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Identification of *Ruminococcus flavefaciens* as the Predominant Cellulolytic Bacterial Species of the Equine Cecum

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Detection and quantification of cellulolytic bacteria with oligonucleotide probes showed that *Ruminococcus flavefaciens* was the predominant species in the pony and donkey cecum. *Fibrobacter succinogenes* and *Ruminococcus albus* were present at low levels. Four isolates, morphologically resembling *R. flavefaciens*, differed from ruminal strains by their carbohydrate utilization and their end products of cellobiose fermentation.

Microbial degradation and fermentation of plant polymers into nutrients is a major function of the equine intestinal ecosystems. Moreover, incomplete fiber utilization can lead to illness and even death of the animal (2). However, information on the fibrolytic community in these nonruminant herbivores is very scarce. *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, and *Ruminococcus albus*, the three main ruminal cellulolytic bacterial species, have been identified based on morphological criteria (4, 7). *F. succinogenes* has been demonstrated with a specific oligonucleotide probe in a pony (19). *Bacteroides* sp., *Bacillus cellulosae dissolvens* (7), *Clostridium* sp., *Eubacterium* sp., and *Butyrivibrio fibrisolvens* have been observed. Protozoa do not seem to play an important role in cellulosis (23), but fungi appear to be strong cellulose degraders (15).

The objectives of our study were to determine the size of the cellulolytic bacterial community in the donkey and pony ceca by culture methods, to detect and quantify with oligonucleotide probes the three major cellulolytic bacterial species usually found in the rumen, to characterize the dominant cellulolytic bacterial strains, and to compare them with ruminal strains.

Three donkeys and three ponies, cecally fistulated, were fed a 70% lucerne-orchard hay and 30% concentrate (43% barley, 40% beet pulp, 10% soybean meal, 5% molasses, 3% minerals) (22) given in two equivalent daily meals. The total cecal contents (200 ml), collected before the morning meal into CO_2-saturated flasks, were serially diluted in an anaerobic mineral solution (5) under O_2-free CO_2 (12). Total viable counts of bacteria were determined in roll tubes on a complete agar medium (18), and the numbers of cellulolytic bacteria were estimated as the most probable number in broth (11) containing a strip of filter paper (Whatman no. 1) as the sole energy source. Culture methods were based on those described by Hungate (12) and Fonty et al. (8), except that the pH was adjusted to 7.3 before autoclaving and a mixture of ruminal fluid and cecum liquor (1:1) replaced ruminal fluid.

Cellulolytic bacteria were also detected and quantified with oligonucleotide 16S rRNA probes. Total RNA was extracted from 50 mg of lyophilized cecal sample after disruption of bacterial cells with zirconium beads (6). The general procedure for RNA isolation and quantitation was based on those previously described (1, 24, 25). The probes S-D-Bact-0338-a-A-18 (which targets eubacteria), S-S-F.succ-0650-a-A-20 (*F. succinogenes*), S-S-R.alb-0196-a-A-18 (*R. albus*), and S-S-R.fla-1269-a-A-20 (*R. flavefaciens*) (21) were labeled at their 5′ ends with T4 polynucleotide kinase (Eurogentec) and [γ-^{32}P]ATP (ICN). The hybridized 16S RNAs were visualized by exposure of the membranes to Hyperfilm MP (Amersham) for 24 h and quantified by liquid scintillation counting (Tricarb 2000 CA; Packard).

Cellulolytic bacterial strains were isolated from the highest-

TABLE 1. Quantification and percentage of *R. albus*, *R. flavefaciens*, and *F. succinogenes* 16S rRNA in cecal contents of ponies and donkeys targeted with specific oligonucleotide probes

Animal	Universal probe (quantification)[a]	*R. albus*[b] (% RNA)[c]	*F. succinogenes*		*R. flavefaciens*	
			Quantification	% RNA	Quantification	% RNA
Donkey1	35.72	<0.01	0.25	0.70	0.68	1.90
Donkey2	29.15	<0.01	0.06	0.21	2.68	9.19
Donkey3	25.10	<0.01	0.28	1.12	1.23	4.90
Pony1	32.59	<0.01	1.40	4.30	1.53	4.69
Pony2	38.87	<0.01	0.27	0.69	0.74	1.90
Pony3	17.83	<0.01	0.05	0.28	0.64	3.59

[a] Quantification is expressed in micrograms of 16S rRNA per 50 mg of lyophilized cecal contents.
[b] *R. albus* was detected at very low levels and was expressed only as a percentage of total 16S rRNA.
[c] % RNA is the percentage of total bacterial 16S rRNA.

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TABLE 2. Utilization of substrates by cecal cellulolytic isolates and by ruminal strains of *R. flavefaciens*

Carbohydrate	Utilization[a] by:				
	AB	AD	PA	PB	*R. flavefaciens*[b]
Pentoses					
L-Arabinose	−	−	−	−	D[c]
Ribose	−	−	−	−	
D-Xylose	+	+	+	+	D
Hexoses					
D-Galactose	+	+	+	+	
D-Glucose	+	+	+	+	D
D-Mannose	ND[c]	+	ND	ND	D
D-Fructose	+	+	+	+	−
Disaccharides					
D-Cellobiose	+	+	+	+	+
D-Maltose	+	+	+	+	−
Lactose	+	+	±	+	D
Sucrose	+	+	+	+	D
Trisaccharides					
Raffinose	+	±	−	+	−
Celluloses					
Filter paper	+	+	+	+	D
MN 300	−	−	−	−	
CMC	−	+	−	−	
Avicel	−	±	−	±	
Other glucose polymers					
Starch	+	−	−	−	−
Dextran 60	+	+	−	−	
Hemicelluloses					
Xylan	+	−	±	+	+
Arabinogalactane	ND	±	ND	ND	
Glucomannan	±	ND	−	−	
Pectic substances					
Pectin	−	+	−	−	+
Galacturonic acid	±	±	−	−	
Sugar-alcohols					
Mannitol	−	+	−	−	−
Dulcitol	−	±	−	−	
Glycerol	−	−	−	−	−
Organic acid					
Lactate	−	−	−	−	

[a] +, the strain maintained its growth on the same substrate after three transfers; ±, the strain could not maintain its growth on the same substrate after one or two transfers; −, the strain did not grow on the substrate after its transfer.
[b] Data from reference 24a.
[c] ND, not determined; D, utilization of the substrate depends on the strain of *R. flavefaciens* (26).

dilution tubes in the most-probable-number assay, serially diluted, and inoculated (12) into a solid medium (11) containing cellobiose (4 g liter^{-1}) as the sole energy source. Colonies grown after 3 days were then transferred to cellulose broth (11). Purity was checked under a phase-contrast microscope. Four representative strains (AB and AD for donkeys and PA and PB for ponies) from the isolates collection were phenotypically characterized and compared with strains FD1 and 007 of *R. flavefaciens*. Their ability to utilize carbohydrates (see Table 2) was determined by using a semisynthetic broth (20) containing 4 g of mono-, di-, or trisaccharides per liter or 1 g of polysaccharides per liter. Cultures were considered positive when the growth was maintained after three subcultures. The end products of cellobiose fermentation were analyzed by high-pressure liquid chromatography HPLC (14) after 72 h of incubation. Oligonucleotide probes targeting the 16S rRNA of *R. flavefaciens*, *R. albus*, and *F. succinogenes* (1, 24, 25) were used for the presumptive identification of the four isolates. RNA extraction of the bacterial cultures and hybridization with the probes were performed as described above for the cecal samples. RNA of each species was extracted from 25 mg of bacterial pellets obtained after centrifugation of a 24-h culture on 0.2% cellobiose.

The concentrations of total viable bacteria ($4.2 \times 10^8 \pm 1.7 \times 10^8$ and $5.7 \times 10^8 \pm 2.4 \times 10^8$ CFU ml^{-1} in the ceca of ponies and donkeys, respectively) and of cellulolytics ($1.6 \times 10^7 \pm 0.4 \times 10^7$ and $1.3 \times 10^7 \pm 0.6 \times 10^7$ bacteria ml^{-1}) showed no significant differences between the two animal spe-

TABLE 3. Major end products of cellobiose fermentation for the ruminal strains *R. flavefaciens* 007 and FD1 and the four cecal isolates

Strain	Concn (mmol/100 mmol of fermented hexoses) of[a]:						
	Acetate	Succinate	Lactate	Formate	Ethanol	Malate	Fumarate
007	55.1 ± 2.0	59.7 ± 2.1	29.7 ± 0.6	13.9 ± 1.1	0	3.3 ± 0.3	0.1 ± 0
FD1	74.9 ± 3.8	51.9 ± 2.5	0.2 ± 0.2	73.6 ± 3.9	0	7.3 ± 0.6	0.4 ± 0
AB	85.6 ± 8.6	0.2 ± 0.2	10.0 ± 5.5	45.3 ± 4.7	36.3 ± 4.1	0	0
AD	59.7 ± 2.3	5.4 ± 1.2	0	48.3 ± 0.6	2.8 ± 9.8	0	0
PA	78.8 ± 4.3	2.1 ± 0.7	0	65.2 ± 3.0	53.3 ± 7.6	0	0
PB	76.2 ± 9.7	34.0 ± 6.2	11.9 ± 4.0	106.4 ± 27.5	55.8 ± 15.2	0	0

[a] Data are means ± standard errors of the mean for triplicate determinations.

cies. Hybridization of rRNA from the cecal contents with specific probes revealed the presence of the three major ruminal cellulolytic bacterial species (Table 1). In all the animals, *R. flavefaciens* was the dominant species. *F. succinogenes* was also detected, whereas the population size of *R. albus* was so small that the amount of detectable 16S rRNA was at the detection limit (ca 1 ng/50 mg of freeze-dried cecal contents). When cultured on cellobiose, the equine strains exhibited long chains of gram-variable cocci resembling *R. flavefaciens*. The carbohydrates used by these isolates are given in Table 2. The four strains fermented cellobiose within the first 24 h of culture and produced mainly acetate, formate, and ethanol but no malate or fumarate, which differentiated them from the ruminal strains FD1 and 007 of *R. flavefaciens* (Table 3). RNAs extracted from the four strains hybridized with the oligonucleotide probe S-S-R.fla-1269-a-A-20 targeting *R. flavefaciens* but not with probes S-S-F.succ-0650-a-A-20 and S-S-R.alb-0196-a-A-18.

Small differences in total viable and cellulolytic counts found between donkeys and ponies suggest that the animal species and other animal factors have a limited impact on the size of the microflora in equines. For the pony cecum, our counts were close to those usually reported in the literature (10, 16, 17, 23) but much higher than those of Goodson et al. (10) (10^4 bacteria g^{-1}). The total and cellulolytic bacterial concentrations in the ceca of equines are about 100-fold lower than those in the rumens (9, 13, 16, 17, 26). Cellulolytic bacteria represented a small percentage of the total anaerobic bacteria in donkeys (2.3%) and ponies (3.8%), reared under our conditions; these percentages are lower than that found by Kern et al. (9%) (16) but similar to those estimated with probes. The three major cellulolytic species commonly found in the rumen were also present in the equine cecum. This finding is novel since these species had never been previously detected together in the equine cecum. The predominance of *R. flavefaciens* demonstrated with probes was consistent with the presumptive morphological identification of our strains. The proportion of *F. succinogenes* we observed was markedly lower than the 12% of total bacterial 16S rRNA found by Lin and Stahl (19) in the cecum of a pony. The diet given to the animals might explain this discrepancy. The equine strains of *Ruminococcus* exhibited metabolic and fermentative differences from the ruminal strains of *R. flavefaciens*. The four strains hybridized with probe S-S-R fla-1269-a-A-20, which suggested that the isolates are genetically closely related to *R. flavefaciens*. These characteristics allowed us to assign cecal isolates AB, AD, and PA to the genus *Ruminococcus*. Characteristics of the 16S rDNA determined by restriction fragment length polymorphism reported elsewhere (7a) showed genetic differences between cecal and ruminal strains. These differences could be due to an evolutionary adaptation of the ruminococci to their ecosystem, which has previously been reported for strains of *F. succinogenes* with the creation of two new lineages (Fibro-A and Fibro-B) (19). Further studies are required to determine whether the relative proportions of the three detected species, particularly the preponderance of *R. flavefaciens*, are generally observed in the equine or whether the equine bacterial cellulolytic community is more diverse. Understanding whether its structure depends on the diet or on other factors linked to the animal characteristics could allow optimization of lignocellulosic compound utilization in equines and therefore contribute to healthier equines.

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