Two SusD-Like Proteins Encoded within a Polysaccharide Utilization Locus of an Uncultured Ruminant Bacteroides Phylotype Bind Strongly to Cellulose


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We demonstrate that two characteristic Sus-like proteins encoded within a polysaccharide utilization locus (PUL) bind strongly to cellulose substrates and interact with plant primary cell walls. This shows associations between uncultured Bacteroidetes-affiliated lineages and cellulose in the rumen and thus presents new PUL-derived targets to pursue regarding plant biomass degradation.

The Bacteroidetes are the most abundant Gram-negative bacteria in gut microbiomes and are commonly associated with degradation of xylan and other noncellulosic polysaccharides (4). However, cellulosytic Bacteroidetes isolates have been described (13), suggesting that these bacteria also contribute toward cellulose degradation in the gut, despite the lack of genes corresponding to common cellulytic enzymes in many rumen isolates (6 and 48 (GH6 and GH48) (3)). Recent metagenomic analyses of rumen microbiomes have revealed the occurrence of polysaccharide utilization loci (PULs) linked to putative GH5 and GH9 cellulases in several uncultured Bacteroidetes phylotypes (12). Bacteroidetes-affiliated PULs are typified by gene clusters that encode lipochromic polysaccharides together with a set of outer membrane lipoproteins (referred to as Sus-like). The Sus-like proteins bear a resemblance to proteins of the starch utilization system (Sus), first identified in the human gut bacterium Bacteroides thetaiotaomicron (14). The so-called SusD-like proteins contribute to saccharide capture, as has been demonstrated for starch and fructan (8,15). Aside from starch and fructan PULs, pectin and hemicellulose PULs have been detected in isolated gut bacteria (3, 9). Nothing is known about the association of PULs with cellulose.

Figure 1A shows a PUL from the dominating uncultured SRM-1 (for “Svalbard reindeer microorganism 1”) phylotype found in the Svalbard reindeer rumen, which exhibits only 91% SRM-1 (for “Svalbard reindeer microorganism 1”) phylotype pendent cloning (LIC) (1) and primers SusD1 lic NT (TTAAGA AGGAGATATACATGTTGACCAGCTGCCCATCGGCA CGCATTC), SusD1 lic CT (AATGGTGTGTGATGTGTCGCCCC ACACCGGGATCTGCGTAGGGGCGTATCC), SusD2 lic NT (TTAAGAAGGATATACATGTTGACCCCTCACTATACGG GAGGGAGACACA), and SusD2 lic CT (AATGGTGTGTGATGTGTCGCCCC ACACCGGGATCTGCGTAGGGGCGTATCC) (overhangs are underlined). Subsequently, recombinant proteins lacking the putative signal peptide and containing a C-terminal His tag were overexpressed in Escherichia coli BL21, purified by immobilized metal affinity chromatography, and dialyzed and concentrated using Vivaspin concentrators. To analyze polysaccharide binding, purified proteins (1 mg/ml), 6% (wt/vol) Sigma cellulose, Avicel (Sigma-Aldrich), filter paper (Whatman), and the insoluble fractions of xylan (Carl Roth GmbH), mannan (Megazyme), or lichenin (Megazyme) were combined in the presence of MES buffer (20 mM; pH 6; final volume, 200 µl) and incubated at 37°C with vertical shaking at 1,000 rpm for 1 h. After centrifugation, the supernatant (referred to as flowthrough) was removed, and the insoluble substrate was resuspended in 200 µl of MES buffer and incubated for 15 min, after which the supernatant was removed by centrifugation (referred to as the wash step). Bound proteins were eluted with 100 µl of 50 mM bis-Tris-propane with 5% Triton (pH 10). Harsher denaturing conditions (100 µl of 8 M urea and boiling for 10 min) were also used (for lichenin, boiling was omitted).

Analysis of the various fractions from the binding experiments by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1B to D) demonstrated that SusD1 and SusD2 bind to various forms of cellulose. Elution with Triton failed (Fig. 1B), whereas elution was achieved with 8 M urea and boiling (Fig. 1C). Elution of SusD2 was incomplete in all cases, suggesting that SusD2 exhibits a different binding mechanism than SusD1 (Fig. 1C). A further difference between SusD1 and SusD2 is that only the former binds to lichenan (β-1,3,β-1,4 β-glucan) (Fig. 1B and C). Studies with SusD1 showed that binding is pH dependent and strongest at pH <8.0 (Fig. 1D) (the pH of the Svalbard reindeer rumen ranges from 6 to 6.75 [11]). Interestingly, both proteins exhibited only weak binding to mannan or xylan. This result is notable because the presence of putative GH26 (mannanase), GH43 (xylodsidase, arabinanase, arabinofuranosidase), and CE7

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(acetyl xylan esterase) enzymes within the SRM-1 PUL (Fig. 1A) suggests such hemicellulosic substrates as potential targets.

To further explore their ability to recognize plant polysaccharides, we investigated binding of SusD1 and SusD2 to the cell wall of *Arabidopsis thaliana* (Fig. 2). Hand-cut sections through the stems of 4- to 5-week-old plants were labeled using a His6 tag-based three-stage procedure essentially as previously described (10), in which binding was detected using a fluorescein isothiocyanate conjugated tertiary antibody. Cellulose-binding CBM3a from *Clostridium thermocellum* (2) was included as a positive control. The binding of SusD2 and that of the positive control, CBM3a, were similar in that both produced widespread labeling across diverse cell types and both produced a characteristic punctate labeling pattern. However, there were subtle differences in the binding of these probes. CBM3a bound predominantly to the adhered faces of adjacent pith parenchyma cell walls, whereas SusD2 binding was more apparent to regions of wall delineating the intercellular spaces. Also, CBM3a bound strongly to epidermal cell walls but weakly to the walls of underlying cortical cells, whereas the reverse was true for SusD2. SusD1 did not bind to equivalent sections (data not shown), confirming that SusD1 and SusD2 have different binding specificities. Interestingly, our data may be taken to indicate that SusD1 has greater binding affinity for lichenan (Fig. 1), a substrate that is scant in cell walls of dicotyledons such as *A. thaliana*.

To our knowledge, these data provide the first experimental evidence linking Sus proteins and PULs to cellulose. Moreover, the difference in binding specificities suggests that SusD1 and SusD2 have complementary functions and are optimized to bind to distinct features of the microstructure of cell walls. The variety of putative glycoside hydrolases encoded within the SRM-1 PUL suggests activities against a broad range of hemicellulosic (GH5, GH26, GH43, CE7) and cellulosic (GH5, GH94) substrates. Preliminary activity data obtained with overexpressed enzymes show that the two GH5 enzymes cleave β-1,4-linked glucose units in various substrates, including Avicel, phosphoric acid-swollen cellulose, lichenan, and glucomannan, and that they produce cellobiose. It remains to be elucidated if insoluble cellulose is a target substrate for this PUL or if SusD binding to cellulose serves the purpose of positioning PUL-linked glycoside hydrolases close to other (hemicellulosic) polysaccharides intertwined with cellulose, i.e., a proximity effect similar to that shown for certain CBMs (6). Interestingly, as previously pointed out (12), one of the Avicel-degrading enzymes extracted from the cow rumen metagenome...
is part of a PUL containing a SusD-like homologue. All in all, available data strengthen the hypothesis that the membrane anchored enzyme systems encoded by PULs are involved in cellulose degradation. Confirmation of this hypothesis would establish a third paradigm for cellulose degradation, next to cellulosomes and free enzyme systems.

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