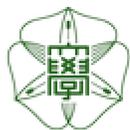
HOKKAIDO UNIVERSITY

Title	Monitoring of gene expression in Fibrobacter succinogenes S85 under the co-culture with non-fibrolytic ruminal bacteria
Author(s)	Fukuma, Naoki M.; Koike, Satoshi; Kobayashi, Yasuo
Citation	Archives of Microbiology, 197(2), 269-276 https://doi.org/10.1007/s00203-014-1049-0
Issue Date	2015-03
Doc URL	http://hdl.handle.net/2115/61012
Rights	The final publication is available at link.springer.com
Type	article (author version)
File Information	68706(Koike).pdf

Instructions for use

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

Monitoring of gene expression in *Fibrobacter succinogenes* S85 under the co-culture with non-fibrolytic ruminal bacteria

Naoki M. Fukuma, Satoshi Koike* and Yasuo Kobayashi

Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan

***Correspondence**: Satoshi Koike, Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan.

Tel & Fax: +81 (11) 706-2812

E-mail: skoike7@anim.agr.hokudai.ac.jp

Key words: rumen bacteria, *Fibrobacter succinogenes,* fiber digestion, co-culture, mRNA expression

15 ## Abstract

16 *Fibrobacter succinogenes* is one of the most pivotal fibrolytic bacterial
17 species in the rumen. In a previous study, we confirmed enhancement of fiber digestion
18 in a co-culture of *F. succinogenes* S85 with non-fibrolytic ruminal strains R-25 and/or
19 *Selenomonas ruminantium* S137. In the present study, mRNA expression level of
20 selected functional genes in the genome of *F. succinogenes* S85 was monitored by
21 real-time RT-PCR. Growth profile of *F. succinogenes* S85 was similar in both the
22 monoculture and co-cultures with non-fibrolytics. However, expression of 16S rRNA
23 gene of *F. succinogenes* S85 in the co-culture was higher ($P < 0.01$) than that of the
24 monoculture. This finding suggests that metabolic activity of *F. succinogenes* S85 was
25 enhanced by coexistence with strains R-25 and/or *S. ruminantium* S137. The mRNA
26 expression of fumarate reductase and glycoside hydrolase genes was up-regulated ($P <$
27 0.01) when *F. succinogenes* S85 was co-cultured with non-fibrolytics. These results
28 indicate the enhancement of succinate production and fiber hydrolysis by *F.*
29 *succinogenes* S85 in co-cultures of *S. ruminantium* and R-25 strains.

30

31 ## Introduction

32 Ruminant animals mostly depend on microbial fermentation within the rumen
33 to acquire energy from plant fibrous materials. In the rumen microbial ecosystem,
34 fibrolytic rumen bacteria such as *Fibrobacter succinogenes, Ruminococcus flavefaciens*
35 and *Ruminococcus albus* have been reported to be involved in ruminal fiber digestion
36 (Flint, 1997; Krause et al., 2003). Several studies employing quantitative PCR

techniques targeting the 16S rRNA gene have revealed the predominance of *F. succinogenes* as compared to other ruminal fibrolytic species (Kobayashi et al., 2008; Mosoni et al., 2011; Lettat et al., 2012; Saro et al., 2012; Koike et al., 2014). Recent genomic studies reported that *F. succinogenes* possesses more abundant and diverse carbohydrate active enzymes, involved in polysaccharide degradation as compared to those of the other ruminococcal species (Berg Miller et al., 2009; Suen, et al., 2011a; 2011b). These findings reveal that *F. succinogenes* is the most pivotal fibrolytic bacterium in the rumen.

On the other hand, the fiber-associated bacterial community in the rumen also consists of a large number of other non-fibrolytic bacteria (Koike et al., 2003; Brulc et al., 2009) which probably play a role in ruminal fiber digestion. The mechanism of the contribution of non-fibrolytic bacteria in ruminal fiber digestion acts in an indirect manner, such as by hydrogen transfer or by cross-feeding of degradation and/or fermentation products derived from fiber (Flint, 1997). To investigate a relationship between fibrolytics and non-fibrolytics several *in vitro* co-culture studies using *F. succinogenes* and non-fibrolytic rumen bacterial species have been performed (Dehority and Scott, 1967; Kudo et al., 1987; Fondevila and Dehority, 1996). These studies revealed that fiber digestion was enhanced by coexistence of *F. succinogenes* with other non-fibrolytic strains. Based on the ecology of fiber-associated rumen bacteria, we had earlier demonstrated that non-fibrolytic group U2 and *Selenomonas ruminantium* can be a core member of the fibrolytic community in the rumen, as well as fibrolytic *F. succinogenes* (Koike et al., 2003; 2007; Koike and Kobayashi, 2009; Koike et al., 2010;

59 2014; Shinkai et al., 2014). Also positive interaction among fibrolytic *F. succinogenes*
60 S85 and non-fibrolytic group U2 bacterium R-25 and/or *S. ruminantium* S137 was
61 confirmed by *in vitro* co-culture studies, which revealed that rice straw digestibility and
62 metabolite production were both enhanced (Sawanon et al., 2011; Fukuma et al., 2012).
63 Although earlier co-culture studies for activation of *F. succinogenes* S85 using
64 conventional approaches such as measurement of fiber digestibility, bacterial growth,
65 fermentation products and enzyme activity have been reported, no direct evidence with
66 regards to an accurate molecular evaluation has been obtained yet. On the other hand,
67 molecular approaches enable us to monitor expression of specific genes that exist in the
68 genome of a bacterium. Béra-Maillet *et al*. (2009) have developed a RT-qPCR method
69 to quantify mRNA expression of functional glycoside hydrolase (GH) genes of *F.*
70 *succinogenes* S85 and have succeeded in specific monitoring of GH genes expression.
71 Thus, we aimed to obtain the molecular evidence for activation of *F. succinogenes* S85
72 in the co-culture with non-fibrolytic strains by quantification of mRNA expression level
73 of functional genes in the genome of this bacterium.
74

75 ## Materials and Methods

76 ### Bacterial strains, medium and incubation conditions

77 *Fibrobacter succinogenes* S85 was purchased from American Type Culture
78 Collection. Rumen bacterium R-25 and *Selenomonas ruminantium* S137 were
79 previously isolated by our research group (Koike *et al.,* 2010; Sawanon *et al.,* 2011).
80 Monoculture, two-member co-culture and three-member co-culture experiments were

performed as previously reported (Fukuma et al., 2012). In brief, *Fibrobacter succinogenes* S85 as a fibrolytic rumen bacterium, and rumen bacterium R-25 and *Selenomonas ruminantium* S137 as non-fibrolytic rumen bacteria were used in this study. Basal medium was prepared anaerobically which composed of (per 100 ml): 7.5 ml of mineral solutions I and II (Bryant and Burkey, 1953), 0.1 ml of 0.1% resazurin, 40 ml of clarified rumen fluid, 39 ml of distilled water, 1 ml of 5% L-cysteine-HCl·H_2O and 5 ml of 8% Na_2CO_3.

Cells were subcultured three times consecutively with the basal media containing rice straw (1.0%; w/v) or cellobiose and glucose (0.5%; w/v of each) as carbon source(s) for *F. succinogenes* S85 or non-fibrolytics, respectively. The OD was adjusted (OD_{660} = 0.2) for each bacterium. This was prepared using anaerobic dilution solution (Bryant and Burkey, 1953) and used as an inoculum. The inoculum was added at a dilution of 0.1 ml to 10 ml of the basal medium containing 0.1 g of rice straw as the sole carbon source, and tubes were incubated at 39°C under anaerobic conditions. Six replicates were used for all four sets: monoculture of *F. succinogenes* S85, two-member co-culture of *F. succinogenes* S85 and strain R-25 or *S. ruminantium* S137, and three-member co-culture, out of which three tubes were used for sampling after 24 h, and the remaining three tubes were used for sampling after 48 h of incubation.

Measurement of metabolites and reducing sugars

After 24 h or 48 h incubation, the cultures of *F. succinogenes* S85 monoculture and three-member co-culture were centrifuged (16,000 ×*g*, 4°C, 10 min) to obtain cell-free supernatant that was used for measurement of metabolites and reducing

103 sugars. Short chain fatty acids were determined by gas chromatography (GC-14B,
104 Shimadzu, Kyoto, Japan). Succinate and D-/L-lactate were measured by commercial
105 assay kits (Megazyme, Wicklow, Ireland). Oligosaccharides derived from rice straw
106 digestion were estimated by measuring the concentration of reducing sugar, as described
107 by Cotta (1988).

108 **Nucleic acid isolation**

109 Bacterial cells adhering to rice straw in the culture were collected after 24 h or
110 48 h incubation using the following procedure. Cultures were centrifuged (377 ×*g*, 4°C,
111 10 min) to precipitate the rice straw particles, and the supernatant containing planktonic
112 bacterial cells was removed. The residue was washed with 10 ml of 0.1 M RNase-free
113 potassium phosphate buffer and re-centrifuged (377 ×*g*, 4°C, 10 min). RNA protect
114 Bacterial Reagent (2 ml) (Qiagen, Hilden, Germany) was added to the washed residue.
115 The rice-straw samples were centrifuged (377 ×*g*, 4°C, 10 min) the supernatant was
116 removed.

117 DNA and RNA were both co-extracted from 0.25 g of the collected rice-straw
118 samples. Two ml stainless-steel tube (Bio medical science, Tokyo, Japan) containing the
119 rice straw sample was flash-frozen in liquid nitrogen and the samples were ground with
120 four pieces of stainless-steel ball (Ø 3.2 mm; TOMY, Tokyo, Japan) for 1 min at a
121 maximum speed using a Mini BeadBeater (BioSpec Products, Bartlesville, OK). The
122 samples were further incubated with 100 μl of RNase-free TE buffer (pH 8.0)
123 containing 3 mg/ml lysozyme (Thermo Fisher Scientific, Waltham, MA) for 5 min at
124 room temperature. Crude nucleic acids containing DNA and RNA were treated with

125 RLT buffer (RNeasy Mini Kit, Qiagen, Hilden, Germany) and ß-mercaptoethanol
126 following the manufacturer's instruction. In order to purify DNA and RNA separately,
127 the nucleic acids extract was divided to two aliquots of 300 µl each. DNA was purified
128 using the RBB+C method purification procedure (Yu and Morrison, 2004). RNA was
129 purified using the RNeasy mini kit with the optional on-column DNase treatment step
130 according to the manufacturer's instructions.

131 Concentration and purity of nucleic acids were evaluated by absorbance at
132 A_{260} and measuring absorbance ratios at A_{260}/A_{280} and A_{260}/A_{230} using the NanoDrop
133 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). RNA integrity was
134 estimated by the band intensities of 23S and 16S rRNA on a 1% [wt/vol] agarose gel by
135 electrophoresis.

136 **Reverse transcription and real-time PCR**

137 Total RNA (0.2 µg) was reverse-transcribed into cDNA using random
138 hexamer primers and 200 U of Superscript III Reverse Transcriptase (Invitrogen,
139 Burlington, Ontario, Canada) according to the manufacturer's instructions. A reverse
140 transcriptase negative control was also included, and generated products were used in
141 subsequent real-time PCRs.

142 The PCR primer sequences used in the present study are shown in Table 1.
143 Genes encoding fumarate reductase (*frd*), cellulolytic enzymes (*cel5C*, *cel5G*, $endA_{FS}$,
144 *cel9G* and *cel51A*) and hemicellulolytic enzymes (*xyn10D* and *xyn11C*) were selected as
145 target genes. Primers for *frd* were newly designed. The genomic sequence of *F.*
146 *succinogenes* S85 was obtained from GenBank (Accession number: CP001792). One of

147 the genes annotated as fumarate reductase was selected from the genome of *F.*
148 *succinogenes* S85, and a primer set was designed using CLC genomics workbench
149 software; version 5.0 (CLC Bio, Cambridge, MA). The copy number of 16S rRNA gene
150 (16S rDNA) and its transcript (i.e., 16S rRNA) was quantified and used as the indices of
151 cell number and metabolic activity of *F. succinogenes* S85, respectively. In order to
152 monitor the growth profile of non-fibrolytic strains, 16S rDNA copy number of *S.*
153 *ruminantium* S137 and strain R-25 was also quantified by using the specific primer sets
154 for respective strains.

155 Preparation of standard template for real-time PCR was performed as
156 described by Koike et al. (2007). The real-time PCR assay was conducted for the
157 absolute quantification of mRNA copy with the standard curve method using a dilution
158 series of standard template. In brief, each target gene was cloned using pGEM-T Easy
159 Vector Systems (Promega, Madison, WI). The concentration of the plasmid was
160 determined using the NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific,
161 Waltham, MA). Copy number of each standard plasmid was calculated using the
162 molecular weight of nucleic acid and the length (base pair) of the cloned standard
163 plasmid. Ten-fold dilution series ranging from 10 to 10^9 copies was prepared for each
164 target.

165 Real-time PCR was performed with a KAPA SYBR FAST qPCR Kit (KAPA
166 Biosystems, Woburn, MA) and a LightCycler 480 System (Roche Applied Science,
167 Mannheim, Germany). Amplification conditions described by Koike et al. (2007) and
168 Béra-Maillet et al. (2009) were used for quantification of 16S rRNA gene and GH genes,

169 respectively. The PCR condition for *frd* was optimized in this study. The melting curve
170 of PCR products was monitored by heating 70°C to 95°C at 0.1°C intervals at the end of
171 the real-time PCR to check for specific amplification. Specific amplification of the
172 target gene was confirmed by the presence of a single peak in each melting curve.

173 Copy number of 16S rDNA and 16S rRNA were quantified, and a ratio of
174 16S rRNA/rDNA under each culture condition of *F. succinogenes* S85 was calculated.
175 The cDNA copy number of target genes encoding fumarate reductase and GHs were
176 normalized by copy number of 16S rDNA derived from the same culture. Extent of
177 gene expression was expressed as the ratio of the copy number of each target gene per
178 10^8 copies of 16S rDNA.

179 **Statistical analysis**

180 Data were expressed as means ± standard deviation. The means for each
181 treatment were subjected to one-way analysis of variance and Tukey's test to detect
182 differences between treatments using GraphPad Prism (ver. 5.0d, GraphPad Software,
183 La Jolla, CA). $P < 0.01$ was regarded as statistically significant.

184

185 Results and discussion

186 Although previous studies (Dehority and Scott, 1967; Kudo et al., 1987;
187 Fondevila and Dehority, 1996; Sawanon et al., 2011; Fukuma et al., 2012) have
188 demonstrated the enhancement of fiber digestion in mixed cultures, there was no direct
189 evidence for activation of fibrolytic bacteria under the co-existence of non-fibrolytics.
190 The present study is the first report of increased fibrolytic and metabolic activity of *F.*

191 *succinogenes* S85 in co-cultures with non-fibrolytics at the molecular level. In the
192 present study, we sampled at 24 h and 48 h after incubation for the monitoring of
193 metabolic activity of *F. succinogenes* S85, because these time points corresponded to
194 the initial phase and middle phase of rice straw digestion by this strain, respectively
195 (Shinkai et al., 2009).

196 Growth profiles of *F. succinogenes* S85, strain R-25 and *S. ruminantium* S137
197 in monoculture or co-culture are shown in Fig. 1 and Table S1. Three strains showed
198 similar growth profile both in monoculture and co-culture conditions. The growth
199 profiles of *F. succinogenes* S85 and *S. ruminantium* S137 were similar, while the growth
200 rate of strain R-25 was lower than the other two strains (Fig. 1). Changes in the 16S
201 rRNA copy number and 16S rRNA/rDNA ratio of *F. succinogenes* S85 in its
202 monoculture and co-culture with non-fibrolytics are shown in Table 2. When *F.*
203 *succinogenes* S85 was incubated with non-fibrolytic bacteria, significant increase in 16S
204 rRNA/rDNA ratio was observed. As the expression of rRNA gene is correlated with
205 protein synthesis, the ratio of rRNA/rDNA has been considered as a useful index for
206 metabolic activity per single cell (Muttray and Mohn, 1999; Muttray et al., 2001;
207 Pérez-Osorio et al., 2010). In the present study, coexistence of strain R-25 did not affect
208 the metabolic activity of *F. succinogenes* S85 after 24 h of incubation, on the other hand
209 *S. ruminantium* S137 enhanced the metabolic activity of *F. succinogenes* S85 at an early
210 stage (24 h after incubation) (Table 2). These differences could be attributed to the
211 lower growth rate of strain R-25 compared to that of *S. ruminantium* S137 (Fig.1). After
212 48 h incubation, 16S rRNA/rDNA ratio in both co-cultures was significantly higher than

213 that of the monoculture, indicating significant positive effects of non-fibrolytic bacteria
214 on metabolic activity of *F. succinogenes* S85.

215 The ratio of 16S rRNA/rDNA at 48 h was numerically lower as compared to
216 the 24 h condition, both in monoculture and co-culture; with the exception of the
217 three-member coculture. However, the degree of decline from 24 h to 48 h was less in
218 the two-member co-culture compared to those of the monoculture. These findings
219 indicate that the metabolic activity of *F. succinogenes* S85 is shown to decline with the
220 incubation time, but co-existing non-fibrolytic bacteria may reduce the decline of
221 metabolic activity of this strain. Furthermore, the increased value of 16S rRNA/rDNA
222 in the three-member co-culture after 48 h (Table 2) suggests that co-existence of both of
223 the strains R-25 and *S. ruminantium* S137 could enhance the metabolic activity of *F.*
224 *succinogenes* S85 synergistically. Reduced activity of *F. succinogenes* S85 in the
225 monoculture could be attributed to the accumulation of metabolites (hydrogen and
226 succinate) and/or oligosaccharides (McGavin et al., 1990; Latham and Wolin, 1977;
227 Williams et al., 1994; Rychlik and May, 2000). Strain R-25 utilizes oligosaccharides
228 and produces lactate, a hydrogen sink, as the main fermentation product (Fukuma et al.,
229 2012). On the other hand, *S. ruminantium* S137 consumes lactate, succinate and
230 oligosaccharides as growth substrates (Sawanon et al., 2011; Fukuma et al., 2012). In
231 the present study, concentrations of oligosaccharides and succinate were significantly
232 lower in the three-member co-culture suggesting the consumption of these metabolites
233 by strains R-25 and *S. ruminantium* S137 (Table 3). In addition, lactate from strain R-25
234 served as a growth substrate for *S. ruminantium* S137 and could be converted into

235 propionate (Table 3). Therefore, hydrogen transfer and crossfeeding of
236 metabolites/oligosaccharides in the three strains may have enhanced the removal of
237 suppression factors for *F. succinogenes* S85, leading to further activation of the strain.
238 When *F. succinogenes* S85 was co-cultured with strain R-25, the mRNA expression of
239 *frd* gene was up-regulated as compared to the monocultures (Table 4). This result
240 corresponds well to an earlier study in which enhanced succinate production of *F.*
241 *succinogenes* S85 was observed with co-existence of the strain R-25 (Fukuma et al.,
242 2012). Also, up-regulation of *frd* expression in the three-member co-culture was found
243 (Table 4), indicating enhancement of succinate production by *F. succinogenes* S85.

244 Majority of GH genes in the genome of *F. succinogenes* S85, have not been
245 characterized with regards to its functional analysis for encoding proteins (Suen et al.,
246 2011b). In order to confirm enhancement of fibrolytic activity of *F. succinogenes* S85
247 under mixed cultures, genes encoding glycoside hydrolases were targeted in this study.
248 In the monoculture of *F. succinogenes* S85, expression level of GH genes at 48 h were
249 lower than those at 24 h with the exception of *xyn11C* (Table 5). Catabolite repression
250 of *F. succinogenes* S85 is well known to be associated with decline of endoglucanase
251 activity related to hydrolytic products of polysaccharides, such as cellobiose (McGavin
252 et al., 1990). Lower expression level of GH genes in *F. succinogenes* S85 monoculture
253 may suggest declined expression of these genes by accumulation of fiber
254 digestion-related products. Upon co-culturing *F. succinogenes* S85 with strains R-25 or
255 *S. ruminantium* S137, six GH genes were found to be up-regulated compared with *F.*
256 *succinogenes* S85 monoculture post 48 h of incubation (Table 5). This may be attributed

257 to consumption of fiber digestion-related products by the non-fibrolytics, resulting in
258 reduction of catabolite repression of *F. succinogenes* S85. Expressions of genes of *frd*
259 and GHs were similar between monoculture and co-culture at 24 h incubation;
260 meanwhile most of these genes were up-regulated in co-culture after 48 h incubation
261 (Tables 4 and 5). These findings suggest that metabolic activity of *F. succinogenes* S85
262 is enhanced by the two non-fibrolytics between 24 h and 48 h after incubation.

263 Among the GH genes quantified in the present study, $endA_{FS}$ and *xyn11C*
264 showed increased expression levels compared to other GH genes at 48 h of incubation
265 (Table 5). Béra-Maillet *et al.* (2000b) monitored GH-genes expression of *F.*
266 *succinogenes* S85 grown on a cellulose filter paper and concluded that these two genes
267 could play a major role in fiber digestion of *F. succinogenes*. Our findings in the present
268 study suggest that the enzymes encoded by $endA_{FS}$ and *xyn11C* also play a key role in
269 digestion of less digestible natural-fiber. The importance of these enzymes has also been
270 characterized by other functional analysis. Enzyme encoded by $endA_{FS}$ gene is known to
271 have multi-functional activity and is able to hydrolyze cellulosic and other xylanosic
272 polysaccharides, such as oat spelt xylan (Cavicchioli and Watson, 1991). Paradis *et al.*
273 (1997) reported that enzymes encoded by *xyn11C* showed maximum increase in
274 xylanolytic activity for birchwood xylan among other characterized xylanase genes of *F.*
275 *succinogenes* S85. Therefore, up-regulation of $endA_{FS}$ and *xyn11C* expression could
276 reasonably be explained with the enhancement of rice straw digestion of *F.*
277 *succinogenes* S85 in co-culture with non-fibrolytics.

278 In conclusion, the expression of 16S rRNA, *frd* and GH genes, are associated

279 and indicative of metabolic and fibrolytic activity of *F. succinogenes* S85, and these
280 were up-regulated under co-cultures with non-fibrolytic bacteria R-25 and *S.*
281 *ruminantium* S137. These results validate the enhancement of succinate production and
282 fiber digestion by *F. succinogenes* S85 under the co-existence with non-fibrolytics at the
283 molecular level.
284

285 **Acknowledgement**

286 This study was supported in part by a Grant-in Aid for Scientific Research
287 (No. 22780238 to S. K. and No. 17380157 to Y. K.) from the Japanese Ministry of
288 Education, Culture, Sports, Science and Technology.
289

290 ## References

291 Berg Miller ME, Antonopoulos DA, Rincon MT, et al (2009) Diversity and strain
292 specificity of plant cell wall degrading enzymes revealed by the draft genome of
293 *Ruminococcus flavefaciens* FD-1. *PLoS ONE* **4**: e6650.

294

295 Béra-Maillet C, Broussolle V, Pristas P, Girardeau J-P, Gaudet G, Forano E (2000a)
296 Characterisation of endoglucanases EGB and EGC from *Fibrobacter succinogenes*.
297 *Biochim Biophys Acta* **1476**: 191–202.

298

299 Béra-Maillet C, Gaudet G, Forano E (2000b) Endoglucanase activity and relative
300 expression of glycoside hydrolase genes of *Fibrobacter succinogenes* S85 grown on
301 different substrates. *Biochim Biophys Acta* **1543**: 77–85.

302

303 Béra-Maillet C, Mosoni P, Kwasiborski A, Suau F, Ribot Y, Forano E (2009)
304 Development of a RT-qPCR method for the quantification of *Fibrobacter succinogenes*
305 S85 glycoside hydrolase transcripts in the rumen content of gnotobiotic and
306 conventional sheep. *J Microbiol Methods* **77**: 8–16.

307

308 Brulc JM, Antonopoulos DA, Berg Miller ME, et al (2009) Gene-centric metagenomics
309 of the fiber-adherent bovine rumen microbiome reveals forage specific glycoside
310 hydrolases. *P Natl Acad Sci USA* **106**: 1948–1953.

311

312 Bryant MP, Burkey LA (1953) Cultural methods and characterization of some of the
313 more numerous groups of bacteria in the bovine rumen. *J Dairy Sci* **36**: 205-217.
314
315 Cavicchioli R, Watson K (1991) Molecular cloning, expression, and characterization of
316 endoglucanase genes from *Fibrobacter succinogenes* AR1. *Appl Environ Microbiol* **57**:
317 359–365.
318
319 Cotta MA (1988) Amylolytic activity of selected species of ruminal bacteria. *Appl*
320 *Environ Microbiol* **54:** 772-776.
321
322 Dehority BA, Scott HW (1967) Extent of cellulose and hemicellulose digestion in
323 various forages by pure culture of the rumen bacteria. *J Dairy Sci* **50**: 1136-1141.
324
325 Flint HJ (1997) The rumen microbial ecosystem -some recent developments. *Trends*
326 *Microbiol* **5**: 483–488.
327
328 Fondevila M, Dehority BA (1996) Interactions between *Fibrobacter succinogenes*,
329 *Prevotella ruminicola*, and *Ruminococcus flavefaciens* in the digestion of cellulose from
330 forages. *J Anim Sci* **74**: 678-684.
331
332 Fukuma N, Koike S, Kobayashi Y (2012) Involvement of recently cultured group U2
333 bacterium in ruminal fiber digestion revealed by coculture with *Fibrobacter*

334 *succinogenes* S85. *FEMS Microbiol Lett* **336**: 17-25

335

336 Huang L, Forsberg CW (1987) Isolation of a Cellodextrinase from *Bacteroides*
337 *succinogenes. Appl Environ Microbiol* **53**: 1034–1041.

338

339 Jun HS, Ha JK, Malburg LM, Verrinder GAM, Forsberg CW (2003) Characteristics of a
340 cluster of xylanase genes in *Fibrobacter succinogenes* S85. *Can J Microbiol* **49**:
341 171–180.

342

343 Kobayashi Y, Shinkai T, Koike S (2008) Ecological and physiological characterization
344 shows that *Fibrobacter succinogenes* is important in rumen fiber digestion - review.
345 *Folia Microbiol* **53**: 195–200.

346

347 Koike S, Yoshitani S, Kobayashi Y, Tanaka K (2003) Phylogenetic analysis of
348 fiber-associated rumen bacterial community and PCR detection of uncultured bacteria.
349 *FEMS Microbiol Lett* **229**: 23–30.

350

351 Koike S, Yabuki H, Kobayashi Y (2007) Validation and application of real-time
352 polymerase chain reaction assays for representative rumen bacteria. *Anim Sci J* **78**:
353 135-141.

354

355 Koike S, Kobayashi Y (2009) Fibrolytic rumen bacteria: Their ecology and functions.

356 *Asian-Aust J Anim Sci* **22**: 131–138.

357

358 Koike S, Handa Y, Goto H, Sakai K, Miyagawa E, Matsui H, Ito S, Kobayashi Y (2010)
359 Molecular monitoring and isolation of previously uncultured bacterial strains from the
360 sheep rumen. *Appl Environ Microbiol* **76**: 1887-1894.

361

362 Koike S, Yabuki H, Kobayashi Y (2014) Interaction of rumen bacteria as assumed by
363 colonization patterns on untreated and alkali - treated rice straw. *Anim Sci J* **85**:
364 524–531.

365

366 Krause DO, Denman SE, Mackie RI, Morrison M, Rae AL, Attwood GT, McSweeney
367 CS (2003) Opportunities to improve fiber degradation in the rumen: microbiology,
368 ecology, and genomics. *FEMS Microbial Rev* **27**: 663-693.

369

370 Kudo H, Cheng KJ, Costerton JW (1987) Interaction between *Treponema bryantii* and
371 cellulolytic bacteria in the in vitro degradation of straw cellulose. *Can J Microbiol* **33**:
372 244-248.

373

374 Latham MJ, Wolin MJ (1977) Fermentation of cellulose by *Ruminococcus flavefaciens*
375 in the presence and absence of *Methanobacterium ruminantium*. *Appl Environ*
376 *Microbiol* **34**: 297-301.

377

378 Lettat A, Nozière P, Silberberg M, Morgavi DP, Berger C, Martin C (2012) Rumen
379 microbial and fermentation characteristics are affected differently by bacterial probiotic
380 supplementation during induced lactic and subacute acidosis in sheep. *BMC Microbiol*
381 **12**: 142.
382
383 Malburg SR, Malburg LM, Liu T, Iyo AH, Forsberg CW (1997) Catalytic properties of
384 the cellulose-binding endoglucanase F from *Fibrobacter succinogenes* S85. *Appl*
385 *Environ Microbiol* **63**: 2449–2453.
386
387 McGavin M, Lam J, Forsberg CW (1990) Regulation and distribution of *Fibrobacter*
388 *succinogenes* subsp. *succinogenes* S85 endoglucanases. *Appl Environ Microbiol* **56**:
389 1235–1244.
390
391 McGavin MJ, Forsberg CW, Crosby B, Bell AW, Dignard D, Thomas DY (1989)
392 Structure of the cel-3 gene from *Fibrobacter succinogenes* S85 and characteristics of
393 the encoded gene product, endoglucanase 3. *J Bacteriol* **171**: 5587–5595.
394
395 Mosoni P, Martin C, Forano E, Morgavi DP (2011) Long-term defaunation increases the
396 abundance of cellulolytic ruminococci and methanogens but does not affect the bacterial
397 and methanogen diversity in the rumen of sheep. *J Anim Sci* **89**: 783–791.
398
399 Muttray A, Mohn W (1999) Quantitation of the population size and metabolic activity

400 of a resin acid degrading bacterium in activated sludge using slot-blot hybridization to
401 measure the rRNA:rDNA ratio. *Microb Ecol* **38**: 348–357.
402
403 Muttray AF, Yu Z, Mohn WW (2001) Population dynamics and metabolic activity of
404 *Pseudomonas abietaniphila* BKME-9 within pulp mill wastewater microbial
405 communities assayed by competitive PCR and RT-PCR. *FEMS Microbiol Ecol* **38**:
406 21–31.
407
408 Rychlik JL, May T (2000) The effect of methanogen, *Methanobrevibacter smithii*, on
409 the growth rate, organic acid production, and specific ATP activity of three predominant
410 ruminal cellulolytic bacteria. *Curr Microbiol* **40**: 176-180.
411
412 Paradis FW, Zhu H, Krell PJ, Phillips JP, Forsberg CW (1993) The *xynC* gene from
413 *Fibrobacter succinogenes* S85 codes for a xylanase with two similar catalytic domains.
414 *J Bacteriol* **175**: 7666–7672.
415
416 Pérez-Osorio AC, Williamson KS, Franklin MJ (2010) Heterogeneous *rpoS* and *rhlR*
417 mRNA levels and 16S rRNA/rDNA (rRNA gene) ratios within *Pseudomonas*
418 *aeruginosa* biofilms, sampled by laser capture microdissection. *J Bacteriol* **192**:
419 2991–3000.
420
421 Saro C, Ranilla MJ, Carro MD (2012) Postprandial changes of fiber-degrading microbes

422 in the rumen of sheep fed diets varying in type of forage as monitored by real-time PCR
423 and automated ribosomal intergenic spacer analysis. *J Anim Sci* **90**: 4487–4494.
424
425 Sawanon S, Koike S, Kobayashi Y (2011) Evidence for the possible involvement of
426 *Selenomonas ruminantium* in rumen fiber digestion. *FEMS Microbiol Lett* **325**:
427 170–179.
428
429 Shinkai T, Ohji R, Matsumoto N, Kobayashi Y (2009) Fibrolytic capabilities of ruminal
430 bacterium *Fibrobacter succinogenes* in relation to its phylogenetic grouping. *FEMS*
431 *Microbiol Lett* **294:** 183-190.
432
433 Shinkai T, Ueki T, Koike S, Kobayashi Y (2014) Determination of bacteria constituting
434 ruminal fibrolytic consortia developed on orchard grass hay stem. *Anim Sci J* **85**:
435 254–261.
436
437 Suen G, Stevenson DM, Bruce DC, et al. (2011a) Complete genome of the cellulolytic
438 ruminal bacterium *Ruminococcus albus* 7. *J Bacteriol* **193**: 5574–5575.
439
440 Suen G, Weimer PJ, Stevenson DM, et al. (2011b) The complete genome sequence of
441 *Fibrobacter succinogenes* S85 reveals a cellulolytic and metabolic specialist. *PLoS*
442 *ONE* **6**: e18814.
443

444 Tajima K, Aminov RI, Nagamine T, Matsui H, Nakamura M, Benno Y (2001)
445 Diet-dependent shifts in the bacterial population of the rumen revealed with real-time
446 PCR. *Appl Environ Microbiol* **67**: 2766-2774.

447

448 Williams AG, Withers SE, Joblin KN (1994) The effect of cocultivation with
449 hydrogen-consuming bacteria on xylanolysis by *Ruminococcus flavefaciens*. *Curr*
450 *Microbiol* **29**: 133-138.

451

452 Yu Z, Morrison M (2004) Improved extraction of PCR-quality community DNA from
453 digesta and fecal samples. *BioTechniques* **36**: 808–812.

454 **Table 1.** List of targeted genes and specific PCR primer sets used in this study.

455

Target strain	Target genes* [Fisuc Locus**]	GH family	Function (Reference for protein characterization)		Sequence (5'-3')	Annealing temp. (°C)	Product size (bp)	Reference for primer set
F. succinogenes S85	16S rRNA	-	Ribosome RNA small subunit	Fw	GGTATGGGATGAGCTTGC	60	446	Tajima et al. 2001
				Rv	GCCTGCCCCTGAACTATC			
	frd	-	Fumarate reductase	Fw	GTTCCTTCAACCAGAACCTC	62	194	This study
	[Fisuc_2493]			Rv	CTTGTATTCCCAAGCACCGA			
	cel5C (cedA)	5	Cellodextrinase	Fw	GGGTCACGATTTCCACCTC	62	200	Béra-Maillet et al., 2009
	[Fisuc_1584]		(Huang and Forsberg, 1987)	Rv	CCCAGAAGATTTCGTCCTTG			
	cel5G (cel3)	5	Endo-glucanase	Fw	AGCGATGGTAAGGTCACTGC	62	240	Béra-Maillet et al., 2009
	[Fisuc_2230]		(McGavin et al., 1989)	Rv	GTGGATGGTGGCGTAGTCC			
	endA$_{FS}$	9	Endo-glucanase	Fw	GGTCCGAACTGGATCTTGG	62	200	Béra-Maillet et al., 2009
	[Fisuc_2362]		(Cavicchioli & Watson 1991)	Rv	TCGCCAGTGTAGAGGTCGTA			
	cel9G (endB)	9	Endo-glucanase	Fw	TTACCAACGGAGCGGTGT	62	206	Béra-Maillet et al., 2009
	[Fisuc_0057]		(Béra-Maillet et al., 2000)	Rv	AGCCGAGCATCAAAGTCG			
	cel51A (celF)	51	Endo-glucanase	Fw	CAAGAACGGTGGCGAATC	62	186	Béra-Maillet et al., 2009
	[Fisuc_3111]		(Malburg et al., 1997)	Rv	CGGGTGTTGTCCCAGTAGAG			
	xyn10D	10	Endo-xylanase	Fw	GGCAAGAACGATGTGACCTT	62	200	Béra-Maillet et al., 2009
	[Fisuc_1791]		(Jun et al., 2003)	Rv	TGTCCTTGCGGTAGTCACTG			
	xyn11C	11	Endo-xylanase	Fw	GCTGAAGTATTGCGGGAAGG	62	193	Béra-Maillet et al., 2009
	[Fisuc_0362]		(Paradis et al., 1993)	Rv	CTATGGCTGGACGGTGGAT			
Strain R-25	16S rRNA		Ribosome RNA small subunit	Fw	CTAGGTGTAGGGGGTATC	60	440	Koike et al., 2010
				Rv	GCTGCCCTCTGTCGTTG			
S. ruminantium S137	16S rRNA		Ribosome RNA small subunit	Fw	TGCTAATACCGAATGTTG	57	513	Tajima et al. 2001
				Rv	TCCTGCACTCAAGAAAGA			

456 * Former name of the gene was written in the parentheses.

457 ** Locus tags refer to the ORF call in the genome sequence of *F. succinogenes* S85 in GenBank (accession no. CP001792).

458

459 **Table 2.** Changes in 16S rRNA copy numbers and 16S rRNA/rDNA ratio of *Fibrobacter succinogenes* S85 in monoculture and in
460 co-cultures with non-fibrolytic strains.
461

Incubation time	16S rRNA Log copy number (g of rice straw)$^{-1}$	16S rRNA/rDNA
24 h		
Monoculture of S85	12.18 ± 0.39	1098 ± 110[b]
Coculture with R-25	12.10 ± 0.28	1018 ± 46[b]
Coculture with S137	12.14 ± 0.15	1713 ± 194[a]
Coculture with R-25 and S137	11.97 ± 0.21	1710 ± 45[a]
P-value	0.7354	< 0.0001
48 h		
Monoculture of S85	11.46 ± 0.10	416 ± 68[d]
Coculture with R-25	11.98 ± 0.48	986 ± 111[c]
Coculture with S137	11.87 ± 0.03	1478 ± 139[b]
Coculture with R-25 and S137	11.76 ± 0.14	2677 ± 300[a]
P-value	0.0749	< 0.0001

462
463 Different letters represent significant difference within a column at a given time point ($P < 0.01$).
464
465

466 **Table 3.** Concentration of organic acids and reducing sugars in the culture of
467 *Fibrobacter succinogenes* S85 monoculture and three-member coculture.
468

	μmol (ml of culture)$^{-1}$	
	24 h	48 h
Acetate		
Monoculture of S85	0.23 ± 0.10	2.25 ± 0.04^{b}
S85 + R-25 + S137	1.19 ± 0.25	3.02 ± 0.10^{a}
P-value	0.0365	0.0084
Propionate		
Monoculture of S85	nd	nd
S85 + R-25 + S137	1.61	2.94
P-value	-	-
D-Lacate		
Monoculture of S85	nd	nd
S85 + R-25 + S137	nd	0.10
P-value	-	-
Succinate		
Monoculture of S85	1.17 ± 0.23	4.87 ± 0.75^{a}
S85 + R-25 + S137	1.04 ± 0.25	0.42 ± 0.29^{b}
P-value	0.5762	< 0.0001
Reducing sugars		
Monoculture of S85	1.01 ± 0.02^{a}	4.89 ± 0.18^{a}
S85 + R-25 + S137	0.24 ± 0.12^{b}	0.38 ± 0.05^{b}
P-value	< 0.0001	< 0.0001

469
470 Different letters represent significant difference within an item at a given time point (*P*
471 < 0.01).
472 nd, not detected.

473 **Table 4.** Expression of fumarate reductase (*frd*) of *Fibrobacter succinogenes* S85 in monoculture and co-cultures with non-fibrolytic
474 strains.
475

	Incubation time	
	24 h	48 h
	Log copy number of transcripts (10^8 copies of 16S rDNA)$^{-1}$	
Monoculture of S85	7.30 ± 0.02	7.19 ± 0.03^{c}
Coculture with R-25	7.50 ± 0.19	7.47 ± 0.04^{ab}
Coculture with S137	7.61 ± 0.26	7.39 ± 0.09^{bc}
Coculture with R-25 and S137	7.40 ± 0.33	7.64 ± 0.18^{a}
P-value	0.3129	0.0004

476
477 Different letters represent significant difference within a column at a given time point ($P < 0.01$).
478

479 **Table 5.** Expression of glycoside hydrolase genes of *Fibrobacter succinogenes* S85 in monoculture and co-cultures with non-fibrolytic
480 strains.
481

Incubation time	Log copy number of transcripts (10^8 copies of 16S rDNA) $^{-1}$						
	cel5C	*cel5G*	*endA$_{FS}$*	*cel9G*	*cel51A*	*xyn10D*	*xyn11C*
24 h							
Monoculture of S85	7.10 ± 0.06	7.10 ± 0.06^{a}	7.44 ± 0.06^{a}	6.91 ± 0.04	6.94 ± 0.07	7.14 ± 0.13	7.19 ± 0.12
Coculture with R-25	7.11 ± 0.10	7.00 ± 0.09^{a}	7.35 ± 0.04^{ab}	6.83 ± 0.05	7.15 ± 0.19	7.23 ± 0.17	7.22 ± 0.12
Coculture with S137	7.14 ± 0.14	6.80 ± 0.20^{a}	7.32 ± 0.03^{bc}	6.90 ± 0.18	7.04 ± 0.27	7.14 ± 0.29	7.13 ± 0.14
Coculture with R-25 and S137	7.07 ± 0.22	6.29 ± 0.33^{b}	7.25 ± 0.03^{c}	6.68 ± 0.25	6.99 ± 0.32	7.00 ± 0.35	6.98 ± 0.33
P-value	0.9053	0.0004	0.0003	0.1665	0.6400	0.6234	0.3573
48 h							
Monoculture of S85	6.79 ± 0.07^{c}	6.90 ± 0.06	7.25 ± 0.01^{d}	6.44 ± 0.16^{c}	6.74 ± 0.03^{c}	6.93 ± 0.02^{c}	7.23 ± 0.02^{b}
Coculture with R-25	7.05 ± 0.03^{b}	6.64 ± 0.05	7.43 ± 0.02^{c}	6.60 ± 0.04^{bc}	7.01 ± 0.07^{ab}	7.08 ± 0.04^{b}	7.48 ± 0.06^{ab}
Coculture with S137	7.05 ± 0.09^{b}	6.78 ± 0.02	7.62 ± 0.09^{b}	6.78 ± 0.07^{ab}	6.94 ± 0.13^{b}	7.04 ± 0.10^{bc}	7.51 ± 0.10^{ab}
Coculture with R-25 and S137	7.26 ± 0.11^{a}	6.91 ± 0.31	7.81 ± 0.03^{a}	6.90 ± 0.13^{a}	7.17 ± 0.13^{a}	7.29 ± 0.06^{a}	7.75 ± 0.25^{a}
P-value	< 0.0001	0.1062	< 0.0001	0.0004	0.0003	< 0.0001	0.0018

482
483 Different letters represent significant difference within a column at a given time point ($P < 0.01$).

484 # Figure legend

485

486 **Fig. 1** Growth profiles of *Fibrobacter succinogenes* S85 (a), strain R-25 (b) and
487 *Selenomonas ruminantium* S137 (c) in *F. succinogenes* S85 monoculture (circle),
488 co-culture with strain R-25 (diamond), co-culture with *S. ruminantium* S137 (triangle)
489 and three-member co-culture (square)

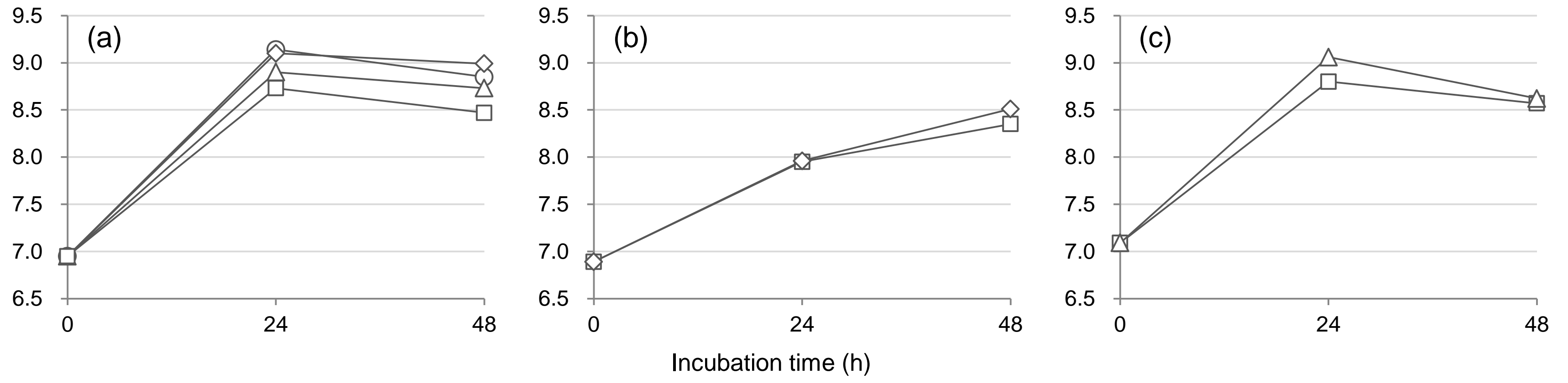

Fig. 1

Table S1. Changes in 16S rDNA copy numbers of *Fibrobacter succinogenes* S85, strain R-25 and *Selenomonas ruminantium* S137 in monoculture of S85 and in co-cultures with non-fibrolytic strains.

Incubation time	Log copy number (g of rice straw)$^{-1}$		
	S85	R-25	S137
24 h			
Monoculture of S85	9.14 ± 0.36	-	-
S85 + R-25	9.10 ± 0.27	7.96 ± 0.15	-
S85 + S137	8.90 ± 0.18	-	9.06 ± 0.01
S85 + R-25 + S137	8.73 ± 0.22	7.95 ± 0.08	8.80 ± 0.21
P-value	0.1814	0.9761	0.2064
48 h			
Monoculture of S85	8.85 ± 0.03	-	-
S85 + R-25	8.99 ± 0.44	8.51 ± 0.25	-
S85 + S137	8.73 ± 0.05	-	8.62 ± 0.54
S85 + R-25 + S137	8.47 ± 0.22	8.35 ± 0.27	8.57 ± 0.68
P-value	0.0154	0.6102	0.9197

Article title: Monitoring of gene expression in *Fibrobacter succinogenes* S85 under the co-culture with non-fibrolytic ruminal bacteria
Journal name: Archives of Microbiology
Author names: Naoki M. Fukuma, Satoshi Koike* and Yasuo Kobayashi
Affiliation: Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan.
E-mail: skoike7@anim.agr.hokudai.ac.jp