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Adherence of *Clostridium thermocellum* to Cellulose

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The adherence of *Clostridium thermocellum*, a cellulolytic, thermophilic anaerobe, to its insoluble substrate (cellulose) was studied. The adherence phenomenon was determined to be selective for cellulose. The observed adherence was not significantly affected by various parameters, including salts, pH, temperature, detergents, or soluble sugars. A spontaneous adherence-defective mutant strain (AD2) was isolated from the wild-type strain YS. Antibodies were prepared against the bacterial cell surface and rendered specific to the cellulose-binding factor (CBF) by adsorption to mutant AD2 cells. By using these CBF-specific antibodies, crossed immunoelectrophoresis of cell extracts revealed a single discrete precipitation peak in the parent strain which was absent in the mutant. This difference was accompanied by an alteration in the polypeptide profile whereby sonicates of strain YS contained a 210,000-molecular-weight band which was missing in strain AD2. The CBF antigen could be removed from cell extracts by adsorption to cellulose. A combined gel-overlay–immunoelectrophoretic technique demonstrated that the cellulose-binding properties of the CBF were accompanied by carboxymethylcellulase activity. During the exponential phase of growth, a large part of the CBF antigen and related carboxymethylcellulase activity was associated with the cells of wild-type strain YS. However, the amounts decreased in stationary-phase cells. Cellobiose-grown mutant AD2 cells lacked the cell-associated CBF, but the latter was detected in the extracellular fluid. Increased levels of CBF were observed when cells were grown on cellulose. In addition, mutant AD2 regained cell-associated CBF together with the property of cellulose adherence. The presence of the CBF antigen and related adherence characteristics appeared to be a phenomenon common to other naturally occurring strains of this species.

Clostridium thermocellum is an anaerobic thermophilic, cellulolytic bacterium which grows on cellulose and its degradation products (9, 12, 15, 28). The extracellular cellulolytic enzyme has been suggested to comprise a constitutive system (6, 9) and to form part of a larger protein complex or aggregate (1, 20). The crude cellulolytic activity has been partially characterized (6, 7, 16, 25), and the purification and properties of two different endo-β-glucanases apparently responsible for at least some of this activity have been described previously (17, 20). At least four additional protein components, however, are considered to be associated with the crude cellulase complex, but the nature of their involvement in cellulose degradation is unknown (1, 20).

It has commonly been believed that stirring of *C. thermocellum* cultures is deleterious to the growth of this organism on the insoluble cellulose substrate. With this in mind, the effect of stirring on cell growth and product distribution was previously investigated (26; T. M. Su, R. Lamed, and J. H. Lobos, Technical Information Series Report No. 81CRD090, General Electric CRD, Schenectady, N.Y., 1981). Surprisingly, it was found that stirring actually increased the rate of growth together with a relative increase in the product ratio of acetic acid versus ethanol. During that study it was observed that cells were tightly bound to the cellulose substrate in the initial stages of fermentation despite vigorous stirring of the cultures. Preliminary ultrastructural evidence indicated that cell surface structures may be involved in the bacterial adherence to cellulose (W. A. Samsonoff, J. H. Prokosh, R. Lamed, J. Lobos, and T. Su, Abstr. Annu. Meet. Am. Soc. Microbiol., 1982, J15, p. 93). These observations prompted the present investigation.

In the present study, we used a combined genetic-immunochemical approach to study the interaction of *C. thermocellum* cells with their

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insoluble cellulosic substrate. After the initial hypothesis that the observed adherence of the organism to cellulose is an important primary event, we proceeded to isolate an adherence-defective mutant of the parent strain. When grown on cellobiose, this mutant lacked the cellulose-binding factor (CBF) found on the parent cell surface and exhibited reduced cell-associated cellulolytic activity.

(Parts of this work were presented at the Annual Meeting of the Israel Society for Microbiology, 27 December 1981 [R. Lamed, R. Kenig, and E. A. Bayer, Isr. J. Med. Sci. **18**:27, 1982]).

MATERIALS AND METHODS

Materials. Alumina (AG-7, 200 mesh) and hydroxyapatite were obtained from Bio-Rad Laboratories (Richmond, Calif.). Methylcellulose (low viscosity), polyethylene imine cellulose (fine mesh), glycogen (type II), Dowex-1-chloride (1 by 4 to 50, 100 to 200 mesh), and polyvinylpolypyrrolidone (cross-linked) were purchased from Sigma Chemical Co. (St. Louis, Mo.). Cellobiose and Celite 545 were obtained from Fluka (Buchs). CAB-O-SIL EH-5 (amorphous-fumed silica) was acquired from Cabot (Boston, Mass.). Polyethylene maleic acid was obtained from Monsanto Inc. (St. Louis, Mo.) as a research sample. Hydroxyethylcellulose (low viscosity) was from Polysciences, (Warrington, Pa.); cellulose acetate (39.8% acetate) was purchased from Schleicher & Schüll (Dassel); and carboxymethylcelluloses (CMC; both low viscosity and insoluble) were obtained from Whatman. Poly-L-lysine (M_r = 12,000) was a generous gift from I. Jacobson, Department of Biophysics, Weizmann Institute of Science. Agarose A was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden).

Organisms. *C. thermocellum* YS, the reference strain used for most of these studies, was originally isolated at the General Electric CRD Center (Schenectady, N.Y.) from soil samples obtained at the hot springs of Yellowstone National Park, *C. thermocellum* LQRI was that studied in a previous work (9), and strain J1 was a reisolate of *C. thermocellum* ATCC 27405.

Media and cultivation conditions. Cultures of 50 ml (unless otherwise stated) were grown in 100-ml serum bottles (Wheaton Scientific, Milville, N.J.). Culture media contained the following additives per 1 liter of distilled water: 0.5 g of $MgCl_2 \cdot 6H_2O$, 1.3 g of $(NH_4)_2SO_4$, 0.5 g of K_2HPO_4, 0.5 g of KH_2PO_4, 5 g of yeast extract, 1 g of cysteine-hydrochloride, 10.5 g of morpholinopropane sulfonic acid, and 2 mg of resazurin. The pH was adjusted to 7.3 with NaOH. In cellobiose-grown cultures, cellobiose (8 g; Fluka) was added to the above stock solution, and in cultures grown on insoluble cellulose, microcrystalline cellulose (0.8% [wt/vol]; Merck & Co., Inc., Rahway, N.J.) was added directly to the serum bottles. The culture medium was treated with a stream of nitrogen gas, and the bottles were sealed with butyl rubber septum-type stoppers (Bellco Glass, Inc., Vineland, N.J.).

A 2-ml inoculum was injected into the culture medium. Cultures were incubated at 60°C in a Tuttnauer shaking bath (Jerusalem, Israel) at 180 strokes per min. Cellobiose-grown cells were washed twice by centrifugation (10,000 × *g*) in phosphate-buffered saline (PBS) containing 0.15 M NaCl and 10 mM potassium phosphate (pH 7.0). Cellulose-grown cells were harvested after 16 to 24 h of growth. Trace amounts of residual cellulose were allowed to settle at atmospheric pressure for 15 min; the supernatant containing the cell suspension was decanted, and the cells were harvested and washed twice with PBS by centrifugation.

Isolation of mutant AD2. A 12-h, cellobiose-grown culture (5 ml) was introduced into a 12-ml serum bottle which included 5 ml of the stock medium containing 1 g of microcrystalline cellulose. The suspension was stirred vigorously for 5 min and allowed to stand for 3 h at room temperature. During this time, the cellulose sedimented together with adhered bacterial cells. A 2-ml portion of the clear supernatant was inoculated into a 5-ml cellobiose-containing growth culture, and the cells were grown for 24 h. The selection and growth cycles were repeated (six times) until the majority of the cells failed to adhere to the cellulose and the resultant supernatant was visibly turbid with bacterial cells.

The procedures described above were performed under strictly anaerobic conditions. Mutant AD2 was one of the several mutant colonies selected by this procedure. The adherence-defective characteristics of this mutant were stable upon alternating cycles of growth in cellobiose- versus insoluble cellulose-containing media.

Adherence assay. A washed cell suspension (1.5 mg of cells in 1 ml of PBS) was brought to a total volume of 3 ml with 1 ml of 20% (wt/vol in PBS) microcrystalline cellulose and 1 ml of PBS (containing the various additives when indicated). The suspension was vortexed for 20 s, and the cellulose containing the adhered bacterial cells was allowed to settle at room temperature for 30 min (or for 60 min if viscous additives were added). The turbidity (absorbance at 400 nm [A_{400}]) of the supernatant was measured and compared with control tubes wherein PBS was substituted for the cellulose suspension. Under the conditions of the assay, addition of cellulose generally caused a 70 to 80% reduction in the initial turbidity of a suspension of wild-type cells.

Preparation of extracellular material. Extracellular (cell-free) material was obtained from the growth cultures by centrifuging the cells at 10,000 × *g* for 30 min. The supernatants were collected, brought to 0.05% NaN_3, and stored at −20°C.

Preparation of cell extracts. Washed cell samples (obtained from 30-ml growth cultures) were suspended in a solution (2 ml final concentration) of 20 mM Tris-hydrochloride buffer (pH 7.5). In some cases, 0.5% (wt/vol) Triton X-100 was included at this point, but neither the cellulolytic activities nor the immunochemical results were significantly altered by this addition. Cell suspensions were sonicated in ice at 70 W in five, 30-s intervals with a standard tapered microtip in a Sonicator Cell Disruptor (Model W-225R; Heat Systems-Ultrasonics, Inc., Plainview, N.Y.). The sonicated samples were centrifuged at 10,000 × *g* for 10 min. The supernatants were collected, brought to 0.05% NaN_3, and stored at −20°C.

Cellulase assay. In vitro determination of endocellu-

lase (carboxymethylcellulase [CMCase]) activity was performed essentially by the method of Miller et al. (13) with CMC as a substrate. The incubation temperature was 60°C, and the time of assay was 30 min. A unit of CMCase activity was defined (within the linear range) as the amount of enzyme which released 1 μmol of reducing sugar (using glucose as a standard) per ml of sample per min under the conditions indicated.

On-plate CMCase overlay. The immunoelectrophoretic plate was placed in an appropriate mold, and a 2-mm overlay (containing 1% agarose and 1.4% CMC in 0.1 M acetate buffer [pH 5.0]; incubated at 50°C) was poured onto the gel. The reaction was carried out at 50°C in a moist environment. After a given period of incubation (usually 5 to 10 h), the plate containing the overlay was cooled to room temperature and immersed in an excess of 2-propanol. After an additional 2 to 3 h incubation period at room temperature, the CMC precipitated, clear areas denoting CMCase activity developed, and the plates were photographed by top illumination on a dark background.

Preparation of reference (anti-whole cell) antibodies. Rabbits were injected intravenously with 1 ml of a washed, mid-exponential-phase, cellobiose-grown, cell culture of *C. thermocellum* YS (A_{400} = 0.8; measured with a Bausch & Lomb Spectronic 20 spectrophotometer, Rochester, N.Y.). Rabbits were immunized on days 0, 3, 6, and 9. Bleeding was performed on day 12. Additional bleedings were performed at weekly intervals, 3 days after a booster consisting of the same cell culture (stored at −20°C) was administered. The antisera were pooled and crude immunoglobulins were prepared by ammonium sulfate precipitation (two precipitation steps). The precipitate was redissolved in a minimal volume of PBS, dialyzed, and adjusted to 10 mg/ml (protein) in PBS.

Preparation of CBF-specific antibodies. To obtain an antibody preparation specific for the CBF, antibodies common to the cell surfaces of both the mutant and wild type were removed from the reference (anti-whole cell) antibody preparation by adsorption onto mutant cells. An exponential-phase culture (2 liter) of cellobiose-grown mutant AD2 cells was washed with 0.15 M NaCl. The centrifuged (8,000 × *g*) pellet was suspended either in 50 ml of the reference antiserum or in 10 ml of the purified immunoglobulin fraction (containing 100 mg of protein). The latter suspension was incubated for 4 h at 4°C and centrifuged at 8,000 × *g* for 20 min, and the supernatant was treated with a second batch of mutant cells. After each adsorption step, the resultant supernatant was routinely tested for agglutinability of wild-type and mutant cells and rocket immunoelectrophoresis of the respective cell sonic extracts was carried out to ensure that no residual common antibody remained. The supernatant, comprising the final adsorbed antibody preparation, was passed through a Millipore HA 0.2-μm filter, and the ammonium sulfate fraction was prepared and adjusted to 10 mg/ml as described above.

Agglutination assay. Cell agglutinability was performed essentially as described by Bayer et al. (2), and the results were analyzed as reported earlier (2, 3).

Immunoelectrophoresis. Two-dimensional (crossed) immunoelectrophoresis of sonicated cell extracts was performed essentially as described previously (3). The intermediate gel contained either buffer alone or 250 μg of the CBF-specific immunoglobulin. The upper gel included 500 μg of the reference immunoglobulin. Electrophoresis of 8-μl samples was carried out in the first dimension for 1.5 h at 200 V and in the second dimension at 200 V for 3 h.

Rocket immunoelectrophoresis was performed by one-dimensional electrophoresis of samples (8 μl) directly into the intermediate (CBF-specific immunoglobulin) and upper (reference immunoglobulin) gels. Electrophoresis was carried out at 200 V for 2 h. Under the above conditions, standard curves of serially diluted samples were used to determine the concentration of the CBF. One unit of CBF-associated antigenic activity was defined as the amount of antigenic material necessary to form a 1.0-cm rocket in the intermediate gel.

Miscellaneous methods. Protein concentrations were estimated spectrophotometrically by the Bradford method (5). Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) was performed as described in the accompanying paper, except that 10% gels were used (8).

RESULTS

Adherence of *C. thermocellum* to cellulose. Cells of *C. thermocellum* were observed by light and scanning electron microscopy to adhere strongly to the insoluble cellulosic substrate. Introduction of cellulose to a suspension of *C. thermocellum* cells caused a rapid clearing of cells from the suspension (Fig. 1). The adherence of *C. thermocellum* to cellulose provided the grounds for the isolation of an adherence-defective mutant of this organism. A spontaneous mutant (AD2) of the parent strain, defective in its capacity to adhere to cellulose, was selected by enriching for cells which failed to adhere to the insoluble substrate (see above; Fig. 1). The procedure consisted of sequential enrichment cycles whereby cellulose was added to a cell suspension and the nonadherent cell fraction was reinoculated into cellobiose medium; the mutant strain was then selected by single-colony isolation. The characteristics of this mutant have remained stable for a period of at least 1 year.

The adherence phenomenon also formed the basis for a rapid adherence assay. A measured amount of cellulose was mixed with a washed cell suspension. After settling of the cellulose, together with the adhered cells, the residual turbidity was measured and compared with that of an identical cell suspension without added cellulose. Table 1 gives the results of such an adherence experiment performed on samples of wild-type and mutant AD2 cells grown either on cellobiose or cellulose. In the presence of cellulose, the turbidity of a washed cell suspension of *C. thermocellum* grown on either substrate decreased to about 30% of the initial A_{400}. In contrast, cellulose had no measurable effect on the turbidity of mutant AD2 cells grown either on cellobiose (Table 1) or glucose (data not shown). However, this lack of interaction was

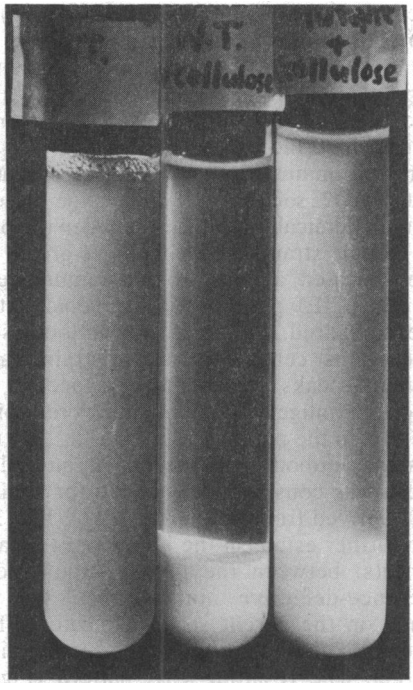

FIG. 1. Three test tubes showing (from left to right) a suspension of wild-type *C. thermocellum* YS cells, a similar suspension to which insoluble cellulose was added, and a suspension of mutant AD2 cells with added cellulose. Note that the cellulose has largely cleared the solution from wild-type cells (middle tube) but failed to do so with the mutant (right tube).

countered by growing the mutant on insoluble cellulose as a substrate; i.e., the property of adherence onto cellulose was regained in the mutant upon altering the composition (carbon source) of the medium.

Adherence of wild-type cells to cellulose was shown to be substrate specific, since the cells failed to adhere to a large variety of other insoluble, uncharged polysaccharides (Table 2). The physical nature of the cellulose was unimportant to the adherence process, since all commercially available forms tested, as well as acid-treated amorphous cellulose, adsorbed the cells specifically. This adherence phenomenon was unaffected by the presence of salts, mild detergents, and changes in temperature (between 4 and 60°C) and pH (between 4 and 9.5). On the other hand, positively charged insoluble polymers adsorbed the cells, probably due, for the most part, to electrostatic interactions. Indeed, in contrast to adherence of cells to cellulose, this adsorption was prevented by salts. It is interesting to note that derivatized cellulosic matrixes, e.g., cellulose acetate, etc., as a rule failed to interact specifically with the bacterial cells.

Numerous monosaccharides (10 to 50 mg/ml) failed to inhibit cellular adherence to cellulose. Interestingly, various cellulose degradation products (e.g., glucose and cellobiose), as well as various soluble cellulose derivatives (e.g., low viscosity forms of methyl-, carboxymethyl-, and hydroxyethylcellulose), also failed to inhibit the adherence of *C. thermocellum* to the insoluble cellulosic substrate. Only polyethylene imine (1 to 10 mg/ml) was found to interfere significantly with the adherence; however, the exact nature of this interference is as yet unknown. Poly-L-lysine (up to 15 mg/ml), another positive-

TABLE 1. Adherence of *C. thermocellum* strains to cellulose

Strain and growth conditions[a]	A_{400} for the following suspensions:	
	Control	Cellulose added
Wild-type		
Cellobiose-grown	0.70	0.21
Cellulose-grown	0.83	0.23
Mutant AD2		
Cellobiose-grown	0.85	0.87
Cellulose-grown	0.70	0.30

[a] Cells grown on the indicated carbon source were harvested at a cellular protein content of approximately 250 μg per ml culture medium. Cellulose-grown cells were obtained from the centrifuged (500 × *g*) supernatant of a vigorously stirred culture containing 0.6% cellulose. Under these growth conditions, more than 90% of the substrate was utilized, and detached cells were readily available. The results described above were representative of similar experiments performed dozens of times.

TABLE 2. Adherence of *C. thermocellum* to various insoluble matrixes

Adhering matrixes[a]	Nonadhering matrixes[a]
Cellulose	Sephadex G-100
Amorphous cellulose[b]	Sepharose 4B
Polyethylene imine cellulose	CMC
Diethylaminomethyl cellulose[c]	Carboxymethylcellulose hydrazide
Dowex-1[c]	Cellulose acetate
Polystyrene surfaces	Starch
CAB-O-SIL EH-5	Polyvinyl alcohol
Bentonite	Silica
Hydroxyapatite	Alumina
Charcoal	Polyvinyl pyrrolidone
	Celite 545

[a] Adhering matrixes were defined as those capable of reducing more than 50% of the initial A_{400}. The conditions used for each matrix were similar to those described in the text for the adherence assay with cellulose.

[b] Phosphoric acid-swollen cellulose.

[c] Adherence to these substances was salt sensitive (inhibited by 1 M NaCl).

ly charged macromolecule, failed to affect the adherence of cells to cellulose.

Antibody-induced agglutination. By using anti-whole cell antibody and a mutant-adsorbed (CBF-specific) antibody preparation, the agglutinability characteristics of the parent strain were compared with those of the adherence-defective mutant (AD2). The kinetics profile of agglutination for this organism was analyzed by the method described previously by Bayer et al. (2, 3) for the antibody-induced agglutination of *Acinetobacter calcoaceticus*. The anti-whole cell antibody preparation presumably recognized several agglutinogenic species on both the parent and mutant surface, since both cell types agglutinated strongly in the presence of this antibody (Fig. 2a). However, fewer agglutinogenic components are located on the cell surface of mutant AD2 than on that of the parent strain as expressed by the different rates of agglutination. As expected, the mutant-adsorbed antibody failed to agglutinate mutant AD2 cells over the entire range of antibody tested (Fig. 2b). The adsorbed antibody still agglutinated the wild-type strain supporting the contention that this antibody preparation is specific for an agglutinogenic component(s) borne exclusively on the parent cell surface.

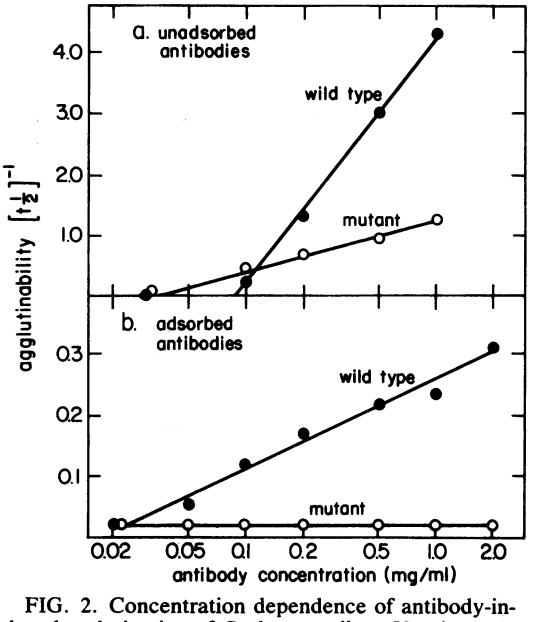

FIG. 2. Concentration dependence of antibody-induced agglutination of *C. thermocellum*. Varying concentrations of the reference antibody (a) or the CBF-specific antibody (b) were used, and the time ($t_{1/2}$) required for the reduction of cell turbidity to one-half its initial value was determined. The reciprocal values, $(t_{1/2})^{-1}$, which are proportional to agglutinability, were calculated for wild-type cells (●) and mutant AD2 cells (○) and plotted against the antibody concentration. Note that the capacity of the reference antibody (a) to agglutinate wild-type cells was much greater than that observed for mutant AD2. The CBF-specific antibodies (b) were less efficient in agglutinating wild-type cells and failed altogether (even at very high concentrations) to agglutinate mutant AD2.

Crossed immunoelectrophoresis of parent and mutant AD2 sonicates. To examine potential immunochemical differences between the parent and mutant strains, the cellobiose-grown cells were sonicated, and the crossed immunoelectrophoretic (CIE) patterns of the sonic extracts were compared. By using the antibodies prepared against cells of the parent strain, various precipitin peaks representing respective cell-associated antigenic components were typically observed in the parent strain (Fig. 3a). By using the same antibody preparation, an altered CIE pattern was consistently obtained for sonic extracts derived from the mutant AD2 (Fig. 3b).

To further establish the immunochemical difference(s) between the parent strain and the adherence-defective mutant, CIE was performed on the parent strain sonicate with the mutant-adsorbed antibody in the second-dimension gel. The resulting CIE pattern (Fig. 3c) revealed that only one CIE peak was observed by using the adsorbed antibody preparation. As would be anticipated, no CIE peaks were visible with the mutant-adsorbed antibody preparation on mutant AD2 sonic extracts, indicating the efficacy of the adsorption process (data not shown). The exact identity of this peak with respect to those shown in the standard pattern (Fig. 3a), however, is uncertain, since the reference peaks have been removed. For this reason, composite CIE of the parent strain sonicate was performed with the mutant AD2-adsorbed antibody contained in an intermediate agarose strip with the anti-whole-cell (parent strain) antibody in the upper gel of the second dimension. The presence of the adsorbed antibody in the intermediate gel should retard (lower) any antigenic peak(s) specific to this particular antibody. Other peaks should remain in the upper gel. By using this course, only one peak (the CBF peak) was retained in the intermediate gel (Fig. 3d). The CIE pattern of sonic extracts derived from *C. thermocellum* LQRI and J1 was strikingly similar to that shown for the wild-type strain YS in Fig. 3d and included the CBF peak in the intermediate gel. It should be noted further that, like strain YS, cells of strains LQRI and J1 were also adherent to cellulose.

The biochemical relationship of the CBF peak to cellulose binding was verified by CIE after adding cellulose to wild-type sonic extracts. After centrifugation of the cellulose, only one peak (the CBF peak) was found to be removed from the CIE pattern (Fig. 3e), indicating that

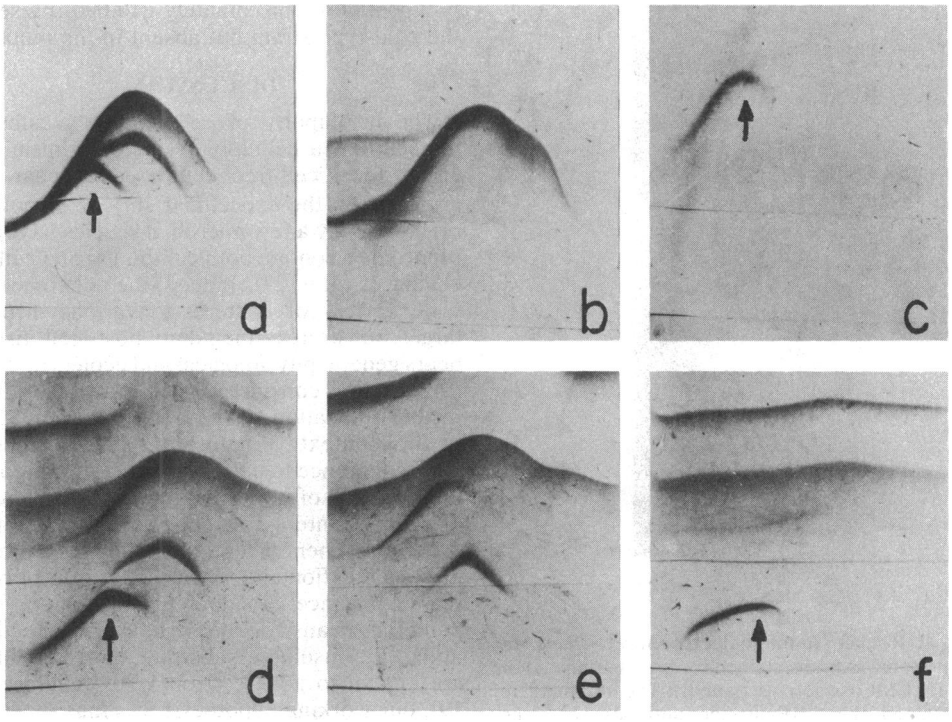

FIG. 3. Crossed immunoelectrophoretic patterns of sonic extracts derived from *C. thermocellum* wild-type (strain YS) and mutant AD2 cells. (a) Wild-type sonic extracts and (b) mutant AD2 sonic extracts with reference antibodies contained in the upper gel of the second dimension. (c) Wild-type sonic extracts with CBF-specific antibodies contained in the second dimension. In gels (a), (b), and (c), no antibodies were added to the intermediate gels. (d) Wild-type sonic extracts as in (a), but in addition to the reference antibodies contained in the upper gel, CBF-specific antibodies were added to the intermediate gel. (e) Cellulose-treated wild-type sonic extracts subjected to CIE as in (d). Note that the peak precipitated by the CBF-specific antibodies in (d) was removed by the addition of cellulose in (e), whereas the other peaks located in the upper gel were relatively unaffected. (f) Regeneration of the cellulose-bound CBF peak by treatment with triethylamine; the gel was subjected to CIE as in (d) and (e). The CBF peak is identified by arrows in the appropriate figures. In each figure, samples were applied to a well at the bottom left-hand corner of the gel; the anode is located at the right in the first dimension and at the top in the second.

this component had adsorbed completely to the cellulose. This peak could be partially regained (eluted) from cellulose by various treatments (Fig. 3f), including 1% triethylamine, 1% ammonium hydroxide, 0.02 M sodium hydroxide, 8 M urea, 1% SDS, and 0.5% Triton X-100. High salt concentrations (4 M KCl) or 1% acetic acid failed to remove any detectable CBF from cellulose.

Association of CMCase activity with the CBF. To determine the possible connection between the anti-CBF antigenic activity and cellulolytic activity, rocket immunoelectrophoresis was combined with a gel-overlay technique for qualitative examination of enzymatic (CMCase) activity directly on the gel (Fig. 4). Under the conditions used, four antigenic peaks (rockets) could be discerned in Fig. 4, lane A: One, the CBF peak, appeared in the intermediate gel precipitated by the CBF-specific (AD2-adsorbed) antibodies, and the other three peaks appeared in the upper gel which contained the reference (anti-whole cell) antibodies. In the CMC-containing overlay (Fig. 4, lane B), enzymatic activity was observed as a clearing (dark area) in the gel where the CMC was hydrolyzed. Interestingly, the enzymes (CMCase) were still active despite interaction with the antibodies and consequent immunoprecipitation. Two immunochemically distinct types of cellulolytic activity were observed. One form was located in the intermediate gel and thus associated with the CBF, and the second form was associated with an unrelated peak located in the upper gel. Clearly, more cellulolytic (CMCase) activity was associated with the CBF peak under the described conditions.

The results were extended to include the wild-type and mutant cells grown and harvested under different culture conditions (Table 3). In general, the mutant produces significantly less CBF antigen than does the wild-type strain. In

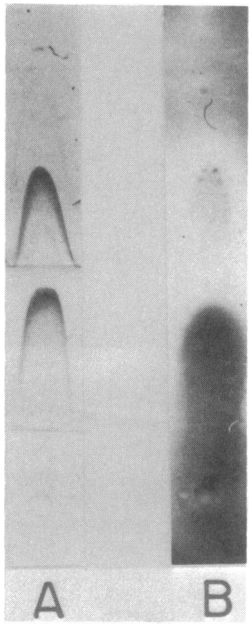

FIG. 4. Rocket immunoelectrophoretograms of wild-type sonic extracts stained for protein (lane A) or treated for CMCase activity (lane B). The intermediate gel contained the CBF-specific antibody, and the upper gel contained the reference antibody preparation. Samples were applied to the well in the bottom gel, and electrophoresis was performed with the anode at the top.

the cellobiose-grown mutant, the amount produced on the surface is so low that it cannot be detected by sensitive immunochemical methods. This feature is accompanied by defective adherence properties in the mutant as described earlier. In addition, the amounts of cell-associated CBF and CMCase activities were consistently higher for exponential-phase cells than those of cells harvested during the stationary phase of growth. This was accompanied by a concomitant increase in the respective extracellular activities during the stationary phase, indicating that the surface antigen(s) and CMCase are apparently released into the extracellular medium upon maturation of the cell cultures. Growth of both mutant and wild-type cells on cellulose results in a marked enhancement of CBF antigen production. Under these conditions, both cell types, including mutant AD2, contain both cell-associated and cell-free CBF antigen accompanied by adherence to cellulose.

SDS-PAGE of sonic extracts. SDS-PAGE of sonic extracts of cellobiose-grown strains YS and mutant AD2 is shown in Fig. 5. The pattern of polypeptides is very similar for both strains, with the exception of a high-molecular-weight polypeptide (approximately 210,000) present in the wild-type strain but absent in the mutant.

DISCUSSION

The insolubility of cellulose as a substrate implies that a cellulolytic microorganism must either form cell-free cellulases or bear such enzymes on the exocellular surface or both. In practice, only a few microbial species have been reported to contain both cell-bound and cell-free cellulases (4, 6, 27). Indeed, the adherence of a wide variety of bacteria to various insoluble target substrates (including host cell tissues) bears general physiological and ecological significance and is considered to be a key factor in the further utilization of these substances (18, 24). In this context, Minato and Suto (14) recently used adherence to cellulose as a criterion for the fractionation of rumen bacteria. It was therefore of primary interest to consider the role of the adherence phenomenon in the process of cellulose degradation and, in particular, to determine the importance of adherence in general as a potential means for the organism to effectively utilize the insoluble substrate. Since cellulases are known to adhere strongly to cellulose (11, 19), our working hypothesis was that adherence of *C. thermocellum* to cellulose may be mediated by cellulases associated with the bacterial cell surface. Our findings indeed support this contention.

During the course of this study, we were able to isolate a mutant (AD2) which, under the described conditions, was much less adherent than the wild type to insoluble cellulose. In an effort to correlate function to structure, the use of the antibody-adsorption procedure was thus designed to emphasize the immunochemical difference(s) between the surfaces of the mutant and wild-type cells. This general approach was previously used to study the cell surface of *A. calcoaceticus* RAG-1 (2, 3, 21, 23).

An immunochemical comparison between *C. thermocellum* YS and AD2 indeed revealed a deficiency in a single major surface antigenic component, the CBF, in the mutant. The following evidence supports this claim: (i) only one consistent antigenic difference was observed between the mutant and wild-type extracts, and (ii) this antigen is associated with the cell surface, since the specific (mutant-adsorbed) antibody was capable of agglutinating wild-type cells. In addition, we found that the CBF fraction eluted from cellulose (Fig. 3f) as well as the isolated CBF (8) successfully inhibited (over 50%) the adherence of the bacterial cells to cellulose.

The immunochemical difference between wild-type and mutant sonicates was accompanied by a single detectable alteration in the polypeptide profile of the two strains, which

TABLE 3. Antigenic and cellulolytic activities of wild-type and mutant AD2 cells of *C. thermocellum*

Strain and growth conditions[a]	Antigenic activity[b]		CMCase activity[b]	
	Cell-associated[c]	Extracellular	Cell-associated[c]	Extracellular[d]
Cellobiose-grown cells				
Wild type				
Exponential phase	54 (240)	43 (1,090)	0.185 (0.85)	0.69 (17.4)
Stationary phase	17 (39)	62 (520)	0.110 (0.25)	1.16 (9.7)
Mutant AD2				
Exponential phase	0 (0)	22 (680)	0.051 (0.18)	0.94 (29.3)
Stationary phase	0 (0)	29 (270)	0.046 (0.13)	1.38 (12.5)
Cellulose-grown cells				
Wild type	88 (290)	120 (790)	0.055 (0.18)	1.24 (13.9)
Mutant AD2	37 (120)	67 (480)	0.045 (0.15)	1.67 (8.2)

[a] Cells were grown on the indicated carbon source as described in the text and harvested during the given phase of growth according to a predetermined growth curve. Exponential-phase cells were harvested at a cellular protein content of 150 to 250 μg of culture medium per ml, and stationary-phase cells were harvested at 400 to 450 μg/ml. Cellulose-grown cells were harvested at a total cellulose-associated protein content (including protein release by treatment with 0.1 M sodium hydroxide) of about 300 μg of broth per ml.

[b] One unit of antigenic activity corresponds to a 1-cm rocket generated by the Laurell technique under the conditions described in the text. One unit of CMCase activity produces 1 μmol of reducing sugar per ml per min. Values for both activities represent the average of duplicate samples derived from duplicate cultures. Values in parentheses represent the respective specific activities standardized as units per milligram of protein (either cell-associated or extracellular) per milliliter of culture medium.

[c] Cell-associated and extracellular activities were determined as described in the text.

[d] In cellulose-grown cells, the extracellular CMCase activity was determined on the entire culture. The cells and residual substrate were centrifuged before color development in the assay. These conditions have been shown to express greater than 80% of the cellulose-adsorbed CMCase activity (unpublished results).

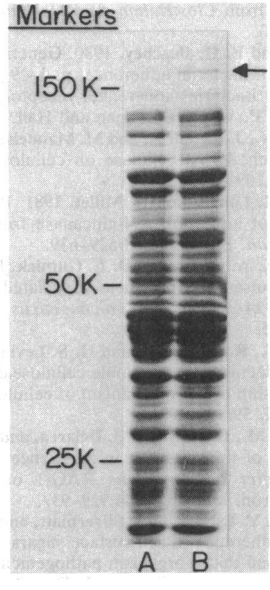

FIG. 5. SDS-PAGE of mutant AD2 (lane A) and wild-type strain YS (lane B) sonic extracts. Note the absence of the large-molecular-weight (ca. 210,000 [210 K]) band in the mutant. The position of molecular weight markers (purified rabbit immunoglobulin G; intact molecule 150,000 [150 K], H-chain 50,000 [50 K], and L-chain 25,000 [25 K]) are indicated in the column at the left.

comprised a lack of a 210,000-molecular-weight band in the mutant sonic extract. Due to the complexity of the SDS-PAGE pattern, however, it is not yet clear whether additional differences in the polypeptide profile exist between the mutant and wild-type cell surfaces. The importance of the 210,000-molecular-weight band has been detailed further (8).

It should be noted that the CBF antigen, as well as other cell surface antigens, was common to all three strains (YS, LQRI, and J1) of *C. thermocellum*, despite the fact that each strain was isolated from a very different clime. Since other characteristics (e.g., adherence to cellulose, cellulolytic activity, agglutinating activity, etc.) are also shared by the three strains, the cell surface features described here may comprise a more general phenomenon common to this species.

Several types of cellulases are known to occur in any given cellulolytic organism (10, 22). The multiple types of this enzyme activity are thought to work in concert to degrade the highly complex quaternary structure of the insoluble substrate into soluble oligosaccharides and glucose. By using rocket immunoelectrophoresis in conjunction with an overlay technique devised to determine the presence of CMCase activity on the gels, we have shown that the majority of the cell-bound CMCase activities is apparently

associated with the CBF. In addition, whereas both the cell-associated CBF peak and related cellulolytic activity were missing in mutant AD2 cells grown on cellobiose, the identical peak was present in the extracellular medium (supernatant fluids) of the mutant. This mutant apparently belongs to the class of surface mutants predicted by Bayer et al. (2) wherein a surface antigen is less tightly associated with the cell surface and is released into the medium such that the corresponding antibody fails to interact with the mutant cell surface. This particular mutant may therefore lack an essential component responsible for anchoring the CBF to the cell surface. Alternatively, organizational aspects or the assembly of the CBF may be altered in cellobiose-grown mutant cells.

Growth of the mutant cells on insoluble cellulose as a substrate led to a reexpression of the CBF on the mutant cell surface, although to a somewhat lesser extent than that in the wild-type strain. The presence of the insoluble cellulosic substrate in the growth medium of mutant cells may therefore induce biosynthesis (or incorporation into the CBF complex) of the proposed anchoring or assembly component(s). In any event, isolation of the different forms of the CBF and comparative biochemical studies should provide further insight into both the adherence phenomenon and the mode of cellulose degradation in this organism.

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