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

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9 **Running title: FISH detection of ruminal cellulolytic bacteria**

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24

25 **ABSTRACT**

26

27 To visualize and localize specific bacteria associated with plant materials, a new
28 fluorescence in situ hybridization (FISH) protocol was established. By using this protocol,
29 we successfully minimized the autofluorescence of orchard grass hay and detected rumen
30 bacteria attached to the hay under a fluorescence microscope. Real-time polymerase chain
31 reaction assays were also employed to quantitatively monitor the representative fibrolytic
32 species *Fibrobacter succinogenes* and *Ruminococcus flavefaciens*, and also total bacteria
33 attached to the hay. *F. succinogenes* was found firmly attached to not only the cut edges but
34 also undamaged inner surfaces of the hay. Cells of phylogenetic group 1 of *F. succinogenes*
35 were detected on many stem and leaf sheath fragments of the hay, even on fragments on
36 which few other bacteria were seen. Cells of phylogenetic group 2 of *F. succinogenes* were
37 often detected on hay fragments coexisting with many other bacteria. On the basis of 16S
38 rDNA copy number analysis, the numbers of bacteria attached to the leaf sheaths were
39 higher than those attached to the stems ($P < 0.05$). In addition, *R. flavefaciens* had a greater
40 tendency than *F. succinogenes* to be found on the leaf sheath ($P < 0.01$) with formation of
41 many pits. *F. succinogenes*, particularly phylogenetic group 1, is suggested to possibly play
42 an important role in fiber digestion, because it is clearly detectable by FISH and is the
43 bacterium with the largest population size in the less easily degradable hay stem.

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49 INTRODUCTION

50

51 *Fibrobacter succinogenes* and *Ruminococcus flavefaciens* are considered to be the
52 predominant cellulolytic bacteria present in the rumens of ruminant animals (16, 17, 18, 27,
53 28). Transmission electron microscopy (TEM) observations of the fibrous materials
54 digested by rumen microbes have shown that *F. succinogenes*- or *R. flavefaciens*-like
55 bacteria are distributed over materials such as fescue and orchard grass, and that sometimes
56 these bacteria account for more than 70% of fiber-attaching bacteria (1, 12). In contrast,
57 when species-specific quantification was carried out, *F. succinogenes* and *R. flavefaciens*
58 accounted for 0.1–6.6% and 1.3–2.9% of total bacteria, respectively (8, 17, 20, 26).
59 Furthermore, in an analysis of fiber-associated rumen bacteria based on a 16S rDNA clone
60 library, only a few clones belonging to *F. succinogenes* or *R. flavefaciens* were obtained,
61 although other species and uncultured bacteria were frequently detected. Thus, the
62 approaches used so far have been inconclusive with respect to clarifying the significance of
63 these cellulolytic species. A new approach allowing both specific visualization and
64 quantification of bacteria, especially fiber-attaching bacteria, might provide more useful
65 information to allow elucidation of their ecology.

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

73 necessary to evaluate the contribution of *F. succinogenes* and its constituent groups to
74 rumen fiber digestion by determining their distribution and quantities.

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

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

88

89 **MATERIALS AND METHODS**

90

91 **Bacterial strains and media**

92 The bacteria used in the present study are shown in Table 1. *F. succinogenes* S85
93 (ATCC 19169) and HM2 (ATCC 43856), *R. flavefaciens* C94 (ATCC 19208) and *R. albus* 7
94 (ATCC 27210) were purchased from the American Type Culture Collection. The *F.*
95 *succinogenes* OS114 strain was newly isolated from sheep rumen in the present study.
96 Strains were maintained either in a filter paper medium or RGC medium (10). The filter

97 paper medium comprised (per liter): yeast extract, 1.2 g (Oxoid Ltd.); Bacto peptone, 2 g
98 (Difco); mineral solution I, 75 ml; mineral solution II, 75 ml; clarified rumen fluid, 300 ml;
99 resazurin (0.1%), 1 ml; NaHCO₃ (8%), 5 ml; L-cysteine hydrochloride, 0.5 g; filter paper
100 (Whatman no. 1) fragments, 3 g; distilled water, 500 ml. Mineral solutions I and II were as
101 described by Bryant and Burkey (10).

102

103 **Rumen samples**

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

119

120 **Fixation**

121 When pure cultures of *F. succinogenes* or *R. flavefaciens* grown in RGC medium not
122 containing filter paper were used, the fixation procedure was as described by Amann et al.
123 (2, 5). When rumen samples or cells grown in filter paper medium were used, sequential
124 fixation was performed by using 3% paraformaldehyde-phosphate-buffered saline (PBS)
125 solution followed by PBS-96% ethanol (1:1 [vol/vol]) with different incubation times as
126 recommended for Gram-positive bacteria. When the fixative solution was changed, tubes
127 were centrifuged at $200 \times g$ for 3 min and the supernatant was carefully removed with a
128 pipette. The fixed samples were stored at -20°C until observation took place, which
129 occurred within 3 days. Glass slides for FISH observation were coated with poly-L-lysine.
130 After the fixed samples were spread on the coated slides, these were air-dried at room
131 temperature.

132

133 **Oligonucleotide probes and in situ hybridization**

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

142 The in situ hybridization procedure was largely the same as described by Amann (2)
143 and Amann et al. (5), but with some modifications. Briefly, sequential dehydration was
144 carried out in 50, 80, 96 and 100% ethanol (3 min each). Hybridizations were performed by

145 using 20-30 μl of a hybridization buffer per field at 46°C for 1.5 hours; probe concentration
146 was 5 ng/ μl . The slides were rinsed in a washing buffer for 20 min at 48°C. The
147 concentration of sodium chloride as a component of the washing buffer was reduced to 900,
148 450, 225, 80, 40 and 7 mM, respectively, as the formamide concentration increased. This
149 was to determine the optimum formamide concentration for obtaining the best fluorescence
150 by using different formamide concentrations (0, 10, 20, 35, 45 and 70%) in hybridizations
151 for the bacteria grown in the filter paper medium.

152 For reducing the autofluorescence of the plant material, 400 μl of toluidine blue O
153 (Division Chroma; 0.05% [wt/vol] in sterilized distilled water with 0.9 M NaCl) was added
154 to the slide samples. The samples were dyed with toluidine blue O for 15 min at room
155 temperature and then rinsed in distilled water until the water became clear. After being
156 air-dried, the samples were incubated in 99.5% ethanol for different periods of time (0.5–15
157 min using 0.5-min intervals) to remove the dye from the bacterial cells but not from the
158 plant material. Then, the samples were immediately washed with distilled water. For
159 different samples, the staining was performed both before (29) and after (as described
160 herein) the probe hybridization to compare the results.

161 Total bacteria were visualized by staining with 4',6-diamidino-2-phenylindole (DAPI;
162 1.5 $\mu\text{g}/\text{ml}$) contained in Vectashield H-1200 (Vector Laboratories, Inc., Burlingame). For
163 microscopic observation of bacteria and their fluorescence signals, a microscope (BX51,
164 Olympus) with a universal reflected-light illuminator (BX-URA2, Olympus) and cooled
165 CCD camera (Cool Snap, Roper Scientific Photometrics) was used. Randomly selected 50
166 and 100 microscopic fields (50 μm squares per field) were employed for observations of in
167 situ sample and rumen contents, respectively. Images were processed with Adobe
168 Photoshop version 6.0.

169

170 **Real-time PCR**

171 Total DNA extraction from the ruminally incubated hay sections associated with bacteria
172 was performed as described previously (15). In brief, each sample (0.35 g) was mixed with
173 0.35 ml of Tris-EDTA buffer (10 mM Tris-HCl [pH8.0], 1 mM EDTA) and 0.7 ml of
174 Tris-buffered phenol (pH 8.0) in a 2-ml screw-capped tube containing 0.25 g of glass beads
175 (diameter, 425 to 600 μ m; Sigma Chemicals, St Louis, MO). After 40 μ l of 10% sodium
176 dodecylsulfate (SDS) was added, the tube was shaken three times for 2 min with 2 min of
177 incubation on ice between shaking. The tube was centrifuged at $16,000 \times g$ for 5 min. DNA
178 in the supernatant was purified with hydroxyapatite chromatography (Hydroxyapatite
179 Bio-Gel HTP Gel; Bio-Rad, Hercules, CA) followed by gel filtration (Microspin S-200R
180 HR Columns; Amersham Pharmacia Biotech, Piscataway, NJ). Purified DNA was eluted
181 into 100 μ l of TE buffer and fluorescently quantified (DyNA Quant 200; Hoefer Pharmacia
182 Biotech, San Francisco, CA) and subjected to PCR. The LightCycler system (Roche,
183 Mannheim, Germany) and FastStart DNA Master SYBR Green I (Roche Applied Science,
184 Mannheim, Germany) were used for the real-time PCR amplification.

185 The 16S rDNA targeted primer sets used in the present study were Fs193f
186 (5'-GGTATGGGATGAGCTTGC-3') and Fs620r (5'-GCCTGCCCCTGAACTATC-3') for
187 *E. succinogenes*, Rf154f (5'-TCTGGAAACGGATGGTA-3') and Rf425r
188 (5'-CCTTTAAGACAGGAGTTTACAA-3') for *R. flavefaciens* (16), and primer 1
189 (5'-CCTACGGGAGGCAGCAG-3') and primer 2 (5'-ATTACCGCGGCTGCTGG-3') for
190 total bacteria (23). The PCR conditions for *E. succinogenes* were as follows: 40 cycles of
191 95°C for 15 s for denaturation, 62°C for 10 s for annealing and 72°C for 18 s for extension.
192 For *R. flavefaciens*, 40 cycles of 95°C for 18 s for denaturation, 55°C for 10 s for annealing

193 and 72°C for 15 s for extension was used. PCR for total bacteria was performed using 35
194 cycles of 95°C for 15 s for denaturation, 60°C for 5 s for annealing and 72°C for 10 s for
195 extension. The denaturation in the first cycle was carried out at 95°C for 10 min and the
196 extension at the end of the last cycle was carried out at 70°C for 15 s. To determine the
197 specificity of the PCR amplification, a melting curve of PCR products was monitored by
198 heating at 70°C to 95°C using 0.1°C intervals.

199 The target 16S rDNA sequences of strains *F. succinogenes* S85 and *R. flavefaciens* C94
200 were PCR-amplified and cloned into pCR2.1 (Invitrogen, Tokyo, Japan) for the use as
201 standard template. The latter standard template was also used for total bacteria. The assay
202 values were obtained with Standard Curve Method using serially diluted standard template
203 ([http://www.appliedbiosystems.co.jp/website/SilverStream/Objectstore/General/04303859r](http://www.appliedbiosystems.co.jp/website/SilverStream/Objectstore/General/04303859rev.B.pdf)
204 [ev.B.pdf](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
205 slope was obtained from the plot of log transformation of serial diluted target copy number
206 versus threshold cycle. Assay reproducibility was assessed by determining inter- and
207 intra-assay variations with five replicates.

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

217 (<http://www.ddbj.nig.ac.jp/Welcome-e.html>), average of the copy numbers for 261 bacterial
218 species is 3.69 ± 2.48 , in which variation within the same species is minimal (copy number
219 of each species ± 1).

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

223

224 **RESULTS**

225

226 **Establishment of the FISH detection protocol**

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

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

241 not allow production of a bacterial fluorescence signal.

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

257

258 **Detection of bacteria on ruminally incubated hay**

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

263 On the leaf sheaths, many *F. succinogenes* group 1 cells were detected in 37 of 50 fields
264 observed (Fig. 2a). Most of the cells showed clear fluorescence signals. The cells were

265 firmly attached to the undamaged inner surfaces of the sheaths (arrowhead 1 in Fig. 2a).
266 Some cells also dispersed and coexisted with many other bacteria on the cut edges of hay
267 fragments (arrowhead 2 in Fig. 2a). For the stems, *F. succinogenes* group 1 cells were
268 detected in 20 of 50 fields observed. Some stem fragments had many *F. succinogenes* group
269 1 cells, which were small with weaker signals in comparison with those on the leaf sheaths.
270 In most cases the cells were dispersed and intermingled with other bacteria. However, there
271 existed well-like structures in the inner tissues of stems that were nearly completely
272 occupied by group 1 cells (Fig. 2b).

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

279

280 **Quantification of bacteria on ruminally incubated hay**

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

286 . The results of real-time PCR assays are shown in Table 4. More than 10^{11} copies of 16S
287 rDNA for total bacteria were monitored per gram of ruminally incubated leaf sheath and
288 stem. The numbers of bacteria attached to the leaf sheaths were higher than those attached

289 to the stems for all the targeted bacterial groups ($P < 0.05$). The leaf sheath to stem ratios
290 were 1.86 for total bacteria, 1.92 for *F. succinogenes* and 5.44 for *R. flavefaciens*, indicating
291 that *R. flavefaciens* has a greater tendency than *F. succinogenes* to be found on the leaf
292 sheath ($P < 0.01$).

293

294 **Detection of bacteria on rumen contents**

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

306

307 **DISCUSSION**

308

309 **FISH detection protocol**

310 FISH detection is a powerful tool for characterizing the localization of a specific
311 bacterium. The method has been used to monitor bacteria of interest in the digesta of
312 humans, pigs and rats. However, it is difficult to use this detection method for digesta rich

313 in plant material such as rumen contents, because the plant material produces strong
314 autofluorescence that hinders the specific detection of bacteria (4).

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

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

337 target rumen bacteria attached to plant fragments.

338

339 **Distribution of fibrolytic bacteria**

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

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

355 *R. flavefaciens* was located along the edges of the pits formed on the leaf sheath (Fig.
356 3a). The pits were confirmed to be formed by *R. flavefaciens* itself in a pure culture study
357 (data not shown). According to the real-time PCR assay values, the number of *R.*
358 *flavefaciens* attached to stems was less than 20% of that attached to leaf sheaths (Table 4).
359 These results clearly indicate that *R. flavefaciens* prefers the leaf sheath, which is more
360 easily degradable than the stem, as a growth substrate. In fact, *R. flavefaciens* was rarely

361 detected by FISH in the ruminally incubated stems (Fig. 3b).

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

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

382

383

384

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TABLE 1. Bacterial strains used in the present study

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

*Phylogenetic groups in *Fibrobacter succinogenes* are defined from the basis of 16S rDNA sequence by Amann *et al.* (3).

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TABLE 2. Oligonucleotide probes and conditions used

Probe specificity	16S rRNA target site ^a	Sequence	Dyes used	% Formamide ^b	Reference
<i>F. succinogenes</i>	650-669	5'-TGCCCCTGAACTATCCAA -3'	Cy3	70	4
<i>F. succinogenes</i> Group 1	628-649	5'-GATCCAGTTCGGACTGCAGAGC-3'	Cy3	70	20
<i>F. succinogenes</i> Group 2	628-649	5'-AACCCAGTTCGGACTGCAGGTC-3'	FITC or Cy5	70	20
<i>F. succinogenes</i> Group 3	628-649	5'-GGTGCAGTCCGAACTGCAGGCC-3'	FITC	20	20
<i>R. flavefaciens</i>	155-171	5'-TACCATCCGTTTCCAGA-3'	Cy3 or FITC	20	This study

^a *Escherichia coli* rRNA numbering (9).

^b Percentage (vol/vol) of formamide in the hybridization.

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TABLE 3. Validation of real-time PCR assays for *Fibrobacter succinogenes*, *Ruminococcus flavefaciens* and total bacteria.

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

^a Serially diluted plasmid was used as a template.

^b Amplification efficiency in each PCR assay was calculated as $E = 10^{(-1/\text{slope})}$.

^c Coefficient of variation was determined with five replicates.

^{xy} Within column, means followed by different letters differ significantly ($P < 0.05$).

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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).

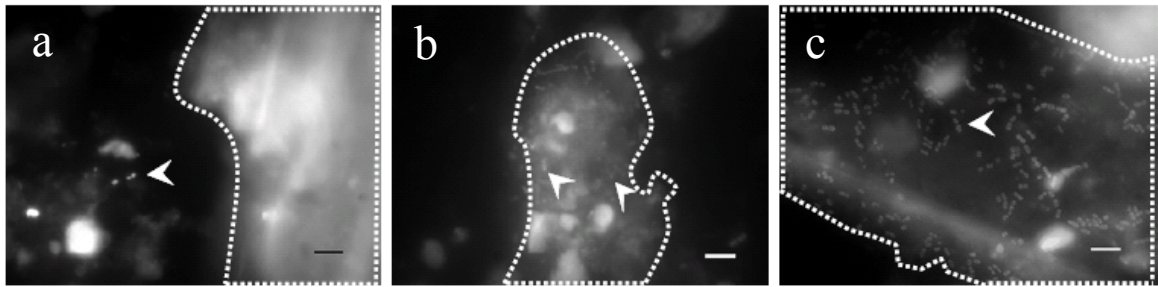
Substrate	Quantities (log copies \pm SD / g of sample)		
	Total bacteria	<i>F. succinogenes</i>	<i>R. flavefaciens</i>
Leaf sheath	11.52 \pm 0.01 ^x	10.36 \pm 0.03 ^x	9.41 \pm 0.05 ^x
Stem	11.25 \pm 0.02 ^y	10.01 \pm 0.09 ^y	8.56 \pm 0.03 ^y
Ratio (Leaf sheath / Stem)	1.86 \pm 0.10 ^a	1.92 \pm 0.36 ^a	5.44 \pm 0.59 ^b

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.

^{x,y}Within column, means followed by different letters differ significantly ($P < 0.05$).

^{a,b}Within row, means followed by different letters differ significantly ($P < 0.01$).

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561 FIG. 1. Comparison between the three different protocols for FISH detection of
562 *Ruminococcus flavefaciens* associated with ruminally incubated leaf sheaths of orchard
563 grass hay. The hay was untreated (a) or treated with toluidine blue O using Weber's method
564 (b) or the method described in the present study (c). *R. flavefaciens* was hybridized with
565 Cy3-labeled probe (arrowheads). Scale bars, 5 μ m.

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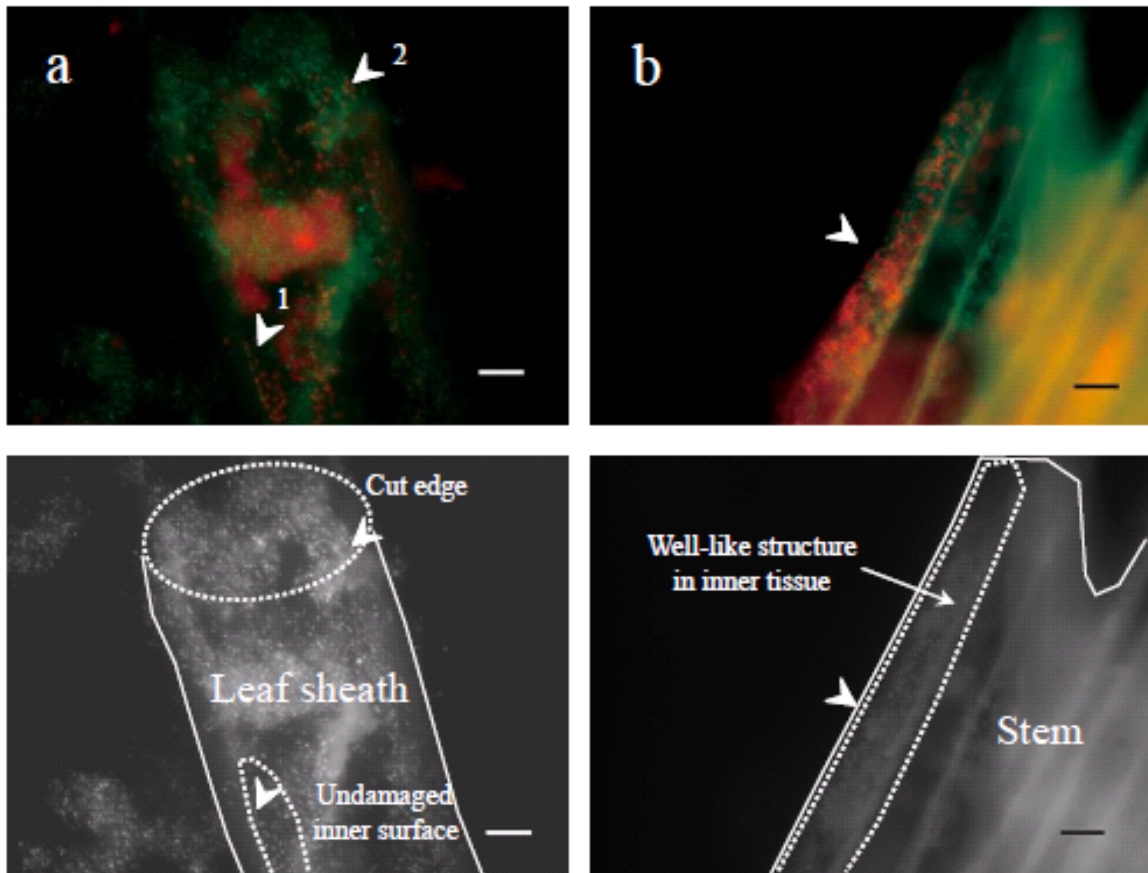
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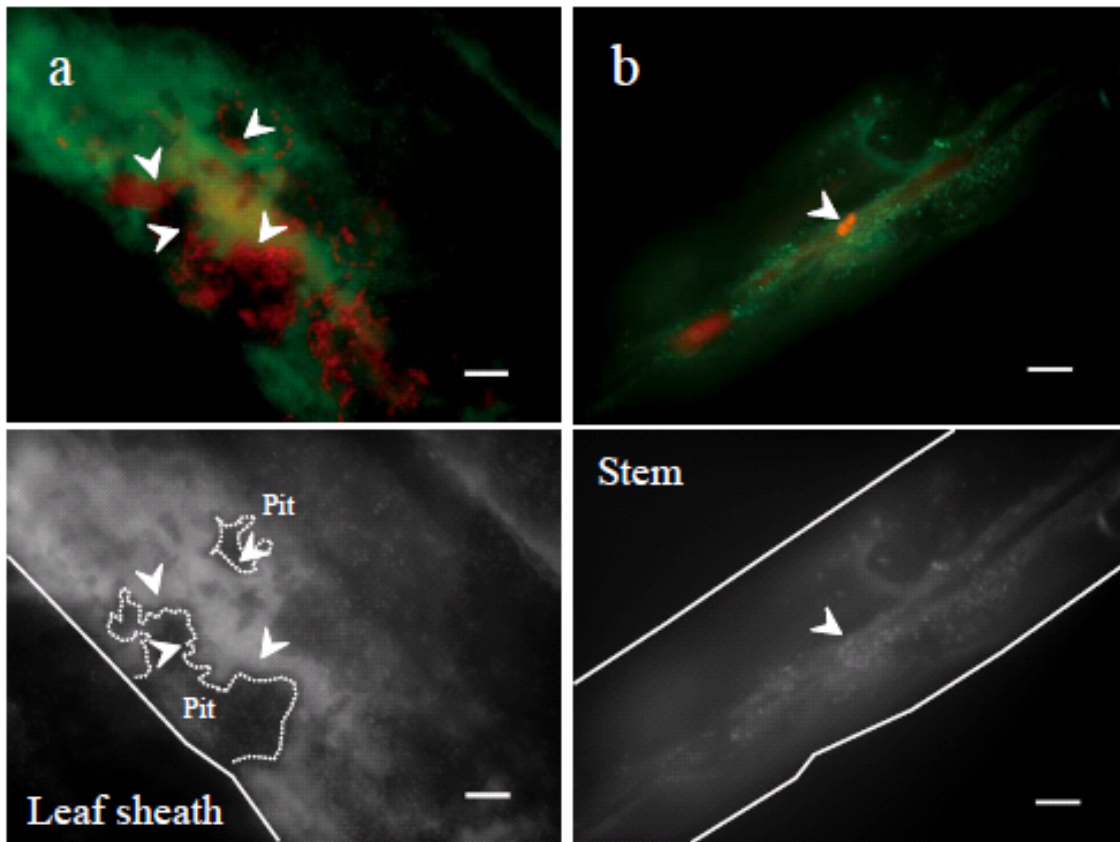
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583 FIG. 2.

584 Detection of *Fibrobacter succinogenes* cells belonging to group 1 on orchard grass hay
 585 incubated in the rumen of a sheep for 24 hours. Upper panels: bacteria on the leaf sheaths
 586 (a) and stems (b) of the ruminally incubated orchard grass hay were hybridized with a
 587 Cy3-labeled *F. succinogenes* group 1 probe (red) and stained with DAPI (green). (a) Cells
 588 tightly adhered to the cell walls of the leaf sheaths (arrowhead 1), or dispersed and
 589 coexisted with many other bacteria (arrowhead 2). (b) Cells were attached to a well-like
 590 structure in the inner tissue of the stem at high density (arrowhead), but were smaller than
 591 the cells attached to the leaf sheaths. Scale bars, 5 μ m. Lower panels: structural outline of
 592 the plant tissue used for observation.



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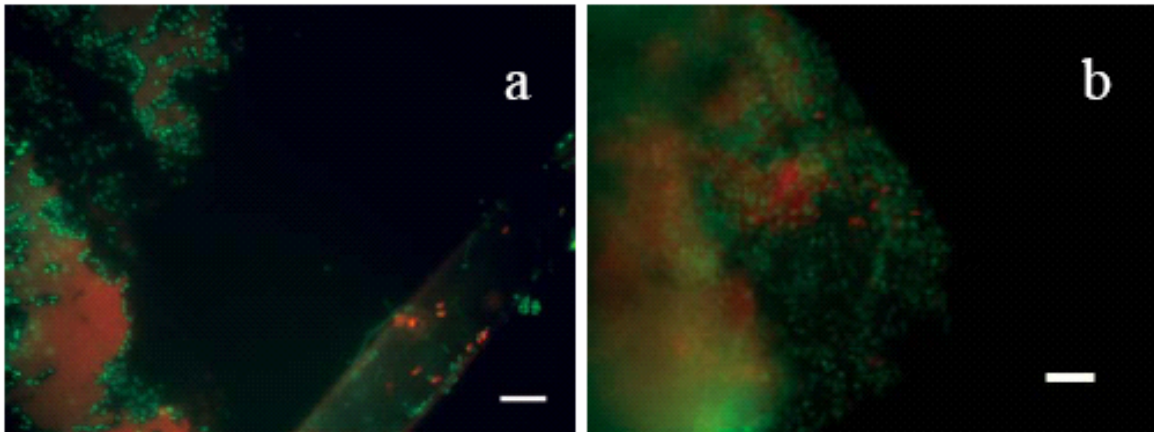
595 FIG. 3.

596 Detection of *Ruminococcus flavefaciens* cells on orchard grass hay incubated in the rumen
 597 of a sheep for 24 hours. Upper panels: bacteria on the leaf sheaths (a) and stems (b) of
 598 ruminally incubated orchard grass hay were hybridized with a Cy3-labeled *R. flavefaciens*
 599 probe (red) and were stained with DAPI (green). (a) Small *R. flavefaciens* cells created
 600 many pits, and were located along edges of the pits (arrowhead). (b) *R. flavefaciens* cells
 601 were rarely detected in stems (arrowhead). Scale bars, 5 μ m. Lower panels: structural
 602 outline of the plant tissue used for observation.

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608 FIG. 4. Detection of *Fibrobacter succinogenes* cells belonging to group 1 (a) and group 2
609 (b) in the fibrous rumen contents. Bacteria attached to the fibrous rumen contents were
610 hybridized with a Cy3-labeled probe for *F. succinogenes* group 1 (red, a) or with an
611 FITC-labeled probe for *F. succinogenes* group 2 (red, b). All bacteria were stained with
612 DAPI (green). Scale bars, 5 μ m.

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