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The Cellular Location of *Prevotella ruminicola* β-1,4-D-Endoglucanase and Its Occurrence in Other Strains of Ruminal Bacteria

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***Prevotella ruminicola* B_14, TC1-1, TF1-3, and TS1-5 all produced immunologically cross-reacting 88- and 82-kDa carboxymethyl cellulases (CMCases). *P. ruminicola* 23, 118B, 20-63, and 20-78 had much lower CMCase activities, and Western blots (immunoblots) showed no cross-reaction with the B_14 CMCase antiserum. *Fibrobacter succinogenes* S85 and *Selenomonas ruminantium* HD4 and D produced CMCases, but these enzymes were smaller and did not cross-react with the B_14 CMCase antiserum. The B_14 CMCase antiserum inhibited the B_14, TC1-1, TF1-3, and TS1-5 CMCase activities and agglutinated these cells, but it had no effect on the other strains or species. On the basis of these results, the B_14 CMCase is a strain-specific enzyme that is located on the outside surface of the cells. *P. ruminicola* B_14 cultures, grown on sucrose, did not have significant CMCase activity, but these cells could bind purified 88- and 82-kDa CMCase but not 40.5-kDa CMCase. Because the 40.5-kDa CMCase is a fully active, truncated form of the CMCase, it appears that the N-terminal domain of the 88-kDa B_14 CMCase anchors the CMCase to the cells. Cells grown on cellobiose produced at least 10-fold more CMCase than the sucrose-grown cells, and the cellobiose-grown cells could only bind 15% as much CMCase as sucrose-grown cells. Virtually all of the CMCase activity of exponentially growing cultures was cell associated, but CMCase activity was eventually detected in the culture supernatant. On the basis of the observation that the 88-kDa CMCase was gradually converted to the 82-kDa CMCase when cultures reached the stationary phase without a change in specific activity, it appears that the 82-kDa protein is probably a proteolytic degradation product of the 88-kDa CMCase.**

Ruminant animals have developed the capacity to digest cellulose by exploiting a symbiotic relationship with cellulolytic ruminal bacteria (7). The enumeration studies of Bryant and Burkey (3) indicated that noncellulolytic bacteria outnumbered the cellulolytic ones, even when wheat straw was the only ingredient in the diet, and later work indicated that there was a cross-feeding of "cellulose fragments" from cellulolytic to noncellulolytic bacteria. *Prevotella ruminicola* is usually described as a starch-degrading bacterium, but it is able to utilize water-soluble cellodextrins (15) and some strains have considerable carboxymethyl cellulase (CMCase) activity (1, 12, 16, 17).

The β-1,4-D-endoglucanase (CMCase) of *P. ruminicola* B_14 degrades carboxymethyl cellulose (CMC) at a rapid rate, but it lacks a cellulose binding domain and cannot degrade crystalline, acid-swollen, or balled milled cellulose at a significant rate. The addition of a cellulose binding domain to the C terminus of the CMCase increased the rate of native cellulose digestion 10-fold, and this finding has been used as a basis for creating a cellulolytic bacterium that can digest cellulose at low pH (10).

P. ruminicola strains have as little as 20% DNA homology, and the B_14 strain is the most genetically distinct strain (11). The B_14 strain produces 88- and 82-kDa CMCases (13), while most other *P. ruminicola* strains produce smaller CMCases (1, 16). The B_14 CMCase was originally described as a cell-free enzyme (12), but more recent work indicates that the activity is primarily cell associated (5). Whole cells degrade large CMC molecules, but there was no direct evidence that the CMCase was located on the outside surface of the cells. The following experiments were designed to examine the occurrence, immunoreactivity, and localization of the *P. ruminicola* B_14 CMCase.

MATERIALS AND METHODS

Strains. *P. ruminicola* TC1-1, TF1-3, and TS1-5 were kindly provided by H. J. Flint (2). *P. ruminicola* 23, 118B, 20-78, 20-63, M384, GA-33, 2202, and D42f were kindly provided by M. Cotta (U.S. Department of Agriculture, Peoria, Ill.). *Bacteroides ovatus*, *Bacteroides distasonis*, *Ruminobacter amylophilus*, and *Selenomonas ruminantium* were kindly provided by T. Miller (N.Y. State Department of Health, Albany).

Media and cultivation conditions. *P. ruminicola*, *B. ovatus*, *B. distasonis*, *R. amylophilus*, *S. ruminantium*, and *Fibrobacter succinogenes* strains were grown as described previously (12). Sugars were prepared anoxically and added from separately sterilized stock solutions, and the final concentration was always 4 mg/ml. Growth was monitored by the increase in optical density (600 nm). *Escherichia coli* pC43 cells were grown aerobically as described previously (12).

Enzyme assays. Cells were harvested by centrifugation (4,000 × *g*, 5°C, 15 min), washed twice in potassium phosphate buffer (50 mM, pH 6.5), and resuspended in potassium phosphate buffer (50 mM, pH 6.5). The suspension was sonicated (Branson model 200 sonifier with microtip; maximum output, 0°C, 5 min), and cell debris was removed by centrifugation (14,000 × *g*, 0°C, 15 min). The whole cells, cell-free supernatants, and cell extracts were assayed for CMCase activity as described previously (12). All assay mixtures were incubated at 37°C for 24 h, and the assays were performed in triplicate.

CMC zymograms. Whole cells were harvested in the mid-log phase by centrifugation (4,000 × *g*, 5°C, 15 min) and resuspended in 1.5× sodium dodecyl sulfate (SDS) loading buffer. Samples were incubated at 55°C for 20 min and loaded onto SDS–8% polyacrylamide gels containing 2% CMC. SDS-polyacrylamide gel electrophoresis was performed as described previously (9) as was the zymogram analysis (5).

Western blotting (immunoblotting). Whole cells were harvested in the mid-log phase by centrifugation (4,000 × *g*, 5°C, 15 min) and resuspended in 1.5× SDS loading buffer. Samples were boiled for 3 min and loaded onto SDS–8% polyacrylamide gels. Western blotting was performed as described previously with antisera prepared against the 40.5-kDa cloned CMCase purified from *E. coli* (12).

Reaction of strains to antisera. Whole cells were harvested in the mid-log phase by centrifugation (4,000 × *g*, 5°C, 15 min) and resuspended in one-half volume of potassium phosphate buffer (50 mM, pH 6.5). Antiserum prepared against the 40.5-kDa form of the *P. ruminicola* CMCase isolated from *E. coli* was reacted with whole cells to ensure that the antiserum did not contain antibodies

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TABLE 1. CMCase activities of various strains of *P. ruminicola*, *S. ruminantium* HD_4, and *F. succinogenes* S85 grown on 4 g of cellobiose per liter

Organism	Sp act (nmol/mg of protein/min)[a]	
	Whole cells	Cell extracts
P. ruminicola		
B_14	117	119
TC1-1	118	133
TF1-3	118	137
TS1-5	105	116
20-78	29	35
20-63	8	12
23	33	34
118B	14	17
S. ruminantium		
HD_4	128	154
D	45	53
F. succinogenes S85	255	282

[a] Standard deviations were less than 10%.

against other *P. ruminicola* proteins (12). An equal volume of antiserum was added to the whole cells, and samples were incubated at 25°C for 48 h.

CMCase purification. Cells (4 liters of *P. ruminicola* or *E. coli*) were harvested by centrifugation (4,000 × *g*, 5°C, 15 min) and resuspended in 100 ml of potassium phosphate buffer (50 mM, pH 6.5). The cells were sonicated (Branson model 200 sonifier with microtip; maximum output, 0°C, 5 min), and cell debris was removed by centrifugation (14,000 × *g*, 0°C, 15 min). Impurities were precipitated by the addition of 15 ml of 30% streptomycin sulfate and incubated on ice for 30 min, and the supernatant was recovered after centrifugation (10,000 × *g*, 4°C, 25 min). Proteins were precipitated by the addition of ammonium sulfate to 40%, followed by incubation on ice for 30 min. The protein pellet was recovered by centrifugation (10,000 × *g*, 4°C, 25 min) and resuspended in 1.5 M ammonium sulfate–500 mM NaCl–50 mM potassium phosphate buffer (pH 6.5). The solution was applied to a phenyl-Sepharose (Pharmacia) column (1.5 by 10 cm), the column was washed with 75 ml of 1.5 M ammonium sulfate–500 mM NaCl–50 mM potassium phosphate buffer (pH 6.5), and proteins were eluted with 50 ml of distilled water. The CMCase-containing fractions were combined and applied to a Hypatite C (Clarkson Chemical Co., Williamsport, Pa.) column (1.5 by 10 cm) and washed with 50 ml of 1 mM potassium phosphate buffer (pH 6.5), and the CMCase was eluted with a 200-ml gradient of 1 to 100 mM potassium phosphate buffer (pH 6.5). The CMCase-containing fractions were combined and applied to a Q-Sepharose column (1.5 by 10 cm), washed with 50 ml of bis-Tris (50 mM, pH 6.5), and the CMCase was eluted with a 200-ml gradient of 0 to 500 mM NaCl–50 mM bis-Tris (pH 6.5). The CMCase-containing fractions were combined and concentrated to 10 ml with a 30,000-molecular-weight-cutoff filter (PTTK; Millipore Corp., Bedford, Mass.). The sample was diluted approximately fivefold with potassium phosphate buffer (50 mM, pH 6.5) and concentrated to 10 ml with the same concentration device.

Stability of the CMCases added to growing *P. ruminicola* cultures. Approximately 25 pmol of purified 88-kDa CMCase or 40.5-kDa CMCase was added to 10 ml of exponential- or stationary-phase *P. ruminicola* B_14 cells growing on either sucrose or cellobiose as the carbon source. At various time points, 1 ml of culture was removed, and the cells were harvested by centrifugation (12,000 × *g*). The cells were washed once in sterile medium and resuspended in potassium phosphate buffer (50 mM, pH 6.5) to yield an optical density (600 nm) of 4.8. The amount of enzyme in the supernatant and on the cells was estimated by Western blotting and determined quantitatively by CMC assays.

Binding of the 88-kDa CMCase to *P. ruminicola* cells. *P. ruminicola* cultures were grown to an optical density (600 nm) of 3.0, and cells were harvested by centrifugation (4,000 × *g*, 5°C, 15 min) and washed twice in potassium phosphate buffer (50 mM, pH 6.5). Cells were resuspended in 10 ml of potassium phosphate buffer (50 mM, pH 6.5). Resuspended cells (50 μl) were added to 450 μl of various concentrations of purified 88-kDa CMCase in 50 mM potassium phosphate buffer (pH 6.5) and incubated at 37°C for 24 h. Cells were harvested by centrifugation (4,000 × *g*, 5°C, 15 min) and washed once in 500 μl of potassium phosphate buffer (50 mM, pH 6.5). The cells were assayed for CMC activity as described previously. All reactions were performed in triplicate.

RESULTS

CMCase activities. *P. ruminicola* B_14 cultures grown on cellobiose had a CMCase specific activity of 117 nmol/mg of protein per min (Table 1). Cell extracts had a similar specific activity, and no activity was detected in the culture supernatant. Strains TC1-1, TF1-3, and TS1-5 had similar CMCase activities, but strains 20-78, 20-63, 23, and 118B had lower CMCase activities. Strains M384, GA-33, 2202, and D42f had no detectable CMCase activity. *B. ovatus* 17624 and *B. distasonis* A111 and 4243, species closely related to *P. ruminicola*, had no detectable CMCase activity. *F. succinogenes* S85, a cellulolytic rumen bacterium, had more CMCase activity than that of B_14, but *S. ruminantium* HD4, a noncellulolytic rumen bacterium, had about the same CMCase activity as that of B_14. *R. amylophilus* 70, a starch-degrading rumen bacterium, did not have any CMCase activity.

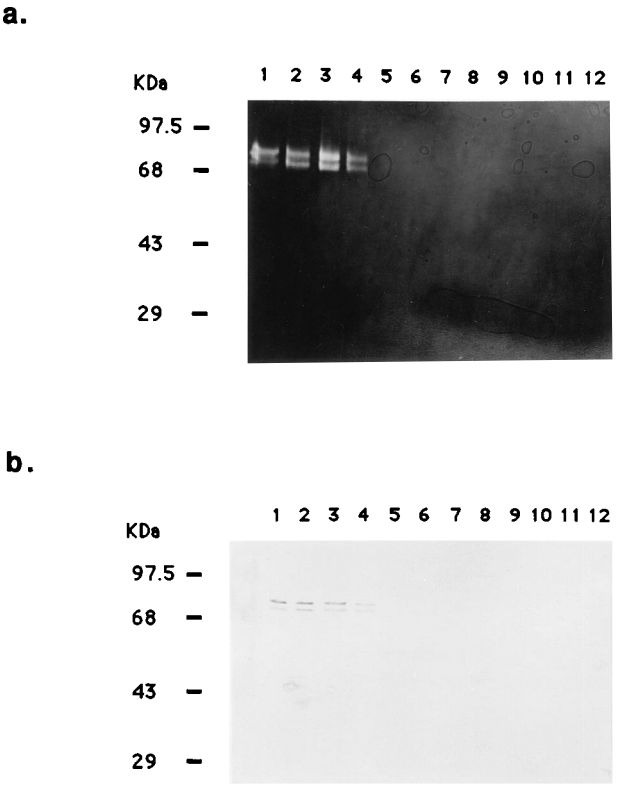

FIG. 1. Zymogram (a) and Western blot (b) of various strains of *P. ruminicola*. Lanes: 1, B_14; 2, TC1-1; 3, TF1-3; 4, TS1-5; 5, 20-78; 6, 20-63; 7, 118B; 8, 23; 9, M384; 10, GA-33; 11, 2202; 12, D42f.

CMC zymograms demonstrated that *P. ruminicola* B_14, TC1-1, TF1-3, and TS1-5 all produced two CMCases having molecular sizes of approximately 88 and 82 kDa (Fig. 1a). No bands were observed with the other *P. ruminicola* strains. *F. succinogenes* S85 produced one CMCase, and its molecular weight was significantly lower than those of the *P. ruminicola* CMCases (Fig. 2a). *S. ruminantium* HD4 produced two CMCases, but the molecular weights were also lower than those of *P. ruminicola*. *S. ruminantium* HD4 produced 60- and 29-kDa CMCases, but strain D produced only a 29-kDa CMCase.

Western blots. *E. coli* clones carrying the *P. ruminicola* B_14 CMCase gene produced a truncated 40.5-kDa CMCase that contained the catalytic domain of the *P. ruminicola* 88- and 82-kDa CMCases (12). Antiserum to the purified *E. coli* 40.5-kDa CMCase reacted with the CMCases of strains B_14, TC1-1, TF1-3, and TS1-5 (Fig. 1b). None of the other *P. ruminicola* strains produced proteins that cross-reacted with the antiserum. No significant cross-reactions were detected with *S.*

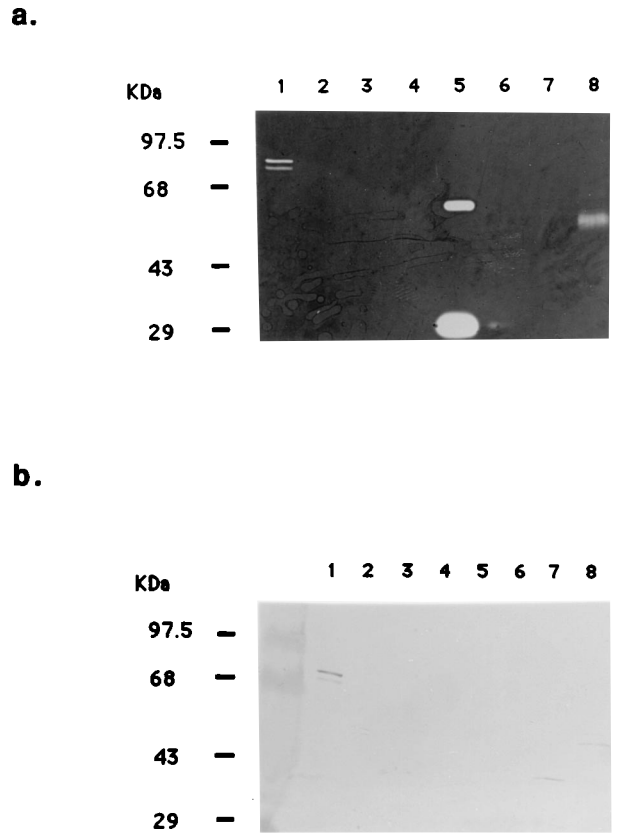

FIG. 2. Zymogram (a) and Western blot (b) of various species of rumen and oral bacteria. Lanes: 1, *P. ruminicola* B_14; 2, *B. ovatus* 17623; 3, *B. distasonis* A111; 4, *B. distasonis* 4243; 5, *S. ruminantium* HD_4; 6, *S. ruminantium* D; 7, *R. amylophilus* 70; 8, *F. succinogenes* S85.

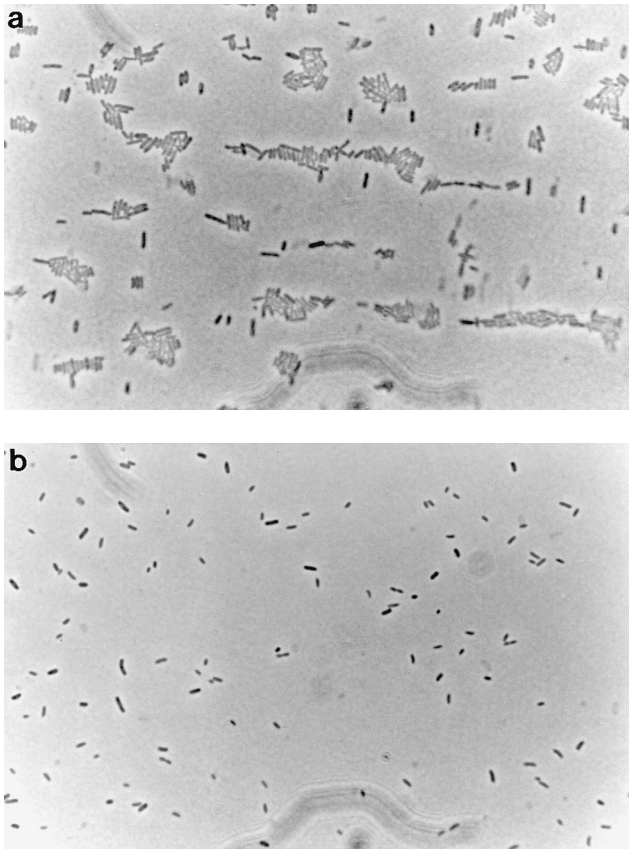

FIG. 4. *P. ruminicola* B_14 cells incubated with (a) and without (b) 40.5-kDa CMCase antisera. Magnification, ×240.

ruminantium HD_4 and D, *F. succinogenes* S85, or the *Bacteroides* species (Fig. 2b).

CMCase assays indicated that the CMCase specific activity remained constant during exponential growth and the stationary phase, but Western blots indicated that the proportion of the 82-kDa CMCase gradually increased when the cultures entered the stationary phase (Fig. 3). On the basis of digital imaging analyses, the 88- and 82-kDa CMCases initially represented 65 and 35% of the total CMCase protein, respectively. After the cultures entered the stationary phase, the 88-kDa CMCase accounted for only 29% of the total CMCase and the 82-kDa CMCase had increased to 71%. CMCases accumulated in the supernatant only when the optical density decreased. Cell lysis had little effect on the total CMCase activity.

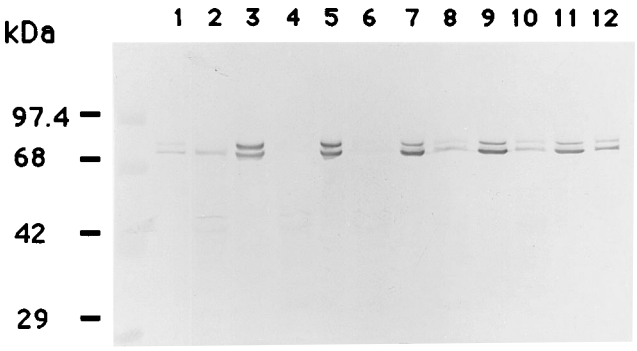

FIG. 3. Western blot of *P. ruminicola* B_14 CMCase at different time points during growth. CMCases from the cell extracts are in the odd lanes, and CMCases from the supernatants are in the even lanes. Time points were 0 h (lanes 1 and 2), 1 h (lanes 3 and 4), 2 h (lanes 5 and 6), 4 h (lanes 7 and 8), 8 h (lanes 9 and 10), and 19 h (lanes 11 and 12).

Reaction of strains to antiserum. Antiserum to the 40.5-kDa CMCase agglutinated B_14 cells (Fig. 4a) and inhibited CMCase activity more than 90%. The 40.5-kDa CMCase antiserum also agglutinated strains TC1-1, TF1-3, and TS1-5 (data not shown), and once again, CMCase activity was inhibited by at least 90%. The 40.5-kDa CMCase antisera had no effect on *P. ruminicola* 20-78, 20-63, 23, or 118B, *F. succinogenes* S85, or *S. ruminantium* HD_4 and D, all of which contained some CMCase activity.

Stability and binding of purified CMCase to *P. ruminicola* cells. B_14 cultures that were growing on sucrose produced little CMCase activity, but these cells were able to bind 88- and 82-kDa CMCases added to the culture supernatant. The decrease in free CMCase was nearly compensated by an increase in cell-bound activity (Fig. 5a). Western blots indicated that the 88- and 82-kDa CMCases were binding to *P. ruminicola* B_14 cells (Fig. 5b) and not just being degraded. When purified 40.5-kDa CMCase was added, no decrease in the cell-free activity was observed, and Western blots indicated that the 40.5-kDa CMCase could not bind to B_14 cells (data not shown).

The binding of the 88-kDa CMCase to sucrose-grown cells was first order with respect to cell density (data not shown).

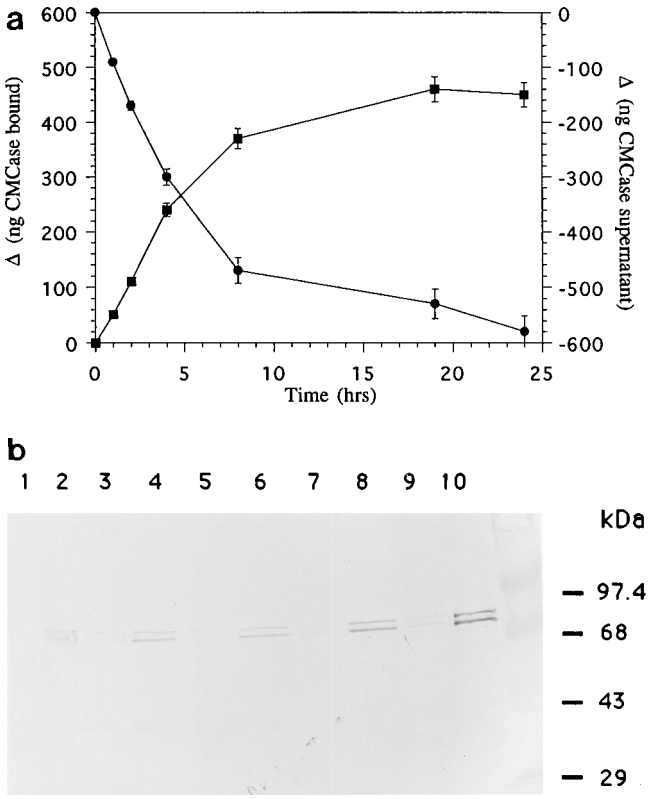

FIG. 5. (a) Change (Δ) of CMCase in the supernatant (●) and bound to the cells (■) when purified 88-kDa CMCase was added to *P. ruminicola* B_14 cells growing exponentially on sucrose; (b) Western blot of *P. ruminicola* B_14 cells with (even lanes) and without (odd lanes) additional purified 88-kDa CMCase added to the supernatant. Cells were harvested at 0 h (lanes 1 and 2), 1 h (lanes 3 and 4), 2 h (lanes 5 and 6), 4 h (lanes 7 and 8), and 8 h (lanes 9 and 10).

When the number of cells was doubled, the CMCase binding was twice as high. By varying the concentration of added CMCase (to a fixed amount of cells), it was possible to demonstrate a saturation (Fig. 6). On the basis of a Lineweaver-Burk plot of CMCase added versus CMCase bound, the maximal binding constant, B_{max}, was 0.209 ng of CMCase per mg of cell protein and the half-maximal binding constant, B_m, was 704 ng of CMCase per ml. When the cells were grown on cellobiose, the B_{max} and B_m values were 0.0218 ng of CMCase per mg of cell protein and 103 ng of CMCase per ml, respectively. The B_{max} and B_m values for cellobiose-grown *P. ruminicola* 23 were 0.0295 ng of CMCase per mg of cell protein and 280 ng of CMCase per ml, respectively.

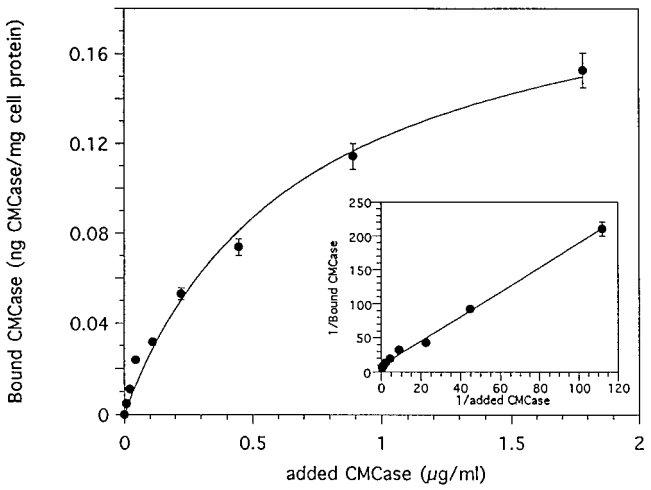

FIG. 6. Amount of 88-kDa CMCase that bound to sucrose-grown cells when the concentration of added 88-kDa CMCase was varied. The inset shows the Lineweaver-Burk plot used to determine the B_{max} and B_m.

DISCUSSION

Recent work has shown that *P. ruminicola* B_14, TC1-1, TF1-3, and TS1-5 have a high degree of 16S rRNA homology (2), and these strains produce identical CMCases (Fig. 1). *P. ruminicola* 20-78, 20-63, 23, and 118B produced less than one-third the CMCase activity of B_14, and Western blots indicated that the other strains that produced CMCases did not react with B_14 CMCase antiserum. These results support the idea that *P. ruminicola* is a highly divergent species that needs further reclassification (2, 11). Strains B_14, TC1-1, TF1-3, and TS1-5 could be a separate species.

The CMCase specific activities of *P. ruminicola* B_14 cultures remained constant during the exponential and stationary phases, but Western blots revealed that the amounts of 88- and 82-kDa CMCases changed when the cultures entered the stationary phase. Digital imaging analyses of the blots indicated that the amount of the 88-kDa CMCase decreased while the amount of the 82-kDa CMCase increased proportionately. On the basis of these results, the 82-kDa CMCase is probably a proteolytic degradation product of the 88-kDa CMCase. *E. coli* clones carrying the *P. ruminicola* CMCase gene produce a fully active 40.5-kDa CMCase (12), but this protein is never observed in *P. ruminicola* cultures.

The DNA sequence of the 40.5-kDa CMCase is preceded by at least four potential *E. coli* promoters, and these promoters occur in the middle of the 88-kDa CMCase open reading frame (13). The 40.5-kDa CMCase, which contains the active site, represents the C-terminal portion of the 88-kDa CMCase, but the function of the N-terminal portion was not known. Because antiserum to the 40.5-kDa CMCase agglutinated *P. ruminicola* B_14 cells and almost completely inhibited the CMCase activity, the C-terminal portion and catalytic domain of the CMCase appear to be on the outside surface of the cell. On the basis of the observation that the 88- and 82-kDa CMCases could bind to the surface of *P. ruminicola* cells and the 40.5-kDa CMCase could not, it seems likely that the N-terminal portion is an anchor that binds the enzyme to the cells. Because there was only a modest decline in activity when the 40.5-kDa CMCase was added to culture supernatants, it appears that the N-terminal region is not required for enzyme stability.

The B_14 CMCase can be purified from cell extracts, indicating that it is not an integral membrane protein. Integral membrane proteins usually have distinct regions of hydrophobicity (4, 6, 14), but much less is known about the binding of peripheral proteins to the bacterial cell wall. Computer analysis (8) of the amino acid sequence indicated that the N-terminal region of the B_14 CMCase did not have any highly hydrophobic regions, but hydrophobicity may not be the primary mechanism of binding.

P. ruminicola B_14 cells grown on sucrose produced little CMCase (5), but these cells could bind purified 88- and 82-kDa CMCases. Cells grown on cellobiose produced 10-fold more CMCase than sucrose-grown cells and did not bind as much additional CMCase. On the basis of these results and the CMCase binding constants, it appeared that cellobiose-grown

cells produced 85% as much CMCase as sucrose-grown cells could bind maximally. Because the concentration of CMCase in the supernatant is much less than the B_m and the amount of CMCase naturally bound to the cells is almost the B_{max} for cellobiose-grown cultures, it is not yet clear whether the natural process of secretion yields the same type of cellular binding. Because *P. ruminicola* 23 had little capacity to bind the $B_1$4 CMCases, CMCase binding appears to be a strain-specific phenomenon.

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