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Author(s)	Shinkai, Takumi; 真貝, 拓三; Ueki, Takaaki et al.
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ORIGINAL ARTICLE

Title: Detection and identification of rumen bacteria constituting a fibrolytic consortium dominated by *Fibrobacter succinogenes*

Authors: Takumi SHINKAI¹, Takaaki UEKI and Yasuo KOBAYASHI

Institute, address, country: *Graduate School of Agriculture, Hokkaido University, Kita, Sapporo, Hokkaido, Japan*

Running Head: RUMEN CELLULOLYTIC CONSORTIUM

¹Current address: National Institute of Livestock and Grassland Science, Tsukuba, Ibaraki 305-0901, Japan

Correspondence: Yasuo Kobayashi, Graduate School of Agriculture, Hokkaido University, Kita-ku, Sapporo-shi 060-8589, Japan.

Phone: +81-11-706-2476; Facsimile: +81-11-706-2476; Email: kyas@anim.agr.hokudai.ac.jp

ABSTRACT

A fibrolytic consortium, dominated by the rumen cellulolytic bacterium *Fibrobacter succinogenes*, was artificially constructed on hay stem to detect and identify rumen bacteria that can potentially interact with *F. succinogenes*. Consortium-bacterial members were determined by DGGE and sequencing analysis targeted bacterial 16S rDNA. An artificial consortium was formed in a 2-step incubation of hay stems; the first step with group 1, 2 or 3 *F. succinogenes* strains, the second step with rumen fluid. After consortium formation, morphologically different bacterial were observed in association with *F. succinogenes*. DGGE exhibited more than 30 bands, the pattern of which depended on the *F. succinogenes* group. Sequencing suggested that *Butyrivibrio fibrisolvens*, *Pseudobutyrvibrio ruminis*, *Clostridium* sp., *F. succinogenes* group 2, *Prevotella ruminicola* and unclassified Bacteroides were prominent in the group 1 consortium and that *Treponema bryantii*, *B. fibrisolvens*, *Acinetobacter* sp, and *Wolinella succinogenes* were prominent in the group 2 consortium. However, in the group 3 consortium, *F. succinogenes*-like bacteria were microscopically undetectable, whereas cellulolytic *Ruminococcus albus* and *F. succinogenes* group 1 were prominent, suggesting that the group 3 cannot be a core member of this consortium. This study is the first attempt to identify bacterial members of a fibrolytic consortium dominated by a specific bacterium.

Key words: bacterial consortium, fiber digestion, *Fibrobacter succinogenes*, rumen bacteria, 16S rRNA gene.

INTRODUCTION

Fibrobacter succinogenes is a predominant cellulolytic bacterium with high cellulolytic activities in the rumen (Lin *et al.* 1994; Koike & Kobayashi 2001; Denman & McSweeney 2006). A recent FISH study indicated a prominent contribution of *F. succinogenes* to the digestion of less degradable fiber (Shinkai & Kobayashi 2007). It is generally accepted that the bacterial population inhabiting plant materials is quite diverse and that some physiologically complementary bacterial species can form intimate associations with other species to digest plant materials (McAllister *et al.* 1994; Koike *et al.* 2003). Such bacterial partnerships are termed “a consortium” and consortia may also occur in the case of *F. succinogenes* in the rumen. Thus, *F. succinogenes*-like cells were observed to be in association with various morphologically different bacterial cells on plant materials (Dinsdale *et al.* 1978; Cheng *et al.* 1984).

The fibrolytic consortium consists not only of cellulolytic bacteria but also non-cellulolytic bacteria (Koike *et al.* 2003). Some of the non-cellulolytic bacteria accelerate fiber digestion by consuming, or drawing away from the consortium, the products of cellulolytic digestion, (Costerton *et al.* 1987; Kudo *et al.* 1987; Fondevila & Deholity 1994; Miron *et al.* 1994; Chen *et al.* 2001). In this way, *Treponema bryantii* increases dry matter digestibility and fermentation products in coculture with *F. succinogenes*, even though *T. bryantii* has no cellulolytic activity (Kudo *et al.* 1987). The symbiotic relationships between *F. succinogenes* and cohabiting bacteria must surely contribute to the progression of fiber digestion in the rumen.

Although traditional co-culture studies have verified symbiotic relationships between specific bacteria, whether such symbiosis actually exists in the rumen is still uncertain due to a lack of ecological evidence and to limitations in methodology. In

addition, co-culture studies present the unavoidable problem that only bacteria that can be cultured can be used as the material for these experiments. Since most of the rumen bacteria cannot be grown in culture, this means that they cannot be assayed in co-culture experiments (Kobayashi 2006). Therefore, to comprehensively detect and identify candidate bacteria that might form a fibrolytic consortium with *F. succinogenes*, alternative methods based on bacterial ribosomal RNA gene sequence, such as denaturing gradient gel electrophoresis (DGGE), are useful (Muyzer *et al.* 1993; Yu & Morrison 2004).

In the present study, to detect and identify bacteria cohabiting with *F. succinogenes* in a bacterial consortium developed on hay stems, a fibrolytic consortium, dominated by *F. succinogenes*, was artificially constructed and employed for bacterial community analysis by DGGE.

MATERIALS AND METHODS

Bacteria, animals and feeding

Strains of *F. succinogenes* S85 (ATCC 19169) and HM2 (ATCC 43856) were purchased from the American Type Culture Collection. The strain OS114 was previously isolated from the rumen of sheep (Kobayashi *et al.* 2008). These three strains were used as representative strains of the phylogenetic groups 1, 3 and 2 of *F. succinogenes*, respectively. *F. succinogenes* strains were anaerobically maintained in Avicel-containing medium (Shinkai *et al.* 2007) and cultured at 37 °C in a Hungate tube.

Four ruminally fistulated wethers (average body weight, 70.5 kg), which had been freely fed orchard grass hay in combination with 200 g of concentrate (Monster 16;

Mercian, Tokyo, Japan) once a day at 09.00 hour for eight weeks, were used as donors of rumen fluid and also of a naturally formed bacterial consortia. All animals received appropriate care as described in the Hokkaido University Guidelines for Animal Experiment.

Preparation of fibrolytic consortia

An outline of the scheme followed for the preparation of the two types of fibrolytic bacterial consortia, natural and artificial, is shown in Figure 1. For preparation of the natural consortium, two grams of orchard grass hay stem, cut longitudinally in 15 mm lengths, were enclosed in 50- μ m nylon mesh bags, placed in the rumen of sheep prior to feeding and incubated for 24 h. The incubated stems were washed twice with 10 mL of anaerobic dilution solution (Bryant & Burkey 1953) and stored at -20°C until DNA extraction for further analysis.

[Figure 1]

The artificial consortium was prepared as follows: A portion of an *F. succinogenes* culture (0.05 mL), grown in Avicel-containing medium ($OD_{645} = 0.10$), was inoculated into 5 mL of a medium containing two pieces of filter paper (Whatman No. 1, 0.5g L⁻¹) instead of Avicel. When this culture started to degrade the filter paper, it was vortexed and an aliquot (0.05 mL) of the culture was transferred into 5 mL of a medium containing orchard grass hay stem prepared as described above (0.1g, 3-6 pieces per tube). This mixture was incubated at 37 °C for 20 h for strain S85 and 24-28 h for the OS114 and HM2 strains. Three pieces of the incubated hay stems were then withdrawn from the tube, placed on a paper towel (Kimwipe S-200; Nippon Paper Crexia, Tokyo, Japan), and a second incubation with 5 mL of rumen fluid was performed for 24 h. The rumen fluid used was the supernatant after centrifugation (2,000 x g, 3 min) of rumen

content taken before feeding. After the second incubation, the stems were withdrawn from the culture, washed twice with 10 mL of anaerobic dilution solution and stored at -20°C. Preparation of the artificial consortium was repeated on three different days.

Scanning electron microscopy

Natural and artificial bacterial consortia developed on the hay stems were fixed overnight in 0.05 mol/L sodium phosphate buffer containing 0.2% (vol/vol) glutaraldehyde (pH 7.0). Fixed stems were washed four times with fresh 0.1 mol/L sodium cacodylate buffer for 15 min. The washed stems were placed in 0.05 mol/L sodium phosphate buffer containing 1% (wt/vol) osmium tetroxide solution for two hours, and then the washing was repeated. After sequential dehydration in a graded ethanol series (50, 75, 90, 95, and 100% [vol/vol], 15 min at each concentration), the stems were exposed to isoamyl acetate for 5 min. The stems were dried by the critical-point method with the Critical Point Dryer HCP-2 (Hitachi, Ltd. Tokyo, Japan) then coated with platinum-palladium alloy with Ion Sputter E101 (Hitachi), and examined using the Low Vacuum Scanning Microscope JSM-5310LV (JEOL, Akishima, Tokyo, Japan).

DNA extraction

Bacterial DNA was extracted as described previously (Koike *et al.* 2003) with some modifications. In brief, hay stems on which consortia were developed were cut into fine pieces with sterilized scissors and placed in a sterilized 2 mL-screw-capped tube containing 0.25 g of glass beads (diameter: 425 to 600 µm; Sigma Chemicals, St Louis, MO, USA). The stems were mixed with 0.35 mL of Tris-EDTA buffer (10 m mol/L

Tris-HCl [pH 8.0], 1 m mol/L EDTA), 0.7 mL of Tris-buffered phenol (pH 8.0) and 40 μ L of 10% sodium dodecylsulfate (SDS). DNA was extracted by shaking the tube horizontally. The extracted DNA was purified by hydroxyapatite chromatography (Hydroxyapatite Bio-Gel HTP Gel; Bio-Rad, Hercules, CA, USA) followed by gel filtration (Microspin S-200R HR Columns; Amersham Pharmacia Biotech, Piscataway, NJ, USA). DNA was quantified by a fluorescent method with DyNA Quant 200 (Hoefer Pharmacia Biotech, San Francisco, CA, USA). The DNA of *F. succinogenes* strains was extracted by a boiling method from a loopful of pure culture.

Denaturing Gradient Gel Electrophoresis

DNA (500 ng), isolated from natural and artificial consortia, was used as a template to amplify the V3 region of bacterial 16S rDNA by PCR with primers 2 and 3 as described by Muyzer *et al.* (1993). In brief, a touch down PCR was performed using the rTaq DNA polymerase system (TOYOBO, Osaka, Japan) and the PCR conditions described by Muyzer *et al.* (1993). The integrity of the PCR products was visually checked by electrophoresis on a 2% agarose gel. The PCR products and the DGGE Marker (NipponGene, Tokyo, Japan) were separated by the Decode Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA, USA) using a 6% (vol/vol) polyacrylamide DGGE gel with a 30 to 70% gradient of denaturant. Electrophoresis was performed at 90 V for 16 h in TAE buffer (pH 7.4) at a controlled temperature of 60 °C. The gel was stained with SYBR Green or silver nitrate and the DGGE gel image was scanned using Lumi Vision PRO 400EX (Aisin Seiki CO., Ltd., Kariya, Japan). The DGGE band profiles obtained were analyzed by clustering via the unweighted pair group method with mathematical averages (UPGMA) using BioNumerics software

(Applied Maths, Inc. Austin, TX, USA).

Cloning and sequencing

The PCR amplicons of the V3 region of the 16S rDNA excised from the DGGE gel were subjected to PCR with primers 1 and 2 (Muyzer *et al.* 1993) using the AmpliTaq Gold system (Applied Biosystems, Foster City, CA, USA). The PCR conditions were as follows: initial denaturation at 94 °C for 9 min then 48 amplification cycles of denaturation at 94 °C for 0.5 min, annealing at 60 °C for 0.5 min and extension at 72 °C for 0.5 min. A final extension was performed at 72 °C for 9.5 min. After purification of the PCR product with the QIAEX II Gel Extract Kit (QIAGEN, Hilden, Germany), the product was ligated with pCR2.1 (Invitrogen Corp, Carlsbad, CA, USA) and introduced into *E. coli* DH5 α . The product cloned into the plasmid was PCR-amplified with primers 2 and 3 (Muyzer *et al.* 1993) to confirm the position of the amplicon on the DGGE gel. The sequence of two clones, screened from a library of each gel band, was determined with a ThermoSequenase cycle sequencing kit (Amersham, Little Chalfont, England) and a DSQ2000L automated DNA sequencer (Shimadzu, Kyoto, Japan). The sequences were compared to those available in the GenBank database using the DDBJ BLAST program (<http://www.ddbj.nig.ac.jp/Welcome-e.html>).

Nucleotide sequence accession numbers

The sequences of the V3 region of the 16S rDNA (ca. 190 bp) obtained from the artificial consortium were deposited in the DDBJ nucleotide sequence database under the accession numbers AB472750 through AB472765.

RESULTS

The morphology of bacteria forming an artificial consortium with three different *F. succinogenes* strains was examined by SEM. The SEM images of the inner surface of orchard grass hay stem before and after formation of an artificial consortium are shown in Figure 2. Before incubation with rumen fluid, short-rod and rod shaped *F. succinogenes* cells covered the inner surface of the orchard grass hay stem (Fig. 2, a-c). After formation of the consortia by incubation with rumen fluid, morphologically different bacteria including spiral bacteria (black arrowhead), curved rods and small cocci were observed over and alongside *F. succinogenes*-like bacterial cells, some of which laid in shallow pits (Fig. 2, d-e, white arrowheads). In the artificial consortium formed with *F. succinogenes* group 3, additional morphologically different bacterial cells were observed that were not seen in consortia of groups 1 and 2 (Fig. 2, f)

[Figure 2]

To further analyze the bacteria in the consortia, DGGE profiles of 16S rDNA obtained from natural and artificial consortia were compared as shown in Figure 3. Some prominent bands obtained by this analysis were specific to the natural or the artificial consortia (Fig. 3a). In the artificial consortia, approximately 30 bands were observed that did not correspond to bands derived from the three strains of *F. succinogenes* tested (Fig. 3b). Although bands originating from the S85 and OS114 strains were clearly observed in the consortium dominated by each strain, only a faint band corresponding to the HM2 strain was detected in the HM2-consortium.

[Figure 3]

To determine similarity between the consortia, a dendrogram was constructed by UPGMA clustering based on the DGGE banding profiles of the natural and artificial consortia (Fig. 4). Natural consortia, obtained from the rumen of four different sheep, all had a similar DGGE profile (with scores ranging from 74.7-84.4%) and this profile

was clearly distinguished from profiles of artificial consortia by a lower level of similarity (< 50%) (Fig. 4a). DGGE profiles of artificial consortia were clustered into three different groups, depended on the *F. succinogenes* strain that was the core member of each consortium (Fig. 4b).

[Figure 4]

Prominent DGGE bands obtained from the artificial consortia were then sequenced in order to identify the bacterial groups to which they belonged (Table 1). Seven out of 16 bands sequenced had more than 97% similarity with the sequences of known bacteria and the similarity of the remaining bands ranged from 86 to 97%. In the artificial consortium dominated by the group 1 strain (S85) of *F. succinogenes*, the sequenced DGGE bands showed the highest similarity to the bacteria *Butyrivibrio fibrisolvens* and/or *Pseudobutyrvibrio ruminis*, *Clostridium* species including *C. polysaccharolyticum* DSM 1801, *F. succinogenes* AS211, *Prevotella ruminicola* Tc2-24 and unclassified *Bacteroides* strain R-23 (100, 98-99, 98, 94 and 90% similarity, respectively). In the consortium dominated by the group 2 strain (OS114) of *F. succinogenes*, prominent DGGE bands were identified as the following bacteria (% similarity): *Acinetobacter* sp. HPC276 (99%), *Treponema bryantii* RUS-1 (89-96%), *Wolinella succinogenes* (87%) and *B. fibrisolvens* M55 (86%). The bands obtained from the consortium dominated by the group 3 strain (HM2) of *F. succinogenes* were identified as the cellulolytic bacteria *Ruminococcus albus* (95-100%), group 1 strains (FE and S85) of *F. succinogenes* (97-98%) and an unclassified *Bacteroides* strain RC-2 (89%).

[Table 1]

DISCUSSION

In the present study, we succeeded in partially identifying bacteria that might

possibly have a symbiotic relationship with *F. succinogenes* by using a culture-independent type of analysis. The bacterial species identified are not only known species such as *T. bryantii* and *B. fibrisolvens*, but also unknown and even uncultured bacteria that show less than 97% similarity in their 16S rDNA sequence with known bacteria. This ecological approach is the first attempt to identify bacterial members of a fibrolytic consortium with a specific cellulolytic bacterium such as *F. succinogenes*. This type of analysis could facilitate the exploration of uncultured bacteria as consortium members.

Traditional culturing studies pointed out the synergism between *T. bryantii* and *B. fibrisolvens* with group 1 strains (BL2, CAO or S85) of *F. succinogenes* (Stanton *et al.* 1980; Kudo *et al.* 1987; Miron & Ben-Ghedalia 1993a, b). The *B. fibrisolvens* detected in artificial consortia containing group 1 or group 2 strains of *F. succinogenes* as a core member is known to have a symbiotic relationship with *F. succinogenes* group 1 strain (S85) (Miron & Ben-Ghedalia 1993a, b). Although *F. succinogenes* has high xylanase activity, it is very inefficient in the utilization of xylan hydrolysis products (Miron & Ben-Ghedalia 1993a). In contrast, *B. fibrisolvens* is known to be active for utilization of xylan and its hydrolysates (Miron *et al.* 1994). These data suggest that hemicellulose utilization should be stimulated when *B. fibrisolvens* and *F. succinogenes* are co-localized within a consortium. Furthermore, protease and tannase activities supplied by *B. fibrisolvens* may promote the fibrolytic activity of *F. succinogenes* if both species coexist on tannin-rich legume plants (Bae *et al.* 1993; McSweeney *et al.* 1999).

In addition to the observation of spiral shaped bacterial cells in the consortia containing either group 1 or group 2 strains (Fig. 2d, e), two DGGE bands, that were identified as *T. bryantii*, were seen at a similar position in both consortia (Fig. 3b, Bands

No. 6 and 7). Therefore, *T. bryantii* is most likely a member of consortia containing either group 1 or group 2 strains of *F. succinogenes*. Previous TEM observations (Dinsdale *et al.* 1978; Cheng *et al.* 1984) support this idea. Thus, *T. bryantii*-like bacterial cells were commonly observed covering *F. succinogenes* group 1-like Gram negative and short rod cellulolytic bacteria on rumen plant materials. Highly motile treponemas may offer motility to non-motile *F. succinogenes* by pushing it to a new substrate, as observed in co-culture with *F. succinogenes* in agar medium (Stanton & Canale-Parola 1980).

Other bacteria identified as *Clostridium* sp. or as unclassified Bacteroidales (Handa *et al.* 2004) could potentially develop a symbiotic relationship in fibrolytic consortia dominated by *F. succinogenes*. However, to confirm this possibility further evaluation by co-culture studies, after such uncultured bacteria are successfully isolated, is obviously necessary. Even if isolation of these bacteria is unsuccessful, an ecological interrelationship could be evaluated by using an improved FISH methodology on plant materials (Shinkai & Kobayashi 2007). This method reflects the metabolic activity of bacteria depending on their 16S rRNA expression.

Although DGGE bands corresponding to HM2 (*F. succinogenes* group 3) were faint in the HM2-consortium, cellulolytic bacteria of *R. albus* and *F. succinogenes* (group 1) were identified as co-members of this consortium by sequencing of prominent DGGE bands. In addition, HM2-like small, rod shaped cells (Fig. 2c) were not seen on the surface of orchard grass hay stem after formation of the consortium (Fig. 2f), suggesting that HM2 levels decrease after formation of the consortium. Strains of *F. succinogenes* belonging to group 3 show a lower digestibility of plant materials than strains of groups 1 and 2 (Shinkai, unpublished data) and group 3 is an ecologically minor group in the

rumen (Shinkai *et al.* 2007). The combined data are consistent with the possibility that group 3 cannot be a core cellulolytic bacterium in a fibrolytic consortium developed in the rumen. Therefore group 3 may simply act as a supporting bacterium for rumen fiber digestion. Differences and similarities between bacterial members of artificial and natural consortia were detected based on the DGGE profiles (Fig. 4a). These data will be discussed elsewhere (Shinkai *et al.*, unpublished results).

In conclusion, by using artificially formed cellulolytic consortia, we have succeeded, for the first time, in detecting and identifying bacteria that have the potential to form a symbiotic relationship with *F. succinogenes* strains belonging to groups 1 and 2. Bacterial species identified include not only predictable consortia members such as *B. fibrisolvans* and *T. bryantii*, whose synergy with *F. succinogenes* has previously been described, but also other bacterial species including uncultured bacteroidales. The two step-incubation method that we used to form fibrolytic consortia on plant materials might be a useful approach for the profiling of candidate bacterial members in a consortium dominated by a specific bacterial species.

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Figure legends

Figure 1 Outline for preparing fibrolytic bacterial consortia that are naturally or artificially developed on hay stems in the rumen.

Figure 2 SEM observation of *Fibrobacter succinogenes* strains grown on orchard grass hay stem (a-c), and artificially formed fibrolytic consortia in which *Fibrobacter succinogenes* strains were core members (d-f). Orchard grass hay stems were incubated with S85 (group 1), OS114 (group 2) or HM2 (group 3) for 20, 24 and 24 hours (a, b and c), respectively. Beside the *F. succinogenes* like bacterial cell with shallow pit (white arrowhead), spiral formed bacterium was observed (black arrowhead). Magnification, $\times 5,000$ (a-c) and $\times 15,000$ (d-f).

Figure 3 DGGE profiles of the V3 region of 16S rDNA amplified from naturally and artificially formed fibrolytic consortia (a) and from artificially formed consortia dominated by three different phylogenetic groups of *Fibrobacter succinogenes* (b). Bands excised and sequenced were indicated by arrowhead. Bands typically detected in naturally and artificially formed consortia are shown by black and white arrows, respectively.

Figure 4 Dendrograms showing relationships among fibrolytic consortia.

Comparisons between naturally and artificially formed consortia (a) and between artificially formed consortia dominated by three different phylogenetic groups of *Fibrobacter succinogenes* (b) were made. The dendrograms were generated by using

Dice coefficient and unweighted pair group method with arithmetic mean (UPGMA) clustering method.

Fig 1.

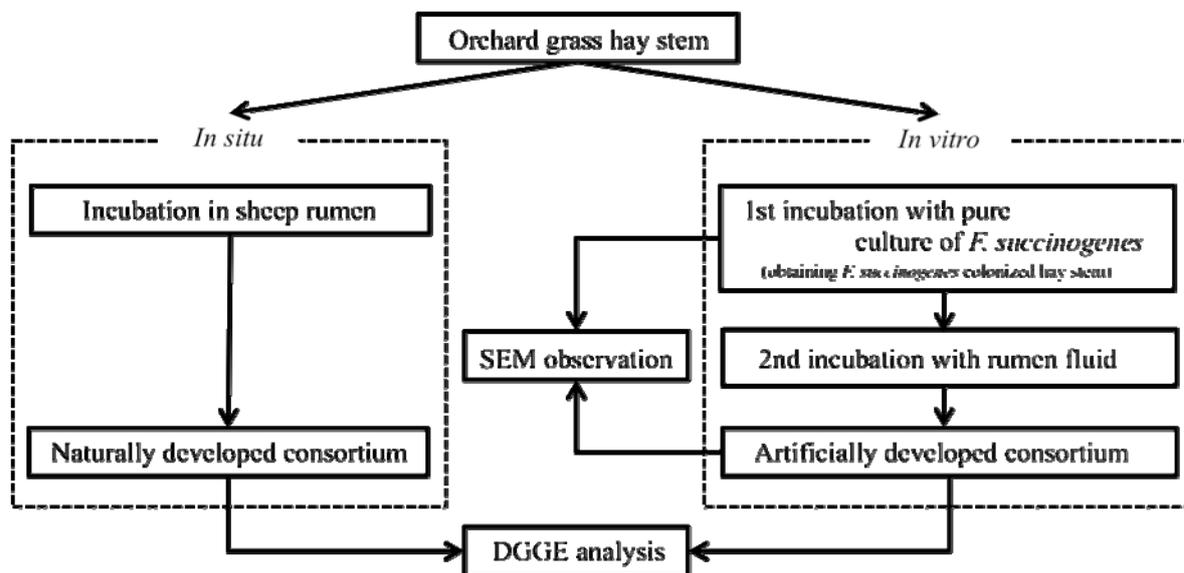


Fig 2.

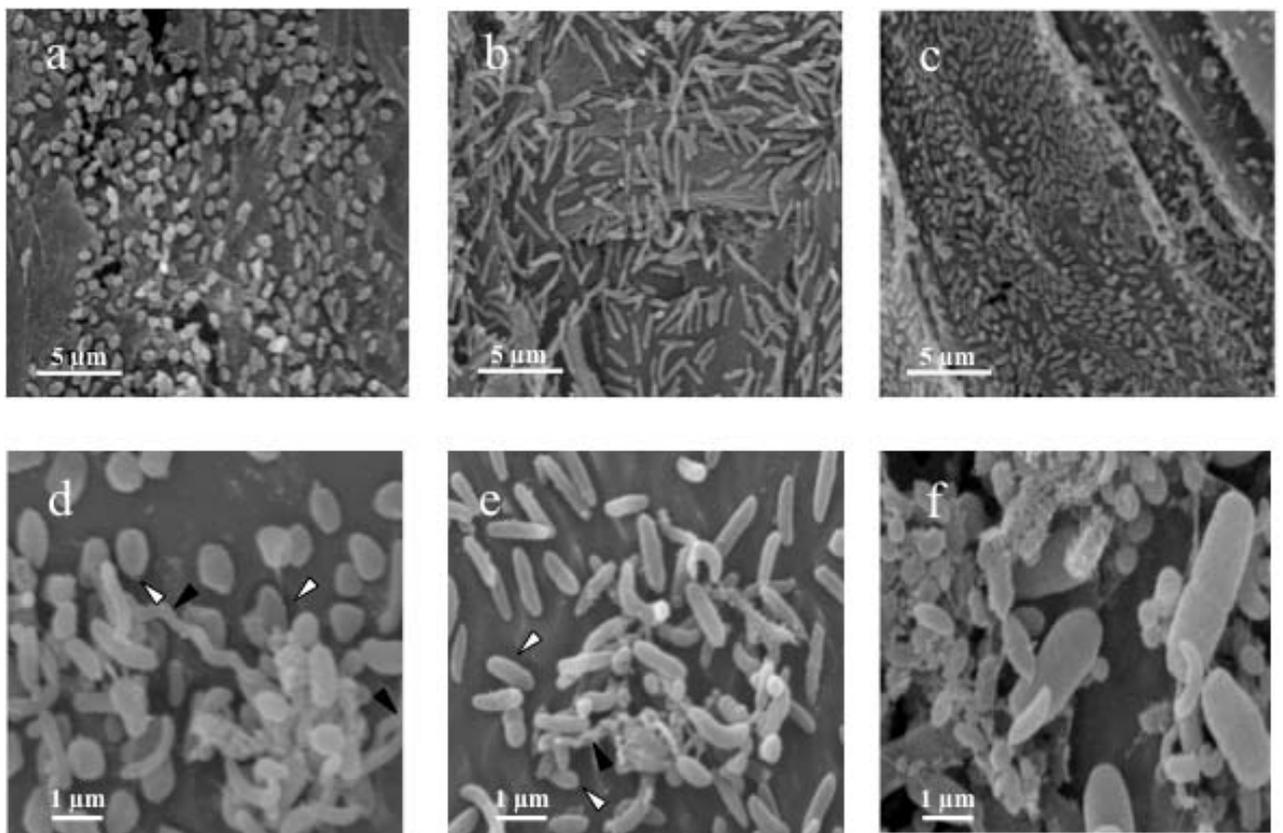


Fig 3.

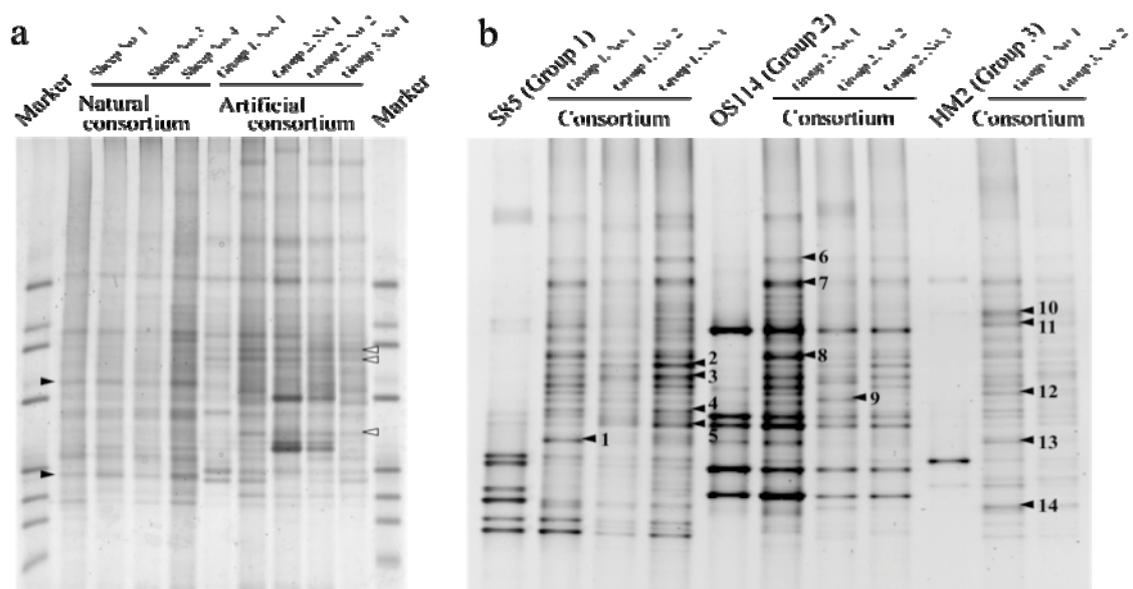


Fig 4.

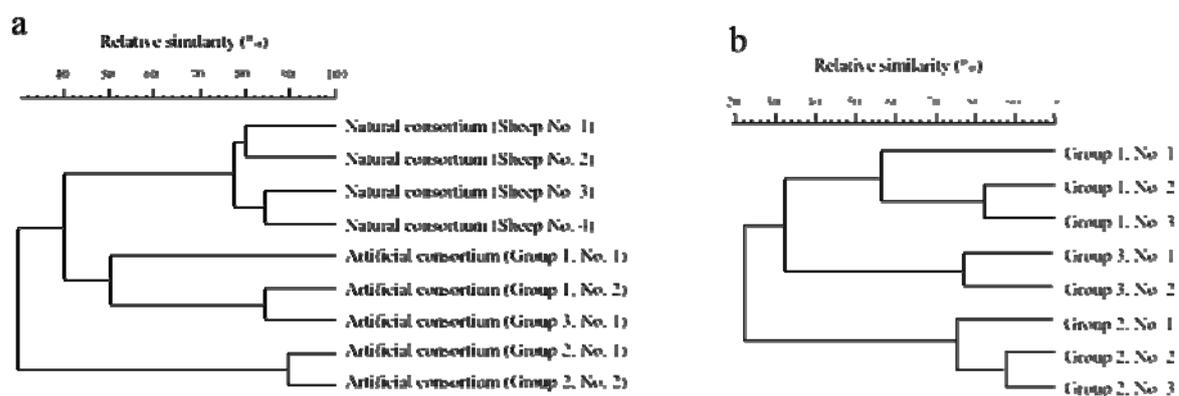


Table 1 Identification of dominant DGGE bands obtained from artificially formed bacterial consortium

DGGE band No.	Phylogenetic group of <i>F. succinogenes</i>	Clone ID	Nearest known bacteria	Similarity (%)
1	1	G1a	<i>Fibrobacter succinogenes</i> AS211 (Group 2)	98
2	1	G1b	<i>Eutyrovibrio fibrisolvens</i> and/or <i>Pseudobutyrovibrio ruminis</i>	100
3	1	G1c	<i>Clostridium polysaccharolyticum</i> DSM 1801	99
4	1	G1d	<i>Clostridium</i> sp.	98
4	1	G1e	Rumen bacterium R-23 (unclassified Bacteroidales)	90
5	1	G1f	<i>Frevotella ruminicola</i> Tc2-24	94
6	2	G2a	<i>Treponema bryantii</i> RUS-1	89
7	2	G2b	<i>Treponema bryantii</i> RUS-1	96
8	2	G2c	<i>Wolinella succinogenes</i> DSMZ 1740	87
8	2	G2d	<i>Eutyrovibrio fibrisolvens</i> M55	86
9	2	G2e	<i>Acinetobacter</i> sp. HPC276	99
10	3	G3a	<i>Fibrobacter succinogenes</i> FE (Group 1)	97
11	3	G3b	<i>Fibrobacter succinogenes</i> S85 (Group 1)	98
12	3	G3c	Rumen bacterium RC-2 (unclassified Bacteroidales)	89
13	3	G3d	<i>Ruminococcus albus</i> F-40	100
14	3	G3e	<i>Ruminococcus albus</i> Ra8	95

和文抄録

Fibrobacter succinogenes を中心とするルーメン内繊維分解コンソーシウム構成細菌
の検出および同定

真貝拓三・上木孝亮・小林泰男

北海道大学大学院農学研究院, 札幌市北区 060-8589

ルーメン内繊維分解性細菌 *Fibrobacter succinogenes* と相互作用をもつ細菌種を検出・同定するため、本菌系統グループ1から3の菌株を中心とする繊維分解コンソーシウムをオーチャードグラス乾草上に人為的に作成した（作成コンソーシウム）。作成コンソーシウムにおいては *F. succinogenes* 様細胞に異なる形態の細菌が付着する様子が電顕観察された。作成コンソーシウムの DGGE 解析では 30 以上のバンドが検出され、そのバンドパターンは主構成員である系統グループの違いを反映していた。グループ1の作成コンソーシウムからは *Butyrivibrio fibrisolvens*, *Pseudobutyrvibrio ruminis*, *Clostridium* 属細菌, *F. succinogenes* グループ2, *Prevotella ruminicola* および未培養の *Bacteroides* 属細菌が、グループ2のそれからは *Treponema bryantii*, *B. fibrisolvens*, *Acinetobacter* 属細菌 および *Wolinella succinogenes* が構成細菌として同定された。一方、グループ3のコンソーシウムでは主構成員であるはずの *F. succinogenes* の観察頻度は下がり、他の繊維分解菌に置き換わっていたため、グループ3の *F. succinogenes* はコンソーシウムで主導的な役割を担い得ないと考えられた。本研究は特定の細菌を中心として構成される繊維分解コンソーシウムに対し、その構成細菌を明らかにしようとした初めての報告である。