Localization of ruminal cellulolytic bacteria on plant fibrous materials as determined by fluorescence in situ hybridization and real-time PCR.

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Localization of ruminal cellulolytic bacteria on plant fibrous materials as determined by fluorescence in situ hybridization and real-time polymerase chain reaction

Running title: FISH detection of ruminal cellulolytic bacteria

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ABSTRACT

To visualize and localize specific bacteria associated with plant materials, a new fluorescence in situ hybridization (FISH) protocol was established. By using this protocol, we successfully minimized the autofluorescence of orchard grass hay and detected rumen bacteria attached to the hay under a fluorescence microscope. Real-time polymerase chain reaction assays were also employed to quantitatively monitor the representative fibrolytic species *Fibrobacter succinogenes* and *Ruminococcus flavefaciens*, and also total bacteria attached to the hay. *F. succinogenes* was found firmly attached to not only the cut edges but also undamaged inner surfaces of the hay. Cells of phylogenetic group 1 of *F. succinogenes* were detected on many stem and leaf sheath fragments of the hay, even on fragments on which few other bacteria were seen. Cells of phylogenetic group 2 of *F. succinogenes* were often detected on hay fragments coexisting with many other bacteria. On the basis of 16S rDNA copy number analysis, the numbers of bacteria attached to the leaf sheaths were higher than those attached to the stems (*P* < 0.05). In addition, *R. flavefaciens* had a greater tendency than *F. succinogenes* to be found on the leaf sheath (*P* < 0.01) with formation of many pits. *F. succinogenes*, particularly phylogenetic group 1, is suggested to possibly play an important role in fiber digestion, because it is clearly detectable by FISH and is the bacterium with the largest population size in the less easily degradable hay stem.
Fibrobacter succinogenes and Ruminococcus flavefaciens are considered to be the predominant cellulolytic bacteria present in the rumens of ruminant animals (16, 17, 18, 27, 28). Transmission electron microscopy (TEM) observations of the fibrous materials 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-attaching bacteria (1, 12). In contrast, when species-specific quantification was carried out, F. succinogenes and R. flavefaciens accounted for 0.1–6.6% and 1.3–2.9% of total bacteria, respectively (8, 17, 20, 26). Furthermore, in an analysis of fiber-associated rumen bacteria based on a 16S rDNA clone library, only a few clones belonging to F. succinogenes or R. flavefaciens were obtained, although other species and uncultured bacteria were frequently detected. Thus, the approaches used so far have been inconclusive with respect to clarifying the significance of these cellulolytic species. A new approach allowing both specific visualization and quantification of bacteria, especially fiber-attaching bacteria, might provide more useful information to allow elucidation of their ecology.

It is generally accepted that F. succinogenes makes a large contribution to fiber digestion, given that this species has a potent ability to solubilize crystalline cellulose and is found in relatively large numbers or biomass in the rumen (13, 26). Although F. succinogenes can be divided into four groups on the basis of 16S rDNA sequences and DNA homology, few descriptions of the corresponding phenotypic characteristics are available (3, 22). The ecology of these groups might differ according to host animal species, gut compartment or feeding conditions (14, 19, 20). Therefore, detailed ecological study is
necessary to evaluate the contribution of *F. succinogenes* and its constituent groups to rumen fiber digestion by determining their distribution and quantities.

Fluorescence in situ hybridization (FISH) is very useful for species- and group-specific detection of bacteria in complex communities such as that in the rumen. However, because of the autofluorescence emitted by plant fibrous materials, FISH has not been effectively used for the detection of fiber-attaching bacteria (4, 29). If FISH were to be available for *F. succinogenes* and ruminococci associated with plant fragments, the obtained images would be useful for characterization of the niches of these bacteria and also for assessment of their physiological significance.

The objectives of this study were (1) to establish a FISH protocol for visualizing the rumen cellulolytic bacteria *F. succinogenes* and *R. flavefaciens* on plant material by minimizing the autofluorescence of the plant fragments, (2) to reveal the localization of these bacteria on the plant material, and (3) to discuss the relationship between FISH-aided localization and real-time polymerase chain reaction (PCR)-aided quantification for the bacteria.

**MATERIALS AND METHODS**

**Bacterial strains and media**

The bacteria used in the present study are shown in Table 1. *F. succinogenes* S85 (ATCC 19169) and HM2 (ATCC 43856), *R. flavefaciens* C94 (ATCC 19208) and *R. albus* 7 (ATCC 27210) were purchased from the American Type Culture Collection. The *F. succinogenes* OS114 strain was newly isolated from sheep rumen in the present study. Strains were maintained either in a filter paper medium or RGC medium (10). The filter
paper medium comprised (per liter): yeast extract, 1.2 g (Oxoid Ltd.); Bacto peptone, 2 g
(Difco); mineral solution I, 75 ml; mineral solution II, 75 ml; clarified rumen fluid, 300 ml;
resazurin (0.1%), 1 ml; NaHCO₃ (8%), 5 ml; L-cysteine hydrochloride, 0.5 g; filter paper
(Whatman no. 1) fragments, 3 g; distilled water, 500 ml. Mineral solutions I and II were as
described by Bryant and Burkey (10).

Rumen samples
A ruminally fistulated wether (castrated male sheep) weighing 68.0 kg was used as a
sample donor. The wether was fed 1200 g orchard grass hay and 200 g concentrate once
daily at 0900 hours, and had free access to water and a mineral block. The wether was
habituated to the feeds for 50 days prior to the sampling. Orchard grass hay in 2 cm-long
fragments, cut from the lower part of the last internode (top to bottom), was manually
divided into stem and leaf sheath fractions and then milled (Dietz Motoren KG,
Dettingen-Teck, Germany) to pass through a 1-mm screen. Each milled hay fraction was
put into a nylon bag (50 mm × 100 mm, 50 µm pore size), placed into the rumen of the
wether prior to feeding and incubated for 24 hours. At the end of that time, the bags were
withdrawn from the rumen, and thoroughly washed in warmed (38°C) saline to recover the
milled sections with attached bacteria. For the in vivo samples, rumen solid contents were
obtained through a rumen fistula prior to feeding. Effort was made to collect representative
samples by mixing the whole rumen contents. Both the ruminally incubated hay fractions
and the rumen contents were immediately transferred to the laboratory and fixed as
described below.

Fixation
When pure cultures of *F. succinogenes* or *R. flavefaciens* grown in RGC medium not containing filter paper were used, the fixation procedure was as described by Amann et al. (2, 5). When rumen samples or cells grown in filter paper medium were used, sequential fixation was performed by using 3% paraformaldehyde-phosphate-buffered saline (PBS) solution followed by PBS-96% ethanol (1:1 [vol/vol]) with different incubation times as recommended for Gram-positive bacteria. When the fixative solution was changed, tubes were centrifuged at 200 × g for 3 min and the supernatant was carefully removed with a pipette. The fixed samples were stored at -20°C until observation took place, which occurred within 3 days. Glass slides for FISH observation were coated with poly-L-lysine. After the fixed samples were spread on the coated slides, these were air-dried at room temperature.

**Oligonucleotide probes and in situ hybridization**

Table 2 lists the probes used in the present study. The species-specific probe and group-specific probes for *F. succinogenes* were the same as described previously (4, 20). A species-specific probe for *R. flavefaciens* was newly designed in the present study. The specificity of the probes was checked with the Probe Match tool of RDP II (http://rdp.cme.msu.edu/index.jsp). Also, the specificity of the probe sequences were confirmed by using the BLAST search tool (http://www.ddbj.nig.ac.jp/Welcome-e.html). The 5’ ends of the oligonucleotide probes were labeled with one of the following dyes: fluorescein isothiocyanate (FITC), Cy3 or Cy5 (Hokkaido System Science, Japan).

The in situ hybridization procedure was largely the same as described by Amann (2) and Amann et al. (5), but with some modifications. Briefly, sequential dehydration was carried out in 50, 80, 96 and 100% ethanol (3 min each). Hybridizations were performed by
using 20-30 μl of a hybridization buffer per field at 46°C for 1.5 hours; probe concentration
was 5 ng/μl. The slides were rinsed in a washing buffer for 20 min at 48°C. The
concentration of sodium chloride as a component of the washing buffer was reduced to 900,
450, 225, 80, 40 and 7 mM, respectively, as the formamide concentration increased. This
was to determine the optimum formamide concentration for obtaining the best fluorescence
by using different formamide concentrations (0, 10, 20, 35, 45 and 70%) in hybridizations
for the bacteria grown in the filter paper medium.

For reducing 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 different periods of time (0.5–15
min using 0.5-min intervals) to remove the dye from the bacterial cells but not from the
plant material. Then, the samples were immediately washed with distilled water. For
different samples, the staining was performed both before (29) and after (as described
herein) the probe hybridization to compare the results.

Total bacteria were visualized by staining with 4',6-diamidino-2-phenylindole (DAPI;
1.5 μg/ml) contained in Vectashield H-1200 (Vector Laboratories, Inc., Burlingame). For
microscopic observation of bacteria and their fluorescence signals, a microscope (BX51,
Olympus) with a universal reflected-light illuminator (BX-URA2, Olympus) and cooled
CCD camera (Cool Snap, Roper Scientific Photometrics) was used. Randomly selected 50
and 100 microscopic fields (50 μm squares per field) were employed for observations of in
situ sample and rumen contents, respectively. Images were processed with Adobe
Photoshop version 6.0.
Real-time PCR

Total DNA extraction from the ruminally incubated hay sections associated with bacteria was performed as described previously (15). In brief, each sample (0.35 g) was mixed with 0.35 ml of Tris-EDTA buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) and 0.7 ml of Tris-buffered phenol (pH 8.0) in a 2-ml screw-capped tube containing 0.25 g of glass beads (diameter, 425 to 600 μm; Sigma Chemicals, St Louis, MO). After 40 μl of 10% sodium dodecylsulfate (SDS) was added, the tube was shaken three times for 2 min with 2 min of incubation on ice between shaking. The tube was centrifuged at 16,000 × g for 5 min. DNA in the supernatant was purified with hydroxyapatite chromatography (Hydroxyapatite Bio-Gel HTP Gel; Bio-Rad, Hercules, CA) followed by gel filtration (Microspin S-200R HR Columns; Amersham Pharmacia Biotech, Piscataway, NJ). Purified DNA was eluted into 100 μl of TE buffer and fluorescently quantified (DyNA Quant 200; Hoefer Pharmacia Biotech, San Francisco, CA) and subjected to PCR. The LightCycler system (Roche, Mannheim, Germany) and FastStart DNA Master SYBR Green I (Roche Applied Science, Mannheim, Germany) were used for the real-time PCR amplification.

The 16S rDNA targeted primer sets used in the present study were Fs193f (5’-GGTATGGGATGAGCTTGC-3’) and Fs620r (5’-GCCTGCCCCTGAACATC-3’) for F. succinogenes, Rf154f (5’-TCTGGAAACGGATGGTA-3’) and Rf425r (5’-CCTTTAAGACAGGAGTTTACAA-3’) for R. flavefaciens (16), and primer 1 (5’-CCTACGGGAGGCAGCAG-3’) and primer 2 (5’-ATTACCGCGGCTGCTGG-3’) for total bacteria (23). The PCR conditions for F. succinogenes were as follows: 40 cycles of 95°C for 15 s for denaturation, 62°C for 10 s for annealing and 72°C for 18 s for extension. For R. flavefaciens, 40 cycles of 95°C for 18 s for denaturation, 55°C for 10 s for annealing
and 72°C for 15 s for extension was used. PCR for total bacteria was performed using 35 cycles of 95°C for 15 s for denaturation, 60°C for 5 s for annealing and 72°C for 10 s for extension. The denaturation in the first cycle was carried out at 95°C for 10 min and the extension at the end of the last cycle was carried out at 70°C for 15 s. To determine the specificity of the PCR amplification, a melting curve of PCR products was monitored by heating at 70°C to 95°C using 0.1°C intervals.

The target 16S rDNA sequences of strains *F. succinogenes* S85 and *R. flavefaciens* C94 were PCR-amplified and cloned into pCR2.1 (Invitrogen, Tokyo, Japan) for the use as standard template. The latter standard template was also used for total bacteria. The assay values were obtained with Standard Curve Method using serially diluted standard template (http://www.appliedbiosystems.co.jp/website/SilverStream/Objectstore/General/04303859rev.B.pdf). Amplification efficiency in each PCR assay was calculated by $10^{(-1/\text{slope})}$, where slope was obtained from the plot of log transformation of serial diluted target copy number versus threshold cycle. Assay reproducibility was assessed by determining inter- and intra-assay variations with five replicates.

Assays for all the experimental samples were performed in triplicate. Assay values for three bacterial groups (two species and total bacteria) were expressed as 16S rDNA copy numbers per g sample. Ratio of assay value for leaf sheath to that for stem was calculated to compare difference of distribution pattern between the bacterial groups. However, direct comparison of bacterial quantity between the groups was avoided, because amplification efficiency differed between the assays (see Results) and 16S rDNA copy number was considered to vary with bacterial species. In fact, the copy number for *F. succinogenes* and *R. flavefaciens* are 3 and 5, respectively (24; Bryan White, personal communication), while those of other rumen bacteria are unknown. When we look a database
average of the copy numbers for 261 bacterial species is 3.69 ± 2.48, in which variation within the same species is minimal (copy number of each species ± 1).

Data for amplification efficiency and bacterial quantity were subjected to ANOVA and Tukey-Kramer’s test to detect differences between assays and samples. Statistical differences were declared at $P < 0.05$.

RESULTS

Establishment of the FISH detection protocol

Figure 1 shows a comparison of FISH detection of *R. flavefaciens* attached to the leaf sheaths of orchard grass hay using three different protocols. When leaf sheaths were not treated with toluidine blue O, they produced strong autofluorescence that totally prevented the detection of bacteria attached to the leaf sheaths (Fig. 1a). Toluidine blue O staining before probe hybridization, as proposed for FISH detection of soil bacteria by Weber et al. (29), allowed partial detection of the target bacteria on the leaf sheaths (Fig. 1b). However, the protocol involving fixation, timing of toluidine blue staining and destaining greatly improved the resolution of the target bacteria attached to the plant material (Fig. 1c). The optimized procedure is as follows.

The toluidine blue staining should occur after probe hybridization (Fig. 1b vs. Fig. 1c). For destaining, the exposure time to 99.5% ethanol (1.5 min) was critical to the specific detection of bacteria attached to the plant material. A longer exposure time resulted in not only the bacteria but also the plant material being destained, which restored the strong fluorescence of the plant material, and hindered bacterial detection. Shorter exposure did
not allow production of a bacterial fluorescence signal.

Using the standard fixation method, \textit{F. succinogenes} cells often had a shrunken morphology and were stained as Gram-positive cells (due possibly to alteration of the cell properties), resulting in insufficient FISH signals being obtained. We thus changed the fixation method from using 3\% paraformaldehyde for Gram-negative bacteria to using 3\% paraformaldehyde, followed by PBS-ethanol for Gram-positive bacteria. This new method gave a 2-3 times stronger signal compared with the former fixation method. The best result was obtained with 3 hours of incubation for each step; longer incubation caused reduction of the signal strength. For the observation of \textit{R. flavefaciens}, fixation using the method of Amann (2) was confirmed to be effective. However, when \textit{R. flavefaciens} was detected together with \textit{F. succinogenes}, the sequential fixation described above for \textit{F. succinogenes} was found to provide satisfactory signals. Optimal formamide concentrations for hybridization are also listed in Table 2. The newly designed probe for \textit{R. flavefaciens} did not react with \textit{R. albus} at all. The specificity of this probe was also confirmed in the rumen fluid supplemented with a pure culture of \textit{R. flavefaciens} by observing that signal counts corresponded to the number of supplemented cells (data not shown).

Detection of bacteria on ruminally incubated hay

Although we attempted to detect groups 1–3 of \textit{F. succinogenes} by FISH, only groups 1 and 2 were detectable on the ruminally incubated hay. For group 2, a few cells only were detected in the supernatant of the fixative solution, but not actually on the hay. Group 3 cells were not detected in any of the samples used (data not shown).

On the leaf sheaths, many \textit{F. succinogenes} group 1 cells were detected in 37\% of 50 fields observed (Fig. 2a). Most of the cells showed clear fluorescence signals. The cells were
firmly attached to the undamaged inner surfaces of the sheaths (arrowhead 1 in Fig. 2a). Some cells also dispersed and coexisted with many other bacteria on the cut edges of hay fragments (arrowhead 2 in Fig. 2a). For the stems, *F. succinogenes* group 1 cells were detected in 20 of 50 fields observed. Some stem fragments had many *F. succinogenes* group 1 cells, which were small with weaker signals in comparison with those on the leaf sheaths. In most cases the cells were dispersed and intermingled with other bacteria. However, there existed well-like structures in the inner tissues of stems that were nearly completely occupied by group 1 cells (Fig. 2b).

Many *R. flavefaciens* cells were detected in the leaf sheaths (in 14 of 50 fields observed). Most were located in a specific area of the sheath along the edge of the pit created by bacterial degradation (Fig. 3a). *R. flavefaciens* cells were rarely detected on the stem fragments (only a few cells were detectable in 5 of 50 fields observed). Unlike on the leaf sheaths, they showed very simple distribution on the stems: only large cells were detected, they were present as pairs, and no colonies were formed (Fig. 3b).

**Quantification of bacteria on ruminally incubated hay**

Validation of real-time PCR assays is summarized in Table 3. Amplification efficiencies were different (*P* < 0.05) between the assays, showing 1.94, 1.81 and 2.02 for *F. succinogenes*, *R. flavefaciens* and total bacteria, respectively, even though all were close to the ideal value (2.0). The assays showed a high degree of reproducibility with minimal intra- and inter-assay variations ranging from 6.0 to 11.6%.

The results of real-time PCR assays are shown in Table 4. More than $10^{11}$ copies of 16S rDNA for total bacteria were monitored per gram of ruminally incubated leaf sheath and stem. The numbers of bacteria attached to the leaf sheaths were higher than those attached
to the stems for all the targeted bacterial groups ($P < 0.05$). The leaf sheath to stem ratios were 1.86 for total bacteria, 1.92 for *F. succinogenes* and 5.44 for *R. flavefaciens*, indicating that *R. flavefaciens* has a greater tendency than *F. succinogenes* to be found on the leaf sheath ($P < 0.01$).

Detection of bacteria on rumen contents

We also detected *F. succinogenes* and *R. flavefaciens* attached to the fibrous material in the rumen contents. Both group 1 and 2 *F. succinogenes* cells were successfully detected, but group 3 cells were not detected. Fluorescence signals obtained from the rumen contents were weaker than those from the ruminally incubated hay samples. In addition, the number of *F. succinogenes* cells detected was drastically lower than the number observed for the ruminally incubated hay (18 of 140 fields vs. 57 of 100 fields in detection frequency). Group 1 cells were attached to fragments on which few other bacteria were seen (Fig. 4a), whereas group 2 cells were usually detected coexisting with other bacteria (Fig. 4b). *R. flavefaciens* cells were detected in 26 of 60 fields observed. As observed for the ruminally incubated hay samples, *R. flavefaciens* cells had stronger signals than *F. succinogenes* in rumen contents fragments.

DISCUSSION

FISH detection protocol

FISH detection is a powerful tool for characterizing the localization of a specific bacterium. The method has been used to monitor bacteria of interest in the digesta of humans, pigs and rats. However, it is difficult to use this detection method for digesta rich
in plant material such as rumen contents, because the plant material produces strong
autofluorescence that hinders the specific detection of bacteria (4).

Toluidine blue O staining has been reported to reduce the autofluorescence of plant
material (25). This dye has been considered useful for the observation of bacteria using Cy3
or fluorescein isothiocyanate (FITC) channels, because the maximum wavelength for
absorption of toluidine blue O (\(\lambda_{\text{max}} \geq 620\) nm) is longer than that of the above commonly
used dyes.

Because bacterial cells as well as plant material are easily stained with toluidine blue O,
FISH signals from the bacteria can be reduced, preventing specific detection of bacteria. In
the present study, however, we were able to successfully remove the dye from bacterial
cells but not from the plant material by optimizing the destaining process. This protocol
was effective for rumen bacteria attached to orchard grass (Figs. 1–4) and other
representative forage materials including alfalfa and rice straw (data not shown). Incubation
of the dyed materials with 99.5% ethanol for 1.5 min reinstated the bacterial fluorescence
signals nearly completely, while maintaining plant material autofluorescence at a low level.
Toluidine blue O staining has been previously used for FISH analysis of soil bacteria mixed
with rice plant fragments by Weber et al. (29), who stained the sample with toluidine blue
O before hybridization to reduce the background signal. These authors found that
dehydration, hybridization and washing after staining could remove the toluidine blue O
from plant material to a considerable extent, as we also found in the present study (Fig. 1b).
We thus carried out hybridization first, followed by staining and destaining. This order
allows definite control over the staining and destaining processes. In addition, we modified
the fixation conditions for \(F.\ succinogenes\) to increase probe permeability and thus improve
the FISH signals. Thus, the established protocol successfully enabled FISH detection of
target rumen bacteria attached to plant fragments.

**Distribution of fibrolytic bacteria**

We successfully detected groups 1 and 2 of *F. succinogenes* associated with orchard grass hay by FISH. Most *F. succinogenes* cells belonged to group 1, and were associated with various types of plant fragments. Although group 1 cells were usually distributed over the plant material including the leaf sheaths and stems of orchard grass hay (Fig. 2) and rumen contents (Fig. 4), in some cases the cells occupied a well-like structure in the inner tissue of orchard grass hay stems (Fig. 2b). In the rumen contents, group 1 cells were often found as a major member of the bacterial community on hay stem-like content (Fig. 4a).

These observations suggest that group 1 of *F. succinogenes* makes a greater contribution to fiber digestion than groups 2 and 3. In fact, the *F. succinogenes* quantified by using real-time PCR is thought to represent group 1, because sequencing revealed that all 20 clones from the PCR products were from group 1 (data not shown). Although little information is available as to the functional differences between the phylogenetic groups of *F. succinogenes*, possession of fibrolytic enzymes and sequence identity for the endoglucanase Cel-3 have been shown to be different between the groups (6). These factors may influence the distribution of each group in the rumen.

*R. flavefaciens* was located along the edges of the pits formed on the leaf sheath (Fig. 3a). The pits were confirmed to be formed by *R. flavefaciens* itself in a pure culture study (data not shown). According to the real-time PCR assay values, the number of *R. flavefaciens* attached to stems was less than 20% of that attached to leaf sheaths (Table 4). These results clearly indicate that *R. flavefaciens* prefers the leaf sheath, which is more easily degradable than the stem, as a growth substrate. In fact, *R. flavefaciens* was rarely
detected by FISH in the ruminally incubated stems (Fig. 3b).

Although *R. flavefaciens* always produces stronger fluorescence signals than *F. succinogenes*, *F. succinogenes* rather than *R. flavefaciens* was frequently visible on stems (Figs. 2b, 3b and 4a). These facts suggest that *R. flavefaciens* cells attaching to stems are not metabolically active enough to be visualized by FISH. This is supported in part by the findings of Miron et al. (21), who noted that the *R. flavefaciens* FD-1 strain adhered to the lucerne cell wall and had only limited digestive activity. It could be difficult to clearly detect the bacterial cells unless they are active. Therefore, the ecology of fiber digestion should be further studied by RNA-based approaches such as FISH detection and quantitative PCR for rRNA and mRNA expression.

To our knowledge, this is the first report describing visualization of fibrolytic bacteria associated with plant material in the rumen by FISH. The protocol we established was effective in determining the cell distribution of two representative species. FISH detection is considered to more accurately reflect cell activity (RNA amount) (5, 7) than real-time PCR assay, which depends on gene copy number (cell number). *R. flavefaciens* was found to colonize the edges of pits formed during digestion of the leaf sheath, whereas *F. succinogenes* group 1 was found to be uniquely present on the less easily degradable stem. These findings strongly indicate the highly potent fibrolytic functions of these two species, even though each species has its own preference for particular plant tissues as a growth substrate. The real-time PCR assays also confirmed the differences in localization between these two species.
REFERENCES


# TABLE 1. Bacterial strains used in the present study

<table>
<thead>
<tr>
<th>Species</th>
<th>Phylogenetic group*</th>
<th>Strain</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Fibrobacter succinogenes</em></td>
<td>Group 1</td>
<td>S85 (ATCC19169)</td>
<td>Bovine rumen</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Group 2</td>
<td>OS114</td>
<td>Ovine rumen</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>Group 3</td>
<td>HM2 (ATCC43856)</td>
<td>Ovine rumen</td>
<td>3</td>
</tr>
<tr>
<td><em>Ruminococcus flavefaciens</em></td>
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<td>C94 (ATCC19208)</td>
<td>Bovine rumen</td>
<td>11</td>
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<tr>
<td><em>Ruminococcus albus</em></td>
<td></td>
<td>7 (ATCC27210)</td>
<td>Bovine rumen</td>
<td>11</td>
</tr>
</tbody>
</table>

*Phylogenetic groups in *Fibrobacter succinogenes* are defined from the basis of 16S rDNA sequence by Amann *et al.* (3).
TABLE 2. Oligonucleotide probes and conditions used

<table>
<thead>
<tr>
<th>Probe specificity</th>
<th>16S rRNA target site</th>
<th>Sequence</th>
<th>Dyes used</th>
<th>% Formamide</th>
<th>Reference</th>
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<tr>
<td><em>F. succinogenes</em></td>
<td>650-669</td>
<td>5'-TGCCCCTGAACTATCCAA -3'</td>
<td>Cy3</td>
<td>70</td>
<td>4</td>
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<td><em>F. succinogenes</em> Group 1</td>
<td>628-649</td>
<td>5'-GATCCAGTTCCGACTGAGGC-3'</td>
<td>Cy3</td>
<td>70</td>
<td>20</td>
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<tr>
<td><em>F. succinogenes</em> Group 2</td>
<td>628-649</td>
<td>5'-AACCCAGTTCCGACTGAGGTC-3'</td>
<td>FITC or Cy5</td>
<td>70</td>
<td>20</td>
</tr>
<tr>
<td><em>F. succinogenes</em> Group 3</td>
<td>628-649</td>
<td>5'-GGTGCAGTCCGAACTGAGGCC-3'</td>
<td>FITC</td>
<td>20</td>
<td>20</td>
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<tr>
<td><em>R. flavefaciens</em></td>
<td>155-171</td>
<td>5'-TACCATCGTCTCCGAA-3'</td>
<td>Cy3 or FITC</td>
<td>20</td>
<td>This study</td>
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</table>

*a* Escherichia coli rRNA numbering (9).

*b* Percentage (vol/vol) of formamide in the hybridization.
TABLE 3. Validation of real-time PCR assays for *Fibrobacter succinogenes*, *Ruminococcus flavefaciens* and total bacteria.

<table>
<thead>
<tr>
<th>Target</th>
<th>DNA used</th>
<th>Quantification range ((10^x\ copy)^a)</th>
<th>Amplification efficiency ((%)^b)</th>
<th>CV ((%)^c) intra-assay</th>
<th>CV ((%)^c) inter-assay</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>F. succinogenes</em></td>
<td>plasmid, rumen DNA</td>
<td>1-9</td>
<td>1.94 ± 0.07 (^x)</td>
<td>7.5</td>
<td>11.6</td>
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<tr>
<td><em>R. flavefaciens</em></td>
<td>plasmid, rumen DNA</td>
<td>1-9</td>
<td>1.81 ± 0.03 (^y)</td>
<td>7.8</td>
<td>6.0</td>
</tr>
<tr>
<td>Total bacteria</td>
<td>plasmid, rumen DNA</td>
<td>4-9</td>
<td>2.02 ± 0.07 (^x)</td>
<td>8.0</td>
<td>10.1</td>
</tr>
</tbody>
</table>

\(^{a}\) Serially diluted plasmid was used as a template.  
\(^{b}\) Amplification efficiency in each PCR assay was calculated as \(E = 10^{(-1/slope)}\).  
\(^{c}\) Coefficient of variation was determined with five replicates.  
\(^{x, y}\) Within column, means followed by different letters differ significantly \((P < 0.05)\).
TABLE 4. Real time PCR quantification of *Fibrobacter succinogenes* and *Ruminococcus flavefaciens* associated with the leaf sheaths and stems of orchard grass hay that had been incubated in an ovine rumen for 24 hours (n=3).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Quantities (log copies ± SD / g of sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total bacteria</td>
</tr>
<tr>
<td>Leaf sheath</td>
<td>11.52 ± 0.01&lt;sup&gt;x&lt;/sup&gt;</td>
</tr>
<tr>
<td>Stem</td>
<td>11.25 ± 0.02&lt;sup&gt;y&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ratio</td>
<td>1.86 ± 0.10&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values in parenthesis are relative proportion of each target species in total bacteria as calculated by dividing the assay value for each species by that for total bacteria.

<sup>x,y</sup>Within column, means followed by different letters differ significantly (*P* < 0.05).

<sup>a,b</sup>Within row, means followed by different letters differ significantly (*P* < 0.01).
FIG. 1. Comparison between the three different protocols for FISH detection of *Ruminococcus flavefaciens* associated with ruminally incubated leaf sheaths of orchard grass hay. The hay was untreated (a) or treated with toluidine blue O using Weber’s method (b) or the method described in the present study (c). *R. flavefaciens* was hybridized with Cy3-labeled probe (arrowheads). Scale bars, 5 μm.
Detection of *Fibrobacter succinogenes* cells belonging to group 1 on orchard grass hay incubated in the rumen of a sheep for 24 hours. Upper panels: bacteria on the leaf sheaths (a) and stems (b) of the ruminally incubated orchard grass hay were hybridized with a Cy3-labeled *F. succinogenes* group 1 probe (red) and stained with DAPI (green). (a) Cells tightly adhered to the cell walls of the leaf sheaths (arrowhead 1), or dispersed and coexisted with many other bacteria (arrowhead 2). (b) Cells were attached to a well-like structure in the inner tissue of the stem at high density (arrowhead), but were smaller than the cells attached to the leaf sheaths. Scale bars, 5 μm. Lower panels: structural outline of the plant tissue used for observation.
FIG. 3.
Detection of *Ruminococcus flavefaciens* cells on orchard grass hay incubated in the rumen of a sheep for 24 hours. Upper panels: bacteria on the leaf sheaths (a) and stems (b) of ruminally incubated orchard grass hay were hybridized with a Cy3-labeled *R. flavefaciens* probe (red) and were stained with DAPI (green). (a) Small *R. flavefaciens* cells created many pits, and were located along edges of the pits (arrowhead). (b) *R. flavefaciens* cells were rarely detected in stems (arrowhead). Scale bars, 5 μm. Lower panels: structural outline of the plant tissue used for observation.
FIG. 4. Detection of *Fibrobacter succinogenes* cells belonging to group 1 (a) and group 2 (b) in the fibrous rumen contents. Bacteria attached to the fibrous rumen contents were hybridized with a Cy3-labeled probe for *F. succinogenes* group 1 (red, a) or with an FITC-labeled probe for *F. succinogenes* group 2 (red, b). All bacteria were stained with DAPI (green). Scale bars, 5 μm.