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Instructions for use

1 **Appl. Environ. Microbiol.**

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3 **Section for publication: Methods**

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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**

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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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INTRODUCTION

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

3

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**

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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

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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; $NaHCO_3$ (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 × *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 μg/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 μm; Sigma Chemicals, St Louis, MO). After 40 μl 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 μl 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   *F. 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 *F. 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

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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

204   ev.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 $\pm$ 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 37of 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

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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 (λmax $\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

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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   endoglucanse 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.

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384

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TABLE 1. Bacterial strains used in the present study

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

*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

Probe specificity	16S rRNA target site [a]	Sequence	Dyes used	% Formamide [b]	Reference
F. succinogenes	650-669	5'-TGCCCCTGAACTATCCAA-3'	Cy3	70	4
F. succinogenes Group 1	628-649	5'-GATCCAGTTCGGACTGCAGAGC-3'	Cy3	70	20
F. succinogenes Group 2	628-649	5'-AACCCAGTTCGGACTGCAGGTC-3'	FITC or Cy5	70	20
F. succinogenes Group 3	628-649	5'-GGTGCAGTCCGAACTGCAGGCC-3'	FITC	20	20
R. flavefaciens	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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522 TABLE 3. Validation of real-time PCR assays for *Fibrobacter succinogenes, Ruminococcus flavefaciens* and total bacteria.

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Target	DNA used	Quantification range (10^x copy)[a]	Amplification efficiency (%)[b]	CV (%)[c]	
				intra-assay	inter-assay
F. succinogenes	plasmid, rumen DNA	1-9	1.94 ± 0.07 [x]	7.5	11.6
R. flavefaciens	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

528 [a] Serially diluted plasmid was used as a template.

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

530 [c] Coefficient of variation was determined with five replicates.

531 [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	*F. succionogenes*	*R. flavefaciens*
Leaf sheath	11.52 ± 0.01^{x}	10.36 ± 0.03^{x}	9.41 ± 0.05^{x}
Stem	11.25 ± 0.02^{y}	10.01 ± 0.09^{y}	8.56 ± 0.03^{y}
Ratio (Leaf sheath / Stem)	1.86 ± 0.10^{a}	1.92 ± 0.36^{a}	5.44 ± 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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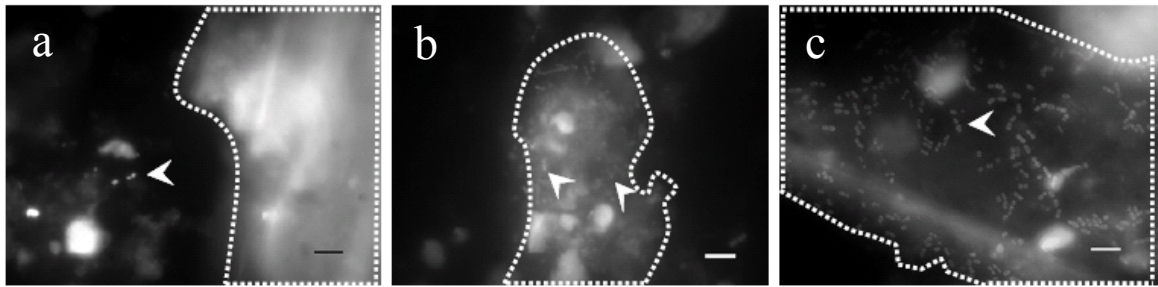
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561 FIG. 1. Comparison between the three different protocols for FISH detection of
562 *Ruminococcus flavefaciens* associated with ruminally incubated leaf sheaths of orchard
563 grass hay. The hay was untreated (a) or treated with toluidine blue O 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 μm.
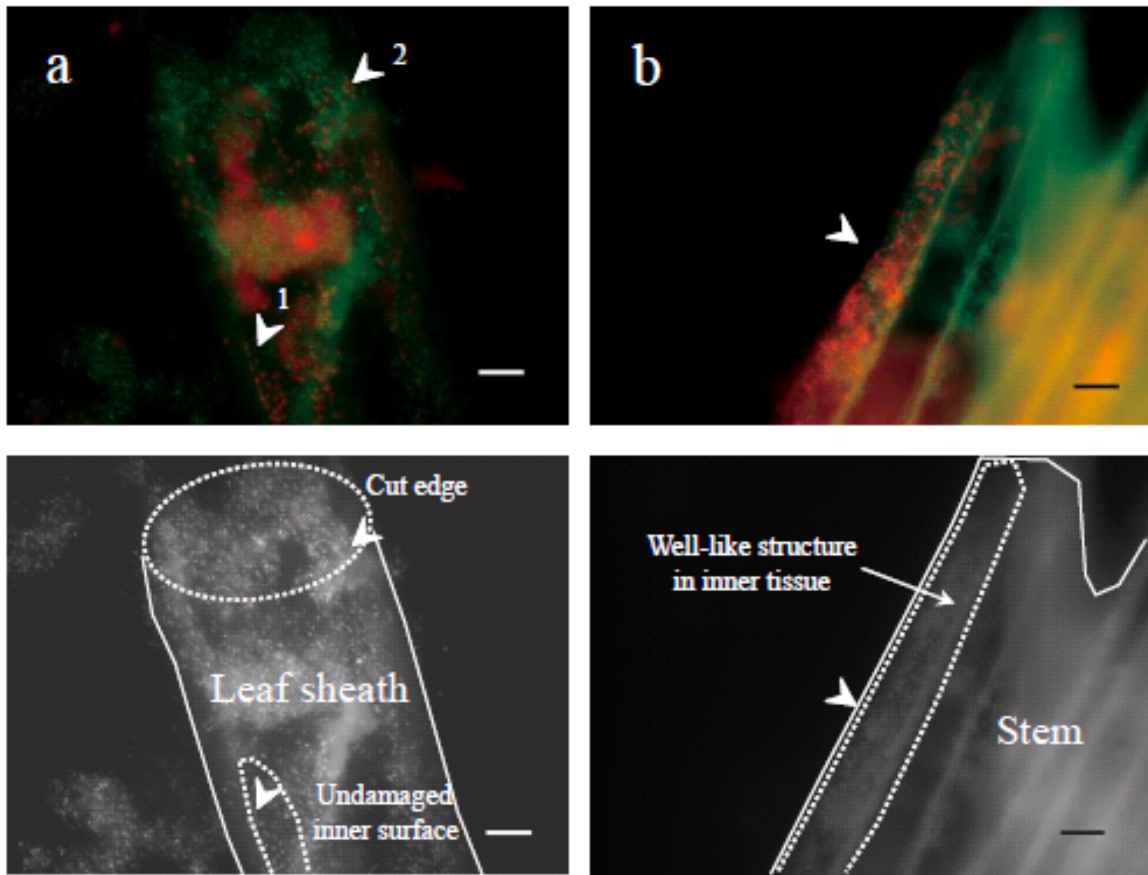
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583   FIG. 2.

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 μm. Lower panels: structural outline of

592   the plant tissue used for observation.

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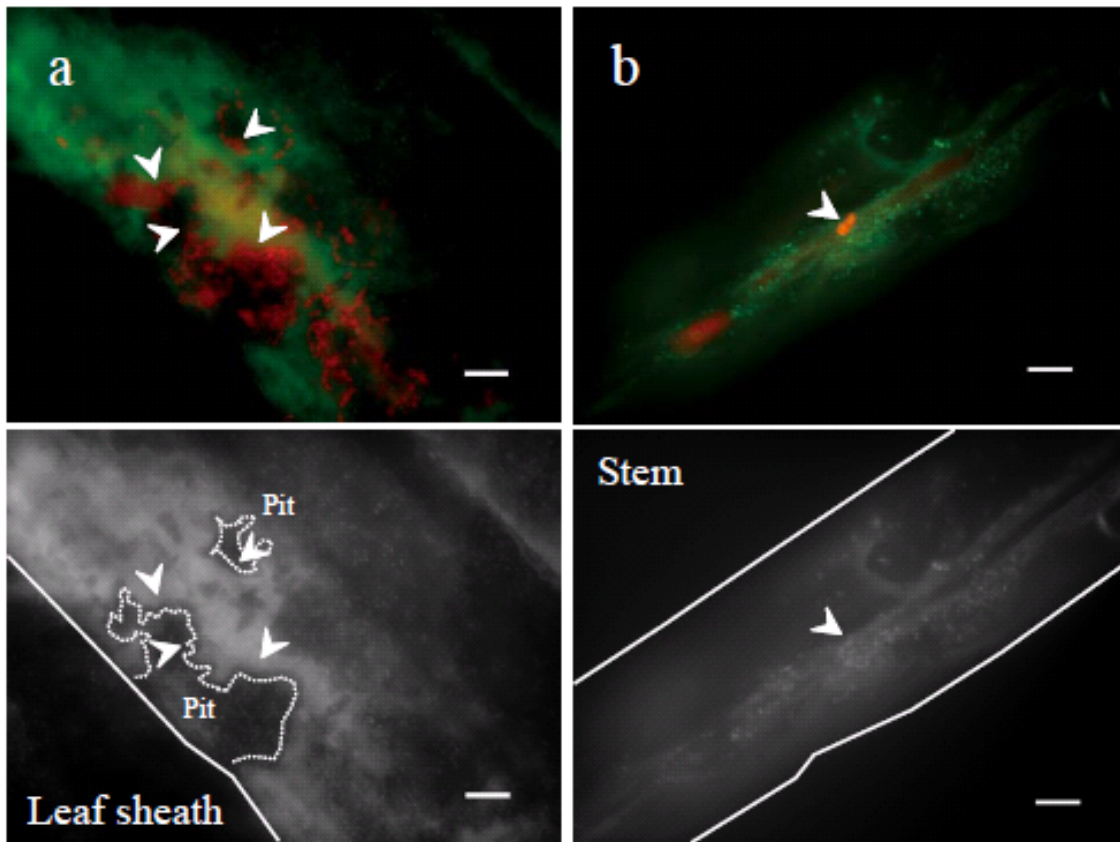
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595   FIG. 3.

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 μm. Lower panels: structural

602   outline of the plant tissue used for observation.

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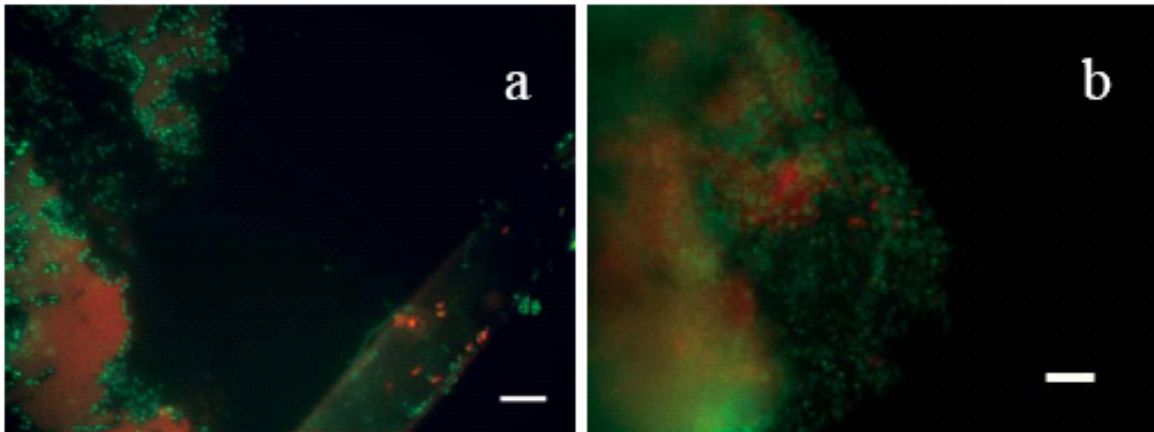
FIG. 4. Detection of *Fibrobacter succinogenes* cells belonging to group 1 (a) and group 2 (b) in the fibrous rumen contents. Bacteria attached to the fibrous rumen contents were hybridized with a Cy3-labeled probe for *F. succinogenes* group 1 (red, a) or with an FITC-labeled probe for *F. succinogenes*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