

Fiber-Degrading Systems of Different Strains of the Genus *Fibrobacter*

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The S85 type strain of *Fibrobacter succinogenes*, a major ruminal fibrolytic species, was isolated 49 years ago from a bovine rumen and has been used since then as a model for extensive studies. To assess the validity of this model, we compared the cellulase- and xylanase-degrading activities of several other *F. succinogenes* strains originating from different ruminants, including recently isolated strains, and looked for the presence of 10 glycoside hydrolase genes previously identified in S85. The NR9 *F. intestinalis* type strain, representative of the second species of the genus, was also included in this study. DNA-DNA hybridization and 16S rRNA gene sequencing first classified the strains and provided the phylogenetic positions of isolates of both species. Cellulase and xylanase activity analyses revealed similar activity profiles for all *F. succinogenes* strains. However, the F_E strain, phylogenetically close to S85, presented a poor xylanolytic system and weak specific activities. Furthermore, the HM2 strain, genetically distant from the other *F. succinogenes* isolates, displayed a larger cellulolytic profile on zymograms and higher cellulolytic specific activity. *F. intestinalis* NR9 presented a higher cellulolytic specific activity and a stronger extracellular xylanolytic activity. Almost all glycoside hydrolase genes studied were found in the *F. succinogenes* isolates by PCR, except in the HM2 strain, and few of them were detected in *F. intestinalis* NR9. As expected, the fibrolytic genes of strains of the genus *Fibrobacter* as well as the cellulase and xylanase activities are better conserved in closely related phylogenetic isolates.

Fibrobacter succinogenes is a major fibrolytic bacterium from the rumen of cattle and sheep. This bacterium is found in large numbers by cultural methods when ruminants are given a poor diet (6), and it is also found to be predominant by the use of molecular tools (37). Pure cultures of *F. succinogenes* strains S85 and A3C digest more cellulose from intact forage than several strains of *Ruminococcus albus* and *R. flavefaciens*, the two other predominant cellulolytic bacterial species in the rumen (9). *F. succinogenes* also shows a great ability to solubilize other cell wall components such as xylans (27).

The enzymatic system of *F. succinogenes* type strain S85 has been studied extensively. This bacterium is known to produce at least seven different glucanases, a cellodextrinase, a cellobiosidase, and both a cellobiase and a cellobiose-phosphorylase (3, 13, 14, 22). *F. succinogenes* S85 produces also at least six xylanases (13), a lichenase (38), an α -glucuronidase (34), and ferulic acid and acetylxylan esterases and an arabinofuranosidase (26). Some of the genes coding for these activities have been isolated (most of them from strain S85) and sequenced (14).

The S85 strain was originally isolated from the bovine rumen (7) and has been maintained as a pure culture in laboratories ever since. Cultivation of the bacterium in laboratory media for nearly 50 years may have led to genotypic changes in the organism. Indeed, it was suggested that when wild strains of *F. succinogenes* are grown under laboratory conditions, physiological changes occur, and variants or mutants are selected (36, 40). Furthermore, recent isolates from cellulolytic bacterial species are sometimes more efficient than the reference strains

(19). In addition, the genus *Fibrobacter* is shown to include strains with a high level of genetic variation, as has also been shown for many other groups of rumen bacteria (18, 32). Two species in the *Fibrobacter* genus are presently known, *F. succinogenes* and *F. intestinalis*, and they show less than 20% total DNA homology, indicating a loose relationship between the species (1). The species *F. intestinalis* comprises strains isolated from the rat and other monogastric animals, but also bovine strains (1). It is therefore probable that several different *Fibrobacter* strains from both species are present in the rumen and that this mixture represents the dominant *Fibrobacter* population. It therefore seems important to check that the S85 *F. succinogenes* strain is truly representative of all rumen *Fibrobacter* strains, particularly when considering its properties related to plant cell wall degradation.

In this work, we compared the phylogenetic relationships and the cellulolytic or xylanolytic activity of several strains of *F. succinogenes* and one strain of *F. intestinalis*. The *F. succinogenes* strains were isolated from different animals (cows and sheep), and from different parts of the world, and the *F. intestinalis* strain was isolated from a rat (1). Several of the *F. succinogenes* strains have been subcultured only a few times since their isolation. The enzyme activities were compared both qualitatively and quantitatively. In parallel, the glycoside hydrolase genes already identified in some strains of *F. succinogenes* were sought by PCR in all the strains studied here.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The nine *Fibrobacter* strains used were type strain *Fibrobacter succinogenes* S85 (ATCC 19169) and strain BL2 (obtained from C. Stewart, Rowett Research Institute, Aberdeen, Scotland), both isolated from the bovine rumen, and strain HM2 (ATCC 43856), isolated from a sheep rumen and belonging to a group different from that of S85 and BL2 (1, 21). *F. succinogenes* strains H, U, and R, isolated from the bovine rumen, were obtained from K.-J. Cheng, Lethbridge, Alberta, Canada. Strain F_E was isolated in our laboratory from a sheep rumen by the roll tube method of Hungate (17).

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TABLE 1. Primer sequences

Protein	Glycoside hydrolase family	Gene	Primer ^a	Sequence (5'-3') ^b	Position (nucleotide)	Position (codon)	T _m (°C)	Size (bp)
EGB	9	<i>endB</i>	endBF*	CCT-TGG-TGG-ACT-TTG-ATG-CG	716	80	62	500
			endBR*	CGA-TTC-CGT-AGG-CGT-CAC-AA	1,196	240	62	
			GH9R	RTC-NCC-NGC-RTC-RWR-VYA-NCC	937	154	68	
CedA	5	<i>cedA</i>	cedAF*	CCA-CCT-CGC-CAG-CAA-TCA-CG	1,292	108	66	713
			cedAR*	GTT-CGG-GGT-TGT-TGT-ATT-GC	1,985	339	60	
Cel3	5	<i>cel3</i>	cel3F*	AGC-GAT-GGT-AAG-GTC-ACT-GC	1,530	452	62	529
			cel3R*	AAG-GAA-TCT-GGA-GGG-TAT-CG	2,039	622	60	
			cel3'F	GAA-AA-Y-GCM-GAT-GGC-AAG	1,137	321	54	
			cel3'R	TCA-TRT-CCG-GTT-CGT-TCA	1,513	447	52	
			cel3'F	GAA-GAA-GCG-GAC-GGC-AAG			54	
EGD	9	<i>celD</i>	celDF*	ACA-ATC-CGA-ACG-ACG-AAA-AG	2,668	331	58	564
			celDR*	GGT-AAT-CGG-CAC-ACT-TCC-AT	3,212	512	60	
			GH9F	GGN-TRB-YWY-GAY-GCN-GGN-GAY	2,187	171	68	
EGE	9	<i>celE</i>	celEF*	CAC-GCT-CCT-TTC-GCT-TTA-CG	160	37	62	562
			celER*	GGC-ATA-AAC-CGC-AAG-ACC-AT	702	218	60	
			celE'F	GGC-TTC-GTT-TGA-CTT-TGC-TG	400	118	60	
			celE'R	CCT-TCT-TGG-CTG-CTT-CGT-AA	931	295	60	
			celGF*	CGT-TTA-TTG-ATG-CTG-TCC-GC	691	188	60	
CelG	5	<i>celG</i>	celGR*	GTG-CTG-CTT-TCA-TTG-CCC-AT	1,233	369	60	562
			celG'F	TTG-AAC-ATC-CAC-CAC-GAA-GG	468	114	60	
			celG'R	TTA-GCA-GCC-GAA-ACG-ACA-TC	1,182	352	60	
			celFF*	TCG-TGA-AGG-GCA-ACA-AGA-AG	1,004	208	60	
			celFR*	GCC-AAC-AGA-GAA-GCG-GAT-TT	1,562	394	60	
EndA	9	<i>endA_{FS}</i>	endA _{FS} F*	GAA-CTT-GAA-CTT-TAC-CGC-AC	1,178	274	58	563
			endA _{FS} R*	TAG-TAA-AGT-CGC-CAG-CAA-TC	1,721	447	58	
			xynCF*	CTC-TAA-GGG-TAA-CGG-CAA-CG	1,696	196	62	
XynC	11	<i>xynC</i>	xynCR*	ATG-GAG-GGC-TGC-TGG-ACA-CG	2,180	285	66	504
			xynC'F	TAA-CAC-CCT-CGC-CAA-TGA-CA	1,285	148	60	
			xynC'R	TAC-CCT-GCT-TGC-TCC-ACT-TG	1,993	384	62	
			mlgF*	TGA-AGC-CCG-TAT-GAA-GAT-GG	282	46	60	
LichA	16	<i>mlg</i>	mlgR*	CGT-CAA-ATG-TCC-AGT-CAC-CC	813	223	62	551

^a Sequences were from *F. succinogenes* S85 genes except for *endA_{FS}*, which was from strain AR1; cel3' primers, which were from an S85/A3c consensus sequence; and the cel3' primer, which was designed from the *F. intestinalis* DR7 sequence. *, described previously (4).

^b R, A or G; N, A or C or G or T; W, A or T; V, A or C or G; Y, C or T; M, A or C; B, C or G or T.

F. intestinalis NR9 (ATCC 43854), the type strain of the second known species of the genus *Fibrobacter*, was isolated from the rat cecum (29). The *Fibrobacter* strains were grown on medium containing 30% rumen fluid (4) and 0.5% filter paper cellulose (Whatman 1MM) for enzyme assays or 0.3% glucose or cellobiose for DNA extraction.

The other rumen strains used were *Ruminococcus albus* 7 (ATCC 27210), *Ruminococcus flavefaciens* 007, and *Prevotella ruminicola* 23 (previously obtained from M. P. Bryant, University of Illinois, Urbana-Champaign). These strains and *Escherichia coli* DH5 α were grown as previously described (10).

Fermentation end product assays. Succinate, acetate, and formate were assayed as described by Matheron et al. (25).

DNA isolation. DNA extraction from *E. coli* was performed with the phenol-chloroform procedure (33). DNA extraction from *P. ruminicola* and from the different strains of *Fibrobacter* was carried out as described previously for *F. succinogenes* (12). DNA from *R. flavefaciens* and *R. albus* was extracted with the Easy DNA kit (Invitrogen, Groningen, The Netherlands), according to the manufacturer's recommendations.

Quantitative DNA-DNA hybridization. The relative DNA concentration for each strain was quantified by spectrophotometry and checked by hybridization to a universal oligonucleotide probe, 5'-ACGGCGGTGT(A/G)C-3' (experimental melting temperature, 46°C), which is complementary to all genes of 16S-like rRNAs (30). The oligonucleotide probe was synthesized by Eurogentec (Seraing, Belgium) and labeled with [γ -³²P]ATP and T4 polynucleotide kinase. The hybridization and washing conditions were as described previously (1). DNA (2 μ g) from each isolate was denatured, divided into three aliquots of 0.7 μ g, and spotted onto nylon membranes (Hybond N+) with a dot-blot device (Bio-Rad). The membranes were then hybridized with [α -³²P]dCTP-radiolabeled total DNA (Ready-to-Go kit from Roche) from each strain and washed under conditions similar to those used previously (1). After autoradiography, either the radioactivity in the membranes was counted with an AMBIS counter, or the spots were cut from the membranes and the radioactivity was counted by scintillation spectrometry.

PCR primers and procedure. The oligonucleotide primers were partly previously designed (4) and purchased from Eurogentec (Seraing, Belgium). The sequence, position, and melting temperature of the primers used to amplify the glycoside hydrolase genes are given in Table 1, as well as the sizes of the amplified PCR fragments. The GH9 degenerate primers were designed to hybridize to family 9 glycoside hydrolase genes. rRNA gene fragments of approximately 1,500 bp were amplified with the 16S rRNA gene bacterial forward primers F8 [5'-AGAGTTTGATC(A/C)TGGCTC-3'] and F500 (5'-CTAACTA CGTGCCAGCAGC-3'), associated with the universal reverse R1492 primer (5'-GNTACTTGTACGACTT-3').

PCRs were carried out with genomic DNA (0.5 μ g) and 1 U of AmpliTaq DNA polymerase (Qbiogene) for glycoside hydrolase genes and the Expand High Fidelity PCR system (Roche) for 16S rRNA gene fragments. Each reaction was subjected to 30 cycles of amplification consisting of 30 s denaturation at 94°C, 2 min of annealing of primers at a temperature 5°C below the melting temperature (Table 1), and 45 s or 2 min of elongation at 72°C for glycoside hydrolase genes and 16S rRNA genes, respectively. A 2-min DNA denaturation at 94°C was performed before the 30 cycles. The final cycle included elongation for 7 min at 72°C. A negative control (without DNA) was included in every PCR procedure.

PCR-generated DNAs were sequenced full length in both directions with an ABI Prism BigDye terminator cycle sequencing kit (Perkin Elmer Corporation, Norwalk, Conn.) with appropriate primers in an ABI Prism 310 genetic analyzer (Perkin Elmer Corporation).

Nucleotide and protein sequence comparison and phylogenetic analyses. Pairwise nucleic acid and protein sequence similarities were estimated with the global alignment programs ALIGNn and ALIGNp, respectively (<http://www.infobiogen.fr>).

The phylogenetic tree was constructed by first performing a multiple alignment with the ClustalW 1.8 program (<http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html>) with all available *Fibrobacter* 16S rRNA gene sequences, with the *Bacteroides fragilis* 16S rRNA gene sequence (EMBL AB050106) used as the

outgroup. Optimization of the alignment was performed manually with sequences of equal lengths (nucleotides 9 to 1315 of the S85 16S rRNA gene sequence) before running the Phylip package 3.5c programs (11). A distance matrix was generated with the Kimura model with the DNAdist program following a bootstrap analysis based on 1,000 replicates with the Seqboot program. Final trees were obtained by neighbor-joining analyses with the Consense (11) and Treeview (31) programs.

Harvesting of cultures and preparation of cells. All *Fibrobacter* strains were subcultured three times on filter paper rumen fluid medium before harvesting. For each strain, three tubes of 10-ml cultures grown for 48 h at 39°C were first centrifuged for 5 min at 500 × g to eliminate filter paper cellulose. Culture media containing free cells were pooled and centrifuged at 12,000 × g for 20 min. The supernatant was concentrated about sixfold with a Macrosep 10K Omega centrifugal device (PALL Life Sciences). The cell pellet was suspended in 1/5 volume of 50 mM sodium phosphate buffer (pH 7.0). The triplicate cultures were repeated at least twice. Supernatants and cells were kept frozen at -20°C.

Enzyme and protein assay. The enzyme preparations were incubated at 39°C for 30 min in 50 mM sodium phosphate buffer, pH 7.0, with 0.5% carboxymethyl cellulose (CMC sodium salt, medium viscosity), 1% Sigmacell 101 cellulose, and 1% oat spelt xylans (all from Sigma Chemical Co., St. Louis, Mo.) or with 1% Avicel PH101 microcrystalline cellulose (Fluka). The amount of enzyme extract and the incubation time were chosen to give conditions in which the measured activity was proportional to these two parameters. Reducing sugars released from polysaccharide hydrolysis were assayed as described previously (12) with glucose and xylose as the standards. Results are given in nanokatals, where 1 kat corresponds to 1 mol of glucose or xylose equivalent produced per s. For each culture and for each substrate, assays were performed at least twice.

Protein was determined by the method of Bradford (5), with bovine serum albumin as the standard.

PAGE and zymogram. Polyacrylamide gel electrophoresis (PAGE) and zymograms for carboxymethyl cellulase (CMCase) and xylanase activity were performed as described previously (10, 12).

Statistical analyses. All data were analyzed by one-way analysis of variance, and the differences between the means of a row were compared with the Student-Newman-Keuls multiple-comparisons test.

Nucleotide sequence accession numbers. Nearly complete 16S rRNA gene sequences were obtained for *Fibrobacter* strains S85 (AJ496032), BL2 (AJ505937), HM2 (AJ496186), H (EMBL AJ496447), R (EMBL AJ505938), U (EMBL AJ496448), F_E (AJ496566), and NR9 (AJ496284) and deposited in the EMBL data library under the indicated accession numbers. PCR-DNA fragments of glycoside hydrolases were also deposited in the EMBL database under the accession numbers AJ548833 (*F. succinogenes* U partial *xynC* gene), AJ548832 (*F. intestinalis* NR9 partial *xynC* gene), and AJ548831 (*F. intestinalis* NR9 partial *celF* gene).

RESULTS

Substrates and end products. All the *Fibrobacter* strains, like the S85 type strain, grew on media containing either glucose, cellobiose or filter paper cellulose as the carbon source (not shown); this is consistent with previous work (24, 25). None of them was able to grow on xylans, possibly because of an inability to utilize xylose, as shown in S85 (23). The fermentation end product patterns of all isolates were similar to that of S85 (succinate, acetate, and formate).

Grouping of strains. The relationships between the eight isolates of *Fibrobacter* were analyzed by DNA-DNA hybridization. The results are presented in Table 2. The estimated genomic DNA similarity determined here between strains S85, HM2, and NR9 were consistent with the values previously obtained with the same technique (1), except for the S85-HM2 DNA similarity, which we found to be higher. The S85 strain presented about 30% DNA similarity with strains BL2 and HM2, whereas 40 to 50% DNA similarity was observed between strains S85 and H, U, R, and F_E. This suggested that the four newly isolated bovine or sheep strains morphologically identified as *F. succinogenes* strains actually belong to this species and are closest to the S85 type strain. Thus, the H, U,

TABLE 2. 16S rRNA gene identity and DNA/DNA hybridization value for the *Fibrobacter* strains^a

Strain	% Identity							
	S85	BL2	H	U	R	F _E	HM2	NR9
S85	100	99.6	99.6	99.4	99.9	99.3	96.1	89.9
BL2	—/32	100	99.3	99.1	99.6	99.0	96.2	89.7
H	47/48	38/—	100	99.7	99.7	99.4	95.6	89.5
U	42/—	36/37	65/77	100	99.6	99.4	95.7	89.6
R	49/61	38/—	—/70	70/64	100	99.3	95.9	89.8
F _E	36/40	39/—	39/29	50/50	58/39	100	95.6	89.4
HM2	37/34	41/—	29/36	31/27	45/27	75/78	100	89.3
NR9	3.5/4	11/—	5/4	9/4	10/3	0/2	5/3	100

^a Values in italics are percent identity between 16S rRNA gene sequences from two strains. Alignments of equal length were made (nucleotides 9 to 1407 from the S85 type strain). The other values are reciprocal DNA/DNA hybridization data with the left value corresponding to use of the DNA from the organism in the first row as the probe. —, not determined.

and R bovine strains presented more than 70% similarity, suggesting that the animal origin could be related to their phylogenetic proximity. In the same way, the F_E and HM2 strains showed about 70% DNA similarity; both of these strains were isolated from the rumen of sheep. Amann et al. (1) have already described subspecies of *F. succinogenes* constituted exclusively of members from one habitat. *F. intestinalis* NR9 was distant from all the other strains (2 to 11% DNA similarity) due to the phylogenetic distance between the two species of the genus *Fibrobacter*.

This grouping was extended by sequencing and comparing the 16S rRNA gene sequence of the strains studied (Table 2). As for Amann et al. (1), the 16S rRNA gene similarity of *F. succinogenes* S85 was greater with strain BL2 (99.6%) than with strain HM2 (96.1%); the lowest value was obtained with *F. intestinalis* NR9 (89.9%). The rRNA gene sequence comparison of the new bovine isolates, like the DNA-DNA similarities, suggested that these strains are closer to the S85 type strain (99.3 to 99.9%) than to strain BL2 (99.0 to 99.6%) or HM2 (95.6 to 95.9%). Surprisingly, the rRNA gene sequence from the F_E strain was closer to that of the recent bovine isolates (99.0 to 99.4%) than to that of the ovine ruminal strain HM2 (95.6%). According to the DNA-DNA reassociation, F_E probably belongs to the same group as HM2, whereas the strain is classified within the bovine ruminal strains with rRNA gene comparison.

A phylogenetic tree was constructed for the rRNA gene sequences from all the *Fibrobacter* strains studied here and available in the nucleotide databases, with *Bacteroides fragilis* (DSM 2151T) as the outgroup sequence (Fig. 1). The 19 *Fibrobacter* sequences included *F. succinogenes* strains S85, A3c, B1, BL2, MC1, HM2, MB4, MM4, GC5, and REH9-1, *F. intestinalis* strains NR9, C1A, DR7, JG1, and LH1, and the *Fibrobacter* sp. strains from this study (H, U, R, and F_E). The tree is consistent with that of Lin and Stahl (20) except for two nodes. The first one, supported by high bootstrap values (85 to 100%), differs in the phylogenetic position of strain HM2, which was closest to strain MB4 in this study. We used a new HM2 16S rRNA gene sequence which was longer and more complete than the previous one (20). The second different node, also supported by a strong bootstrap value (100%), consists of the phylogenetic position of the GC5/REH9-1 branch,

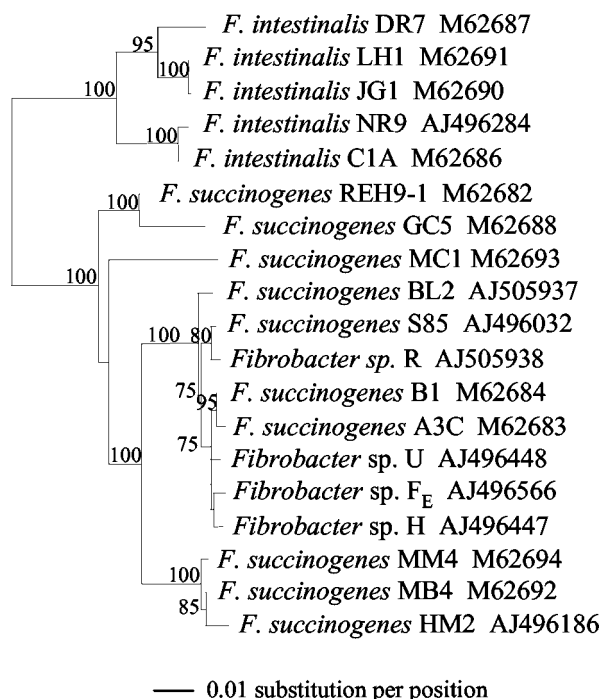


FIG. 1. Phylogenetic tree for 16S rRNA gene sequences from *Fibrobacter* strains. Phylogenetic relationships were based on ClustalW alignments, optimized manually, and analyzed with the Phylip programs. A total of 1,000 bootstrap replicates were performed, and bootstrap values greater than 75 are indicated above each branch. rRNA gene sequences are shown with their species and EMBL or GenBank accession numbers.

which was found to be distant from the other *F. succinogenes* strains. Additional new 16S rRNA gene sequences in the phylogenetic analysis could have resulted in reorganization of this genetic branch.

Zymograms. Six *Fibrobacter* strains representative of different phylogenetic groups (Fig. 1) were compared by zymograms for CMCCase and xylanase activities (Fig. 2). Extracellular culture fluid (ECCF) and cells were analyzed separately.

The CMCCase activity pattern observed for cells (Fig. 2A, P) was different from that of the ECCF (Fig. 2A, S). The activity profiles obtained for strains U and R were very similar. The strains studied all presented a majority of bacterial CMCases of between 60 and 120 kDa (Fig. 2A, P). Several lower bands of activity could also be detected for strains F_E and HM2, at approximately 35 and 45 kDa, but were not detected or detected only weakly for the other strains. The NR9 profile did not differ from those of the *F. succinogenes* strains.

Comparison of the extracellular CMCases from the six strains revealed three types of profiles (Fig. 2A, S). Strains S85, U, and R presented extracellular CMCases of between 35 and 150 kDa, with two major bands at about 50 and 95 kDa, whereas the F_E and HM2 strain profiles included an additional band at 60 kDa. The CMCCase profile of *F. intestinalis* NR9 did not correspond to any of those of the *F. succinogenes* strains.

The xylanase activity of cells and ECCF of all strains was also examined by zymograms (Fig. 2B). The xylanolytic profile of each strain revealed fewer bands than for the CMCases. Again, the strain U and R profiles looked similar. The *F.*

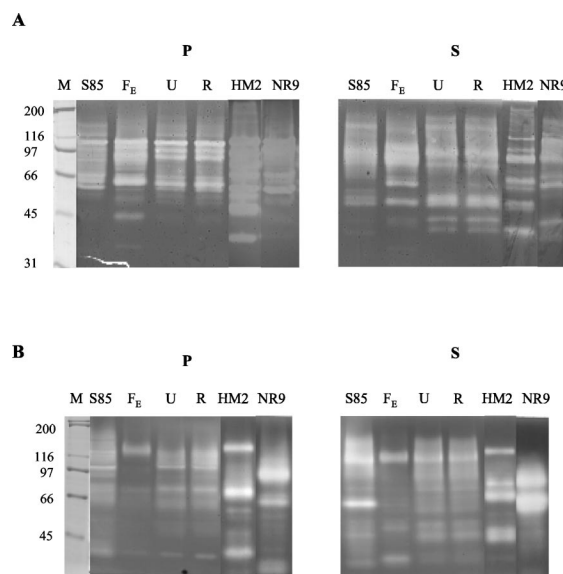


FIG. 2. CMCCase (A) and xylanase (B) zymogram analyses of bacterial (P) and extracellular (S) proteins from several strains of the genus *Fibrobacter*. Lanes M, broad-range molecular mass standards (in kilodaltons) (Bio-Rad). Protein migration was performed with a 10% acrylamide gel.

succinogenes strains revealed several xylanases of between 35 and 130 kDa, while *F. intestinalis* NR9 showed two large xylanases at about 65 and 95 kDa. Several lower-molecular-mass xylanases were weakly detected.

The cells (Fig. 2B, P) could be separated into three different profiles: the first of these included strains S85, U and R, the second one included strains F_E and HM2, and the last profile was specific for *F. intestinalis* NR9. Except for strains U and R, the extracellular xylanase profiles (Fig. 2B, S) were different for each strain studied. Type strain S85 presented a major xylanase band at about 65 kDa which could correspond to the known *F. succinogenes* xylanase XynB (about 65 kDa) or XynC (63 kDa) (13).

Quantification of cellulase and xylanase activities of cells and ECCF. The CMCCase, cellulase, and xylanase activities were quantified with CMC, Sigmacell, Avicel, and oat spelt xylans as the substrate. The relative CMCCase and xylanase activities of cells and ECCF in an equal volume of 48-h cultures of the *Fibrobacter* strains are reported in Fig. 3. The activity distribution between bacteria and ECCF was generally similar for all the strains. The relative CMCCase activity of each strain was mostly extracellular (60 to 75%) (Fig. 3A). The Sigmacellase relative activity of each strain showed the same profile (not shown). The xylanase activity (Fig. 3B) was also mostly extracellular (55 to 70%) for each strain except for strain U. This activity was almost totally extracellular (90%) for NR9.

The CMCCase, Sigmacellase, Avicelase, and xylanase specific activities of the cells and ECCF were calculated. Bacterial activities are reported in Table 3. Strains HM2 and NR9 showed significantly higher (double) CMCCase and Sigmacellase specific activity than the other strains. A weak Avicelase activity could be detected for all the strains. The xylanolytic activity of all the strains was much higher than the cellulolytic

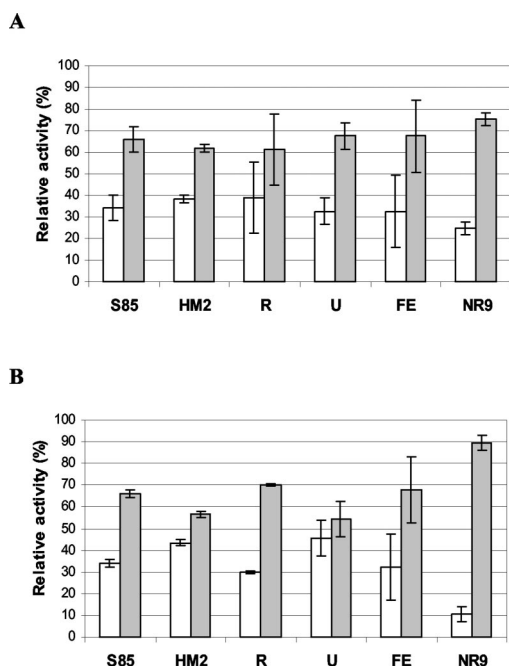


FIG. 3. CMCCase (A) and xylanase (B) activity distribution between cells (□) and ECCF (■) for several strains of *Fibrobacter* grown on filter paper cellulose. The CMCCase activities varied between 3.6 and 10.7 nkat in cells in the initial culture and from 7.0 to 20.0 nkat in ECCF. The xylanase activities varied between 8.3 and 42.7 nkat of total culture volume in cells and from 17.3 to 175.0 nkat in ECCF.

activity except for F_E, which showed the lowest xylanase specific activity.

The ECCF specific activities were almost similar to those of bacteria measured with the same substrates (Table 4). As for the bacteria, Avicelase activity was weakly detectable, suggesting that neither ECCF proteins nor harvested cells are able to degrade crystalline cellulose efficiently. The highest activity (45.1 nkat of xylose equivalent/mg of protein) was obtained for *F. intestinalis* NR9 ECCF; this could signify a particular ability of this strain to degrade hemicellulosic substrates compared to the *F. succinogenes* ruminal strains.

In conclusion, the fibrolytic activities of the recently isolated *F. succinogenes* strains (U, R, and F_E) were similar, according to the nature of the substrate, to those of the S85 type strain except for F_E, which showed the lowest levels of activity. The

TABLE 3. Enzyme specific activities of bacteria grown on cellulose^a

Strain	Mean sp act (nkat eq/mg of protein) ± SD			
	CMC	Xylans	Sigmacell	Avicel
S85	2.5 ± 0.5 ^a	15.5 ± 2.9 ^a	2.6 ± 0.4 ^{d,e,f}	0.10 ± 0.04 ^a
U	2.1 ± 0.6 ^a	12.6 ± 4.6 ^a	1.8 ± 0.6 ^{a,d}	0.07 ± 0.01 ^a
R	1.9 ± 0.7 ^a	12.0 ± 4.4 ^a	1.4 ± 0.4 ^{a,e}	0.06 ± 0.01 ^a
HM2	3.8 ± 0.4 ^b	15.3 ± 1.0 ^a	3.6 ± 1.1 ^{b,c,f}	0.12 ± 0.02 ^a
F _E	1.3 ± 0.7 ^a	4.0 ± 1.5 ^b	1.1 ± 0.8 ^a	0.08 ± 0.01 ^a
NR9	4.1 ± 0.6 ^b	16.9 ± 1.8 ^a	4.3 ± 0.6 ^b	0.13 ± 0.07 ^a

^a Specific activities are given as nanokatals of glucose (incubation with CMC or cellulose) or xylose (incubation with xylans) equivalent produced per milligram of protein. Each value is the mean of assays for at least two different cultures. Values bearing different letters within a row differ significantly (*P* < 0.05).

TABLE 4. Enzyme specific activities of ECCF of strains grown on cellulose^a

Strains	Mean sp act (nkat eq/mg of protein) ± SD			
	CMC	Xylans	Sigmacell	Avicel
S85	2.4 ± 0.4 ^a	18.2 ± 2.3 ^b	3.3 ± 0.4 ^{c,d}	0.17 ± 0.04 ^a
U	2.9 ± 0.8 ^a	13.0 ± 1.4 ^a	2.6 ± 0.5 ^{a,d}	0.12 ± 0.04 ^a
R	3.3 ± 0.5 ^a	11.1 ± 1.6 ^a	2.4 ± 0.7 ^{a,d}	0.06 ± 0.06 ^a
HM2	4.3 ± 0.5 ^b	16.9 ± 1.5 ^b	2.7 ± 1.4 ^{a,d}	0.18 ± 0.04 ^a
F _E	2.3 ± 0.2 ^a	7.1 ± 1.4 ^c	1.4 ± 0.5 ^a	0.05 ± 0.02 ^a
NR9	5.0 ± 0.2 ^b	45.1 ± 0.9 ^d	5.6 ± 1.1 ^b	0.17 ± 0.06 ^a

^a See Table 3, footnote a.

cellulase activities of HM2 and NR9 were usually higher than those of the other *Fibrobacter* strains that were more distant phylogenetically.

Detection of glycoside hydrolase genes. We searched for copies of the 10 glycoside hydrolase genes from *F. succinogenes* S85 in the genomes of the eight other strains by PCR. These genes, which were studied previously (4), include eight cellulase genes (*endB*, *cedA*, *cel3*, *celD*, *celE*, *celF*, *celG*, and *A_{FS}*), the *xynC* gene, encoding a xylanase; and the lichenase gene *mlg*. To detect homologous genes in all the strains, several pairs of primers had to be used (Table 1). The primers were chosen to amplify DNA fragments of approximately 500 bp, or 700 bp in the case of *cedA*, *celG*, and *xynC* (Table 1). These fragments were located in regions of the gene coding for the catalytic domain of the enzymes except for the *celF* gene.

Table 5 reports the detection of the genes in the *Fibrobacter* strains. With the exception of *celG*, which was not specifically detected in strain F_E, all the glycoside hydrolase genes studied were detected in the strains phylogenetically closest to S85 (BL2, H, U, R, and F_E) (Table 5, Fig. 1). Only two hydrolase genes were specifically detected in *F. succinogenes* HM2, the *celF* cellulase gene and the lichenase gene *mlg*. In addition, only two cellulase genes, *celF* and *cedA*, and the *xynC* xylanase gene were detected by PCR in *F. intestinalis* NR9.

celF is the only glycoside hydrolase gene to be specifically detected in all strains of the two *Fibrobacter* species. The PCR-DNA fragment of *celF* amplified from *F. intestinalis* NR9 was sequenced and showed 80.9% identity with the corresponding sequence (about 580 nucleotides) of *celF* from *F. succinogenes* S85 (Table 6). The 192-amino-acid translated protein sequence

TABLE 5. PCR detection of glycoside hydrolase genes in the *Fibrobacter* strains

Gene	Amplification ^a in strain:							
	S85	BL2	H	U	R	F _E	HM2	NR9
<i>endB</i>	+	+	+	+	+	+	-	-
<i>cel3</i>	+	+	+	+	+	+	NS	NS
<i>celD</i>	+	+	+	+	+	+	-	-
<i>celE</i>	+	+	+	+	+	+	NS	-
<i>celF</i>	+	+	+	+	+	+	+	+
<i>celG</i>	+	+	+	+	+	NS	-	NS
<i>endA_{FS}</i>	+	+	+	+	+	+	-	NS
<i>cedA</i>	+	+	+	+	+	+	-	+
<i>mlg</i>	+	+	+	+	+	+	+	-
<i>xynC</i>	+	+	+	+	+	+	NS	+

^a +, specific amplification; -, no PCR product; NS, nonspecific amplification.

TABLE 6. Partial glycoside hydrolase gene and deduced amino acid sequence comparisons for several *Fibrobacter* isolates^a

Gene	<i>Fibrobacter</i> strains compared	DNA PCR fragment identity (%)	Protein identity (%)
<i>celF</i>	S85-NR9	80.9	83.2
	S85-U	98.1	99.2
<i>xynC</i>	S85-NR9	92.9	93.8
	U-NR9	94.0	94.6
	S85-A3c	93.6	97.2
<i>cel3</i>	S85-REH9-1	84.5	89.8
	S85-DR7	72.6	72.3

^a The DNA identities of partial sequences were calculated based on alignments of equal length with *F. succinogenes* S85 bases 1004 to 1581 for *celF*, 1285 to 2012 for *xynC*, and 1530 to 2058 for *cel3* and the corresponding sequences (amplified by PCR or available in the GenBank database) in the other strains.

EGF showed 83.2% identity between the two strains. As the *xynC* xylanase gene was also detected in *F. intestinalis* NR9, the sequence of the PCR fragment (725 nucleotides) was determined and compared with that of *F. succinogenes* S85 and U (Table 6). The partial *xynC* gene sequence from *F. succinogenes* S85 showed 98.1% DNA identity (99.2% over 242 amino acids) with strain U and 92.9% (93.8% amino acid identity) with *F. intestinalis* NR9; this is in agreement with the phylogenetic relationships of these strains (Fig. 1). The PCR-DNA fragment of *xynC* from *F. succinogenes* U showed 94% DNA identity (94.6% amino acid identity) with *F. intestinalis* NR9.

The *cel3* endoglucanase gene of *F. succinogenes* S85 was previously sequenced from several *F. succinogenes* ruminal isolates, S85, A3c, and REH9-1, and from porcine cecum strain *F. intestinalis* DR7 (20). Based on the comparison of a 530-bp sequence, surrounded by the positions of the *cel3R* and *cel3F* primers (Table 1) and encoding a part of the endoglucanase catalytic domain, *cel3* presented 93.6% identity (97.2% protein identity) with the corresponding sequence from the genetically close *F. succinogenes* strain A3c but only 84.5% (89.8% protein identity) with REH9-1 and 72.6% (72.3% protein identity) with *F. intestinalis* DR7 (Table 6). This finding is consistent with the phylogenetic distances between these strains (Fig. 1). The DNA and amino acid sequences of *cel3*/EG3 appeared more divergent between the two *Fibrobacter* species (about 72% identity) compared to *celF* and *xynC* glycoside hydrolase sequences (81 to 93%). This result could explain the fact that even the degenerate primer *cel3'*, designed with oligonucleotide sequences conserved between *F. succinogenes* S85, A3c, and REH9 and *F. intestinalis* DR7, did not allow the gene to be detected in *F. succinogenes* HM2 or *F. intestinalis* NR9, which are genetically related to strains REH9-1 and DR7, respectively.

The degenerate family 9-specific glucoside hydrolase primers that we designed allowed the *endB* and *celD* genes to be detected in six strains (S85, BL2, H, U, R, and F_E) of *F. succinogenes* (Table 5), but did not enable these genes to be detected specifically in the genome of *F. succinogenes* HM2 or *F. intestinalis* NR9. In fact, modifications of the hybridization temperature during the PCR procedures to detect several glycoside hydrolase genes of *F. succinogenes* F_E and HM2 and *F. intestinalis* NR9 produced nonspecific PCR fragments after amplification (Table 5).

The primers for four *F. succinogenes* glycoside hydrolase

genes (*endB*, *cel3*, *celF*, and *xynC*) did not allow these genes to be amplified in other fibrolytic ruminal species such as *R. albus*, *R. flavefaciens*, and *Prevotella ruminicola* (not shown). This confirms their specificity for the *Fibrobacter* fibrolytic system. PCR was negative for all glycoside hydrolase gene-specific primers with *E. coli* DH5 α DNA as a negative control.

DISCUSSION

Previous DNA hybridization and 16S rRNA gene similarity analyses indicate broad genetic diversity in the genus *Fibrobacter* (1, 21). This work was carried out, first, to extend such studies by characterizing new ruminal *Fibrobacter* isolates and, second, to determine whether strain S85 is a good model for ruminal *F. succinogenes* and whether it has been subjected to phenotypic alteration over time.

Both DNA-DNA hybridization and 16S rRNA gene sequence comparisons were used to determine phylogenetic relationships between the *Fibrobacter* strains studied, as it is known that 16S rRNA gene sequence analysis is insufficient to discriminate among closely related organisms (35, 39). Thus, as four *Fibrobacter* isolates (H, U, R, and F_E) had been proposed by morphological criteria to belong to the same species (*F. succinogenes*), the phylogenetic positions of seven *F. succinogenes* strains had to be distinguished in the present study. Equal-length, nearly complete (1,400 bp) 16S rRNA gene sequences were compared and associated with DNA-DNA hybridization to evaluate the similarity of the strains. A good correlation was observed except for strain F_E (Fig. 1), which appeared to be close to *F. succinogenes* HM2 by DNA-DNA reassociation but more distant from the same strain by rRNA gene sequence comparison. Noncongruence between prokaryotic whole-genome and 16S rRNA gene sequences or between genotyping methods has already been documented (15, 19). Nevertheless, our results suggest that all of the newly studied strains belong to *F. succinogenes* and are distinct from *F. intestinalis*. Furthermore, the phylogenetic tree confirms the genetic diversity of *Fibrobacter* isolates.

The fibrolytic systems of the eight *Fibrobacter* strains studied, including *F. intestinalis* NR9, revealed common characteristics, such as qualitative cellulolytic and xylanolytic system (visualized by zymograms), homogenous specific activities towards four different polysaccharide substrates, and common hydrolase genes. However, some strains, such as HM2 and NR9, which are more distantly related to S85 than the others presented specific phenotypic characteristics.

The qualitative and quantitative analyses of the fibrolytic activities could be correlated for several strains with respect to the substrate, especially for phylogenetically related *F. succinogenes* strains S85, U, and R. CMCCase zymograms of cells and ECCF revealed common activity patterns for these strains, which are consistent with similar CMCCase specific activities. The cell xylanase specific activities of these strains were also concordant with the qualitative profiles observed in zymograms. However, an extracellular xylanase of *F. succinogenes* S85, appearing as a strong band on the xylan zymogram with an apparent molecular mass of 65 kDa, could explain why the specific activity of the S85 ECCF was higher than that of strains H and U.

The lowest specific activity measured on xylans was that of

strain F_E and was well correlated with the xylan zymogram, as very few and weakly detectable bands of activity were observed. On the contrary, the highest xylanase activity was obtained from *F. intestinalis* NR9, which was concordant with the strong bands of activity on the zymogram.

All of the *Fibrobacter* cells and ECCF studied presented a very low common activity on crystalline cellulose. Forsberg et al. have shown previously (13) that disrupted cell extracts or ECCF were not able per se to degrade crystalline cellulose efficiently, whereas bacteria can grow well on this substrate. This property thus seems to be associated with living bacteria.

The CMCase, sigmacellase, and xylanase activity distribution showed that major enzyme activities of all strains were extracellular for all the substrates studied. This is in agreement with a previous study that showed major endoglucanase and xylanase activities of strain S85 associated with membrane vesicles in the ECCF of cellulose-grown cultures (16).

Molecular study of endoglucanase, cellodextrinase, lichenase, and xylanase genes in eight *Fibrobacter* isolates provided evidence for the presence of homologous genes in all *F. succinogenes* strains studied (with one exception, *celG*). The sequence of the partial *xynC* gene amplified from strain U confirmed its homology with the *F. succinogenes* S85 type strain and suggested that the glycoside hydrolase genes of the type strain could be widespread in other close phylogenetic isolates. Moreover, conserved DNA fragments from *celF* and *xynC* were detected in the most distantly related strain, *F. intestinalis* NR9. These genes were conserved between the two strains, whose DNA-DNA similarity is less than 5%. The EGF endoglucanase from *F. succinogenes* S85 is a complex modular enzyme displaying CMCase activity and comprising a cellulose binding module preceding a catalytic domain homologous to that of the family 51 glycoside hydrolase (28). The ubiquitous detection of the partial *celF* gene in all *Fibrobacter* strains studied was probably due to particularly strict conservation of the *celF* primer sequences, chosen in the N-terminal coding region of EGF, whose function is unknown and which has no homology with any sequence present at this time in the databases. Similar nucleotide conservation was observed with the *xynC* primers except that no amplification was obtained with *F. succinogenes* HM2 DNA, suggesting nucleotide substitutions in the HM2 primer targeted sequences or absence of the gene in this strain which may possess homologous xylanases.

The choice of pairs of primers is of particular importance when assessing the distribution of glycoside hydrolase genes in the *Fibrobacter* genus, and this is illustrated by the fact that, despite the use of several pairs of primers, we did not detect the *celE* and *celG* genes in several strains, probably because of mismatches in the sequences targeted by the chosen primers. Similarly, the *cel3* gene was not detected in strains HM2 and NR9, whereas the gene was present in other closely related strains (20). This gene may be widespread in *Fibrobacter* strains, but this was not demonstrated here. Several sets of PCR primers were also necessary to detect homologous xylanase genes in several strains of another ruminal bacterium, *Butyrivibrio fibrisolvens* (8).

The experimental findings presented here suggest that *F. succinogenes* strains could be considered a homogeneous group in terms of phylogeny but also because of similar patterns of activities and glycoside hydrolase components. Strains HM2

and F_E presented some peculiarities, with more complex zymogram profiles for HM2 and lower glycanase specific activities for F_E. A previous study, which compared the polypeptide patterns of 15 strains of *F. succinogenes* by sodium dodecyl sulfate-polyacrylamide gel electrophoresis demonstrated homogeneous patterns between the strains (2). However, many of these strains were isolated from the same cow. A previous work demonstrated that the carbon metabolism of strains S85, H, and HM2 of *F. succinogenes* was very similar (24, 25), including some unusual features, also suggesting marked homogeneity in the carbon metabolism of *F. succinogenes* (23, 25).

The S85 type strain of *F. succinogenes* is thus a good model for studying the fibrolytic properties of the species, as the strain has conserved its enzymatic characteristics compared to several phylogenetically close isolates and is representative of recent isolates. Furthermore, our study provides evidence for different enzymatic activities of the *F. intestinalis* isolate with significantly higher xylanolytic specific activity and extracellular location. This may be related to the origin of the strain, and its function could have been adapted during evolution to the nature of the substrates available in the host digestive tract (monogastric versus polygastric animals).

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