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Characteristics of the Endoglucanase Encoded by a *cel* Gene from *Bacteroides succinogenes* Expressed in *Escherichia coli*

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A *cel* gene from *Bacteroides succinogenes* inserted into the vector pUC8 coded for an enzyme which exhibited high hydrolytic activity on carboxymethylcellulose, *p*-nitrophenylcellobioside, and lichenan and low activity on laminarin and xylan. The enzyme was not synthesized by the *Escherichia coli* host when cells were cultured in complex medium containing added glucose. In the absence of added glucose, the endoglucanase and cellobiosidase activities synthesized were partitioned into the periplasmic space during growth, and practically all enzyme was located in the periplasm when the stationary phase of growth was reached. The enzyme exhibited 17- and sixfold higher K_m values for the hydrolysis of carboxymethylcellulose and lichenan, respectively, than did the extracellular endoglucanase complex from *B. succinogenes*. The Cel endoglucanase had a pH optimum similar to that of the *B. succinogenes* enzyme except that the range was narrower, and the Cel endoglucanase was more readily inactivated on exposure to high temperature, detergents, and certain metals. Its activity was stimulated by calcium and magnesium. Nondenaturing polyacrylamide gel electrophoresis at different acrylamide concentrations revealed the presence of three endoglucanase components, two with molecular weights of 43,000 and one with a molecular weight of 55,000.

The anaerobic ruminal bacterium *Bacteroides succinogenes* is one of the major cellulolytic organisms in the bovine rumen. It predominates in the rumen of animals receiving a high-fiber diet (5, 13). In pure culture it digests forage more completely than other cellulolytic ruminal bacteria (10) and has been shown to have high fiber-digesting activity (18).

The endoglucanase activity of *B. succinogenes* has been shown to be caused by a multiplicity of enzymic components (28). To determine whether this multiplicity of endoglucanases is a result of either posttranslational modification of a single gene product or synthesis by a number of different genes, the genes in question have been cloned into *Escherichia coli*. Six distinct cellulase genes have been identified which code for endoglucanase activity (9). A 6.6-kilobase-pair (kbp) DNA insert from one of the most active clones (BC14) was previously mapped with restriction enzymes and, through a sequential subcloning strategy, was reduced to a 1.9-kbp fragment (clone RE3) which still retained cellulase activity as determined by screening with the Congo red staining method (9, 31). The cellulase gene contained in this cloned insert has been designated *cel* to conform with the nomenclature for the gene introduced by Cornet et al. (8). The original clone, BC14, expresses an endoglucanase activity which is glucose repressible and possesses low xylanase activity. The endoglucanase encoded by the gene in RE3 has been characterized in more detail, and the information is presented herein.

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MATERIALS AND METHODS

Bacterium, chimeric plasmids, and growth conditions. The bacterium used in this study was *Escherichia coli* K-12 strain RR1 (22) harboring any one of five plasmids, pUC8, pBC14, pO-64, pLE1, or pRE3 (9). *E. coli* was grown in a complex broth medium (2YT) consisting (per liter) of 16 g of tryptone, 10 g of yeast extract, 5 g of sodium chloride, and 50 mg of ampicillin. The ampicillin was filter sterilized and added to the cooled sterile medium. When glucose and agar were included in the medium, they were added at concentrations of 2.0 and 1.5%, respectively. The pH of the medium was 7.0. Growth was monitored by measuring the turbidity of the culture at a wavelength of 675 nm with a 1-cm cuvette.

Preparation of cells for enzyme assays. Cultures were harvested by centrifugation at 16,000 × *g* for 10 min at 4°C and washed twice in 0.05 M sodium phosphate buffer (pH 6.5). The cells were suspended in the same buffer and either assayed directly or disrupted by ultrasonication (four treatments, 15 s each at 200 W with 0.5 min of cooling on ice). Cells were subjected to osmotic shock to cause the release of periplasmic proteins, as described by Cornelis et al. (7). Cells harvested at 4°C were washed in 0.01 M Tris chloride buffer (pH 8.0) and suspended in a half volume of a 25% (wt/vol) sucrose solution in 0.01 M Tris chloride buffer (pH 8.0)–1 mM disodium EDTA at 22°C. After 10 min of slow shaking, the cells were sedimented by centrifugation, suspended in the same volume of ice-cold distilled water, and shaken for 10 min. The cells were centrifuged, the supernatant fluid was saved for the periplasmic fraction, the cells were washed once, and the supernatant fluid was included in

TABLE 1. Cellulolytic and xylanolytic activities of *Escherichia coli* strain RR1 harboring plasmid pBC14 and various subclones[a]

Plasmid	Insert size (kbp)	Sp act (nmol/min per mg of protein) of:					Halo[b] formation
		Endoglucanase	Cellobiosidase	β-Glucosidase	Xylanase	Xylosidase	
pUC8/RR1[c]	0	0.05	0.05	0.3	0.05	0.05	−
pBC14	6.6	0.89[d]	0.95	0.29	0.10	0.15	++
pO64	4.9	20.4	30.0	0.45	0.10	0.14	+++
pBal31-1	2.2	22.8	23.0	0.69	0.22	0.27	+++
pRE3	1.9	21.0	25.1	0.05	0.22	0.05	+++

[a] Cells were grown in 2YT broth to the late exponential phase of growth (optical density at 675 nm, 2.0).
[b] Cell extract equivalent to 1.2 ml of culture (optical density at 675 nm, 2.0) was placed in 0.5-cm-diameter wells cut in agar containing 0.1% CMC and was incubated overnight at 37°C before staining with Congo red.
[c] Plasmid-host combination with no insert in the plasmid.
[d] Values taken from a single experiment.

the periplasmic fraction. The cells were sonicated in 0.05 M sodium phosphate and centrifuged at 49,000 × *g* for 30 min to separate the cytoplasmic and membrane fractions.

***Bacteroides succinogenes* endoglucanase.** The extracellular enzyme from *B. succinogenes* prepared as described by Schellhorn and Forsberg (28) was generously provided by H. E. Schellhorn. Before use, the enzyme preparation was centrifuged at 100,000 × *g* to sediment membranes.

Enzyme assays. Assays for endoglucanase, xylanase, cellobiosidase, and Avicel hydrolysis were performed as described by Schellhorn and Forsberg (28). Low-viscosity carboxymethylcellulose (CMC; Sigma Chemical Co., St. Louis, Mo.) was used for the endoglucanase assays. Acid-swollen cellulose, lichenan (Sigma), and laminarin (Sigma) hydrolyses were tested at 1% (wt/vol) concentrations in 0.05 M sodium phosphate buffer (pH 6.5). Digests were tested for reducing sugar by using the Nelson-Somogyi reagent (1). Cyclic phosphodiesterase and β-galactosidase were assayed as described by Neu and Heppel (23) except that for the β-galactosidase assay *p*-nitrophenyl-β-D-galactoside was used as the substrate and 0.05 M sodium phosphate (pH 7.5) was the buffer. β-Lactamase activity was assayed as described by Sargent (25) with the modifications suggested by Sawai et al. (27). One unit of enzyme activity was defined as that amount of enzyme catalyzing the formation of 1 nmol of reducing sugar (or product) per min. Specific activity was defined as units per milligram of protein. CMC depolymerizing activity was determined as described by Schellhorn and Forsberg (28) with the exception that the enzyme-substrate mixture was incubated without shaking at 39°C. At intervals, 1-ml samples were removed, the reactions were stopped by heating at 100°C for 10 min, and 0.5-ml volumes were used for viscosity measurements.

Analytical methods. Polyacrylamide gel electrophoresis was performed as described by Laemmli (19). Nondenaturing gels were electrophoresed under the same conditions as sodium dodecyl sulfate (SDS) gels with the exception that SDS was omitted. Zymograms were prepared by using an agarose gel overlay technique described by Beguin (2). Ferguson plots were performed by the method of Hendrick and Smith (17). Endoglucanase bands in the gels were identified by zymogram analysis, and molecular weight markers were identified by Coomassie blue staining. Gels of 5, 7, 10, 12, and 15% were used to establish a curve of the log relative mobility (R_m) against the polyacrylamide concentration (in percent [wt/vol]). From the slope of this curve, for endoglucanase bands relative to slopes for proteins of known molecular weights, the molecular weight of each endoglucanase component was determined.

Protein concentrations were determined by the method of Lowry et al. (21). Glucose levels were determined with glucose oxidase (4), cellobiose levels were determined by high-pressure liquid chromatography (14), and total carbohydrate levels were determined by the method of Dubois et al. (12).

RESULTS

Hydrolytic activities and regulation of the *cel*-encoded endoglucanase. The pBC14 insert coding for the *cel* gene from *B. succinogenes* has been subcloned to remove non-*cel* DNA from the insert (9). The data in Table 1 demonstrate that selective size reduction of the cloned 6.6-kbp segment of DNA from *B. succinogenes* to 1.9 kbp increased expression of the gene. The endoglucanase activity of different cell extracts of BC14 varied from a low of 0.46 up to 14.1 nmol/min per mg of protein. However, the endoglucanase activities of the subclones appeared to be stably expressed, in particular the RE3 activity. The β-lactamase activities of RE3 and BC14 were tested and were found to be 48.7 and 53.9 μmol of ampicilloic acid formed per min per mg of protein, respectively, thereby indicating that the plasmid copy numbers were similar. At the same time, the endoglucanase activity of RE3 was at least double that of the BC14 clone.

High *p*-nitrophenylcellobiosidase activity was exhibited by the Cel endoglucanase. A low level of xylanase activity was also detected, although it did not exceed 1% of the endoglucanase activity. High lichenanase activity (159 nmol/min per mg of protein) was also exhibited in extracts of cells expressing the *cel* gene. Thus, the hydrolytic activity on lichenan was sixfold higher than that on CMC. In contrast to this level of activity, the *B. succinogenes* extracellular nonsedimentable endoglucanase exhibited similar activities on CMC and lichenan. The Cel enzyme also cleaved laminarin, although the activity was only 1% of the endoglucanase activity. *E. coli* RR1 harboring pUC8 without a DNA insert from *B. succinogenes* exhibited no hydrolytic activity on CMC, *p*-nitrophenylcellobioside, xylan, or lichenan.

The inclusion of glucose in the culture medium strongly repressed expression of the endoglucanase and cellobiosidase activities, whereas the inclusion of glycerol in the medium did not influence activities (Table 2).

Cellular distribution of the endoglucanase and cellobiosidase activities. The cellular distribution of endoglucanase and cellobiosidase activities is shown in Table 3 for a culture of RE3 in the late exponential phase of growth. At this stage of growth, 40 to 60% of the endoglucanase and cellobiosidase activities was located in the periplasmic compart-

TABLE 2. Influence of growth on glucose on the *cel*-encoded endoglucanase, cellobiosidase, and β-galactosidase activities of *E. coli* RR1[a]

Incubation time (h)	Sp act (nmol/min per mg of protein)					
	Endoglucanase		Cellobiosidase		β-galactosidase	
	2YT	2YT + G[b]	2YT	2YT + G	2YT	2YT + G
5	26	8	9	3	373	70
8	21	0.1	6	0.1	561	76
10	18	0.1	6	0.1	829	77

[a] Cells were disrupted by ultrasonication.
[b] Glucose (G) was included in the growth medium at a final concentration of 2% (wt/vol).

ment. The validity of this separation was supported by the high cyclic phosphodiesterase activity and low β-galactosidase activity detected in the periplasmic fraction. A small portion of the endoglucanase (5 to 10%) was associated with a membrane fraction. Very little of the enzymic activities was released into the culture fluid during growth.

The influence of the stage of growth on the cellular distribution of endoglucanase, cellobiosidase, and β-galactosidase is illustrated in Fig. 1. As growth progressed, greater proportions of the endoglucanase and cellobiosidase activities were located in the periplasmic compartment such that, by the stationary phase of growth, practically all of these activities were located in the periplasmic space. Similar profiles were observed when cells were grown in the same medium with glycerol added to give a final concentration of 2%. Whole cells of RE3 did not exhibit either endoglucanase or cellobiosidase activity. Toluene-treated cells did not exhibit endoglucanase activity, although cellobiosidase activity of the treated cells was equivalent to that of ultrasonically disrupted cells.

Hydrolytic characteristics of the periplasmic endoglucanase. The periplasmic endoglucanase from clone RE3 released less reducing sugar per unit of fluidity change of CMC than the extracellular cellulase from *B. succinogenes* (Fig. 2). This indicated that the Cel enzyme cleaved CMC in a more random fashion than the *B. succinogenes* endoglucanase complex.

The Cel endoglucanase exhibited higher K_m and V_{max} values than the *B. succinogenes* endoglucanase for the hydrolysis of CMC, lichenan, and *p*-nitrophenylcellobioside. The Lineweaver-Burk plots were all linear with the exception of that for nitrophenylcellobioside hydrolysis by *B. succinogenes*, which was biphasic with K_m values of 0.15 and 1.02 mM (Table 4). Hydrolysis of *p*-nitrophenylcellobioside by the Cel enzyme released cellobiose and *p*-nitrophenol, and no glucose was detected. The Cel endoglucanase preparation cleaved laminarin; however, the activity was only 1% of that exhibited on CMC.

The Cel enzyme exhibited much lower activity on acid-swollen cellulose than the *B. succinogenes* enzyme when they were standardized to have equal activities on CMC (Fig. 3).

Physiological properties of the endoglucanase. Binding of the endoglucanase to acid-swollen cellulose was tested by measuring the amount of enzyme remaining in the supernatant after centrifugation of the enzyme-cellulose reaction mixture. The *B. succinogenes* extracellular endoglucanase exhibited 40% binding within 10 min, after incubation at 4°C. The Cel enzyme did not bind to acid-swollen cellulose when incubated under identical conditions.

These endoglucanase preparations were tested for the effect of pH on activity. The *B. succinogenes* enzyme complex exhibited high activity over a wide pH range with a maximum pH of between 6.0 and 6.3. The Cel enzyme exhibited a narrower pH range with the maximum pH at 5.9.

The *B. succinogenes* endoglucanase preparation had maximum activity at 47°C. When the enzyme samples were preincubated without substrate for 10 min at various temperatures and then assayed at 39°C, it was observed that the activity was decreased as the preincubation temperature was increased, with a 50% loss in activity at 60°C. The Cel enzyme also exhibited a maximum activity at 47°C, but the maximum was sharper. The enzyme was less stable than that from *B. succinogenes*, being completely inactivated by incubation at 60°C.

Four types of detergents were tested to determine their effects on the Cel and *B. succinogenes* endoglucanases. SDS and cetramide, both at 0.1% completely inactivated the Cel enzyme, while the dipolar ionic detergent Zwittergent 3-10 (Calbiochem-Behring, La Jolla, Calif.) was only inhibitory at the 1% concentration. Triton X-100 at 0.1 and 1.0% had no effect. Only SDS strongly inactivated the *B. succinogenes* enzyme.

The metals mercury, copper, and zinc, each at a concentration of 0.1 mM, reduced the activity of both the *B. succinogenes* and Cel enzymes, although the Cel enzyme was more strongly inhibited. Manganese at a higher concentration also had an inhibitory effect on both enzymes. Calcium had no effect on the *B. succinogenes* enzyme, but stimulated the Cel enzyme by 82% at 10 mM. In a separate experiment, EDTA at either 1 or 10 mM inhibited the *B. succinogenes* enzyme by approximately 35% and the Cel enzyme by approximately 72%. Magnesium chloride at 10 mM partially prevented the inhibitory effect of EDTA.

Molecular weight of the Cel endoglucanases. Ferguson plots of the relative mobilities of the periplasmic Cel enzymes and the *B. succinogenes* enzyme complex were calculated. The Cel enzyme had three activity bands (Fig. 4, lane B). The

TABLE 3. Cellular distribution of endoglucanase and cellobiosidase activities in RE3 at the mid-exponential phase of growth

Compartment	Activity (%)[a] of enzyme:			
	Endoglucanase	Cellobiosidase	Cyclic phosphodiesterase	β-Galactosidase
Extracellular fluid	1.8 (—)	7.0 (—)	6.5 (—)	0 (0.05)
Periplasm	38.3 (348.4)	60.5 (163.9)	78.7 (151.3)	6.9 (630.6)
Cytoplasm	51.5 (31.8)	23.3 (4.3)	13.1 (1.7)	75.9 (474.9)
Membrane	8.3 (6.5)	9.3 (2.2)	1.6 (0.3)	17.2 (136.1)
Spheroplast sonicate	55.0 (18.7)	29.3 (3.2)	18.8 (1.6)	90.0 (239.6)

[a] All percentage values are expressed as percentages of the total extracellular and cellular fractions. Specific activity (shown in parenthesis) is expressed as nanomoles of product per minute per milligram of protein. —, No specific activity.

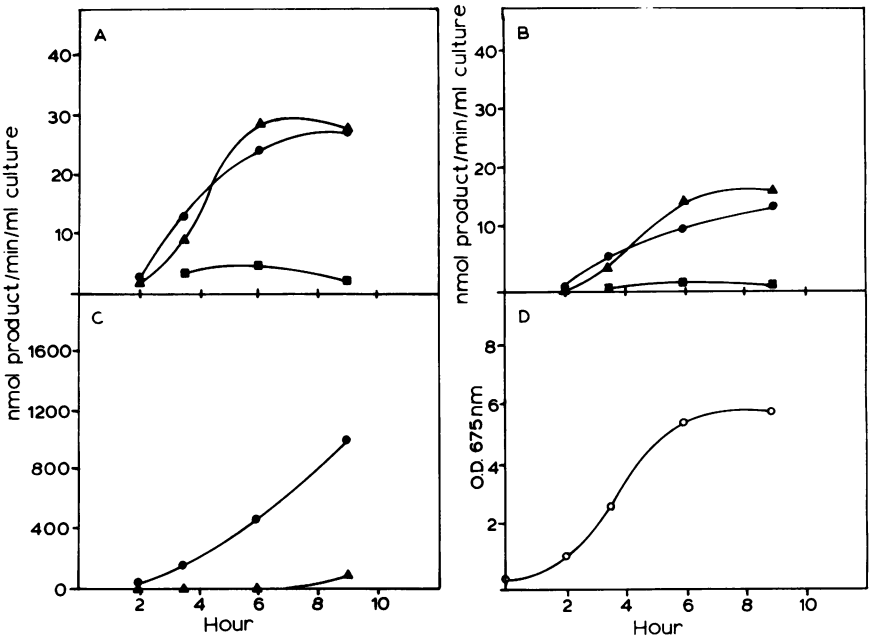

FIG. 1. Distribution of endoglucanase, cellobiosidase, and β-galactosidase activities in various cell fractions of RE3 as demonstrated by osmotically shocking cells taken from different stages of growth. (A) Endoglucanase activities of the following fractions: ultrasonicated cells (●), periplasmic fluid (▲), and osmotically shocked cells (cytoplasm) (■). (B) The same three fractions tested for cellobiosidase. (C) Ultrasonicated cell (●) and periplasmic (▲) fractions tested for β-galactosidase. The cytoplasmic fraction is not shown but was essentially identical to the ultrasonicated cells. (D) Optical density (○) of the growing culture measured at 675 nm.

two faster-migrating bands had molecular weights of 43,000. They migrated as a doublet, equidistantly apart at all gel concentrations, which suggests that they had slightly different charges. The slower-migrating band had a molecular weight of 55,000.

The *B. succinogenes* enzyme (Fig. 4, lane A) showed four major activity bands which had molecular weights of 41,500, 45,000, 64,000, and 83,000. The molecular weight of the slowest-migrating component was calculated from the front of the band because it formed a smear. Since the curves from the plots for the 45,000- and 83,000-molecular-weight proteins intersected at a gel concentration of 0%, these two proteins probably had similar charges.

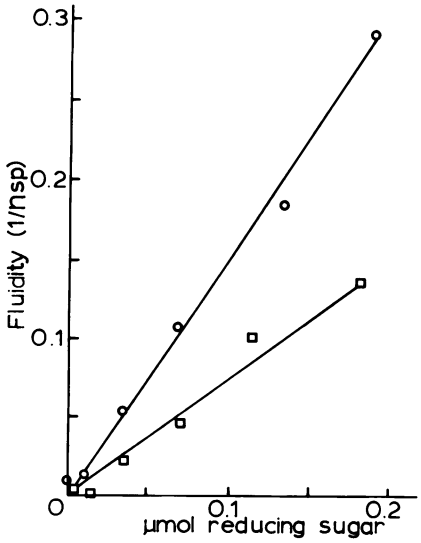

FIG. 2. Relationship between change in fluidity and reducing-sugar production during the hydrolysis of CMC by endoglucanases from *B. succinogenes* (□) and RE3 (○).

DISCUSSION

Syntheses of *B. succinogenes cel*-encoded endoglucanase and the cellobiosidase activities occurred in *E. coli* during growth in the absence of glucose but were inhibited in the presence of glucose. Since the *cel* gene is expressed in opposite orientations in pUC8 and pUC9 host vectors (B. Crosby, unpublished data) and growth of bacteria on glucose did not influence plasmid copy number, this observation suggests that expression of the gene is subject to catabolite repression and infers that cyclic AMP may be important in regulating the cellulase gene in *B. succinogenes*; however, cyclic AMP has not been detected in anaerobic bacteria tested (29, 30). Repression has been observed in *E. coli* in

TABLE 4. Kinetics of substrate hydrolysis by the periplasmic Cel endoglucanase and the *B. succinogenes* extracellular endoglucanase complex[a]

Substrate	Value for indicated endoglucanase:			
	Cel		*B. succinogenes*	
	K_m	V_{max}	K_m	V_{max}
CMC	79.4	4,066	4.6	743
Lichenan	4.3	1,960	0.75	917.4
p-Nitrophenyl-cellobioside	11.6	313	0.15, 1.02[b]	46.2, 76.9[b]

[a] Substrate concentrations were expressed as millimolar concentrations, while V_{max} values were expressed as specific activity. The K_m and V_{max} values were derived from Lineweaver-Burk plots.

[b] K_m *and* V_{max} values from a biphasic Lineweaver-Burk plot.

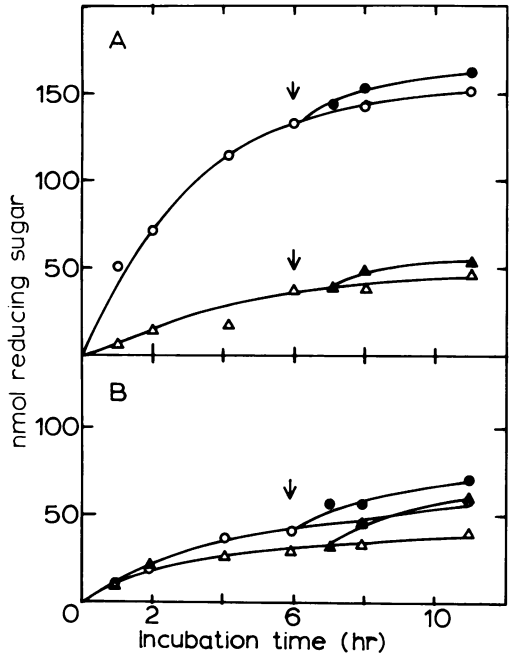

FIG. 3. Comparative activities of enzymes on CMC and acid-swollen cellulose. Equal activities of 0.13 U of *B. succinogenes* (A) and RE3 periplasmic (B) enzymes were incubated with CMC. After 6 h an amount of enzyme equal to that originally used was added, and the effects were noted (△). Enzyme at 100-fold the activity applied to the CMC was incubated with acid-swollen cellulose (○). At 6 h an equal amount of enzyme was added, and the effects were noted (●).

the absence of cyclic AMP and *crp* (32), and there is no reason to preclude this mechanism in *B. succinogenes*. When *B. succinogenes* was grown in the presence of glucose or cellobiose, a reduction in endoglucanase activity was noted (16). This indicates some form of catabolite regulation. The mechanism of regulation of cellulases in bacteria is poorly understood (34); therefore, expression of the *B. succinogenes cel* gene will provide an excellent model system for exploration of the regulation of cellulase induction.

The endoglucanase and cellobiosidase activities coded for by the *cel* gene were secreted into the periplasmic space of *E. coli*. Most of the secretion occurred during the late exponential phase of growth. This suggests that *B. succinogenes* possesses a sequence on the gene which codes for a signal peptide that is fully functional in *Escherichia coli* (33). Endoglucanases cloned from other bacteria into *E. coli* have reportedly been partially secreted into the periplasmic space (6, 8, 15, 26); however, it has not been the practice to examine the effect of growth phase on the extent of secretion as we have done in this study.

Activity of the Cel endoglucanase encoded by the *cel* gene in pUC8 was lower than that reported for cellulase genes from *Cellulomonas fimi* (15) and *Clostridium thermocellum* (8) but higher than that reported for a cellulase gene from *Thermomonospora* sp. strain YX (6).

The ability of the *cel*-encoded enzyme to hydrolyze CMC, *p*-nitrophenylcellobioside, and lichenan and to hydrolyze CMC in a more-random fashion than the *B. succinogenes* endoglucanase complex suggests that the enzyme cleaves glucans with less specificity than the major endoglucanase(s) of the *B. succinogenes* cellulase complex. The higher K_m values for hydrolysis by the Cel endoglucanase were probably a reflection of this lack of substrate specificity. The difference in hydrolytic properties between the *cel* gene product and the *B. succinogenes* endoglucanase was further exemplified by the inability of the former to bind to acid-swollen cellulose under conditions in which the enzyme(s) of the latter do bind. The very different nature of this enzyme was also exhibited in higher detergent sensitivity, a narrower pH range for activity, and lower temperature stability. The obvious differences between the Cel endoglucanase and the complex from *B. succinogenes* might have occurred because the Cel enzyme is a minor component of the cellulase complex or perhaps because it lacks proper glycosylation or other posttranslational modification. Langsford et al. (20) have reported, for example, that glycosylated *Cellulomonas fimi* cellulases bind to cellulose, but once they are deglycosylated, they no longer bind.

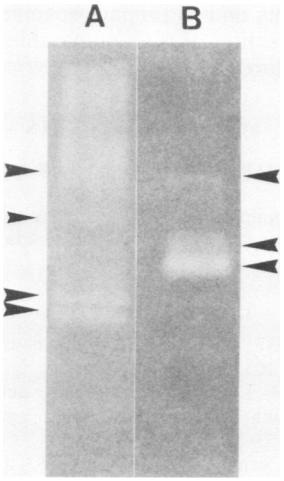

FIG. 4. Zymogram of the *B. succinogenes* extracellular endoglucanase (lane A) and the Cel periplasmic endoglucanase (lane B). The arrows indicate the major activity bands observed on a 10% polyacrylamide gel run under nondenaturing conditions.

The ability of the Cel endoglucanase to hydrolyze the aglycone bond of *p*-nitrophenyl-β-D-cellobioside and lichenan, a mixed glucan containing β-1,4 and β-1,3 linkages, indicates that it is a unique enzyme which possesses properties in common with both exoglucanases (11, 15) and endoglucanases (11). The low activity of the Cel endoglucanase on laminarin, a predominantly β-1,3-linked glucan, suggests that lichenan is predominantly cleaved at the β-1,4 linkages, although no experimental evidence is available to support this contention.

It is worth noting that a gene coding for a lichenanase which lacks hydrolytic activity on CMC has been cloned from *B. succinogenes* (J. E. Irvin, P. J. Wood, R. M. Teather, and W. Crosby, Abstr. Annu. Meet. Am. Soc. Microbiol., 1986, O-73, p. 273).

Nondenaturing gel electrophoresis of the periplasmic Cel endoglucanase revealed the presence of three components, two with different charges and each having a molecular weight of 43,000 and one with a molecular weight of 55,000. Similar results were obtained for the *celB* gene from *Clostridium thermocellum* in that two active peptides with molecular weights of 55,000 and 53,000 were detected, which was attributed to posttranslational proteolysis (3, 24).

We are presently purifying the *cel* gene products to allow more detailed characterization of the separate endoglu-

canase components and to prepare specific antibody which will allow identification of the comparable enzyme from *B. succinogenes* cultures.

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