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Cell Surface Enzyme Attachment Is Mediated by Family 37 Carbohydrate-Binding Modules, Unique to *Ruminococcus albus*▽‡

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The rumen bacterium *Ruminococcus albus* binds to and degrades crystalline cellulosic substrates via a unique cellulose degradation system. A unique family of carbohydrate-binding modules (CBM37), located at the C terminus of different glycoside hydrolases, appears to be responsible both for anchoring these enzymes to the bacterial cell surface and for substrate binding.

Ruminococcus albus is widely recognized as one of the specialist cellulose-degrading bacteria resident in the rumena and gastrointestinal tracts of herbivores. Most isolates have been shown to utilize cellulose, xylan, and cellobiose as carbon sources and found to produce a wide range of enzymatic activities, including β-glucosidase, β-xylosidase, α-galactosidase, α-arabinosidase, cellulase, polygalacturonase, and β-1,4-xylanase activities (2, 3, 5). Interestingly, many of these glycoside hydrolases bear a recently described family 37 carbohydrate-binding module (CBM37), which appears to be exclusive to *R. albus*. These ~100-residue modules were first identified at the C-terminal ends of an exoglucanase (Cel48A) and a processive endocellulase (Cel9B) from *R. albus* strain 8 (1) and appear to be nondiscriminatory in their carbohydrate-binding properties, recognizing a variety of polysaccharides, including cellulose, xylan, chitin, and lichenan (21). This breadth of adhesive properties makes the CBM37 family unique among the CBM families known to date. A preliminary examination of the draft genome sequence for *R. albus* strain 8 suggests that CBM37 modules, which are grouped into three major subtypes, are present in numerous *R. albus* polysaccharide-degrading enzymes and other nonenzymatic proteins from this bacterium (http://blast.jcvi.org/rumenomics/index.cgi).

Previous studies have shown that effective cellulose hydrolysis by *R. albus* strains is conditional on the provision of micromolar concentrations of phenylacetic and phenylpropionic acids (9, 17, 18). These compounds appear to influence capsule formation by the bacterium, and cellulase activity is retained as high-molecular-mass complexes on the bacterial cell surface. In the absence of phenylacetic and phenylpropionic acids, the adhesion of the bacterium to cellulose (and its hydrolysis) is negatively affected. Additionally, cellulase activity is secreted into the culture medium and, by size exclusion chromatography, is shown to be present in a form suggesting that there is no aggregation of activity into larger, multiprotein complexes (17). Although it was long believed that these characteristics were attributable to a cellulosomal mode of enzyme organization, the identification of CBM37 modules (rather than dockerins) in these two key enzymes suggests that the CBM37 modules might play some role(s) in protein retention to the bacterial cell surface. In the present study, we present evidence to validate this hypothesis, and we propose that an additional function for the CBM37 family is the attachment of the parent protein to the bacterium's cell surface.

Three different CBM37 modules from *R. albus*, Cel5G (C-terminal module of AAT48117), Cel9C (AAT48118), and Cel48A (AAR01217), were used in this study, and they map to different branches within the major subgroup of CBM37 modules (see Fig. S1 in the supplemental material). The three CBM37s were cloned and fused to the C-terminal end of a recombinant maltose-binding protein (the resulting fusion proteins are hereinafter referred to as MBP-CBM5G, MBP-CBM9C, and MBP-CBM48A, respectively) (Table 1) and expressed in *Escherichia coli* as described earlier (21). MBP was required for solubility of the fusion partner and also served as a recognition tag. As a control, the MBP was fused to the catalytic module of Cel5G (hereinafter referred to as MBP-CD5G). Cellobiose-grown cells of *R. albus* 8 were harvested by centrifugation and washed using previously described procedures (1). Aliquots of the cell suspension were mixed with any one of the four different fusion proteins followed by mouse anti-MBP antibody and fluorescein isothiocyanate-conjugated goat anti-mouse antibody, according to the method described by Orgad et al. (12). The cells were then examined by fluorescence microscopy (Fig. 1). All three MBP-CBM fusion proteins could attach to the cell surface; however, the MBP-CD5G fusion protein did not. These results suggest that the attachment of the recombinant protein to the surface of *R. albus* 8 cells is mediated via the CBM37 module.

Next, we employed several mutant strains of *R. albus* 8

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TABLE 1. Oligonucleotide primers and plasmids used in this study

MBP fusion protein[a]	Primer name	Nucleotide sequence	Comment
MBP-CD5G	F-GH5G-CD	ATATGAATTCGCAACATCAGCAGTGAATGACACC	Catalytic domain of Cel5G (without CBM37)
	R-GH5G-CD	CCCCAAGCTTTTAAGGTGTTTCGGGATCAATGATTATC	
MBP-CBM5G	F-Cel5G-CBM	ATATGAATTCGCTATAAATGTTATGGCGAAAGATG	CBM37 of Cel5G
	R-Cel5G-CBM	CCCCAAGCTTTTACTTTACAGTGATAGTCACAGCG	
MBP-CBM48A	F-GH48A-CBM	ATATGAATTCGATGATAAGACTTATCCTACCAAC	CBM37 of Cel48A
	R-GH48A-CBM	CCCCAAGCTTTTAAACTGTAACGTTAACTACAGA	
MBP-CBM9C	F-GH9C-CBM	ATATGAATTCGATCGTTTCGGCGGTTCGAATCCTG	CBM37 of Cel9C
	R-GH9C-CBM	CCCCAAGCTTTTACTTTATAGTAACAGTACAAGCACG	

[a] Forward (F-) and reverse (R-) primers contain EcoRI and HindIII cleavage sites, respectively (underlined).

isolated by selective enrichment for defective adhesion to cellulose. Proteomic analysis of these mutant strains showed that they possess significantly less than wild-type amounts of the cell-associated CBM37-bearing enzymes Cel9B and Cel48A and are also defective in terms of cellulose hydrolysis kinetics compared to the wild-type strain (1). Following cultivation using cellobiose-containing medium (the same as that used for the wild-type strain), all three mutant strains appeared to be unable to bind the MBP-CBM fusion proteins, because no fluorescent label was detectable by microscopy (data not shown). To further validate this negative result, a cell-based enzyme-linked immunosorbent assay (ELISA) was employed, essentially as described by Rosok et al. (15), using lyophilized and resuspended bacterial cells (*R. albus* 8 or its ADM-2 mutant). The results, presented in Fig. 2, confirmed that the MBP-CBM5G recombinant protein can attach to the cell surface of wild-type *R. albus* 8 cells but cannot bind to the cell surface of the mutant strains. These findings suggest that the mutants all lack a key feature on the cell surface that is necessary for the attachment of *R. albus* proteins via the CBM37 module.

Previous research showed that cell-free enzyme extracts are often not as effective as the intact cells in cellulose degradation, demonstrating that essential factors that may be missing in the extracellular medium are present in the bacterium (20). In an earlier report (13), transmission electron microscopic visualization of ruthenium red-labeled *R. albus* cells indicated an extensive "coat" layer, described as a compact mat of polysaccharide fibers external to the cell wall. This polysaccharide coat, or "glycocalyx," was considered in subsequent works to mediate adhesion of the cells to cellulose (14, 19). In a later work, it was shown that most of the cellulases and xylanases in *R. albus* SY3 were associated with the capsular and cell wall fraction but were severely reduced on the surfaces of an adhesion-defective mutant (7). Two enzymes critical to effective

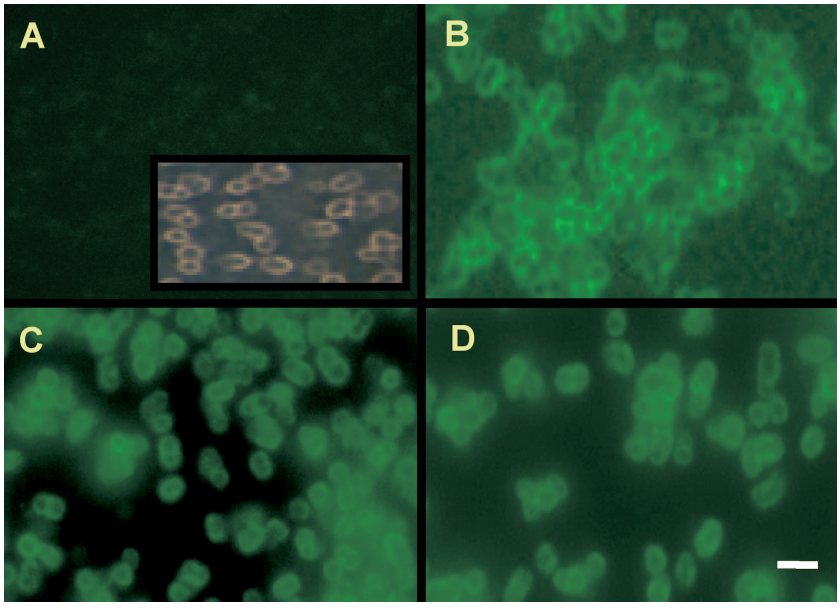

FIG. 1. Binding of different CBM37 fusion proteins to *R. albus* 8 cells by fluorescence microscopy. Log-phase *R. albus* cells interacted with the MBP control (MBP-CD5G lacking CBM37) (A), MBP-CBM5G (B), MBP-CBM48A (C), and MBP-CBM9C (D). All test proteins were expressed as fusion proteins with MBP at the N terminus. The labeled cells were subjected to interaction with mouse anti-MBP antibody followed by fluorescein isothiocyanate-conjugated donkey anti-mouse antibody and then visualized by fluorescence microscopy. The inset in panel A shows a phase-contrast micrograph of the *R. albus* 8 cells used for these studies. Bar = 2 μm.

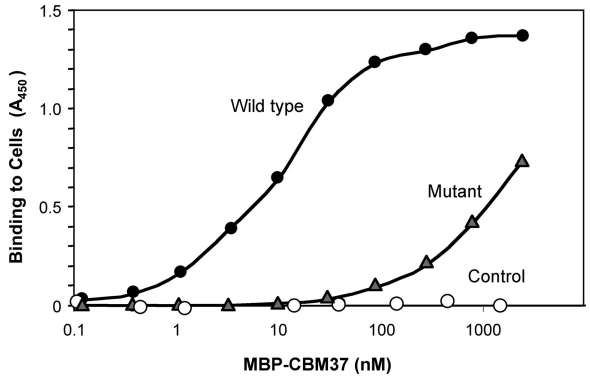

FIG. 2. ELISA of the binding of CBM37 to the whole cells of *R. albus* 8. ELISA plates, containing attached *R. albus* 8 or its ADM-2 mutant, were reacted with the test protein, MBP-CBM37 from Cel5G (MBP-CBM5G), at the indicated final concentrations and labeled with mouse anti-MBP-horseradish peroxidase antibodies. MBP was used as the control.

solubilization by *R. albus* 8 (Cel48A and Cel9B) were previously demonstrated to be cell-associated proteins and to bind strongly to cellulose (1), presumably via the C-terminal CBM37 modules each enzyme bears. The results of the present study demonstrate that the same module serves to secure the enzymes to the cell surface. Consequently, we propose that CBM37 may function as a shuttle to convey the parent enzyme between the bacterial cell surface and the polysaccharide substrate.

Preliminary scanning electron microscopy of cationized ferritin-treated *R. albus* 8 versus its adherence-defective mutants clearly revealed a protuberance-laden surface in the wild-type cells as opposed to a smooth surface in the mutant ADM-2 (see Fig. S2 in the supplemental material), but it is as yet unclear whether the CBM37-binding component relates directly to this finding. Preliminary work (not shown) has also demonstrated that a polysaccharide-containing cell extract of *R. albus* 8 (obtained by a combination of lysosyme and DNase, followed by proteinase K treatments) is highly inhibitory to CBM37 binding to the bacterial cell surface. In contrast, similar extracts derived from the *R. albus* adherence-defective mutants or from *Ruminococcus flavefaciens* failed to inhibit the binding. Further work to identify the suspected cell wall carbohydrate component is currently being pursued.

In conclusion, *R. albus* cellulases are indeed known to be released into the medium during growth (16) and are subsequently bound to the cellulose fibers, yet cellulose digestion is facilitated by the proximity of the cells to the cellulose fibers (6). The model proposed here suggests that the CBM37 acts as a shuttle which transfers the appended enzymes from the bacterial surface to the plant cell wall. Another alternative might be that the CBM37 has two separate carbohydrate-binding sites, as shown previously for other CBMs (4). In this case, one site would bind to the plant cell wall and the other to the bacterial polysaccharide capsule. It remains to be seen, however, whether one or more of the remaining CBM37-bearing nonenzymatic proteins produced by this bacterium might play some role in the binding of the cells to the substrate. Previous research has already demonstrated that more than one mechanism is involved with the adhesion of *R. albus* to the substrate (8, 10, 11), but the localization of the glycanases at the interface appears to be mediated largely by CBM37.

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