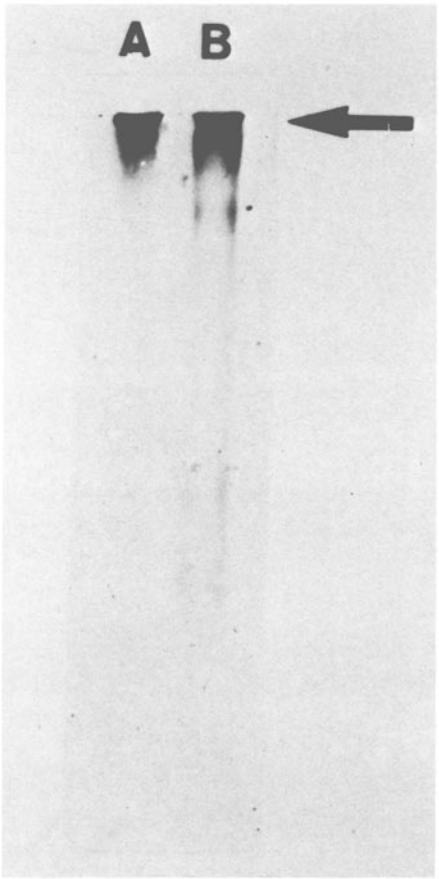

Fig. 2. Native polyacrylamide gel electrophoretogram of concentrated growth medium from *C. thermocellum* (panel A) and *B. cellulosolvens* (panel B). Electrophoresis was carried out in the absence of SDS. Arrow indicates the interface between the stacking (3%) and separation (6%) gels.

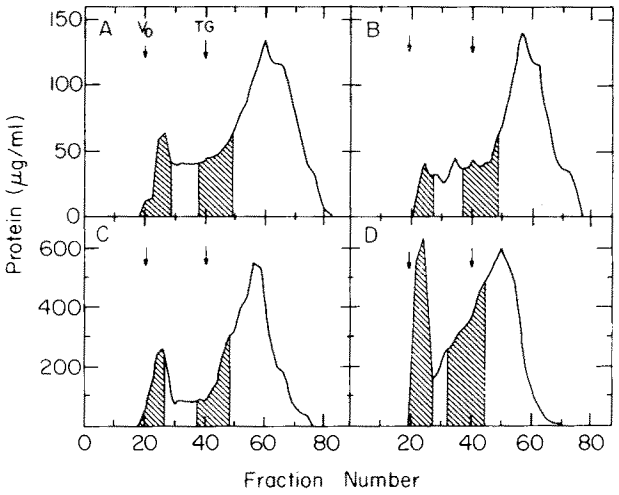

Fig. 3. Gel filtration analysis of cell-associated and cell-free material of *B. cellulosolvens*. Whole-cell sonic extracts and cell-free growth fluids derived from *B. cellulosolvens* grown on either cellulose- or cellobiose-containing medium were fractionated by gel filtration on Sepharose 4B. The cross-hatched areas designate fractions that were reactive with the GSI lectin. The elution positions of the void volume (V_0) and the 670-kDa protein thyroglobulin (TG) are shown. Panel A, extracellular material from cellulose-grown cells; Panel B, extracellular material from cellobiose-grown cells; Panel C, cell-associated material from cellulose-grown cells; Panel D, cell-associated material from cellobiose-grown cells.

(Fig. 2) indicated the presence of very high molecular mass entities that barely penetrated the interface of the stacking and separating gels. Upon gel filtration on Sephacryl S200, the concentration eluted in the void volume fraction.

Whole-cell sonicates and cell-free supernatant fluids, derived from cellobiose- and cellulose-grown *B. cellulosolvens*, were fractionated by gel filtration on Sepharose 4B. The fractions obtained were analyzed for protein and for their interaction with the αGal-specific lectin in a semiquantitative dot blot assay. In all cases (Fig. 3), two regions of lectin-binding activity were observed that apparently corresponded to aggregate forms eluting at about 700 kDa and in the void volume (>10 MDa).

SDS-PAGE of the carbohydrate-containing fractions from extracellular material derived from cellobiose-grown cells (Fig. 3B, 700-kDa region) showed four major bands (M_r230, 140, 100, and 84 kDa) and a number of minor bands (Fig. 4). The very high molecular weight region (>10 MDa) showed a similar SDS-PAGE pattern (data not shown). Lectin-based staining of the blotted material indicated that the 230-kDa band was mainly labeled. PAS staining (not shown) verified the latter as the major carbohydrate-containing component. An essentially identical picture emerged for the analogous material from cellulose-grown cells (Fig. 3A). Anti-cellulosome antibody (from *C. thermocellum*) also cross-reacted mainly with the 230-kDa band (Fig. 4).

Regarding cell-associated material, the labeling pattern was different from that of the extracellular polypeptide pattern. Coomassie staining of SDS-PAGE gels showed a relatively complex set of polypeptides. Lectin staining revealed a strong signal that coincided with a comparatively weak Coomassie-stained doublet at about 310 kDa and 290 kDa. The latter two bands could be segregated by gel filtration (data not shown); both were localized in separate outermost fractions of the 700-kDa region. Interestingly, the 290-kDa band eluted in the larger

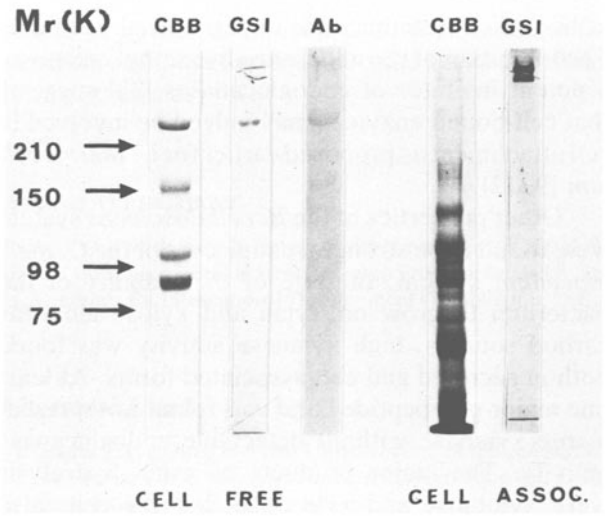

Fig. 4. Interaction of *B. cellulosolvens* protein components with lectin and with anticellulosome antibodies. Cells were grown on cellobiose-containing medium, and the lectin-binding fraction of cell-free material was prepared by gel filtration chromatrography (see Fig. 3B, 700-kDa region of shaded area). The cell-associated preparation consisted of the total sonic extract obtained from washed cells grown on the same medium. Protein fractions were separated by SDS–PAGE, blotted onto nitrocellulose paper prior to labeling with either the GSI lectin or anticellulosomal antibody (Ab). Coomassie-stained samples (CBB) are provided for reference.

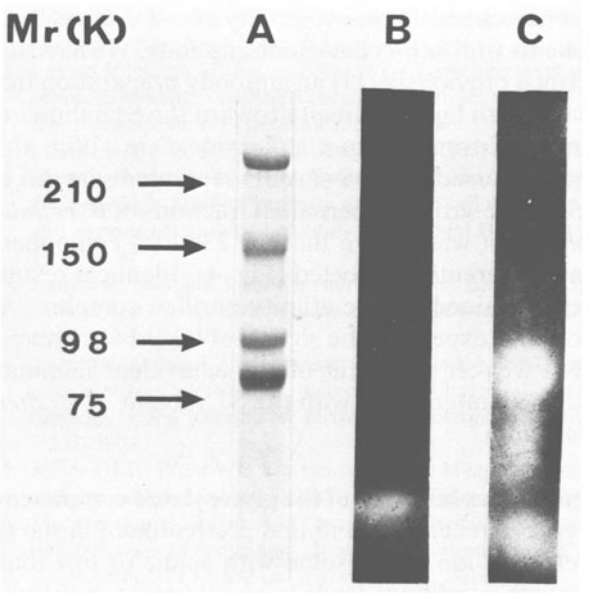

Fig. 5. Detection of endoglucanase and xylanase activities in extracellular protein complexes of *B. cellulosolvens*. The 700-kDa region of the cell-free growth culture derived from cellobiose-grown cells (Fig. 3B) was pooled, concentrated and separated by SDS–PAGE. The endoglucanase- and xylanase-containing bands were detected in gels that included the corresponding substrate. The gels were stained subsequently by the Congo red procedure. Panel A, Coomassie brilliant blue staining; Panel B, CMCase activity; Panel C, xylanase activity.

molecular-mass fraction, and the 310-kDa band followed in the lower-sized fraction, again suggesting that these glycopolypeptides occur in solution as complexes.

Affinity chromatography of extracellular material. Samples of the supernatant fluids (20 times concentrated) from cellobiose-grown cells (containing 3.4 and 9.6 units/ml endoglucanase and xylanase activity, respectively), were adsorbed to either microcrystalline cellulose or lectin–Sepharose (GSI) resin. In either case, the majority of enzymatic activity was adsorbed to the respective matrix; cellulose adsorbed 96% of the endoglucanase activity and 94% of the xylanase activity; the lectin-Sepharose adsorbed 68% and 70% of the corresponding activities. Free galactose (0.1 *M*) completely inhibited the binding to the lectin–Sepharose matrix.

Elution of the material was less than quantitative for both processes. Partial elution from the lectin–Sepharose could be effected by 1 *M* galactose, Triethylamine (pH 11) succeeded in releasing about 20% of the adsorbed material from cellulose. In either instance, three major polypeptide bands were reproducibly eluted (the 230-kDa glycosylated component, the 100-kDa and 84-kDa components), and one major component (140 kDa) failed to be adsorbed by both affinity resins. The data thus indicate that at least three major components comprise a cellulose-binding, αGal-containing enzyme complex in *B. cellulosolvens*.

Localization of enzyme activity. Since both xylanase and endoglucanase activities are associated with the protein complex that is adsorbed and eluted from both cellulose and lectin affinity resins, it was of interest to identify which proteins were responsible for this activity. With the Congo red procedure together with the appropriate substrate (CMC or xylan), it was found that none of the major components expressed endoglucanase activity (Fig. 5). Rather, a relatively minor band ($M_r \sim 50$ kDa) appeared to be an endoglucanase. In the case of xylanase activity, one of the major bands (84 kDa) displayed a strong signal, and a variety of other minor and smaller polypeptide components exhibited lower levels of xylanase activity. It has been reported that $CaCl_2$ and ascorbic acid significantly enhance the endoglucanase activity in this bacterium, but it was not feasible to include these additives in the zymogram assay.

Immunochemical crossreactivity of cell-derived components with anti-cellulosome antibody. We have described previously [11] an antibody preparation that reacts with high specificity toward the S1 subunit of the cellulosome from *C. thermocellum*. With this antibody used on SDS–PAGE-separated samples of cellobiose-grown supernatant fractions of *B. cellulosolvens*, it was shown that the 230-kDa component was preferentially labeled (Fig. 4). Identical results were obtained for the affinity-purified complex. As would be expected, the signals obtained were somewhat weaker than that of the equivalent immunochemical interaction with the S1 subunit of *C. thermocellum*.

Anomalous behavior of the glycosylated component. We have recently found that pretreatment of the *C. thermocellum* cellulosome with acidic or low ionic strength conditions leads to an alteration in the mobility of the S1 subunit in SDS–PAGE [17]. It was, therefore, of interest to determine whether a similar effect could be observed for components of the *B. cellulosolvens* system. Indeed, dialysis of the cell-free supernatant fluids against distilled water or pH 4.5 acetate buffer caused a similar anomalous electrophoretic effect in the glycosylated 230-kDa component. As for the *C. thermocellum* system, the usual SDS–PAGE profile of the latter component could be rectified by simple adjustment of the pH or ionic strength.

Discussion

The plant cell wall, the natural target of attack by cellulolytic bacteria, is degraded by the concerted action of several enzymes. These enzymes must bind initially to their target substrate and are effective only through their combined synergistic operation. A unique advantage for the enzyme-bearing microorganism must exist in such a highly competitive ecological environment. In many cases, the primary selective advantage is gained by the adhesion of the bacterial cell to the substrate, a process accompanied by the localization of critical hydrolytic enzymes at the cell–substrate interface.

Bacteroides cellulosolvens is an efficient crystalline cellulose-degrading microorganism that binds tightly to cellulose derivatives and to xylan. Most of its cellulolytic enzymes appear to be maintained in a cell-associated form [21]. The specificity of substrate adherence for this bacterium was broader than that of the well-characterized anaerobic thermophilic strain, *Clostridium thermocellum*, which exhibits a rather strict preference for underivatized cellulose. The inhibition of the adherence by methyl cellulose, a potent inhibitor of endoglucanases [22] suggests that cell-bound enzymes may indeed be involved in cell attachment as proposed earlier for *C. thermocellum* [9, 12].

Other properties of the *B. cellulosolvens* system were found to be strongly reminiscent of the *C. thermocellum* system. In spite of the inability of the bacterium to grow on xylan and xylose alone as carbon sources, high xylanase activity was found both in secreted and cell-associated forms. At least one major polypeptide band was found to represent a true xylanase without detectable endoglucanase activity. The major products of xylan hydrolysis were xylobiose and xylotriose, but the cells also appear to contain an independent β-xylosidase which eventually converts these products to xylose (unpublished results). The xylanolytic activities appear to be physically associated into the cellulosome-like complex analogous to the situation in *C. thermocellum* [17].

Also noteworthy is the presence in the complex of a high-molecular-weight αGal-containing glycoconjugate(s) whose properties are strikingly similar to those of the S1 subunit of *C. thermocellum*. In this regard, neither glycoconjugate appears to exhibit enzymatic activity alone, but both exhibit an unusual pH- or salt-dependent anomalous effect in their electrophoretic mobility properties in SDS–PAGE [16]. At least one endoglucanase was also detected in gels, although the complex itself appeared to account for at least 70% of the total endoglucanase activity of the extracellular material, indicating, perhaps, the presence of other undetected cellulases in the complex. It should be noted that the incubation conditions used for these zymograms were not ideal; there may be additional enzymes in the complex that may be susceptible to the electrophoretic conditions, and their presence may be masked. This is especially true regarding CMCase activity; one should, therefore, not compare the observed levels of xylanase activities with those of endoglucanase activities. Nevertheless, the data do provide some indication of the presence of the respective activities.

The results presented in this communication strongly support previous reports concerning the nature of anaerobic degradation of cellulose mediated by exocellular complexes of hydrolytic enzymes. Additional work on the molecular level is required in order to better understand the intra- and intermolecular interactions among the cellulosome components, the cell wall, and the cellulosic substrate. It

is also of particular interest to further examine the generality of these findings for other types of cellulolytic organisms.

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