RESEARCH LETTER

Fibrolytic capabilities of ruminal bacterium *Fibrobacter succinogenes* in relation to its phylogenetic grouping

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Abstract

To characterize the fibrolytic function of *Fibrobacter succinogenes* strains in relation to their phylogenetic grouping, 32 strains were newly isolated from the rumen of sheep. All new strains were classified into phylogenetic groups 1 or 2 including a novel subgroup of group 2. Importantly, the majority of the strains belonging to group 1 were isolated from ruminally incubated hay. Although almost complete degradation of Avicel was observed among all strains, significantly lower digestibility of three different forages was recorded for strain HM2 of group 3 than for the strains of groups 1 and 2. In a comparison of all strains, two group 1 strains showed significantly higher digestibility of alfalfa and orchard grass hays, while two strains of the novel subgroup of group 2 had lower digestibility of orchard grass hay. Adhesion ability of each strain did not necessarily associate with the extent of digestibility. Maximum growth on Avicel was higher in group 1 than in group 2 strains, and two group 1 strains showed a shorter lag time. The results suggest that the ecological prominence of group 1 is due to a mixture of strains that are diverse in their fibrolytic capability making this group highly adaptable to any forage.

Introduction

Bacteria of the *Fibrobacter succinogenes* species are considered to be one of the most widespread cellulolytic bacteria in the rumen and even the cecum of mono-gastric animals (Stewart & Flint, 1989; Lin & Stahl, 1995b; Miron *et al.*, 2001). Their high fibrolytic activity against various fibers including crystalline cellulose and their high abundance in the rumen may have contributed to a prominent role in rumen fiber digestion (Halliwell & Bryant, 1963; Dehority & Scott, 1967; Koike & Kobayashi, 2001; Michalet-Doreau *et al.*, 2001, 2002).

This species is phylogenetically classified into four groups (group 1–4) based on the 16S rRNA gene sequence of currently available isolates (Amann *et al.*, 1992) that are originated from the rumen of cattle (groups 1 and 2) and sheep (groups 1, 3 and 4). Three of the phylogenetic groups (groups 1–3) vary in their abundance on ruminally incubated plant materials and in whole rumen digesta in a manner that is influenced by factors such as host animal species and feeding conditions (Lin *et al.*, 1994; Koike *et al.*, 2004). However, phylogenetic group 4 strains have not been detected in these ecological studies.

Because DNA–DNA hybridization values among the phylogenetic groups of *F. succinogenes* are quite low (Amann *et al.*, 1992), physiological differences among the groups are expected. Although the only differences that have so far been reported among the phylogenetic groups are differences in cell morphology, vitamin requirements and yellow pigment production (Amann *et al.*, 1992), functional analysis has revealed that 16S rRNA gene classification partially reflects enzymatic differences among *F. succinogenes* strains that would affect fiber digestion (Lin & Stahl, 1995a). Therefore, to accurately understand the biological roles of *F. succinogenes* strains, a comparison of the fibrolytic capabilities of the different phylogenetic groups, as has been performed for other rumen fibrolytic bacteria such as *Ruminococcus albus* and *Ruminococcus flavefaciens*, is necessary (Krause *et al.*, 1999). Despite the recognized importance of *F. succinogenes* in rumen fiber digestion (Kobayashi *et al.*, 2008), fibrolytic comparisons among *F. succinogenes* strains have been difficult to accomplish. This has been mainly due to the limited

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culture collection of this species which, in turn, is due to the difficulties in strain isolation and in maintenance of their fibrolytic capacity under laboratory conditions (Latham *et al*., 1971; Stewart *et al*., 1981; Béra-Maillet *et al*., 2004).

In the present study, to functionally characterize *F. succinogenes* strains in relation to their phylogenetic grouping, *F. succinogenes* strains were newly isolated from the rumen and subjected to phylogenetic classification and preliminary functional analysis.

Materials and methods

Isolation of *F. succinogenes* strains and growth conditions

The *F. succinogenes* strains S85 (ATCC 19169), GC5 (ATCC 51216) and HM2 (ATCC 43856) were purchased from the American Type Culture Collection. Other *F. succinogenes* strains were newly isolated from the rumen of two sheep fed either alfalfa hay, orchard grass hay or rice straw once daily *ad libitum* at 09:00 hours for 2 weeks. Rumen fluid, taken at the time of feeding and passed through two layers of surgical gauge, was used as a bacterial source. Ruminally incubated forage stems were used as an additional bacterial source. Thus, 2-cm-long forage stems of the above three forages were ruminally incubated for 15 min before feeding and washed in saline, prewarmed to 37 °C, until the supernatant became clear.

These isolation sources were inoculated into a cellulose medium and anaerobically incubated at 39 °C until bacterial colonies were observed on filter paper (36–48 h). The cellulose medium was prepared as described by Bryant (1972) with some modifications. The medium comprised (per liter): filter paper, 0.5 g (rectangular-shaped, Whatman No. 1); yeast extract, 1.2 g (Oxoid); Bacto peptone, 2 g (Difco); mineral solution I, 75 mL; mineral solution II, 75 mL; clarified rumen fluid, 300 mL; resazurin (0.1%), 1 mL; $NaHCO_3$ (8%), 5 mL; L-cysteine hydrochloride, 3 g; and distilled water, 500 mL. Mineral solutions I and II were as described by Bryant & Burkey (1953). After incubation, the filter paper was removed from the culture and vortexed in an ice-cold anaerobic dilution solution (Bryant & Burkey, 1953) to separate cellulolytic bacterial cells from the filter paper. The solution containing the bacterial cells was appropriately diluted and used to make the rumen fluid–glucose–cellobiose agar (RGCA) roll tubes containing 1.2% Bacto agar (Bryant & Burkey, 1953) that were used to separate single colonies. This agar content was found to be the minimal amount required for stabilization of the medium layer in the tube and for growth of the less motile *F. succinogenes* strains. Colonies developed after a 48–72-h incubation were randomly picked and transferred into an RGCA slant followed by addition of cellulose medium. Strains showing degradation of the filter paper were checked for their purity by microscopy and maintained in a cellulose medium containing 0.05% (w/v) of Avicel (Avicel, PH-101, Asahi Chemical Industry Co. Ltd, Osaka, Japan) instead of filter paper. The strains were subjected to PCR with *F. succinogenes* species-specific primers (Koike & Kobayashi, 2001) and the identity of the PCR-positive strains was further confirmed by 16S rRNA gene sequencing. Cells of *F. succinogenes* strains, grown at late-log phase ($OD_{645\,nm}$, 0.3–0.4) in the above medium containing Avicel, were used for physiological assays.

Bacterial 16S rRNA gene sequencing and phylogenetic analysis

The 16S rRNA gene of the *F. succinogenes* strains was amplified in two series of PCR using the universal primer pairs: 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 515R (5′-ATTACCGCGGGGCTGG-3′), and 530F (5′-GTGCCAGCM GCCGCGG-3′) and 1392R (5′-ACGGGCGGTGTGTRC-3′), with the AmpliTaq Gold system (Applied Biosystems, Foster City, CA). The PCR conditions were as follows: initial denaturation at 94 °C for 9 min, 48 cycles of amplification at 94 °C for 0.5 min, at 58 °C (for 27F and 515R) or 60 °C (for 530F and 1392R) for 0.5 min and at 72 °C for 1 min. Final extension was performed at 72 °C for 9 min. After purification of the PCR product using the QIAEX II Gel Extraction Kit (QIAGEN, Hilden, Germany), the product was cloned into pCR2.1 (Invitrogen) and introduced into *Escherichia coli* DH5α.

The complete sequence of the 16S rRNA genes (*c*. 1400 bp) was determined with a ThermoSequenase cycle sequencing kit (Amersham, Little Chalfont, UK) and a DSQ2000L automated DNA sequencer (Shimadzu, Kyoto, Japan). The obtained sequences were compared with the available sequences in the GenBank database using NCBI BLAST (http://www.ncbi.nlm.nih.gov/) and the sequence match tool in RDPII (http://rdp.cme.msu.edu/). The phylogenetic tree was constructed using the multiple sequence alignment software CLUSTAL W available in DDBJ (http://www.ddbj.nig.ac.jp/index-e.html). A distance matrix was generated with the Kimura model. To evaluate the reliability of the branching, bootstrap analysis was carried out with data that had been resampled 1000 times.

Preparation of test forage materials

Stems of alfalfa hay, orchard grass hay and rice straw were milled to pass through a 1.5-mm screen (Dietz Motoren KG, Dettingen-Teck, Germany), soaked in distilled water at 60 °C for 1 h and the supernatant was then removed. This washing process was repeated three times. After overnight incubation of these stems in distilled water at room temperature the supernatant was again removed. The stems from which soluble components had been removed were oven-dried

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at 60 °C and used for measurements of digestibility and bacterial adhesion.

Digestibility and adhesion ability

Five strains (S85, RS230, RS233, RS225 and OS117) were chosen from group 1 as representative strains of the five subgroups (see Results) and were used in physiological assays together with all five strains of group 2 (GC5, AS211, OS114, AL227 and AL225) and one strain of group 3 (HM2). Fiber degradation capability was determined as described by Miron *et al.* (1989) with some modifications. Briefly, bacterial cells were inoculated into 10 mL of the Avicel-containing medium (0.25%, w/v), or one of the forage stems described earlier (0.5%, w/v) as the sole carbon source and were incubated at 37 °C. Incubations were performed in triplicate for 30, 60, 90, 120 and 150 h and the disappearance of dry matter (DM) was assayed.

Bacterial adhesion was determined as reported by Mitsumori & Minato (1993) with some modifications. Thus, bacterial cells, grown in 10 mL resazurin-free medium containing Avicel were mixed with 0.5 g of each fiber source (Avicel and three different forages) and were incubated at 37 °C for 15 min. The proportion of attached cells was calculated photometrically by subtraction of the absorbance before and after addition of the fiber source.

Bacterial growth characteristics

The bacterial culture (0.1 mL), grown at log phase ($OD_{645\,nm}$, 0.32–0.39) in Avicel-containing medium was inoculated into 10 mL of medium containing Avicel or cellobiose (0.05%, w/v) that was used as the sole carbon source. Bacterial growth was monitored in triplicate by measuring the $OD_{645\,nm}$ every 2 h from the start of incubation. The OD values observed during the log-growth phase were used for calculation of the growth rate of each strain in Avicel ($OD_{645\,nm}$, 0.25–0.35) or cellobiose ($OD_{645\,nm}$, 0.2–0.5) media. The growth rate was calculated by dividing the difference between the initial and final OD by the time period between the two OD readings. Lag time and maximum OD were also recorded.

Statistical analyses

Data on digestibility, adhesion ability and the growth characteristics of *F. succinogenes* strains were subjected to ANOVA followed by the Turkey–Kramer test using the GLM procedure of SAS. Phylogenetic groups were compared with Student's *t*-test. Correlation between adhesion ability and forage digestibility was evaluated by the Pearson product–moment or the Spearman rank analysis. Statistical significance was defined as $P < 0.05$.

Nucleotide sequence accession numbers

The 16S rRNA gene sequences of newly isolated *F. succinogenes* strains are deposited in the DDBJ nucleotide sequence databases under the accession numbers AB275483–AB275515.

Results

The *F. succinogenes* strains isolated from the rumen of sheep fed three different forages are shown in Table 1. Out of a total of 339 isolates, 32 were ultimately identified as *F. succinogenes* based on their 16S rRNA gene sequence. All of the isolates possessed filter paper-degrading ability and were of rod or coccoid morphology. All strains also retained Avicel-degrading activity, which they degraded at approximately the same rate. The isolated strains were affiliated with phylogenetic group 1 (28 strains) or group 2 (4 strains) and no group 3- or 4-related strains were isolated. All of the group 1 strains and half of the group 2 strains were obtained using ruminally incubated hay as an isolation source.

The phylogenetic position of all known *F. succinogenes* strains, including the newly isolated strains, is shown in Fig. 1. The phylogenetic tree indicated a novel subgroup (sub. B) of group 2 and the branching of this group was supported by a 99% bootstrap value. Among the group 2 strains only the strains of this subgroup (AL225 and AL227) originated from rumen fluid. These strains produced a yellow pigment when cultured with Avicel. Although reliably high bootstrap values were not necessarily obtained, the strains of group 1 were divided into five subgroups (sub. a through sub. e). The branch dividing sub. a from the other groups was supported by a 100% bootstrap value, while the other branches showed values ranging from 3% to 33%. From these five subgroups, strain S85 (sub. c) and the four newly isolated strains of RS230

Table 1. Number of *Fibrobacter succinogenes* strains newly isolated from the rumen of sheep fed three different forages

		Isolation source	
Forage	Phylogenetic groups	*In situ* incubated hay	Rumen fluid
Alfalfa hay	Group 1	8 (75)	0 (33)
	Group 2	1 (75)	2 (33)
	Group 3	0 (75)	0 (33)
	Group 4	0 (75)	0 (33)
Orchard grass hay	Group 1	9 (99)	0 (43)
	Group 2	1 (99)	0 (43)
	Group 3	0 (99)	0 (43)
	Group 4	0 (99)	0 (43)
Rice straw	Group 1	11 (53)	0 (36)
	Group 2	0 (53)	0 (36)
	Group 3	0 (53)	0 (36)
	Group 4	0 (53)	0 (36)
Total *F. succinogenes* isolates		30 (227)	2 (112)

The total number of isolates is shown in parentheses.

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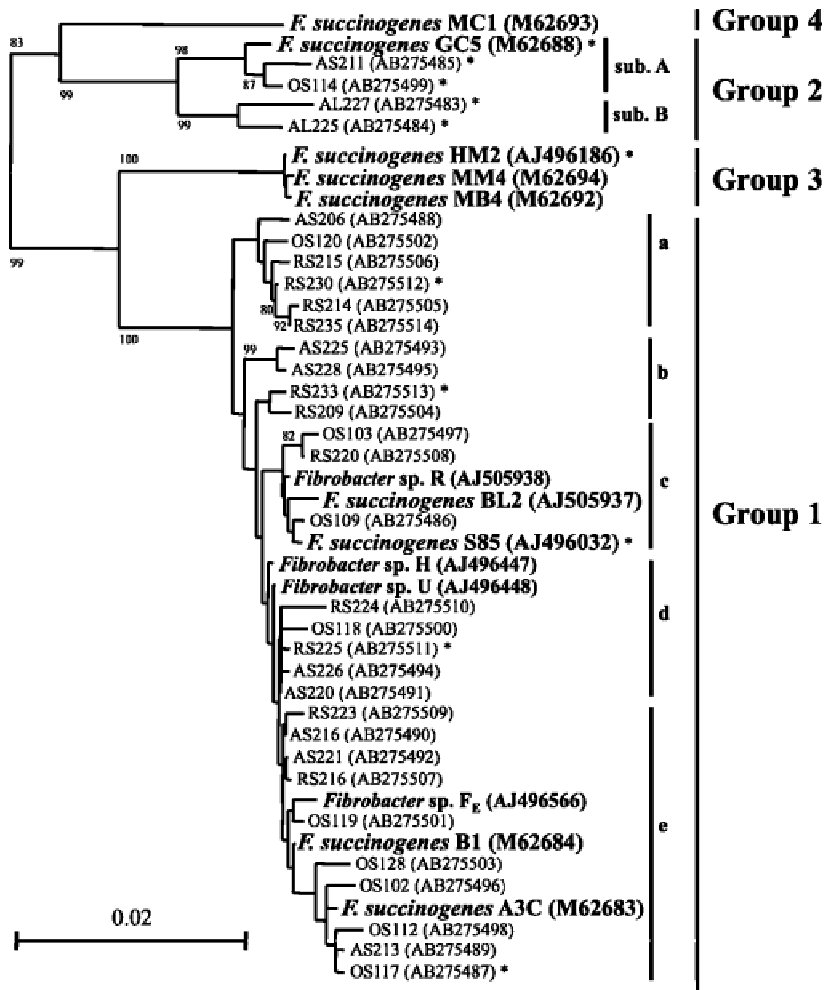

Fig. 1. Phylogenetic relationships of newly and previously isolated *Fibrobacter succinogenes* strains based on their16S rRNA gene sequences. A phylogenetic tree of all *F. succinogenes* strains was constructed as described in the Materials and methods section. The sequence of *Bacteroides fragilis* (accession number M11656) was used as an outgroup. Numbers ($\geq$80) given at the nodes show bootstrap value representing the confidence percentage given from 1000 trees. The strains in bold type are previously described known isolates, while others are strains newly isolated in the present study. Strain numbers that are preceded with 'AS', 'OS' or 'RS' indicate that the strains were isolated from ruminally incubated alfalfa hay, orchard grass hay or rice straw, respectively. Strain numbers that are preceded with 'AL' were isolated from the rumen fluid of sheep fed alfalfa hay. Strains with an asterisk were used for physiological assays.

(sub. a), RS233 (sub. b), RS225 (sub. d) and OS117 (sub. e) were chosen as group1-representative strains and were used in physiological analysis, together with all of the group 2 strains and strain HM2 of group 3 (11 strains in total).

Figure 2 shows the time-course of disappearance of DM for the strains of each phylogenetic group. Active Avicel degradation was similar for all three groups. Although the strains of groups 1 and 2 induced a similar disappearance of alfalfa hay, orchard grass hay and rice straw over incubation time, the values observed for the group 3 strain HM2 were far lower than those for the strains of groups 1 and 2.

Table 2 shows the disappearance of DM induced by each strain after 150 h incubation. No difference was observed in Avicel disappearance among the strains, although significant differences did exist among the strains and subgroups in the disappearance of three types of forage. The group 1 strains RS225 (sub. d) and OS117 (sub. e) showed significantly higher DM disappearance of alfalfa hay and orchard grass hay than the other strains ($P < 0.05$). The sub. B strains of group 2 (AL225 and AL227) showed significantly lower DM disappearance of orchard grass hay than the other strains with the exception of the HM2 strain of group 3 ($P < 0.05$). When the mean value for the group 1 strains was compared with that for group 2 strains, no significant difference was detected. The group 3 strain HM2 showed the lowest disappearance of all three forages ($P < 0.05$).

The ability of the different strains to adhere to Avicel and to three different forages is shown in Table 3. All strains adhered most strongly to Avicel (72.8–102.1%) among the tested materials. Group 2 strains adhered more highly to alfalfa hay and orchard grass hay (58.9% and 66.2%, respectively) than group 1 strains (37.2% and 38.7%, respectively) ($P < 0.05$). Although the group 3 strain HM2 could adhere to alfalfa hay and orchard grass hay as strongly as groups 1 and 2 strains, significantly lower adhesion was recorded for rice straw ($P < 0.05$). Strains belonging to phylogenetic group 1 showed a considerable variation in their ability to adhere to fiber sources. For example, the strain RS230 (sub. a) adhered 1.2–2.7-fold more strongly to alfalfa or orchard grass than other strains of group 1, and the

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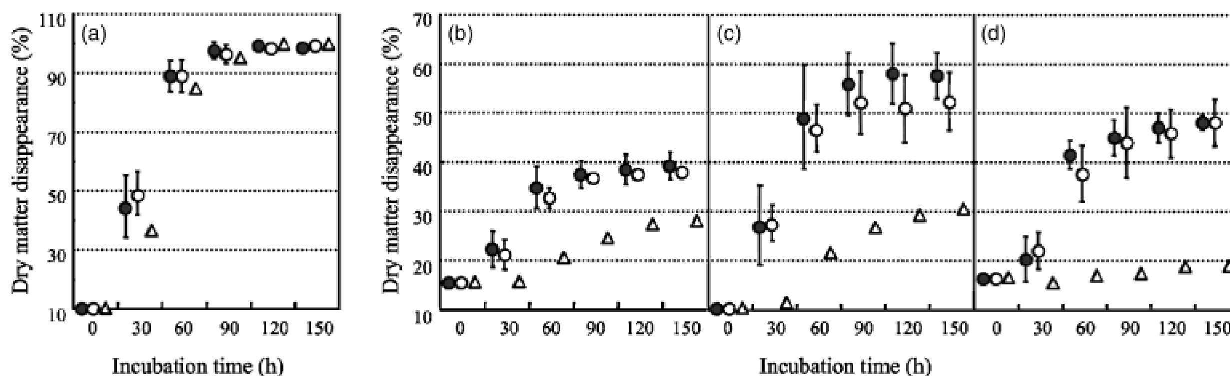

Fig. 2. Time course of disappearance of DM in different forage sources by *Fibrobacter succinogenes* strains. Time course of dry matter disappearance was determined for Avicel (a), alfalfa hay (b), orchard grass hay (c) and rice straw (d) during incubation with *F. succinogenes* strains belonging to the phylogenetic groups 1 (closed circle), 2 (open circle) or 3 (triangle). Each value represents the mean ± SD for five strains of group 1 (S85, RS230, RS233, RS225 and OS117), five strains of group 2 (GC5, AS211, OS114, AL227 and AL225) and one strain of group 3 (HM2). All determinations were performed in triplicate.

Table 2. Ability of the *Fibrobacter succinogenes* strains* to digest Avicel and three forage materials

		Disappearance of DM (%) ± SD			
Phylogenetic groups†	Strains	Avicel	Alfalfa hay	Orchard grass hay	Rice straw
Group 1					
Sub. c	S85	97.7 ± 1.5	37.1 ± 1.3^{B}	52.5 ± 0.2^{D}	45.7 ± 1.7^{C}
Sub. a	RS230	100.5 ± 2.0	36.3 ± 1.3^{B}	55.2 ± 2.1^{CD}	48.8 ± 0.3^{ABC}
Sub. b	RS233	98.7 ± 0.8	38.3 ± 1.0^{B}	56.0 ± 0.9^{BC}	48.3 ± 1.6^{BC}
Sub. d	RS225	98.1 ± 0.6	41.9 ± 1.2^{A}	62.7 ± 1.1^{A}	47.9 ± 1.6^{BC}
Sub. e	OS117	98.1 ± 1.4	42.1 ± 0.6^{A}	62.6 ± 0.9^{A}	49.6 ± 2.6^{ABC}
Group 1 average		98.6 ± 1.1	39.1 ± 2.7	57.8 ± 4.6	48.1 ± 1.5
Group 2					
Sub. A	GC5	97.6 ± 1.2	36.7 ± 0.6^{B}	56.5 ± 0.1^{BC}	52.7 ± 0.5^{A}
	AS211	101.6 ± 2.1	38.5 ± 1.7^{B}	55.2 ± 1.2^{CD}	51.4 ± 1.7^{AB}
	OS114	98.0 ± 0.4	38.0 ± 0.1^{B}	58.3 ± 0.8^{B}	49.7 ± 0.6^{AB}
Sub. B	AL227	99.6 ± 1.1	37.1 ± 0.4^{B}	46.5 ± 0.5^{E}	41.1 ± 1.3^{D}
	AL225	99.5 ± 1.0	38.7 ± 0.7^{B}	45.8 ± 0.7^{E}	45.7 ± 0.9^{C}
Group 2 average		99.3 ± 1.6	37.8 ± 0.9	52.5 ± 5.9	48.1 ± 4.7
Group 3	HM2	99.7 ± 0.6	27.8 ± 0.6^{C}	30.4 ± 0.5^{F}	18.9 ± 0.3^{E}

*Disappearance of DM of each fiber material was determined for each cultured strain after 150-h incubation.

†Phylogenetic groups of *Fibrobacter succinogenes* were defined on the basis of the 16S rRNA gene sequence (Amann *et al*., 1992).

Within a column, means followed by different capital letters differ significantly ($P < 0.05$).

adherence was of comparable strength to group 2 strains. Adhesion ability of the tested strains was not significantly correlated with digestibility of three forages ($r = -0.008$ and -0.292 for alfalfa and orchard grass, respectively; $\rho = 0.497$ for rice straw, $P > 0.10$).

Table 4 shows a comparison of the lag time, growth rate and maximum growth of *F. succinogenes* on Avicel and cellobiose that were obtained from growth curves. Although there was no difference in the mean growth rate of the three phylogenetic groups on any substrate, significant differences were observed in the lag time and the maximum growth among strains and even among groups. When cultured on Avicel, strain HM2 of group 3 showed a longer lag time than strains of groups 1 and 2. The RS225 (sub. d) and OS117 (sub. e) strains had a shorter lag time than other group 1 strains ($P < 0.05$). On average, group 1 showed higher maximum growth than group 2 (0.54 vs. 0.44, $P < 0.05$).

Discussion

We have successfully isolated a range of *F. succinogenes* strains for phylogenetic and functional analysis. The use of ruminally incubated hay as an inoculation source allowed a more efficient isolation of *F. succinogenes* strains (30 out of 227 strains) than rumen fluid (2 out of 112 strains, Table 1). Because all strains of group 1 and the sub. A of group 2 were

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Table 3. Ability of *Fibrobacter succinogenes* strains to adhere to Avicel and three forage materials[†]

		Percentage of adherence (%) ± SD			
Phylogenetic groups[‡]	Strains	Avicel	Alfalfa hay	Orchard grass hay	Rice straw
Group 1					
Sub. c	S85	72.8 ± 9.1^{D}	31.1 ± 3.8^{CD}	32.5 ± 3.6^{D}	58.8 ± 5.1^{AB}
Sub. a	RS230	94.2 ± 2.5^{AB}	48.6 ± 9.9^{ABCD}	67.9 ± 4.0^{AB}	51.5 ± 7.9^{ABCD}
Sub. b	RS233	83.7 ± 4.2^{BCD}	28.0 ± 3.5^{D}	25.3 ± 4.6^{D}	52.0 ± 4.9^{ABCD}
Sub. d	RS225	83.8 ± 0.4^{BCD}	39.8 ± 12.0^{BCD}	29.3 ± 3.7^{D}	39.6 ± 2.6^{CD}
Sub. e	OS117	79.9 ± 4.8^{CD}	38.6 ± 11.8^{BCD}	38.6 ± 2.5^{CD}	45.8 ± 3.0^{BCD}
Group 1 average		82.3 ± 7.7	37.2 ± 8.1	38.7 ± 17.0	49.5 ± 7.2
Group 2					
Sub. A	GC5	101.2 ± 2.5^{A}	66.6 ± 12.9^{A}	77.3 ± 2.0^{A}	60.3 ± 1.2^{AB}
	AS211	102.1 ± 3.7^{A}	60.1 ± 3.0^{AB}	60.4 ± 9.5^{AB}	69.6 ± 10.0^{A}
	OS114	84.8 ± 6.0^{BCD}	57.0 ± 12.5^{ABC}	65.3 ± 12.4^{AB}	40.3 ± 5.4^{CD}
Sub. B	AL227	96.0 ± 3.5^{AB}	56.0 ± 7.9^{ABC}	63.5 ± 4.2^{AB}	57.1 ± 3.5^{ABC}
	AL225	93.2 ± 5.9^{ABC}	54.9 ± 8.6^{ABC}	64.7 ± 9.2^{AB}	37.5 ± 12.1^{D}
Group 2 average		95.5 ± 7.0*	58.9 ± 4.7*	66.2 ± 6.5*	53.0 ± 13.7
Group 3	HM2	85.9 ± 4.6^{BCD}	39.9 ± 5.9^{BCD}	57.0 ± 8.9^{BC}	14.9 ± 3.3^{E}

[†]Bacterial adherence to each fiber type was determined after 15-min incubation at 37 °C with three replicates.
[‡]Phylogenetic groups of *Fibrobacter succinogenes* were defined on the basis of their16S rRNA gene sequence (Amann *et al.*, 1992).
Within a column, means followed by different capital letters differ significantly ($P < 0.05$).
*Values were significantly different between the phylogenetic groups 1 and 2 ($P < 0.05$).

Table 4. Growth characteristics of *Fibrobacter succinogenes* strains grown on Avicel and cellobiose

		Avicel			Cellobiose		
Phylogenetic groups	Strains	Lag time (h)	Growth rate ($\times 10^{-1}$)	Maximum OD	Lag time (h)	Growth rate ($\times 10^{-1}$)	Maximum OD
Group 1							
Sub. c	S85	34^{FG}	0.11^{C}	0.49^{BC}	20^{BC}	0.24^{CDE}	0.56^{BCD}
Sub. a	RS230	25^{CD}	0.15^{ABC}	0.54^{AB}	12^{AB}	0.13^{FG}	0.61^{A}
Sub. b	RS233	22^{BC}	0.17^{ABC}	0.54^{AB}	11^{A}	0.15^{EFG}	0.60^{AB}
Sub. d	RS225	18^{A}	0.19^{AB}	0.59^{A}	16^{ABC}	0.23^{CDEF}	0.57^{ABC}
Sub. e	OS117	18^{A}	0.25^{A}	0.55^{AB}	12^{AB}	0.25^{CD}	0.64^{AB}
Group 1 average		23.3 ± 6.6	0.17 ± 0.05	0.54 ± 0.04*	13.9 ± 3.4	0.20 ± 0.06	0.60 ± 0.03
Group 2							
Sub. A	GC5	31^{EF}	0.17^{ABC}	0.45^{CD}	21^{C}	0.12^{G}	0.53^{D}
	AS211	29^{E}	0.18^{ABC}	0.45^{CD}	21^{BC}	0.27^{BC}	0.54^{CD}
	OS114	28^{DE}	0.19^{ABC}	0.38^{D}	16^{ABC}	0.50^{A}	0.60^{AB}
Sub. B	AL227	19^{AB}	0.22^{AB}	0.56^{AB}	9^{A}	0.35^{B}	0.61^{A}
	AL225	29^{E}	0.13^{BC}	0.38^{D}	16^{ABC}	0.31^{BC}	0.62^{A}
Group 2 average		27.1 ± 4.5	0.18 ± 0.03	0.44 ± 0.07	16.7 ± 4.8	0.31 ± 0.14	0.58 ± 0.04
Group 3	HM2	37^{G}	0.15^{ABC}	0.43^{CD}	61^{D}	0.16^{DEFG}	0.61^{A}

Within a column, means followed by different capital letters differ significantly ($P < 0.05$).
*Values were significantly different between the phylogenetic groups 1 and 2 ($P < 0.05$).

isolated from ruminally incubated hay but not from rumen fluid (Fig. 1), this isolation method is judged useful for efficient accumulation of the dominant strains of *F. succinogenes*. The success of this method may be due to the fast adhesion of *F. succinogenes* to plant materials (Koike *et al.*, 2003). However, it could be argued that only easily cultured *F. succinogenes* strains were isolated by this method. Thus, for example, because group 1 strains constituted the majority of the new isolates in the present study, these isolates might simply be a mixture of strains that are easily cultivated under lab conditions. Nevertheless, the number of *F. succinogenes* strains obtained by this method was sufficient for a functional comparison between the strains of groups 1 and 2. This culture collection should expand future studies of *F. succinogenes*, because the 16S rRNA gene sequence is available in GenBank only for 13 isolates of *F. succinogenes*.

The phylogenetic tree constructed based on the 16S rRNA gene sequences of all *F. succinogenes* strains suggested that AL225 and AL227 represent a novel subgroup of group 2 (sub. B) (Fig. 2), that produce a yellow pigment in Avicel

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culture. These data support the idea that 16S rRNA gene-based grouping partially reflects physiological differences. Production of a yellow pigment has been described by Kopecný & Hodrová (1997). This research group showed that the yellow pigment produced by *R. flavefaciens* has a strong affinity for cellulosic materials, which facilitates the association of bacterial endoglucanase. However, it is to be characterized whether the pigment acts similarly as observed in *R. flavefaciens*.

Although a similar complete digestion of Avicel was observed for all of the strains, differences in ability to digest forage materials were observed among strains and even among subgroups (Table 2). The HM2 strain of group 3 was less potent than the other strains for forage digestion, although forage digestion did not necessarily correlate to adhesion ability. This lack of correlation between digestion and adhesion ability was also supported by the fact that the RS225 and OS117 strains, that were the most potent for digestion, showed a similar or a lower adherence to alfalfa hay and orchard grass hay than the least potent strain HM2 (Table 3). Such different responses to forages among the strains can be attributed to heterogeneity of forage components including hemicellulose and to the diversity of bacterial enzymatic activities that degrade these components, as has been pointed out by Miron (1991). Although we did not determine bacterial activity against hemicellulose and pectin, it is possible that differences in type and expression of a wide range of enzymes might be the main factor responsible for the different potencies of bacterial strains in digestion of forages that was observed in the present study. In support of this hypothesis, the strain HM2 of group 3, which was the least potent strain in the present study, has been shown to lack the gene *xynC* that encodes the primary xylanase and which is inductively expressed, in strain S85 of group 1 (Béra-Maillet *et al*., 2004). However, it should be noted that the strain HM2 could not be a representative but just a reference of group 3.

Differences in growth characteristics on Avicel were also observed among the different strains and groups (Table 4). Thus, the HM2 strain of group 3 had a far longer lag time than the other strains before initiating growth, while the RS225 (sub. d) and OS117 (sub. e) strains of group1 had a shorter lag time. Maximum growth on Avicel was significantly greater in group 1 than in group 2. A short lag time and high growth are characteristics that are indicative of the growth superiority of group 1, in particular the superiority of specific strains or subgroups such as RS225 (sub. d) and OS117 (sub. e). This growth superiority would, in turn, give group 1 a competitive advantage in the rumen environment.

Strain S85 of group 1 has been shown to digest various plant fibers (Dehority & Scott, 1967; Morris & van Gylswyk, 1980; Miron & Ben-Ghedalia, 1993), suggesting that it has a fibrolytic capability adaptable to a wide range of fibrous substrates. If all group 1 strains possess this functional capability, possibly attributable to the expression of a variety of fibrolytic enzymes, this would explain the ecological superiority of group 1. The high abundance of this group associated with liquid, solid and ruminally incubated plant materials has been demonstrated (Shinkai & Kobayashi, 2007; Shinkai *et al*., 2007). In contrast, group 2 strains tend to prefer easily digestible forage tissues such as orchard grass hay in the rumen (Shinkai & Kobayashi, 2007; Shinkai *et al*., 2007).

The 16S rRNA gene sequences of *F. succinogenes* strains share 96–100% identity (Amann *et al*., 1992; this study) that is close to a criterion (97% identity) to define as the same species. However, we revealed some physiological differences among phylogenetic groups, subgroups and even strains of *F. succinogenes* in the present study. Because similar discussion is in progress for *Butyrivibrio fibrisolvens* (Paillard *et al*., 2007), further genetic and physiological information is necessary for possible reclassification of *F. succinogenes*.

This is the first report describing physiological characterization of variety of *F. succinogenes* strains in relation to their phylogenetic grouping. Phylogenetic group 1 consists of a variety of strains including potentially highly fibrolytic strains that are tentatively classified as sub. d and sub. e, and whose fibrolytic ability would facilitate the ecological dominance of group 1 in the rumen. It can be concluded from the physiological data in this study and from previously reported ecological evidence that the *F. succinogenes* group 1 strains contribute to rumen fiber digestion to a greater extent than the other groups.

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