Isolation and Identification of Cellulose-Binding Proteins from Sheep Rumen Contents[▽]

Atsushi Toyoda,^{1,2*} Wataru Iio,¹ Makoto Mitsumori,³ and Hajime Minato¹

College of Agriculture, Ibaraki University, Ami, Ibaraki 300-0393, Japan¹; United Graduate School of Agricultural Science, Tokyo University of Agriculture and Technology, Fuchu City, Tokyo 183-8509, Japan²; and National Institute of Livestock and Grassland Sciences, Tsukuba, Ibaraki 305-0901, Japan³

Received 8 August 2008/Accepted 6 January 2009

To extend our understanding of the mechanisms of plant cell wall degradation in the rumen, cellulose-binding proteins (CBPs) from the contents of a sheep rumen were directly isolated and identified using a metaproteomics approach. The rumen CBPs were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and some CBPs revealed endoglucanase activities toward carboxymethyl cellulose. Using mass spectrometry analyses, four CBPs were identified and annotated as known proteins from the predominant rumen cellulolytic bacterium *Fibrobacter succinogenes*: tetratricopeptide repeat domain protein, OmpA family protein, fibro-slime domain protein, and cellulose-binding endoglucanase F (EGF). Another CBP was identified as the cellulosomal glycosyl hydrolase family 6 exoglucanase, Cel6A, of *Piromyces equi*. *F. succinogenes* cells expressing EGF were found to be major members of the bacterial community on the surface or at the inner surface of hay stems by immunohistochemical analyses using anti-EGF antibody. The finding that four of the five CBPs isolated and identified from sheep rumen contents were from *F. succinogenes* indicates that *F. succinogenes* is significantly involved in cellulose degradation in the rumen.

Efficient plant cell wall degradation depends on cooperation between microorganisms that produce fibrolytic enzymes and the ruminant, which provides an anaerobic fermentation chamber. These microorganisms include symbiotic cellulolytic bacteria, fungi, and protozoa, which are able to digest cellulosic plant materials and produce energy for the host animal. Many fibrolytic enzymes have been isolated from rumen microorganisms, and the genes encoding these enzymes have been cloned and sequenced (3, 10, 20, 21, 30, 34, 38, 41). However, the precise mechanisms of lignocellulose degradation in the rumen microbial ecosystem are not yet fully understood. In order to extend our understanding of the mechanisms of lignocellulose degradation in the rumen, it is necessary to study fiber digestion in the rumen biochemically, genetically, and proteomically.

Fibrobacter succinogenes, *Ruminococcus flavefaciens*, and *Ruminococcus albus* are considered to be the predominant cellulolytic bacteria present in the rumen (24–26, 48, 50). Transmission electron microscopy observations of plant fibers digested by rumen microbes have shown that *F. succinogenes*- or *R. flavefaciens*-like bacteria are distributed over materials such as fescue and orchard grass and that sometimes these bacteria account for more than 70% of fiber-associated bacteria (1, 6). It is generally accepted that *F. succinogenes* contributes greatly to fiber digestion, given that this species has a potent ability to solubilize crystalline cellulose (14, 47). In contrast, when the proportions of certain species in the rumen microbial population were examined using species-specific hybridization probes, *F. succinogenes* and *R. flavefaciens* ac-

counted for 0.1 to 6.6% and 1.3 to 2.9% of total rumen bacteria, respectively (5, 26, 29, 47). Furthermore, in an analysis of fiber-associated rumen bacteria based on a 16S rRNA gene clone library, only a few clones belonging to *F. succinogenes* or *R. flavefaciens* were obtained although other species and uncultured bacteria were frequently detected (24, 50). Molecular cloning and probing techniques have provided us with the ability to detect single cells and have revealed the microbial diversity of the rumen (7, 9, 44, 49, 58). Wilmes and Bond (54) proposed the term “metaproteomics” to describe the characterization of the entire protein component at the microbial community level. To improve our knowledge of plant cell wall degradation in the rumen, metaproteomic research promises to provide information on gene expression in the rumen microbial community. To the best of our knowledge, there have been two reports to date on the use of metaproteomics to characterize complex microbial ecosystems, illustrating the feasibility of this approach in analyzing such microbial ecosystems as a natural acid mine drainage microbial biofilm community (43) and an activated sludge microbial system (54).

The mechanism involved in the adhesion and digestion of plant cell walls by *F. succinogenes* has been studied extensively, and five endoglucanases (30, 32–34), one cellobiosidase (16), one celldextrinase (17), four xylanases (20, 57), two acetyl xylan esterases (23), and two cellulose-binding proteins (CBPs) (13) have been purified or cloned, and the catalytic properties of the enzymes have been determined. Although these studies have provided valuable information about many of the cellulases and hemicellulases of *F. succinogenes*, the actual contribution of these enzymes to the degradation of feedstuff in the rumen remains unclear. Some fibrolytic enzymes possess carbohydrate-binding modules (CBMs), which play an important role in assisting the enzymes to hydrolyze cellulosic materials (2, 56). Additionally, the adhesion of bacterial cells to cellulose

* Corresponding author. Mailing address: College of Agriculture, Ibaraki University, Ami, Ibaraki 300-0393, Japan. Phone and fax: 81 29 888 8584. E-mail: atoyoda@mx.ibaraki.ac.jp.

[▽] Published ahead of print on 16 January 2009.

appears to be a prerequisite for efficient cellulose hydrolysis by bacteria (27). In the present study, we used a metaproteomic analysis to focus on the identification of CBPs in the rumen contents in order to extend our knowledge of the fibrolytic systems in the rumen.

The objectives of this study were the following: (i) to isolate CBPs directly from the sheep rumen contents, (ii) to identify and annotate the rumen CBPs using liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS), and (iii) to reveal the localization of cellulose-binding endoglucanase F (EGF) of *F. succinogenes*, one of the rumen CBPs, in rumen fibrous materials.

MATERIALS AND METHODS

Rumen samples. A ruminally fistulated female sheep weighing 47.0 kg was used as a sample donor. The sheep was fed 260 g of timothy, 110 g of alfalfa hay cube, and 95 g of concentrate twice daily at 0900 h and 1600 h and had free access to water and a mineral block. The sheep was habituated to the feeds for 60 days prior to sampling. Rumen contents were obtained through a rumen fistula prior to feeding at 0800 h. Every effort was made to collect representative samples by mixing the whole-rumen contents. The rumen contents were immediately transferred to the laboratory and treated as described below.

Isolation of CBPs from rumen samples. Rumen CBPs were prepared from 1.7-liter volumes of rumen contents. Rumen contents were diluted with an equal volume of ice-cold phosphate-buffered saline (PBS) including protease inhibitors and minced in a blender (MX2050; Braun, Yokohama, Japan) 10 times at 4°C for 10 s each time. The minced rumen contents were solubilized with Triton X-100 at a final concentration of 1% and incubated at 4°C for 5 h with agitation. The solubilized rumen contents were centrifuged at $10,000 \times g$ for 10 min (Avanti HP-25; Beckman Coulter, Fullerton, CA). The supernatant was subjected to ultracentrifugation (Optima XL-80K; Beckman Coulter) at $100,000 \times g$ for 30 min and was then collected. When such an amorphous cellulose as acid-swollen cellulose (ASC) is used as a substratum for binding CBPs, contamination of the CBPs by nonspecific proteins occurs. Therefore, we used Avicel crystalline cellulose as a substratum for binding CBPs in the present study. Avicel cellulose PH-101 (8.5 g) was added to the supernatant, and the mixture was incubated at room temperature overnight with agitation. After incubation, the Avicel cellulose was sedimented and washed six times with PBS. The proteins (CBPs) bound to the Avicel cellulose were eluted with 50 ml of 8 M urea, dialyzed against water, and concentrated using an Amicon Ultra-15 device (Millipore, Tokyo, Japan). CBPs were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The detection of carboxymethyl cellulase (CMCase) activities was carried out as described previously (51, 52). In a preliminary experiment, CBPs aggregated after the removal of Triton X-100 by dialysis against water or by treatment with Bio-Beads SM-2 (Bio-Rad, Tokyo, Japan), and Triton X-100 did not affect the binding ability of CBPs to cellulose. The binding of CBPs to Avicel cellulose was thus performed in the presence of Triton X-100.

LC-ESI-MS/MS and the identification of proteins. Proteins were identified as previously described by Shevchenko et al. (45). CBPs (20 µg) were concentrated using a two-dimensional clean-up kit (GE Healthcare, Piscataway, NJ). Precipitate was dissolved in SDS sample buffer and subjected to SDS-PAGE. After Coomassie brilliant blue staining, the SDS-PAGE gel was cut into 30 pieces (sections) according to molecular weight. Separated proteins stained with Coomassie brilliant blue were excised from the gel, cut into pieces, destained twice with a solution containing 50 mM NH_4HCO_3 in 50% (vol/vol) acetonitrile, and dehydrated in 100% acetonitrile. Reduction of the protein samples was achieved with 100 µl of 10 mM dithiothreitol in 100 mM NH_4HCO_3 for 1 h at 56°C, and alkylation was then performed with 100 µl of 55 mM iodoacetamide in 100 mM NH_4HCO_3 for 45 min at room temperature in the dark. Next, the gel pieces were washed twice for 5 min alternately with 100 mM NH_4HCO_3 and acetonitrile and then completely dried under reduced pressure. The dried gel pieces were rehydrated in appropriate volumes of trypsin solution (25 µg trypsin/ml and 5 mM CaCl_2 in 50 mM NH_4HCO_3) at 4°C for 10 min, followed by removal of the excess trypsin solution. The rehydrated gel pieces were covered with 50 µl of 50 mM NH_4HCO_3 and incubated at 37°C overnight. After incubation, the supernatant containing digested peptides was transferred to a new tube. The gel pieces were washed first with 20 µl of 20 mM NH_4HCO_3 and 20 µl of 50% (vol/vol) acetonitrile in 5% (vol/vol) formic acid. The supernatants from each extraction were pooled in the new tube, and the combined supernatant was concentrated to 10 µl

under reduced pressure. The concentrated solution containing peptides was mixed with 10 µl of 0.1% trifluoroacetic acid, and the resultant solution was analyzed by LC-ESI-MS/MS (LCQ Deca XP; Thermo Fisher Scientific K.K., Yokohama, Japan) in positive ion mode. The spectrum data were submitted for protein identification, and a database search was carried out using MASCOT, version 1.9 (Matrix Science, Ltd., London, United Kingdom) in the NCBI non-redundant database. The search parameters were the following: all entries as taxonomy; enzyme, trypsin; one missed cleavage allowed; variable modification of carbamidomethyl (cysteine); oxidation (methionine); peptide tolerance, 2.0 Da; MS/MS tolerance, 0.8 Da; peptide charges, 1⁺, 2⁺ and 3⁺; monoisotopic. Only significant hits as defined by the MASCOT probability analysis ($P < 0.05$) were accepted.

Western immunoblotting analysis. Western immunoblotting was performed as previously described (51) except that polyclonal antiserum against EGF of *F. succinogenes* S85 (1:1,000 dilution) was used as the primary antibody (38). The secondary antibody and detection were as described previously (51).

Fixation. To fix the rumen samples, sequential fixation was performed using 3% paraformaldehyde-PBS solution followed by PBS–96% ethanol (1:1 [vol/vol]) with different incubation times, as recommended for gram-positive bacteria (46). When the fixative solution was changed, the tubes were centrifuged at $200 \times g$ for 3 min, and the supernatant was carefully removed with a pipette. The fixed samples were stored at –20°C until observation, which occurred within 3 days. The fixed samples were spread on coated slides (Superfrost; Matsunami, Osaka, Japan), which were then air dried at room temperature.

Immunohistochemistry. The fixed rumen samples on the slides were incubated with PBS containing 5% bovine serum albumin for 1 h at room temperature. After incubation, the samples were incubated for 12 h with the rabbit anti-EGF antibody. The samples were washed with PBS and incubated for 12 h with fluorescence-conjugated anti-rabbit antibody (Alexa488; Molecular Probes, Inc., Eugene, OR). After incubation, the samples were washed again with PBS. To reduce the autofluorescence of the plant material, 400 µl of toluidine blue O (Division Chroma) (0.05% [wt/vol]) in sterilized distilled water with 0.9 M NaCl) was added to the slide samples. The samples were dyed with toluidine blue O for 15 min at room temperature and then rinsed in distilled water until the water became clear. After being air dried, the samples were incubated in 99.5% ethanol for 1.5 min to remove the dye from the bacterial cells but not from the plant material and were then immediately washed with distilled water. Total bacteria were visualized by staining with Vectashield H-1200 mounting medium (Vector Laboratories, Inc., Burlingame, CA) containing 4',6'-diamidino-2-phenylindole (DAPI; 1.5 µg/ml). For microscopic observation of the bacteria and their fluorescence signals, we used a microscope (IX71; Olympus, Tokyo, Japan) and cooled charge-coupled-device camera (Penguin 600CL; Pixera Corporation, San Jose, CA). Images were processed with Adobe Photoshop, version 6.0, and Microsoft Power Point, version 2003.

RESULTS

Isolation and detection of CBPs from rumen contents. To investigate cellulose degradation in the rumen, we attempted to isolate CBPs from rumen contents. Solubilized rumen contents were centrifuged, and the supernatant was mixed with Avicel cellulose. The proteins bound to the Avicel cellulose were eluted and pooled as CBPs. The profile of rumen CBPs is shown in Fig. 1A. The CBPs were composed of proteins having various molecular masses. Two proteins of approximately 170 kDa were prominent and were detected repeatedly. We observed the CMCase activity of rumen CBPs by spotting CBPs into agarose gel containing carboxymethyl cellulose (CMC). As shown in Fig. 1B, rumen CBPs revealed CMCase activity. We also performed zymogram analysis of CMCase activity in the rumen CBPs. As shown in Fig. 1C, when the rumen CBPs were eluted from Avicel at 40°C, broad CMCase activities by several enzymes were detected although most of these activities were eliminated by incubating the rumen CBPs for elution from Avicel at 95°C.

Identification of CBPs by LC-ESI-MS/MS. To identify and annotate the rumen CBPs, we first separated the CBPs by SDS-PAGE. After protein separation, the SDS-PAGE gel was

FIG. 1. Detection of rumen CBPs and their endoglucanase activities. (A) Proteins bound to Avicel cellulose were eluted and separated by SDS-PAGE. Lane M, molecular mass standards. (B) Proteins were stained with silver. CBPs were spotted on agarose gel containing CMC. (C) After incubation, the agarose gel was stained with 0.1% Congo red and destained with 0.1 M sodium chloride. Zymogram analysis of CBPs for endoglucanase was performed. Proteins bound to Avicel cellulose were eluted with 8 M urea, incubated at 40°C or 95°C, and separated by SDS-PAGE gels containing 0.1% CMC. After incubation, gels were stained with 0.1% Congo red and destained with 0.1 M sodium chloride. CBPs were eluted at 95°C and 40°C, as indicated. Lanes HM and LM, molecular mass standards.

processed as described above, and the resultant peptide mixtures from each gel piece were subjected to LC-ESI-MS/MS. A total of five proteins were identified (Table 1). The rumen CBPs included four CBPs of *F. succinogenes* S85: a tetratricopeptide repeat (TPR) domain protein, an OmpA family protein, a Fibro-slime domain protein, and EGF. We also identified the cellulosomal glycoside hydrolase family 6 (GH6) exoglucanase, Cel6A, of *Piromyces equi*. The domain structures of these CBPs are shown in Fig. 2. The TPR domain protein of *F. succinogenes* S85 possessed an N-terminal domain belonging to toluene X, a middle domain of TPR, and a C-terminal domain of unknown function (PRK10049 domain; predicted outer membrane protein domain registered in the NCBI conserved domain database). The OmpA family protein of *F. succinogenes* S85 included an N-terminal domain of pectinesterase and a C-terminal domain of OmpA. The Fibro-slime domain protein of *F. succinogenes* S85 contained a Fibro-slime domain at the middle region, and the EGF of *F. succinogenes* S85 had two CBMs belonging to families 30 and 11 (CBM30 and CBM11, respectively) and one GH51 catalytic module. Cel6A of *P. equi* is composed of an N terminus, two N-terminal

CBM10s, and one C-terminal catalytic module of GH6. The amino acid sequences of all of the corresponding peptides (13 to 19 amino acid residues) were 100% identical to their matching *F. succinogenes* S85 genomic sequences (data not shown). Although the same protein was identified from different gel sections, this may be due to the analysis of the degradation products from the same protein by proteases present in the rumen (40).

Detection of EGF of *F. succinogenes* in rumen contents. To confirm the existence of *F. succinogenes* and EGF in fibrous materials in the rumen, we performed immunoblot analysis and immunohistochemistry using anti-EGF antibody. As shown in Fig. 3A, EGF was detected in the present rumen CBPs. This result is consistent with the protein identification data obtained by LC-ESI-MS/MS. Additionally, EGF-expressing bacteria were observed in the rumen contents (Fig. 3B). These EGF signals localized on the surface or at the inner surface of plant fibrous materials. Furthermore, DAPI staining revealed many microbes attached to the surface of plant materials from rumen contents, as previously reported by Shinkai and Kobayashi (46).

TABLE 1. Identification of CBPs from rumen-soluble fractions by LC-ESI-MS/MS

SDS-PAGE gel section no. ^a	Accession no.	Protein name or description	Organism	MW ^b	pI ^b
3, 5, 6, 8, 11, 12	gi 156072342	TPR domain protein	<i>F. succinogenes</i>	83,900	5.51
6, 7	gi 156072350	Fibro-slime domain protein	<i>F. succinogenes</i>	170,752	5
7, 15, 16	gi 156072340	OmpA family protein	<i>F. succinogenes</i>	56,567	4.86
8, 9	gi 1620001	Cellulose binding protein (EGF)	<i>F. succinogenes</i>	118,828	7.81
8	gi 33620325	Cellulosomal GH6, exoglucanase Cel6A	<i>P. equi</i>	54,555	4.86

^a Sections 5, 10, 11, 14, 19, and 25 approximately correspond to 200, 116, 97, 66, 45, and 30 kDa, respectively.

^b Values are theoretical. MW, molecular weight.

FIG. 2. Domain structures of CBPs isolated and identified from sheep rumen contents.

DISCUSSION

To date, various cellulolytic microbes have been isolated from the rumen of ruminants, and many cellulolytic enzymes and genes encoding the enzymes from these rumen microbes have been characterized (3, 10, 13, 14, 16, 17, 20, 23, 30, 32–34, 38, 41, 51, 52, 57). The present work was carried out, first, to extend studies on the digestion of plant cell walls in the rumen by directly isolating CBPs from the rumen contents of sheep

and, second, to identify and annotate these proteins using LC-ESI-MS/MS.

When the rumen CBPs were separated by SDS-PAGE, they were found to consist of various proteins, including two deeply stained proteins with molecular masses of approximately 170 kDa (Fig. 1A). The CBPs in sections 6 and 7 of the SDS-PAGE gel of rumen CBPs were analyzed by LC-ESI-MS/MS and identified as a Fibro-slime domain protein of *F. succinogenes* with a similar molecular mass (Table 1). Jun et al. (21) reported that the Fibro-slime domain protein of *F. succinogenes* was a CBP and named it CBP-2. CBP-2 was also identified as the 180-kDa cellulose-binding glycoprotein described by Gong et al. (13) and was annotated as the Fibro-slime domain protein from analysis of the N terminus and internal amino acid sequence (21). Thus, the present 170-kDa CBP seems to correspond to the 180-kDa CBP described by Gong et al. (13). The Fibro-slime domain is present in nine other putative proteins from *F. succinogenes*, at least one putative protein from *F. intestinalis* (42), and several hypothetical proteins from the slime mold *Dictyostelium discoideum* (8) as well as some proteobacteria. Interestingly, these proteins had no similarity in regions other than the Fibro-slime domain. The Fibro-slime protein was one of the dominant proteins in the present rumen CBPs (Fig. 1A). Therefore, the protein of *F. succinogenes* S85 may be importantly involved in the adhesion of the bacterium to cellulose in the rumen. Nevertheless, the role of the Fibro-slime protein in this adhesion and in the digestion of cellulose in the rumen remains obscure.

The TPR domain proteins of *F. succinogenes* have been described previously (21); however, to the best of our knowledge, the type of TPR domain protein identified in the present study (Table 1) has not been previously described. The TPR domain protein consists of a toluene X domain, a TPR domain,

FIG. 3. Detection of EGF of *F. succinogenes* in the rumen CBPs (A) and on the rumen fibrous materials (B). The arrowhead in panel A indicates EGF. The top images in panel B show rumen contents that were incubated with anti-EGF antibody or the preimmune serum. Bacteria attached to the fibrous rumen contents are shown in green. The lower six panels show rumen contents as observed by phase-contrast or fluorescence microscopy. Arrowheads indicate that EGF signals associated with fibrous materials. All bacteria were stained with DAPI (blue). Bars, 50 μ m (B, top) and 10 μ m (B, bottom).

and a predicted outer membrane protein domain (Fig. 2), as does the *F. succinogenes* S85 TPR domain protein identified in the present study (Fig. 2). Of the CBPs identified as *F. succinogenes* S85 TPR domain proteins by Jun et al. (21) (CBP-3 and CBP-7), CBP-7 was identical in molecular mass and isoelectric point to the TPR domain protein identified in the present study; however, with nine consecutive TPRs, it was different in domain structure from our TPR domain protein, which had only 2.75 consecutive TPRs. The TPR is a 34-amino-acid repeated motif (11) that frequently forms tandem arrays (28). Since CBP-7 was present only in the parent *F. succinogenes* S85 strain and was absent from the adhesion-defective mutant Ad4, the TPR motifs of CBP-7 and the present TPR domain protein may be involved in the adhesion of the bacterium to cellulose. Further research is required to determine whether this assumption is correct. Additionally, the toluene X domain has been discovered in the outer membrane proteins and transport proteins (TbuX/TodX/FadL) from bacteria (22, 42). TbuX seems to play an important role in the catabolism of toluene in *Ralstonia pickettii* PKO1 (22). An apparent function of TodX is likely to be involved in facilitating the delivery of exogenous toluene inside the cells of *Pseudomonas putida* F1 (53). FadL is known to be required for the binding and transport of long-chain fatty acids in *E. coli* (4). However, the function of the toluene X domain in the TPR domain protein of *F. succinogenes* S85 remains unknown.

One CBP from the present sheep rumen content was identified as an OmpA family protein of *F. succinogenes* (Table 1). The OmpA family protein has multiple domains, including an N-terminal pectinesterase domain and a C-terminal OmpA-like domain. Another OmpA family protein of *F. succinogenes* was identified as CBP-8 (21), which has multiple domains, including four parallel β -helix repeats (19) and a C-terminal OmpA-like domain. Additionally, although pectinesterase is also produced by *Butyrivibrio fibrisolvens* (55), the OmpA family protein identified in the present study may be one of the components necessary for plant cell wall degradation, including pectinolysis, in the rumen. It remains to determine the location of the CBM of the OmpA family protein from *F. succinogenes*.

One CBP from the present sheep rumen content was identified as Cel6A of *P. equi* (Table 1). It had multiple domains, including CBM10 and GH6 (15). *P. equi* was originally discovered in the equine cecum (12); however, the present study suggests the possibility that *P. equi* may be a rumen cellulolytic fungus.

Some of the rumen CBPs exhibited CMCase activities (Fig. 1B and C), which were broadly detected in the zymogram gel (Fig. 1C). However, only one endoglucanase of *F. succinogenes*, EGF (approximately 120 kDa), was identified among the rumen CBPs (Table 1). Therefore, other endoglucanase activities may be derived from novel proteins in the rumen. In our previous study, EGF was isolated as one of the crystalline cellulose-binding proteins from *F. succinogenes* S85 culture lysate (36, 37), and EGF and endoglucanase 2 (EG2) were found to be encoded by the same gene, *celF* (31–33). The domain structure of EGF has been well researched; it consists of CBM30, CBM11, and GH51 catalytic modules (21, 38). Additionally, EGF located in the outer membrane of *F. succinogenes* binds to the ASC (21). EGF exhibits a higher

activity against ASC than crystalline cellulose and may be one of the components involved in the adhesion of *F. succinogenes* S85 to amorphous cellulose (21). Furthermore, among the seven endoglucanases in *F. succinogenes* S85, only EGF and endoglucanase G have CBMs (18). Since it is known that the removal of CBMs from endoglucanase remarkably reduces catalytic activities with various polysaccharides, endoglucanases with CBMs may play an important role in the digestion of cellulosic materials in the rumen (2, 56).

The present immunohistochemical analysis using anti-EGF antibody showed that EGF was present in both the solid and liquid portions of rumen contents (Fig. 3B). The fact that EGF was identified in many sections of the SDS-PAGE gel (Table 1) is consistent with the finding that cell-free cellulases are more digestible than cell-associated cellulases in the rumen (40). Additionally, *F. succinogenes* cells expressing EGF were found to be a major component of the bacterial community on the surface or at the inner surface of hay stems (Fig. 3B). This finding supports the report by Shinkai and Kobayashi (46) that *F. succinogenes* cells were detected on many stem and leaf sheath fragments of hay, while *R. flavefaciens* cells were rarely detected on stem fragments. Although a dense microbial population was observed on stem fragments (Fig. 3B), the development of this microbial community on hay may be due to the juxtapositional adhesion to hay of microbes utilizing intermediary metabolites with cellulolytic bacteria, the attraction of physiologically related microbes to the adherent cellulolytic bacteria, and the subsequent growth of these microbes (6, 35). Therefore, *F. succinogenes* cells may be importantly involved in the digestion of hay in the rumen.

To the best of our knowledge, the present metaproteomic study is the first report on rumen CBPs from sheep rumen contents. However, because the numbers of identified CBPs were limited, further research is required to isolate protein spots of rumen CBPs individually using two-dimensional electrophoresis and to determine the amino acid sequences of the CBPs in order to isolate the gene encoding these rumen CBPs.

ACKNOWLEDGMENTS

We are grateful to T. Shinkai (National Institute of Livestock and Grassland Sciences, Tsukuba, Japan) for instruction on immunohistochemical techniques. We thank S. Watanabe and M. Hashimoto (Hitachi High-Tech Science Systems Corporation Proteome Analysis Laboratory) for operating the LC-ESI-MS/MS instrument and analyzing MS/MS spectra.

This work was supported in part by Grant-in-Aid for Encouragement of Young Scientists (no. 18688015) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

REFERENCES

1. Akin, D. E. 1980. Evaluation by electron microscopy and anaerobic culture of types of rumen bacteria associated with digestion of forage cell walls. *Appl. Environ. Microbiol.* **39**:242–252.
2. Arai, T., R. Arai, A. Tanaka, S. Karita, T. Kimura, K. Sakka, and K. Ohmiya. 2003. Characterization of a cellulase containing a family 30 carbohydrate-binding module (CBM) derived from *Clostridium thermocellum* CelJ: importance of the CBM to cellulose hydrolysis. *J. Bacteriol.* **185**:504–512.
3. Barros, M. E., and J. A. Thomson. 1987. Cloning and expression in *Escherichia coli* of a cellulase gene from *Ruminococcus flavefaciens*. *J. Bacteriol.* **169**:1760–1762.
4. Black, P. N. 1991. Primary sequence of the *Escherichia coli* *fadL* gene encoding an outer membrane protein required for long-chain fatty acid transport. *J. Bacteriol.* **173**:435–442.
5. Briesacher, S. L., T. May, K. N. Grigsby, M. S. Kerley, R. V. Anthony, and J. A. Paterson. 1992. Use of DNA probes to monitor nutritional effects on

- ruminal prokaryotes and *Fibrobacter succinogenes* S85. *J. Anim. Sci.* **70**:289–295.
6. Cheng, K.-J., C. S. Stewart, D. Dinsdale, and J. W. Costerton. 1983. /1984. Electron microscopy of bacteria involved in the digestion of plant cell walls. *Anim. Feed Sci. Technol.* **10**:93–120.
 7. Eckburg, P. B., E. M. Bik, C. N. Bernstein, E. Purdom, L. Dethlefsen, M. Sargent, S. R. Gill, K. E. Nelson, and D. A. Relman. 2005. Diversity of the human intestinal microbial flora. *Science* **308**:1635–1638.
 8. Eichinger, L., et al. 2005. The genome of the social amoeba *Dictyostelium discoideum*. *Nature* **435**:43–57.
 9. Favier, C. F., E. E. Vaughan, W. M. de Vos, and A. D. L. Akkermans. 2002. Molecular monitoring of succession of bacterial communities in human neonates. *Appl. Environ. Microbiol.* **68**:219–226.
 10. Forsberg, C. W., J. Gong, L. M. Malburg, Jr., H. Zue, A. Iyo, K.-J. Cheng, and P. J. Krell. 1993. Cellulases and hemicellulases of *Fibrobacter succinogenes* and their role in fiber digestion, p. 125–136. In K. Shimada, K. Ohmiya, Y. Kobayashi, S. Hoshino, K. Sakka, and S. Karita (ed.), *Genetics, biochemistry and ecology of lignocellulose degradation*. Uni Publishers, Tokyo, Japan.
 11. Goebel, M., and M. Yanagida. 1991. The TPR snap helix: a novel protein repeat motif from mitosis to transcription. *Trends Biochem. Sci.* **16**:173–177.
 12. Gold, J. J., Heath, I. B., and T. Bauchop. 1988. Ultrastructural description of a new chytrid genus of caecum anaerobe, *Caecomyces equi* gen. nov., sp. nov., assigned to the *Neocallimastixaceae*. *Biosystems* **21**:403–415.
 13. Gong, J., E. E. Egbosimba, and C. W. Forsberg. 1996. Cellulose-binding proteins of *Fibrobacter succinogenes* and the possible role of a 180 kDa cellulose-binding glycoprotein in adhesion to cellulose. *Can. J. Microbiol.* **42**:453–460.
 14. Halliwell, G., and M. P. Bryant. 1963. The cellulolytic activity of pure strains of bacteria from the rumen of cattle. *J. Gen. Microbiol.* **32**:441–448.
 15. Harhangi, H. R., A. C. Freelove, W. Ubhayasekera, M. van Dinther, P. J. Steenbakkers, A. Akhmanova, C. van der Drift, M. S. Jetten, S. L. Mowbray, H. J. Gilbert, and H. J. Op den Camp. 2003. Cel6A, a major exoglucanase from the cellulosome of the anaerobic fungi *Piromyces* sp. E2 and *Piromyces equi*. *Biochim. Biophys. Acta* **1628**:30–39.
 16. Huang, L., C. W. Forsberg, and D. Y. Thomas. 1988. Purification and characterization of a chloride-stimulated cellobiosidase from *Bacteroides succinogenes* S85. *J. Bacteriol.* **170**:2923–2932.
 17. Huang, L., and C. W. Forsberg. 1988. Purification and comparison of the periplasmic and extracellular forms of the cellobiosidase from *Bacteroides succinogenes*. *Appl. Environ. Microbiol.* **54**:1488–1493.
 18. Iyo, A. H., and C. W. Forsberg. 1996. Endoglucanase G from *Fibrobacter succinogenes* S85 belongs to a class of enzymes characterized by a basic C-terminal domain. *Can. J. Microbiol.* **42**:934–943.
 19. Jenkins, J., O. Mayans, and R. Pickersgill. 1998. Structure and evolution of parallel β -helix proteins. *J. Struct. Biol.* **122**:236–246.
 20. Jun, H. S., J. K. Ha, L. M. Malburg, Jr., G. A. Verrinder, and C. W. Forsberg. 2003. Characteristics of a cluster of xylanase genes in *Fibrobacter succinogenes* S85. *Can. J. Microbiol.* **49**:171–180.
 21. Jun, H. S., M. Qi, J. Gong, E. E. Egbosimba, and C. W. Forsberg. 2007. Outer membrane proteins of *Fibrobacter succinogenes* with potential roles in adhesion to cellulose and in cellulose digestion. *J. Bacteriol.* **189**:6806–6815.
 22. Kahng, H. Y., A. M. Byrne, R. H. Olsen, and J. J. Kukor. 2000. Characterization and role of *ibuX* in utilization of toluene by *Ralstonia pickettii* PKO1. *J. Bacteriol.* **182**:1232–1242.
 23. Kam, D. K., H. S. Jun, J. Ha, G. D. Inglis, and C. W. Forsberg. 2005. Characteristics of adjacent family 6 acetyl xylan esterases from *Fibrobacter succinogenes* and the interaction with the XynE xylanase in hydrolysis of acetylated xylan. *Can. J. Microbiol.* **51**:821–832.
 24. Koike, S., and Y. Kobayashi. 2001. Development and use of competitive PCR assays for the rumen cellulolytic bacteria: *Fibrobacter succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens*. *FEMS Microbiol. Lett.* **204**:361–366.
 25. Krause, D. O., B. P. Dalrymple, W. J. Smith, R. I. Mackie, and C. S. McSweeney. 1999. 16S rDNA sequencing of *Ruminococcus albus* and *Ruminococcus flavefaciens*: design of a signature probe and its application in adult sheep. *Microbiology* **145**:1797–1807.
 26. Krause, D. O., S. E. Denman, R. I. Mackie, M. Morrison, A. L. Rae, G. T. Attwood, and C. S. McSweeney. 2003. Opportunities to improve fiber degradation in the rumen: microbiology, ecology, and genomics. *FEMS Microbiol. Rev.* **27**:663–693.
 27. 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**:267–272.
 28. Lamb, J. R., S. Tugendreich, and P. Hieter. 1995. Tetratricopeptide repeat interactions: to TPR or not to TPR? *Trends Biochem. Sci.* **20**:257–259.
 29. Lin, C., B. Flesher, W. C. Capman, R. I. Amann, and D. A. Stahl. 1994. Taxon-specific hybridization probes for fiber-digesting bacteria suggest novel gut-associated *Fibrobacter*. *Syst. Appl. Microbiol.* **17**:418–424.
 30. Malburg, L. M., Jr., A. H. Iyo, and C. W. Forsberg. 1996. A novel family 9 endoglucanase gene (*celD*), whose product cleaves substrates mainly to glucose, and its adjacent upstream homolog (*celE*) from *Fibrobacter succinogenes* S85. *Appl. Environ. Microbiol.* **62**:898–906.
 31. Malburg, S. R. C., L. M. Malburg, Jr., T. Liu, A. H. Iyo, and C. W. Forsberg. 1997. Catalytic properties of the cellulose-binding endoglucanase F from *Fibrobacter succinogenes* S85. *Appl. Environ. Microbiol.* **63**:2449–2453.
 32. McGavin, M., and C. W. Forsberg. 1988. Isolation and characterization of endoglucanases 1 and 2 from *Bacteroides succinogenes* S85. *J. Bacteriol.* **170**:2914–2922.
 33. McGavin, M., and C. W. Forsberg. 1989. Catalytic and substrate-binding domains of endoglucanase 2 from *Bacteroides succinogenes*. *J. Bacteriol.* **171**:3310–3315.
 34. McGavin, M. J., C. W. Forsberg, B. Crosby, A. W. Bell, D. Dignard, and D. Y. Thomas. 1989. Structure of the *cel-3* gene from *Fibrobacter succinogenes* S85 and characteristics of the encoded gene product, endoglucanase 3. *J. Bacteriol.* **171**:5587–5595.
 35. Minato, H., and T. Suto. 1978. Technique for fractionation of bacteria in rumen microbial ecosystem. II. Attachment of bacteria isolated from bovine rumen to cellulose powder *in vitro* and elution of bacteria attached therefrom. *J. Gen. Appl. Microbiol.* **24**:1–16.
 36. Mitsumori, M., and H. Minato. 1993. Presence of several cellulose-binding proteins in cell lysate of *Fibrobacter succinogenes* S85. *J. Gen. Appl. Microbiol.* **39**:273–283.
 37. Mitsumori, M., and H. Minato. 1995. Distribution of cellulose-binding proteins among the representative strains of rumen bacteria. *J. Gen. Appl. Microbiol.* **41**:297–306.
 38. Mitsumori, M., H. Minato, T. Sekizaki, I. Uchida, and H. Itoh. 1996. Cloning, nucleotide sequence and expression of the gene encoding the cellulose-binding protein 1 (CBP1) of *Fibrobacter succinogenes* S85. *FEMS Microbiol. Lett.* **139**:43–50.
 39. Reference deleted.
 40. Miwa, T., S. Takagi, and T. Hino. 2000. Degradation of the cellulase of ruminal bacteria by the protease of coexisting bacteria *in vitro*. *Anim. Sci. J.* **71**:157–163.
 41. Ohara, H., J. Noguchi, S. Karita, T. Kimura, K. Sakka, and K. Ohmiya. 2000. Sequence of *egV* and properties of EgV, a *Ruminococcus albus* endoglucanase containing a dockerin domain. *Biosci. Biotechnol. Biochem.* **64**:80–88.
 42. Qi, M., K. E. Nelson, S. C. Daugherty, W. C. Nelson, I. R. Hance, M. Morrison, and C. W. Forsberg. 2005. Novel molecular features of the fibrolytic intestinal bacterium *Fibrobacter intestinalis* not shared with *Fibrobacter succinogenes* as determined by suppressive subtractive hybridization. *J. Bacteriol.* **187**:3739–3751.
 43. Ram, R. J., N. C. VerBerkmoes, M. P. Thelen, G. W. Tyson, B. J. Baker, R. C. Blake, I. I., M. Shah, R. L. Hettich, and J. F. Banfield. 2005. Community proteomics of a natural microbial biofilm. *Science* **308**:1915–1920.
 44. Satokari, R. M., E. E. Vaughan, H. Smidt, M. Saarela, J. Mättö, and M. de Vos. 2003. Molecular approaches for the detection and identification of bifidobacteria and lactobacilli in the human gastrointestinal tract. *Syst. Appl. Microbiol.* **26**:572–584.
 45. Shevchenko, A., M. Wilm, O. Vorm, and M. Mann. 1996. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal. Chem.* **68**:850–858.
 46. Shinkai, T., and Y. Kobayashi. 2007. Localization of ruminal cellulolytic bacteria on plant fibrous materials as determined by fluorescence *in situ* hybridization and real-time PCR. *Appl. Environ. Microbiol.* **73**:1646–1652.
 47. Stahl, D. A., B. Flesher, H. R. Mansfield, and L. Montgomery. 1988. Use of phylogenetically based hybridization probes for studies of ruminal microbial ecology. *Appl. Environ. Microbiol.* **54**:1079–1084.
 48. Stewart, C. S., and H. J. Flint. 1989. *Bacteroides (Fibrobacter) succinogenes*, a cellulolytic anaerobic bacterium from the gastrointestinal tract. *Appl. Microbiol. Biotechnol.* **30**:433–439.
 49. Suau, A., R. Bonnet, M. Sutren, J. Godon, G. R. Gibson, M. D. Collins, and J. Dore. 1999. Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. *Appl. Environ. Microbiol.* **65**:4799–4807.
 50. Tajima, K., R. I. Aminov, T. Nagamine, H. Matui, M. Nakamura, and Y. Benno. 2001. Diet-dependent shifts in the bacterial population of the rumen revealed with real-time PCR. *Appl. Environ. Microbiol.* **67**:2766–2774.
 51. Toyoda, A., K. Yoda, Y. Nakamura, and H. Minato. 2001. Presence of several cellulose-binding proteins in culture supernatant and cell lysate of *Eubacterium cellulosolvens* 5. *J. Gen. Appl. Microbiol.* **47**:321–328.
 52. Toyoda, A., and H. Minato. 2002. Identification of the cellulose-binding and the cell wall-binding domains of *Eubacterium cellulosolvens* 5 cellulose-binding protein A (CBPA). *FEMS Microbiol. Lett.* **214**:113–118.
 53. Wang, Y., M. Rawlings, D. T. Gibson, D. Labbe, H. Bergeron, R. Brousseau, and P. C. K. Lau. 1995. Identification of a membrane protein and a truncated LysR-type regular associated with the toluene degradation pathway in *Pseudomonas putida* F1. *Mol. Gen. Genet.* **246**:570–579.
 54. Wilmes, P., and P. L. Bond. 2004. The application of two-dimensional polyacrylamide gel electrophoresis and downstream analyses to a mixed community of prokaryotic microorganisms. *Environ. Microbiol.* **6**:911–920.
 55. Wojciechowicz, M., K. Heinrichova, and A. Ziotecki. 1982. An exopeptidase

- lyase of *Butyrivibrio fibrisolvens* from the bovine rumen. J. Gen. Microbiol. **128**:2661–2665.
56. Yoda, K., A. Toyoda, Y. Mukoyama, Y. Nakamura, and H. Minato. 2005. Cloning, sequencing, and expression of a *Eubacterium cellulosolvens* 5 gene encoding an endoglucanase (Cel5A) with novel carbohydrate-binding modules, and properties of Cel5A. Appl. Environ. Microbiol. **71**:5787–5793.
57. Zhu, H., F. W. Paradis, P. J. Krell, J. P. Phillips, and C. W. Forsberg. 1994. Enzymatic specificities and modes of action of the two catalytic domains of the XynC xylanase from *Fibrobacter succinogenes* S85. J. Bacteriol. **176**:3885–3894.
58. Zoetendal, E. G., A. D. L. Akkermans, and W. M. de Vos. 1998. Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. Appl. Environ. Microbiol. **64**:3854–3859.