Applied and Environmental Microbiology, Dec. 2006, p. 7559–7566 Vol. 72, No. 12
0099-2240/06/$08.00+0 doi:10.1128/AEM.01632-06
Copyright © 2006, American Society for Microbiology. All Rights Reserved.

Studies of the Extracellular Glycocalyx of the Anaerobic Cellulolytic Bacterium *Ruminococcus albus* 7[▽]

Paul J. Weimer,[1,2]* Neil P. J. Price,[3] Otini Kroukamp,[4]† Lydia-Marie Joubert,[4]‡ Gideon M. Wolfaardt,[4]† and Willem H. Van Zyl[4]

U.S. Dairy Forage Research Center, Agricultural Research Service, U.S. Department of Agriculture, Madison, Wisconsin[1]; Department of Bacteriology, University of Wisconsin—Madison, Madison, Wisconsin[2]; National Center for Agricultural Utilization Research, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois[3]; and Department of Microbiology, University of Stellenbosch, Stellenbosch, South Africa[4]

Received 13 July 2006/Accepted 2 October 2006

Anaerobic cellulolytic bacteria are thought to adhere to cellulose via several mechanisms, including production of a glycocalyx containing extracellular polymeric substances (EPS). As the compositions and structures of these glycocalyces have not been elucidated, variable-pressure scanning electron microscopy (VP-SEM) and chemical analysis were used to characterize the glycocalyx of the ruminal bacterium *Ruminococcus albus* strain 7. VP-SEM revealed that growth of this strain was accompanied by the formation of thin cellular extensions that allowed the bacterium to adhere to cellulose, followed by formation of a ramifying network that interconnected individual cells to one another and to the unraveling cellulose microfibrils. Extraction of 48-h-old whole-culture pellets (bacterial cells plus glycocalyx [G] plus residual cellulose [C]) with 0.1 N NaOH released carbohydrate and protein in a ratio of 1:5. Boiling of the cellulose fermentation residue in a neutral detergent solution removed almost all of the adherent cells and protein while retaining a residual network of adhering noncellular material. Trifluoroacetic acid hydrolysis of this residue (G plus C) released primarily glucose, along with substantial amounts of xylose and mannose, but only traces of galactose, the most abundant sugar in most characterized bacterial exopolysaccharides. Linkage analysis and characterization by nuclear magnetic resonance suggested that most of the glucosyl units were not present as partially degraded cellulose. Calculations suggested that the energy demand for synthesis of the nonprotein fraction of EPS by this organism represents only a small fraction (<4%) of the anabolic ATP expenditure of the bacterium.

The anaerobic cellulolytic bacterium *Ruminococcus albus* is one of the most important agents of fiber fermentation in the rumen (17, 48, 51). As in the cases of many other anaerobic cellulolytic bacteria, cellulose degradation by *R. albus* involves adherence to cellulose, presentation of cellulolytic enzymes directly at the microbial cell surface, uptake of cellodextrin oligomers, and intracellular phosphorolytic cleavage to the common catabolic intermediate, glucose-1-phosphate (25). Adherence and surface-localized hydrolysis presumably enhance the concentration of cellulolytic enzymes at the cellulose surface, allow the organism first access to hydrolytic products, and reduce predation by grazing protists (6, 50). The cellulolytic apparatus in several anaerobic cellulolytic bacteria appears to be organized in the form of a specific cell surface organelle, the cellulosome (1, 2, 36). Detailed studies of the cellulosomes of *Clostridium thermocellum* (a species phylogenetically related to *R. albus*) have revealed that these complexes consist of a spatially organized array of at least 22 different catalytic proteins (exoglucanases, endoglucanases, and hemicellulases), a number of which have carbohydrate-binding modules (CBMs) supported on a CBM-containing structural protein, scaffoldin (1, 2). Evidence is accumulating that *R. albus* contains several homologs of cellulosomal proteins and displays some cell surface features characteristic of cellulosomes (32, 36).

Adherence to cellulose appears to be an important physiological property of *R. albus*. Mosoni and Gaillard-Martinie (33) have reported that an adherence-defective mutant of *R. albus* 20 displayed slower degradation of cellulose and a longer lag period prior to cellulose degradation, and Miron et al. (29) have reported that adherence-defective mutants of *R. albus* SY3 degraded several types of cellulose more slowly and produced lower titers of cellulase and xylanase than did wild-type cells. However, even among wild-type *R. albus* isolates, the relationship between adherence and cellulolytic capability varies substantially by strain, leading Miron et al. and Morrison and Miron (27, 32) to suggest that multiple mechanisms may be involved in adherence. Structures thought to be involved in adherence by this species include pilin-like proteins associated with fimbriae (32, 38, 40, 41), CBMs of polysaccharide hydrolases (8, 27, 28, 32, 52), and an extracellular glycocalyx (6, 8, 33, 37, 49). Despite the importance of adherence in anaerobic cellulolysis, the characteristics of these glycocalyces are largely unknown. The purpose of this study was to characterize the composition and structure of the glycocalyx of *R. albus* 7.

* Corresponding author. Mailing address: USDA-ARS-USDFRC, 1925 Linden Drive West, Madison, WI 53706. Phone: (608) 890-0075. Fax: (608) 890-0076. E-mail: pjweimer@wisc.edu.

† Present address: Department of Chemistry and Biology, Ryerson University, Toronto, Ontario, Canada.

‡ Present address: Cell Sciences Imaging Facility, Stanford University, Stanford, Calif.

▽ Published ahead of print on 6 October 2006.

MATERIALS AND METHODS

Cultures. *Ruminococcus albus* 7 was grown at 39°C under a CO_2 atmosphere in modified Dehority medium (49) with Sigmacell 50 microcrystalline cellulose (SC50) (3 g/liter; Sigma, St. Louis, MO) as the sole fermentable carbohydrate source, yeast extract (0.5 g/liter) as a source of supplemental nutrients, and 3-phenylpropionic acid (25 μM) to facilitate the attachment of cells to cellulose fibers (44). Culture tubes or vials were incubated in an upright position and were gently mixed once or twice daily. Cultures (24 to 48 h old, depending on the experiment) were harvested by centrifugation (12,000 × *g*; 25 min; 5°C). The resulting pellets, hereafter referred to as "whole-culture pellets," contained both planktonic and adherent cells, as well as glycocalyx and substantial amounts of residual cellulose substrate, but did not contain soluble fermentation products or soluble extracellular proteins.

Microscopy. For fluorescence microscopy, cultures grown for 48 h on SC50 were stained with *Bac*Light bacterial viability probe (Invitrogen, Carlsbad, CA) and visualized with a Nikon Eclipse E400 epifluorescence microscope equipped with a multipass filter set appropriate for viewing 4′,6-diamidino-2-phenylindole (DAPI), as well as excitation/barrier filter sets of 465 to 495/515 to 555 nm, and 540 to 580/600 to 660 nm. Images were captured with a Nikon Coolpix 990 digital camera mounted on the microscope. For variable-pressure scanning electron microscopy (VP-SEM) studies, SC50 in the culture medium was replaced by Whatman (Brentford, United Kingdom) no. 3 filter paper disks (nominal thickness, 0.39 mm; ~0.5-cm diameter; 30 disks per 10 ml of culture), and after 24 to 48 h of incubation, the partially degraded disks containing adherent bacteria and glycocalyx were recovered by decantation and then gently rinsed with water in a petri dish. These fully hydrated samples were placed onto carbon-conductive double-sided adhesive tape (Ted Pella, Redding, CA) mounted on aluminum stubs and evacuated in a sputter coater chamber (model S150A; BOC Edwards) until the vacuum reached a constant value (~7 × 10^5 Pa), after which sputter coating with Au continued at a current of 15 to 17 mA for 2 min. SEM was carried out in VP mode (specimen chamber pressure, 50 to 60 Pa) with a LEO 1450VP scanning electron microscope (Carl Zeiss SMT, Inc., Thornwood, NY) operated at 7 kV at a working distance of 5 mm.

Chemical analyses. Chemical analyses were performed on cellulose fermentation residues that either were untreated or were treated with various chemical agents, according to the fractionation scheme shown in Fig. 1. By using pure SC50 microcrystalline cellulose powder (lacking measurable quantities of nonglucosyl sugars) as the substrate, we took advantage of the fact that any nonglucosyl materials associated with the fermentation residue would have to be a component of the bacterial cells or the glycocalyx rather than having originated from the cellulose substrate. Freeze-dried whole-culture pellets (6 to 15 mg, weighed to 0.001 mg), with or without prior boiling for 1 h in neutral detergent solution (ND) (12) to remove bacterial cells, followed by exhaustive washing in water, were placed in 1.5-ml microcentrifuge tubes. The solids were first suspended in 1.00 ml H_2O and then centrifuged (12,000 × *g*; 10 min), and the supernatants were discarded. The pellet was resuspended in 600 μl of 0.1 N HCl, 0.1 N NaOH, or 0.1 N NaOH with 0.1% (wt/vol) $NaBH_4$ (the last to minimize oxidative peeling reactions of polysaccharides) and then was incubated in a 70°C water bath for 1 h with frequent mixing. The suspension was then neutralized with 600 μl of 0.1 N NaOH or 0.1 N HCl and centrifuged as described above. The supernatants were assayed colorimetrically for protein (4), using commercial Coomassie Plus reagent (Bio-Rad, Hercules, CA) with lysozyme as a standard; for soluble carbohydrate, using phenol-sulfuric acid (9) with D-glucose (Glc) as a standard; and for uronic acids, using the 3-phenylphenol/borate/sulfuric acid method (3) with D-galacturonic acid as the standard and with correction for minor interference by hexoses. Amino sugars were assayed colorimetrically by the method of Sandford et al. (42), using a 5-h incubation in the presence of NaOCl (1:10 dilution of Clorox) at 39°C prior to addition of the $NaNO_2$ and amylose/KI; chondroitin sulfate (70% isomer A plus 30% isomer C; Na salt; Sigma catalog no. C9819) and hyaluronic acid (Na salt; Fluka) were used as standards. Qualitative tests for the presence of curdlan (β-1,3-glucan) were performed using the aniline blue staining method of Kenyon and Buller (20).

Carbohydrate linkage analysis. Residual ND-treated samples (containing glycocalyx and residual cellulose) were treated with trifluoroacetic acid (TFA) (2 N; 110°C; 2 h). Aldononitrile acetate derivatives were prepared by the method of Price (39). Hydroxylamine-dimethylaminopyridine reagent (0.2 ml) was added, and the samples were reacted on a hot block (60°C) for 30 min. The reaction mixtures were cooled and then treated with acetic anhydride (0.5 ml; 60°C; 30 min) to complete the peracetylation. The reaction mixtures were quenched with water (2 ml) and extracted with ethyl acetate (1 ml). The upper layer was used directly for gas chromatography/mass spectrometry (GC/MS) analysis (1-μl injection). Permethylation linkage analysis was performed as described by Hakomori (15). GC/MS analyses of the derivatized samples employed a Hewlett-Packard 6890N gas chromatograph (Agilent Technologies, Palo Alto, CA) equipped with an HP 7683 autoinjector. The gas chromatograph was interfaced with an HP 5973 series mass spectrometer configured in electron impact mode. Chromatography was accomplished with a capillary HP-1 column (25 m long; 0.2-mm diameter), using helium as the carrier gas at a flow rate of 0.8 ml/min. The oven temperature was ramped over a linear gradient from 150 to 250°C at 4°C/min. Mass spectra were recorded in positive-ion mode over the range 50 to 500 *m/z*.

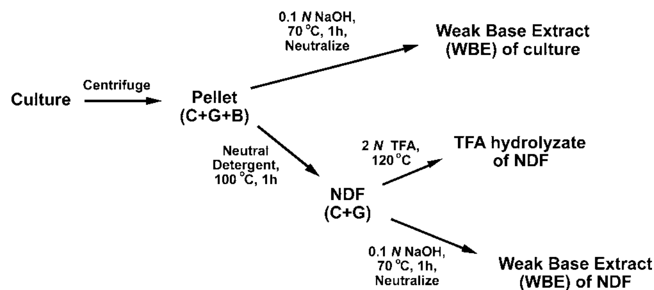

FIG. 1. Fractionation scheme for glycocalyx-containing cellulose residue from *R. albus* 7 grown on cellulose as the sole fermentable energy source. B, bacterial cells; C, cellulose; G, glycocalyx; NDF, neutral detergent fiber residue.

To preferentially cleave 1,6 linkages, the ND-treated residue (40 mg) was subjected to mild sulfuric acid-catalyzed acetolysis (acetic anhydride-glacial acetic acid-concentrated sulfuric acid, 100:100:1 [vol/vol/vol]; 60°C; 2 h) (21). The resulting peracetylated oligosaccharides were partitioned into ethyl acetate and back washed three times with water. The ethyl acetate fraction was analyzed by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) on a Bruker-Daltonic Omniflex instrument (Bruker, Billerica, MA) operating in reflectron mode. Samples were dried under a lamp on a conventional 49-place (7 by 7) stainless steel target. Ion sources 1 and 2 were 19.0 kV and 14.0 kV, with lens and reflector voltages of 9.20 and 20.00 kV, respectively. The matrix used was 2,5-dihydroxybenzoic acid. A 200-ns pulsed ion extraction was used, with matrix suppression up to 200 Da. Excitation was at 337.1 nm, typically at 60% of a 150-μJ maximum output, and 80 shots were accumulated.

For proton nuclear magnetic resonance (NMR) spectrometry, the acetylated oligosaccharides generated by acetolysis were redissolved in deuterated chloroform. NMR spectra (proton, COSY, TOCSY, and HMQC) were recorded on a Bruker Avance 500-MHz instrument using pulse sequences supplied by Bruker.

RESULTS

Visualization of cells and glycocalyces. Young (24-h) cultures of *R. albus* 7 displayed growth only within the sedimented cellulose powder, which became yellow due to bacterial pigment production. Older (48-h) cultures displayed more intense pigmentation of the sediment and turbidity in the overlying liquid phase, indicating the presence of planktonic cells only late in fermentation. Under fluorescence microscopy, extensive colonization of cellulose fibers was observed, and both cellulose-adherent and planktonic cells freshly removed from actively growing cultures and stained with *Bac*Light bacterial-viability stain exhibited the green fluorescence characteristic of viable cells.

Because the high water content of biofilms affords little structural integrity under the high-vacuum, dehydrating conditions of conventional SEM (5, 18, 47), VP-SEM was used to visualize specimens in a saturated chamber atmosphere. Operation under humid conditions reduced the risk of producing artifacts in extracellular polymeric substances (EPS) that would result from a collapse of the biofilm's three-dimensional

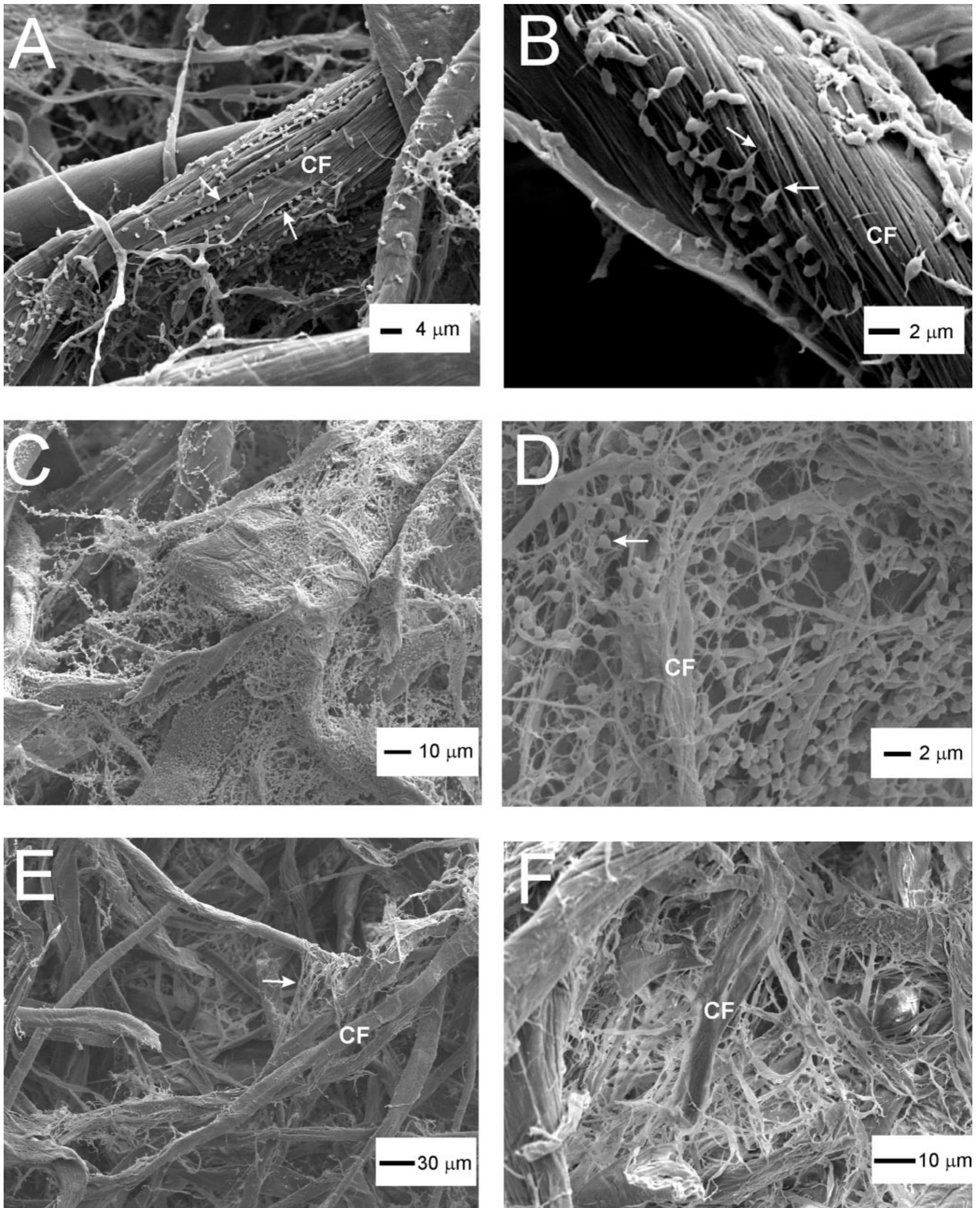

FIG. 2. Variable-pressure scanning electron micrographs of *R. albus* 7 cells and associated glycocalyx adhering to cellulose fibers (CF). (A) Young culture (24 h) showing individual cells localized primarily in crevices (arrows) of fibers. (B) Cells from young (24-h) cultures showing discrete cellular appendages (arrows) prior to formation of a distinct glycocalyx. (C and D) Older cultures (48 h) showing greater coverage of the fibers and formation of a ramifying network of bacterial cells (arrow), glycocalyx, and partially degraded cellulose. (E) Residual cellulose covered with an adherent glycocalyx (arrow) following removal of bacterial cells by boiling them in neutral detergent solution. (F) Residual cellulose, after being boiled for 2 h in 2 N TFA, showing cellulose fibers at various extents of degradation and without adherent glycocalyx.

TABLE 1. Weak-base (0.1 N NaOH) and weak-acid extraction of protein, carbohydrate, and uronic acids from residues of *R. albus* 7 cellulose fermentations before or after being boiled in ND solution

Component	Composition of residue (mg/g residue) ± SEM[a]	
	Untreated	ND-treated
Weak-base extraction		
Protein	51.95 ± 0.82	0.65 ± 0.29
PS-CHO[b]	10.25 ± 1.13	9.66 ± 1.73
Uronic acids	NT[c]	0.32 ± 0.12
Weak-acid extraction		
Protein	NT	0.05 ± 0.06
PS-CHO[b]	NT	3.06 ± 0.43

[a] Results are mean values of duplicate samples, each assayed in duplicate.
[b] Phenol sulfuric acid-reactive carbohydrate.
[c] NT, not tested.

structure. VP-SEM revealed that in the early stages of colonization, cells were localized in crevices and discontinuities along the length of the fiber and were not preferentially localized at the fiber ends (Fig. 2A). Isolated individual cells, generally coccoid in shape and often in the process of cell division, were common during the early phase (24 h) of colonization and growth. These young cultures also displayed discrete cellular extensions or appendages that appeared to directly contact the surfaces of the cellulose fibers (Fig. 2B). Cells were not aligned along the crystallographic axes of the cellulose fibers, as has been demonstrated for another ruminal cellulolytic bacterium, *Fibrobacter succinogenes* (22, 50). Older cultures displayed more extensive colonization as a monolayer of cells interconnected with one another and to the cellulose fibers via an extensive threadlike network of extracellular glycocalyx material (Fig. 2C and D).

Boiling of whole-culture pellets in ND solution for 1 h yielded a residue that initially retained the yellow pigmentation associated with the partially degraded cellulose, although this pigmentation faded over subsequent sample preparations for chemical analysis. The residue was not stained by aniline blue, a specific stain for β-1,3-glucans (20). VP-SEM of the ND-treated residue revealed a nearly complete absence of adherent cells from the surfaces of the cellulose fibers, while the bulk of the glycocalyx material appeared to remain intact as a cobweb-like material (Fig. 2E). Intimate association of the glycocalyx with the cellulose, along with the unraveling of the cellulose microfibrils from the larger cellulose fibers as degradation proceeded, made it difficult to visually distinguish the point of transition between the glycocalyx and the microfibrils. The glycocalyx material was not removed from the ND-treated residue by dimethyl sulfoxide or cold TFA. Treatment of the ND-treated residue with Bmim[dicyanamide], an ionic liquid capable of solubilizing pure cellulose (10), were unsuccessful at removing the glycocalyx or at solubilizing the underlying cellulose, suggesting that the glycocalyx adhered tightly to, and was protective of, the cellulose fibers. More aggressive treatment with trifluoroacetic acid (2.0 N; 100°C; 2 h), removed both cells and glycocalyx and left a residue containing partially degraded microfibrils (Fig. 2F), suggesting some chemical degradation or solubilization of the partially degraded cellulose, in accord with the ability of this treatment to hydrolyze amorphous, but not crystalline, cellulose (31).

Chemical analyses of cultures and cellulosic residues. Whole-culture pellets resulting from centrifugation of 48-h-old cultures of *R. albus* 7 contained weak base (0.1 N NaOH/0.1% $NaBH_4$; 70°C; 1 h)-extractable (WBE) protein and WBE phenol-sulfuric acid-reactive carbohydrate (WBE-PS-CHO) in a ratio of 1:5 (Table 1). Boiling of these whole-culture pellets in ND solubilized 98.8% of the WBE protein but <10% of the WBE-PS-CHO. Uronic acids were also found to be present in the WBE-PS-CHO at a concentration 5.2% of that of the neutral sugars, but the specific uronic acid residues were not identified. Separate extraction of the ND-treated residue with 0.1 N HCl released considerably smaller amounts of carbohydrate and almost none of the protein, relative to that removed by the weak-base treatment (Table 1). Detectable levels of amino sugars were not released by alkali or acid treatment.

Resistance of glycocalyx to hydrolysis. ND-treated residue, containing glycocalyx and residual cellulose, was not hydrolyzed by refluxing in formic acid (0.2 N or 2 N) or in 0.2 N TFA. Component monosaccharides from 2 N TFA-hydrolyzed samples were analyzed by GC/MS as their aldononitrile acetates. Glc was dominant (77.6%), along with substantial amounts of xylose (Xyl) (14.6%) and mannose (Man) (4.8%). Smaller amounts of rhamnose (Rha) (1.7%), arabinose (Ara) (0.9%), and galactose (Gal) (0.3%) were also detected. Although 2 N TFA does not hydrolyze crystalline cellulose (31), it can hydrolyze amorphous cellulose or cellulose oligomers that may be formed during microbial or enzymatic cellulose hydrolysis. To determine if the monosaccharide residues in the glycocalyx were equally accessible to TFA, the release of neutral sugars from a separate batch of ND-treated residue was quantified at different concentrations of TFA (Fig. 3). Monosaccharides were released from the residue in a linear fashion with TFA concentration. However, even at the highest TFA concentration, less than 1% of the sugars were released, indicating that the carbohydrate component of the glycocalyx represented only a small fraction of the cellulose fermentation residue. Of the sugars released, Xyl represented a higher proportion of

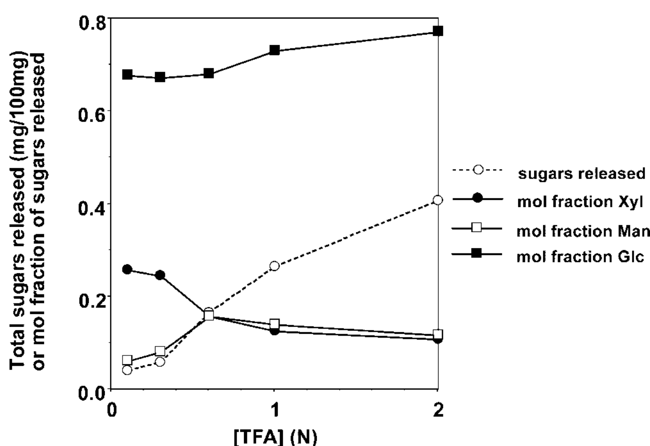

FIG. 3. Release of sugars associated with the *R. albus* 7 glycocalyx by different concentrations of trifluoroacetic acid (120°C; 90 min). The cellulose fermentation residue was first boiled in ND solution to remove bacterial cells (Fig. 1).

TABLE 2. GC/MS of permethylated aldonitrile acetates generated from ND-treated residues of *R. albus* 7 cellulose fermentation

RT (min)[a]	Designation	Deduced linkage	Integral (area units)	Normalized ratio[b]
8.1	2,3,6-MeMan	1,4	853,748	1
9.1	2,6-MeHexose	1,3,4	515,534	0.60
9.8	3,6-MeHexose	1,2,4	1,109,842	1.30
10.3	2,3-MeHexose	1,4,6	1,373,673	1.61
10.7	6-MeHexose	1,2,3,4	469,368	0.55
11.3	2-MeHexose	1,3,4,6	1,539,852	1.80
12.2	3-MeHexose	1,2,4,6	1,801,627	2.11
13.0	6-Deoxyhexose		1,413,711	1.66

[a] RT, retention time.
[b] The integrated peak areas were normalized to the unbranched 4-MeMan peak at 8.1 min.

these monosaccharides at low TFA concentrations than at high TFA concentrations, suggesting that Xyl was present either as side chains on another polysaccharide or in a polymer located closer to the outer boundary of the glycocalyx.

Monosaccharide linkages were deduced by GC-MS analysis of the permethylated aldononitriles (Table 2). A peak at 8.1 min was assigned to 4-linked Man residues. Most other peaks were designated as "hexose" because of the difficulty in distinguishing Man and Glc; these hexoses also appear to be 1,4 linked. The presence of peaks corresponding to dimethylated (9.1, 9.8, and 10.3 min) and monomethylated (10.7, 11.3, and 12.2 min) derivatives suggests multiple glycosidic branching. However, such peaks may also result from undermethylation due to the poor solubility of the polysaccharide in the permethylation reaction mixture. The smaller peaks in the 7- to 8-min range likely correspond to Xyl- and Ara-peracetylated aldononitriles, whose presence confirmed the compositional analysis. The putative Rha derivative displayed *m/z* values of 117, 129, and 231, characteristic of 6-deoxyhexoses.

Despite its insolubility in, or resistance to, several acids and solvents, the ND-treated residue was readily solubilized by sulfuric acid-catalyzed acetolysis, a procedure known to preferentially cleave 1,6-glycosidic linkages while leaving 1,2, 1,3, and 1,4 linkages intact (21). Peracetylated oligosaccharides generated by the acetolysis procedure were extracted with ethyl acetate and washed with water prior to analysis by MALDI-TOF MS. Twenty-four peaks from *m/z* 701.19 to *m/z* 7,329.47 due to $[M+Na]^+$ ions were detected in positive-ion mode, corresponding to a series of peracetylated hexose-containing oligosaccharides of degree of polymerization (DP) 2 to 25 (Fig. 4). The mass difference between these peaks (289 atomic mass units) corresponds to a mass difference of one triacetylhexose residue. Hence, these data indicate that the acid-stable core oligosaccharides are comprised of hexose sugars. The pentose (Xyl and Ara) and deoxyhexose (Rha) residues are removed by the mild acetolysis procedure, suggesting

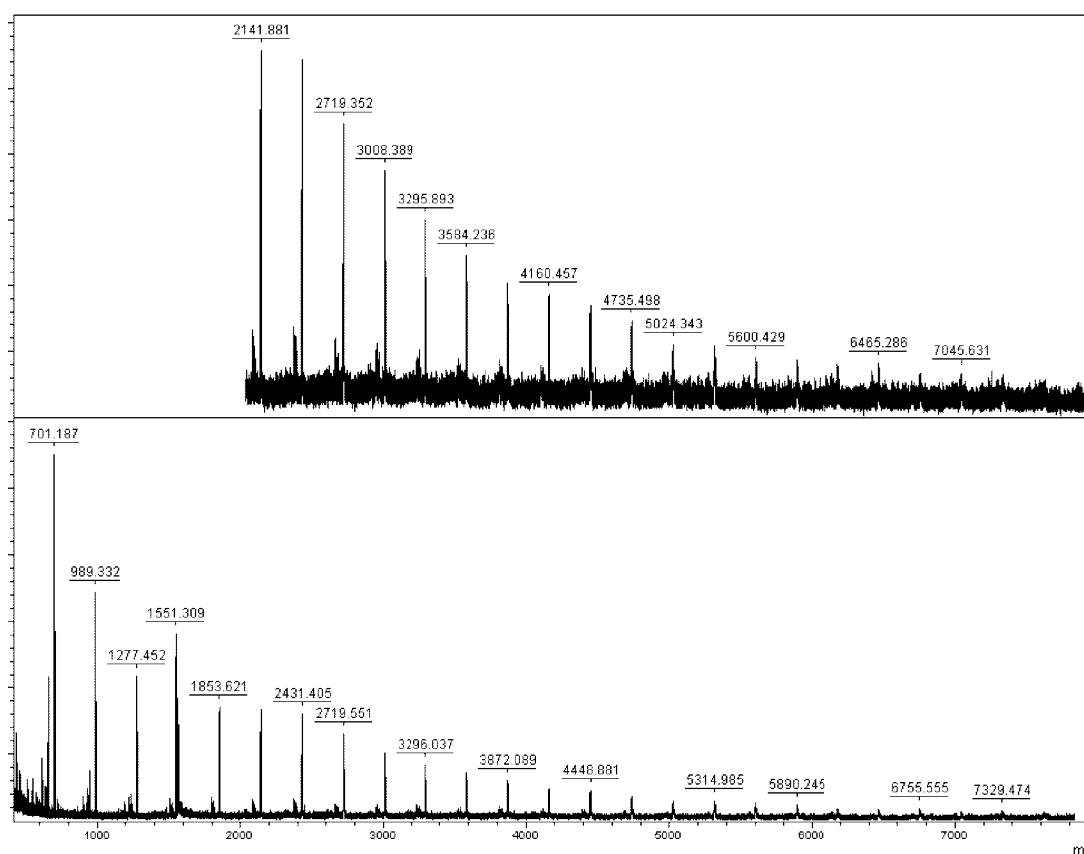

FIG. 4. MALDI-TOF MS analysis of peracetylated oligosaccharides generated by mild acetolysis of the neutral detergent fiber residue (cellulose plus glycocalyx) (Fig. 1) of the *R. albus* 7 cellulose fermentation. (Top) Expanded plotting range showing the presence of higher-DP components. (Bottom) Reduced plotting range showing dominance of low-DP components.

TABLE 3. Estimated ATP demands for biosynthesis of cellular components of *R. albus* 7, calculated from macromolecular components produced from degradation of 1 g of cellulose

Component	ATP demand (mmol ATP per g cellulose degraded)	
	Whole culture	ND-insoluble residue
Protein	5.72	0.09
Carbohydrate	0.216	0.149
Lipid	0.031	
DNA	0.042	
RNA	0.064	
Total	5.834	0.239

that they are 1,6 linked to the main hexose-containing backbone. NMR data confirmed these findings and established that both α- and β-linked glycosidic linkages are present in the hexose-containing backbone.

Biosynthetic demand. Calculations of culture composition revealed that cellulose fermentation produced 0.166 g weak-base-extractable protein, 0.023 g weak-base-extractable carbohydrate, and 0.0103 g of extracellular ND-insoluble carbohydrate per g of cellulose degraded (1.1-g hexose equivalents). Assuming similarities in intracellular composition between *R. albus* and other bacteria (46), such a conversion of cellulose to cell material should have also produced ~0.09 g of nucleic acids plus lipids. Based on this estimated yield of macromolecular components and known ATP demands for biosynthesis in bacteria (46), the anabolic cost of biosynthesis of each component can be calculated (Table 3). The energy cost of synthesizing the ND-insoluble component of the glycocalyx was estimated at ~4% (equal to 0.239/6.073) of the energy recovered from the cellulose fermentation. These calculations excluded any protein that might have been present in the glycocalyx but that would have been removed upon being boiled in ND.

DISCUSSION

Like many species of anaerobic cellulolytic bacteria, *Ruminococcus albus* attaches to a solid surface via an extracellular polymeric substance of its own making—the accepted definition of a biofilm (6). This biofilm appears to be stabilized by thin extensions of the bacterial cells (morphologically similar to the "anchor-like appendages" produced by *Acinetobacter* sp. strain Tol 5 [18]) that may be of particular importance early in growth on cellulose and by an EPS-containing material that is laid down later in the fermentation. However, the biofilm produced by *R. albus* differs in several respects from most bacterial biofilms that have been studied intensively over the past two decades. Most strikingly, *R. albus* adheres, not to an inert surface (e.g., glass or silicon nitride), but only to the growth substrate (viz., insoluble cellulose or complex plant cell wall matrices). The *R. albus* biofilm is distinguished further by the fact that the bacterial cells form a near monolayer on the degrading fiber surface, consistent with the notion that the adherent cells have direct access to the products of polysaccharide hydrolysis (25, 50). The monolayer arrangement of the cells renders certain microscopic and spectroscopic techniques, so useful in characterizing the three-dimensional structures of more conventional biofilms (24, 34), both less practical and less informative. Moreover, because the plant cell walls serve as both substrate and substratum, the products of plant cell wall hydrolysis during the course of polysaccharide degradation have the potential both to be utilized in EPS synthesis (thus contributing to EPS structure and function) and to contaminate the EPS during extraction and analysis (if they have not been previously incorporated into the EPS).

To avoid the problem of substrate contamination in analyzing EPS composition, we took advantage of the adherence of EPS to the pure cellulose substrate to separate the EPS from the culture medium and nonadherent bacteria. Boiling of the resulting solids (containing residual cellulose, adherent bacterial cells, and EPS) in neutral detergent solution to remove the adherent bacteria yielded residual cellulose with the adherent EPS. The strong adherence of the EPS to cellulose complicated EPS compositional analysis. While treatment with alkali solubilized the EPS, it is possible that some partially degraded cellulose was also extracted by this treatment. Nevertheless, the use of a pure cellulose substrate permitted the identification of any nonglucan material as intrinsic components of the EPS, rather than of the original substrate. While the detergent treatment was effective at removing adherent bacterial cells, it would also be expected to remove the vast majority of any protein associated with the EPS. Consequently, the EPS analyzed was primarily the polysaccharide fraction, and the extracellular protein content of the native glycocalyx could not be determined with accuracy due to the presence of the adherent bacteria. Analysis of the extracellular polysaccharide recovered in this fashion revealed an abundance of Man and Xyl units, with much smaller amounts of Rha, Ara, and Gal. Among gram-positive bacteria, Man has been identified as a substantial component of only a few exopolysaccharides from strains in the *Bacillus* (23, 26, 43) and *Propionibacterium* (14) lineages. By contrast, the exopolysaccharides of the lactic acid bacteria *Lactobacillus*, *Lactococcus*, and *Streptococcus*, which have received considerable study due to their food and industrial use, contain primarily Gal, Glc, and Rha (13), and Gal has been reported to be the major monosaccharide associated with the cellulosome complex of the cellulolytic anaerobic bacterium *Clostridium thermocellum* (11). The presence of Xyl in the EPS of *R. albus* is particularly interesting, in that production of this pentose from cellulose would require hexosyl decarboxylation or carbon skeleton rearrangements of the original glucan substrate. Among bacteria, Xyl has been reported as a substantial component in the EPS of only a few gram-negative species (cyanobacteria [16, 30, 35] and *Rhizobium* [7]).

While some partially degraded cellulose potentially could have been removed by TFA treatment, it did not appear that the EPS contained an abundance of β-1,4-linked Glc residues. The 1,4 linkages, based on chromatographic retention times, were restricted to Man residues, and the Glc residues instead appeared to be distributed among several hexosyl fractions having various degrees of substitution (Table 2). Undermethylation of the recalcitrant EPS could have masked the presence of short cellulose chains incorporated into the EPS, and MALDI-TOF MS indicated that hexosyl chains of up to DP 26 were detected. However, NMR spectra suggested that these fractions contained both α- and β-linkages, the former not characteristic of cellulose.

Linkage analysis (Table 2) and the lack of staining of the ND-treated residue by aniline blue suggested that the EPS did not contain significant amounts of β-1,3-glucans. Kenyon and Buller (20) demonstrated that the aerobic gram-positive cellulolytic bacterium *Cellulomonas flavigena* KU produces curdlan, a β-1,3-glucan, and that this polymer is a component of the extracellular polymeric matrix of the bacterium during growth on cellulose. Interestingly, *C. flavigena* produces considerably more curdlan when grown on cellobiose (19), under which conditions the organism grows in a purely planktonic mode and would appear to have little need for an EPS that adheres to cellulose, unless its synthesis was a specific strategy for binding to newly encountered cellulose. By contrast, Mosoni and Gaillard-Martinie (33) reported that *R. albus* 20 forms only a very thin and irregular glycocalyx during growth on cellobiose. We have not systematically examined glycocalyx production by *R. albus* 7 during growth on cellobiose, largely because the lack of a cellulose matrix did not allow application of the same recovery techniques used for cellulose-grown cells. However, we have not observed Man or Xyl residues in 80% ethanol precipitates of extracted whole-culture pellets of *R. albus* 7 cells grown on cellobiose, suggesting the lack of a glycocalyx in cells grown on soluble substrates. More definitive studies of glycocalyx synthesis on soluble substrates by *R. albus* will require not only alternative isolation procedures, but also that comparisons between cellulose- and soluble-sugar-grown cells be conducted at equivalent growth rates to remove the confounding effect of growth rate in cells batch cultured on cellulose versus cellobiose. In this regard, certain "cellulose-specific" genes (e.g., those encoding the major exoglucanase CelS and the scaffoldin protein CipA) in the phylogenetically related *Clostridium thermocellum* have been shown to be expressed during growth in cellobiose-limited chemostats at low dilution rates ($<0.06\ h^{-1}$), but not at higher dilution rates (45).

Because *R. albus* 20 has been shown to contain a glycosylated protein, GP25 (since renamed PilA1) (41), that may be involved in binding of this strain to cellulose (33), it is worth considering if the monosaccharide residues observed on acid treatment of the *R. albus* 7 cellulose fermentation were merely released from a similar glycoprotein. However, because the amount of nonglucan carbohydrate present in the cellulose fermentation residues was substantial (Table 1), and because boiling of the residue in neutral detergent solution resulted in removal of essentially all of the protein but retention of this carbohydrate, it is likely that the nonglucan carbohydrate in *R. albus* 7 is present in a form distinct from that associated with a GP25 homolog, although its exact form and distribution remain to be elucidated.

The adherence of *R. albus* to cellulose has been under intensive investigation for over a decade, but most research effort has focused on the roles of proteins, particularly the noncellulosomal processive endocellulases Cel9B and Cel48A (8), the fimbria-associated PilA1 that shows homology to the pilin proteins of gram-negative bacteria (38, 40, 41), and a variety of proteins of unknown function (28). The involvement of glycocalyces and their carbohydrate moieties in the adherence process, while assumed from earlier electron microscopic studies (6, 22, 33, 37), has received relatively little attention. Our data provide further evidence for a role of the glycocalyx in biofilm formation and stabilization by *R. albus*, as proposed by Mosoni and Gaillard-Martinie (33), who suggested that the glycocalyx strengthens adherence to cellulose in the later stages of colonization. Our study further shows that VP-SEM is a valuable tool for the visualization of biofilms in situ in a fully hydrated state, as previously reported by Ishii et al. (18). In *R. albus* 7, EPS appears to interconnect individual cells, which themselves are likely bound to cellulose via CBMs of cellulosome components and perhaps via fimbriae (27, 32). Development of EPS, CBMs, and fimbriae may contribute to the adherence of cells to cellulose by increasing the total number of cell-cellulose interactions, in much the same way as individual hydrogen bonds between cellulose chains additively stabilize the crystalline regions of cellulose microfibrils. Moreover, the EPS may provide additional points of contact and adherence among the cellulose fibers themselves, as seems likely from the avidity with which the EPS remains attached to cellulose after cells are removed from fermentation residues by boiling them in neutral detergent solution. In either case, the glycocalyx of *R. albus* potentially has a central role in optimizing the contact of cells with the cellulose fiber, a prerequisite for rapid and effective cellulose hydrolysis by this species.

ACKNOWLEDGMENTS

This research was supported by the Office of International Programs, Agricultural Research Service, U.S. Department of Agriculture, and by USDA-ARS CRIS project 3655-41000-004-00D.

We thank the Claude Leon Foundation for financial support of L.-M.J., C. L. Odt (USDA-ARS) and the University of Stellenbosch microscopy facility for technical assistance, R. D. Hatfield and L. R. Lynd for helpful discussions, and D. M. Stevenson for assistance with graphics.

Mention of a specific product does not constitute an endorsement or warranty of such product over similar products that may also be suitable.

REFERENCES

1. **Bayer, E. A., H. Chanzy, R. Lamed, and Y. Shoham.** 1998. Cellulose, cellulases and cellulosomes. Curr. Opin. Struct. Biol. **8:**548–557.
2. **Béguin, P., and P. M. Alzari.** 1998. The cellulosome of *Clostridium thermocellum*. Biochem. Soc. Trans. **26:**178–185.
3. **Blumenkrantz, N., and G. Asboe-Hansen.** 1973. New method for quantitative determination of uronic acids. Anal. Biochem. **54:**484–489.
4. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
5. **Chenu, C., and A. M. Jaunet.** 1992. Cryoscanning electron microscopy of microbial extracellular polysaccharides and their association with minerals. Scanning **14:**360–364.
6. **Costerton, J. W., K. J. Cheng, G. G. Geesey, T. I. Ladd, J. C. Nickel, M. Dasgupta, and T. J. Marrie.** 1987. Bacterial biofilms in nature and disease. Annu. Rev. Microbiol. **41:**435–464.
7. **Datta, C., and P. S. Basu.** 1999. Production of extracellular polysaccharides by a *Rhizobium* species from root nodules of *Cajanus cajan*. Acta Biotechnol. **19:**59–68.
8. **Devillard, E., D. E. Goodheart, S. K. Karnati, E. A. Bayer, R. Lamed, J. Miron, K. E. Nelson, and M. Morrison.** 2004. *Ruminococcus albus* 8 mutants defective in cellulose degradation are deficient in two processive endocellulases, Cel48A and Cel9B, both of which possess a novel modular architecture. J. Bacteriol. **186:**136–145.
9. **Dubois, M., K. A. Giles, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colorimetric method for determination of sugar and related substances. Anal. Chem. **28:**350–356.
10. **Forsyth, S. A., D. R. MacFarlane, R. J. Thomson, and M. von Itzstein.** 2002. Rapid, clean, and mild *O*-acetylation of alcohols and carbohydrates in an ionic liquid. Chem. Commun. **2002:**714–715.
11. **Gerwig, G. J., J. P. Kamerling, J. F. G. Vliegenthart, E. Morag, R. Lamed, and E. A. Bayer.** 1993. The nature of the carbohydrate-peptide linkage region in glycoproteins from the cellulosomes of *Clostridium thermocellum* and *Bacteroides cellulosolvens*. J. Biol. Chem. **268:**26956–26960.
12. **Goering, H. K., and P. J. Van Soest.** 1970. Forage fiber analysis (apparatus, reagents, procedures and some applications). Agriculture handbook 379. U.S. Department of Agriculture, Washington, D.C.

13. **Goh, K. K. T., D. R. Haisman, and H. Singh.** 2005. Development of an improved procedure for isolation and purification of exopolysaccharides produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483. Appl. Microbiol. Biotechnol. **67:**202–208.
14. **Gorret, N., C. M. G. Renard, M. H. Famelart, J. Maubois, and J. L. Doublier.** 2003. Rheological characterization of the EPS produced by *Propionibacterium acidi-propionici* on milk microfiltrate. Carbohydr. Polym. **51:** 149–158.
15. **Hakomori, S.** 1964. A rapid permethylation of glycolipid, and polysaccharide catalyzed by methylsulfinyl carbanion in dimethyl sulfoxide. J. Biochem. **55:**205–208.
16. **Huang, Z., Y. Liu, B. S. Paulsen, and D. Klavens.** 1998. Studies on polysaccharides from three edible species of *Nostoc* (Cyanobacteria) with different colony morphologies: comparison of monosaccharide compositions and viscosities of polysaccharides from field colonies and suspension cultures. J. Phycol. **34:**962–968.
17. **Hungate, R. E.** 1966. The rumen and its microbes. Academic Press, New York, N.Y.
18. **Ishii, S., J. Koki, H. Unno, and K. Hori.** 2004. Two morphological types of cell appendages on a strongly adhesive bacterium, *Acinetobacter* sp. strain Tol 5. Appl. Environ. Microbiol. **70:**5026–5029.
19. **Kenyon, W., S. Esch, and C. S. Buller.** 2005. A curdlan-type exopolysaccharide produced by *Celluomonas flavigena* KU forms part of an extracellular glycocalyx involved in cellulose degradation. Antonie Leeuwenhoek **87:**143–148.
20. **Kenyon, W. J., and C. S. Buller.** 2002. Structural analysis of the curdlan-like exopolysaccharide produced by *Cellulomonas flavigena* KU. J. Ind. Microbiol. Biotechnol. **29:**200–203.
21. **Kobayashi, H., N. Shibata, and S. Suzuki.** 1986. Acetolysis of *Pichia pastoris* IFO 0948 strain mannan containing alpha-1,2 and beta-1,2 linkages using acetolysis medium of low sulfuric acid concentration. Arch. Biochem. Biophys. **245:**494–503.
22. **Kudo, H., K. J. Cheng, and J. W. Costerton.** 1987. Electron microscopic study of the methylcellulose-mediated detachment of cellulolytic rumen bacteria from cellulose fibers. Can. J. Microbiol. **33:**267–271.
23. **Larpin, S., N. Sauvageot, V. Pichereau, J. M. Laplace, and Y. Auffray.** 2002. Biosynthesis of exopolysaccharide by a *Bacillus licheniformis* strain isolated from ropy cider. Int. J. Food Microbiol. **77:**1–9.
24. **Lawrence, J. R., G. D. W. Swerhone, G. G. Leppard, T. Araki, X. Ahang, M. M. West, and A. P. Hitchcock.** 2003. Scanning transmission X-ray, laser scanning, and transmission electron microscopy mapping of the exopolymeric matrix of microbial biofilms. Appl. Environ. Microbiol. **69:**5543–5554.
25. **Lynd, L. R., P. J. Weimer, W. H. van Zyl, and I. S. Pretorius.** 2002. Microbial cellulose utilization: fundamentals and biotechnology. Microbiol. Mol. Biol. Rev. **66:**506–577.
26. **Manca, M. C., L. Lama, R. Improta, E. Esposito, A. Gambacorta, and B. Nicolaus.** 1996. Chemical composition of two exopolysaccharides from *Bacillus thermoantarcticus*. Appl. Environ. Microbiol. **62:**3265–3269.
27. **Miron, J., D. Ben-Ghedalia, and M. Morrison.** 2001. Adhesion mechanisms of rumen cellulolytic bacteria. J. Dairy Sci. **84:**1294–1309.
28. **Miron, J., J. Jacobovitch, E. A. Bayer, R. Lamed, M. Morrison, and D. Ben-Ghedalia.** 2001. Subcellular distribution of glycanases and related components in *Ruminococcus albus* SY3 and their role in cell adhesion to cellulose. J. Appl. Microbiol. **91:**677–685.
29. **Miron, J., E. Morag, E. A. Bayer, R. Lamed, and D. Ben-Ghedalia.** 1998. An adhesion-defective mutant of *Ruminococcus albus* SY3 is impaired in its capability to degrade cellulose. J. Appl. Microbiol. **84:**249–254.
30. **Moreno, J., M. A. Vargas, J. M. Madiedo, J. Munoz, J. Rivas, and M. G. Guerrero.** 2000. Chemical and rheological properties of an extracellular polysaccharide produced by the cyanobacterium *Anabaena* sp. ATCC 33047. Biotechnol. Bioeng. **67:**283–290.
31. **Morrison, I. M., and D. Stewart.** 1998. Plant cell wall fragments released on solubilisation in trifluoroacetic acid. Phytochemistry **49:**1555–1563.
32. **Morrison, M., and J. Miron.** 2000. Adhesion to cellulose by *Ruminococcus albus*: a combination of cellulosomes and Pil-proteins? FEMS Microbiol. Lett. **185:**109–115.
33. **Mosoni, P., and B. Gaillard-Martinie.** 2001. Characteristics of a spontaneous adherence-defective mutant of *Ruminococcus albus*. Arch. Microbiol. **176:** 52–61.
34. **Neu, T. R., and J. W. Lawrence.** 1999. In situ characterization of extracellular polymeric substances (EPS) in biofilm systems, p. 21–48. *In* J. Wingender, T. R. Neu, and H.-C. Fleming (ed.), Microbial extracellular substances. Springer-Verlag, Berlin, Germany.
35. **Nicolaus, B., A. Panico, L. Lama, I. Romano, M. C. Manca, A. DeGiulio, and A. Gambacorta.** 1999. Chemical composition and production of exopolysaccharides from representative members of heterocystous and non-heterocystous cyanobacteria. Phytochemistry **52:**639–647.
36. **Ohara, H., S. Karita, T. Kimura, K. Sakka, and K. Ohmiya.** 2000. Characterization of the cellulolytic complex (cellulosome) of *Ruminococcus albus*. Biosci. Biotechnol. Biochem. **64:**254–260.
37. **Patterson, H., R. Irvin, J. W. Costerton, and K.-J. Cheng.** 1975. Ultrastructural and adhesion properties of *Ruminococcus albus*. J. Bacteriol. **122:**278–287.
38. **Pegden, R. S., M. A. Larson, R. J. Grant, and M. Morrison.** 1998. Adherence of the gram-positive bacterium *Ruminococcus albus* to cellulose and identification of a novel form of cellulose-binding protein which belongs to the Pil family of proteins. J. Bacteriol. **180:**5921–5927.
39. **Price, N. P. J.** 2004. Acyclic sugar derivatives for GC/MS analysis of ^{13}C-enrichment during carbohydrate metabolism. Anal. Chem. **76:**6566–6574.
40. **Rakotoarivonina, H., G. Jubelin, M. Hebraud, V. Gaillard-Martinie, E. Forano, and P. Mosoni.** 2002. Adhesion to cellulose of the Gram-positive bacterium *Ruminococcus albus* involves type IV pili. Microbiology **148:**1871–1880.
41. **Rakotoarivonina, H., M. A. Larson, M. Morrison, J.-P. Girardeau, B. Gaillard-Martinie, E. Forano, and P. Mosoni.** 2005. The *Ruminococcus albus pilA1-pilA2* locus: expression and putative role of two adjacent *pil* genes in pilus formation and bacterial adhesion to cellulose. Microbiology **151:**1291–1299.
42. **Sandford, P. A., A. J. Nofziger, and A. Jeanes.** 1971. Reaction of sodium hypochlorite with amines and amides: a new method of quantitating polysaccharides containing hexosamines. Anal. Biochem. **44:**111–121.
43. **Seo, W. T., G. G. Kahng, S. H. Nam, S. D. Choi, H. H. Suh, S. W. Kim, and Y. H. Park.** 1999. Isolation and characterization of a novel exopolysaccharide-producing *Paenibacillus* sp. WN9 KCTC 8951P. J. Microbiol. Biotechnol. **9:**820–825.
44. **Stack, R. J., and R. E. Hungate.** 1984. Effect of 3-phenylpropanoic acid on capsule and cellulases of *Ruminococcus albus* 8. Appl. Environ. Microbiol. **48:**218–223.
45. **Stevenson, D. M., and P. J. Weimer.** 2005. Expression of 17 genes in *Clostridium thermocellum* during fermentation of cellulose or cellobiose in continuous culture. Appl. Environ. Microbiol. **71:**4672–4678.
46. **Stouthamer, A. H.** 1973. A theoretical study on the amount of ATP required for synthesis of microbial cell material. Antonie Leeuwenhoek **39:**545–565.
47. **Sutherland, I. W.** 2001. The biofilm matrix—an immobilised but dynamic microbial environment. Trends Microbiol. **9:**222–227.
48. **Van Gylswyk, N. O.** 1970. The effect of substituting a low protein hay on the cellulolytic bacteria in the rumen of sheep and on the digestibility of cellulose and hemicellulose. J. Agric. Soc. **74:**169–180.
49. **Weimer, P. J., A. H. Conner, and L. F. Lorenz.** 2003. Solid residues of *Ruminococcus* cellulose fermentations as components of wood adhesive formulations. Appl. Microbiol. Biotechnol. **63:**29–34.
50. **Weimer, P. J., and C. L. Odt.** 1995. Cellulose degradation by ruminal microbes: physiological and hydrolytic diversity among ruminal cellulolytic bacteria. Am. Chem. Soc. Symp. Ser. **618:**291–304.
51. **Weimer, P. J., G. C. Waghorn, C. L. Odt, and D. R. Mertens.** 1999. Effect of diet on populations of three species of ruminal cellulolytic bacteria in lactating dairy cows. J. Dairy Sci. **82:**122–134.
52. **Xu, Q., M. Morrison, E. A. Bayer, N. Atamna, and R. Lamed.** 2004. A novel family of carbohydrate-binding modules identified with *Ruminococcus albus* proteins. FEBS Lett. **566:**11–16.