

# Competition among three predominant ruminal cellulolytic bacteria in the absence or presence of non-cellulolytic bacteria

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**Competition among three species of ruminal cellulolytic bacteria – *Fibrobacter succinogenes* S85, *Ruminococcus flavefaciens* FD-1 and *Ruminococcus albus* 7 – was studied in the presence or absence of the non-cellulolytic ruminal bacteria *Selenomonas ruminantium* or *Streptococcus bovis*. Co-cultures were grown under either batch or continuous conditions and populations were estimated using species-specific oligonucleotide probes to 16S rRNA. The three cellulolytic species co-existed in cellobiose batch co-culture, but inclusion of either *Sel. ruminantium* or *Str. bovis* yielded nearly a monoculture of the non-cellulolytic competitor. In cellobiose chemostats, *R. albus* completely dominated the triculture, but *R. flavefaciens* became predominant over *F. succinogenes* and *R. albus* when *Sel. ruminantium* was co-inoculated into the chemostats. Similar effects on competition were observed in the presence of *Str. bovis* at a lower (0.021 h<sup>-1</sup>), but not at a higher (0.045 h<sup>-1</sup>) dilution rate. In cellulose batch co-cultures, *R. albus* was more abundant than both *F. succinogenes* and *R. flavefaciens*, regardless of the presence of the non-cellulolytic species. Co-existence among the three cellulolytic species was observed in almost all cellulose chemostats, but *Sel. ruminantium* altered the relative proportions of the cellulolytic species. *R. albus* and *R. flavefaciens* were found to produce inhibitors that suppressed growth of *R. flavefaciens* and *F. succinogenes*, respectively. These data indicate that interactions among cellulolytic bacteria, while complex, can be modified further by non-cellulolytic species.**

Keywords: cellobiose, cellulose, competition, *Fibrobacter succinogenes*, *Ruminococcus*

## INTRODUCTION

Numerous cellulolytic microbial species have been identified in the rumen, but it is generally agreed that ruminal cellulolysis is carried out primarily by three species of bacteria: *Fibrobacter succinogenes*, *Ruminococcus flavefaciens* and *Ruminococcus albus* (Hungate, 1966; Dehority, 1993). Because these species are nutritional specialists that differ in fermentation end products, and because cellulose is a major component of the diets of forage-fed ruminants, the relative populations of these three species can potentially impact on the ratios of volatile fatty acids available to the animal, an important determinant of animal performance. Several studies have examined competition among these cellulolytic species. Odenyo *et al.* (1994a, b) used oligonucleotide probes to species-specific segments of 16S rRNA to quantify specific populations in binary (two-

membered) and ternary (three-membered) batch cultures grown on cellobiose, cellulose or alkaline hydrogen peroxide-treated wheat straw. Their data indicated that *R. albus* 8 generally out-competed *R. flavefaciens* FD-1, due to production by the former of a bacteriocin-like substance. Fondevila & Dehority (1996) presented evidence that cellulose digestion was reduced when strains of *F. succinogenes* A3c and *R. flavefaciens* B34b were grown together in batch culture and suggested that an inhibitor was produced by one of the two species. Mosoni *et al.* (1997) reported that *R. flavefaciens* FD-1 became detached from cellulose in the presence of *R. albus* 20.

Within a permissive range of ruminal pH, digestion of cellulose is considered to be a first-order process that is limited by the available surface area of cellulose rather than by the hydrolytic capabilities of the cellulolytic

species (Waldo *et al.*, 1972; Weimer *et al.*, 1990). Studies with paired combinations of these species in chemostats revealed that substrate limitation intensifies competition for cellobiose, resulting in monocultures dominated by strains having a higher affinity for substrate (Shi & Weimer, 1997). By contrast, cellulose-limited chemostats inoculated with paired species in different combinations yielded stable co-cultures in which the two species displayed niche specialization (Shi *et al.*, 1997).

In the rumen, competition among cellulolytic bacteria is complicated by potential interactions with non-cellulolytic species. While specific interactions among cellulolytic and non-cellulolytic species have been characterized in binary culture (Scheifinger & Wolin, 1973; Stanton & Canale-Parola, 1980; Pavlostathis *et al.*, 1990) the effects of non-cellulolytic species on the outcome of competition among the cellulolytic species have not been characterized. The purpose of this study was to determine the effects of two important ruminal non-cellulolytic species, *Selenomonas ruminantium* and *Streptococcus bovis*, on the outcome of competition among the predominant cellulolytic species in defined co-culture under conditions of substrate excess and substrate limitation.

## METHODS

**Cultures and growth conditions.** Bacteria used in this study were *Ruminococcus albus* 7, *Ruminococcus flavefaciens* FD-1, *Fibrobacter succinogenes* S85, *Selenomonas ruminantium* D, GA192, HD4 and H18, and *Streptococcus bovis* JB-1. All cultures were maintained at  $-80^{\circ}\text{C}$  in 50% (v/v) glycerol until required. For competition studies, pure cultures of *R. albus*, *R. flavefaciens* and *F. succinogenes* were combined in a sterile serum vial and then inoculated simultaneously into the culture vessels; where indicated, non-cellulolytic strains were also co-inoculated with the cellulolytic triculture. Individual pure cultures were combined at equal volumes (0.5 ml per strain) from cultures grown to mid-exponential phase on the substrate of interest (cellobiose or cellulose). No attempts were made to provide identical cell numbers in the inocula, because this could not be immediately assessed in cellulose-grown cultures due to the abundance of adherent cells. However, previous experiments (Shi *et al.*, 1997; Shi & Weimer, 1997) revealed that the outcome of competition was independent of inoculum size.

Competition experiments with *Str. bovis* used strain JB1 (pregrown on cellobiose) in both batch and continuous culture. Batch culture competition experiments with *Sel. ruminantium* (also pregrown on cellobiose) used strain D, while in continuous culture all four strains of *Sel. ruminantium* were used to minimize the chance that a single strain may give an anomalous result. Thus, direct comparisons of batch versus continuous culture could not be made in cultures inoculated with *Sel. ruminantium*, although comparisons across substrate (cellobiose versus cellulose) within a culture mode (batch or continuous) could be made, as could comparisons of tricultures in the presence or absence of *Sel. ruminantium* within a culture mode.

Modified Dehority Medium (MDM), described previously (Weimer *et al.*, 1991), included either Sigmacell 20 microcrystalline cellulose ( $4\text{ g l}^{-1}$ ; Sigma) or cellobiose ( $4\text{ g l}^{-1}$ ) as carbon and energy source. All incubations were conducted

anaerobically under  $\text{CO}_2$  at  $39^{\circ}\text{C}$ . Batch cultures were conducted in 158 ml serum bottles (containing 100 ml medium), each fitted with a butyl stopper and an aluminium crimp seal. Continuous cultures were performed in a system described previously (Weimer *et al.*, 1991). The cellulose-containing medium in the reservoir was homogenized by stirring and diffusive gas sparging with  $\text{CO}_2$ , and was delivered as a  $\text{CO}_2$ -segmented slurry to a stirring fermenter (875 ml working volume) by a peristaltic pump. Cellobiose continuous cultures were conducted in a stirring reactor (139 ml working volume), continuously fed cellobiose-containing MDM. Both cellulose and cellobiose reactors were continuously sparged with filter-sterilized, humidified  $\text{CO}_2$ .

**Substrate and fermentation product assays.** For batch cultures, the entire culture was collected after incubation for 24 h (cellobiose) or 48 h (cellulose). Chemostat samples (28 ml for cellulose, 6 ml for cellobiose) were first collected after the reactor had received three dilutions of feed medium. Subsequent samples were removed from reactors at 6–20 h intervals over a 3 d period. These samples were then analysed and those showing constant fermentation product concentrations were used to define the steady state, under the assumption that this stability in product ratios results from a stabilization of the microbial population (whose individual species vary markedly with respect to product ratios).

Samples were analysed for pH using a Corning model 320 pH meter, residual soluble sugars by a phenol/sulfuric acid method (Dubois *et al.*, 1956) and fermentation acids and ethanol by HPLC (Weimer *et al.*, 1991). Concentrations of cellulose in the reactor or reservoir were measured by a modified neutral detergent fibre method (Weimer *et al.*, 1990).

**Quantification of relative populations of three cellulolytic species using oligonucleotide probes.** Three samples were taken from triplicate batch cultures and from each steady-state continuous culture. The procedures used for RNA isolation, hybridization and detection were similar to those of Shi *et al.* (1997). Samples from cellulose cultures were separated into adherent and non-adherent bacterial populations by filtering through a 47 mm diameter polycarbonate membrane ( $3\ \mu\text{m}$  pore size; Poretics). This method is based on microscopic observation that none of the three cellulolytic strains forms chains or clumps when growing in the planktonic mode with cellulose as energy source, although we observed that *R. flavefaciens* and *R. albus* form chains and clumps, respectively, when grown in batch culture on cellobiose. The filtrate (containing non-adherent cells) and filter cake (containing adherent cells and rinsed with deionized water into a 50 ml centrifuge tube) were recovered separately by centrifugation at  $4000\text{ g}$  for 30 min.

**RNA isolation.** RNAs were separated from other cellular components using a low-pH phenol extraction procedure modified from Odenyo *et al.* (1994a) that minimized extraction of DNA (Wallace, 1987). All reagents were prepared in diethylpyrocarbonate (DEPC)-treated water. Cell pellets ( $4000\text{ g}$ , 30 min) were transferred to 2 ml screw-cap conical tubes (Sarstedt) along with 0.5 g zirconium beads ( $\sim 0.1\text{ mm}$  diam., heated overnight at  $160^{\circ}\text{C}$ ),  $700\ \mu\text{l}$  50 mM sodium acetate/10 mM EDTA (pH 5.1),  $50\ \mu\text{l}$  20% (w/v) SDS and  $700\ \mu\text{l}$  saturated phenol (pH 4.3; Amresco). The tubes were shaken twice for 2 min in a bead beater at  $4^{\circ}\text{C}$ . These tubes were then heat-shocked in a  $65^{\circ}\text{C}$  water bath for 10 min, followed by another two cycles (2 min each) in the bead beater. The suspensions were centrifuged at  $12000\text{ g}$  at  $4^{\circ}\text{C}$  for 5 min. The aqueous phases were transferred to new 1.7 ml microcentrifuge tubes and extracted twice with each of

the following reagents in order: saturated phenol; phenol (pH 4.3)/chloroform (1:1, v/v) and chloroform. RNAs were precipitated by adding 2 vols absolute ethanol and 0.1 vols 3 M sodium acetate, and frozen at  $-80^{\circ}\text{C}$  overnight. RNA pellets were collected by centrifugation (12000 g,  $4^{\circ}\text{C}$ ) for 20 min, washed with 70% ethanol and centrifuged. Pellets of purified RNA were dissolved in 50–100  $\mu\text{l}$  water.

The purities and concentrations of the RNAs were determined by reading absorbance at 260 and 280 nm in a Beckman DU series 600 spectrophotometer. RNA solutions having  $A_{260}/A_{280}$  ratios  $\geq 1.7$  were used for slot-blots and hybridizations; otherwise extractions with phenol/chloroform (1:1, v/v) and chloroform were repeated to obtain purer preparations. Purified RNA was diluted to  $\sim 5\text{ ng }\mu\text{l}^{-1}$  and stored in microcentrifuge tubes (50  $\mu\text{l}$  per tube) at  $-80^{\circ}\text{C}$ .

**Hybridizations.** RNA samples (4–40  $\mu\text{l}$ , containing 20–200 ng RNA) were diluted to 100  $\mu\text{l}$  with denaturation solution (DEPC-treated water/10  $\times$  SSC/formaldehyde, 5:3:2, by vol.), then incubated at  $65^{\circ}\text{C}$  for 15 min. RNA loading buffer (2  $\mu\text{l}$  of 1 mM EDTA, 0.4% bromophenol blue, 50% glycerol) was then added and the entire 102  $\mu\text{l}$  solution was slot-blotted onto a Nytran membrane (Schleicher & Schuell) prewetted with 10  $\times$  SSC. RNAs were cross-linked onto the membrane by UV radiation (Stratalinker 1800; Stratagene) at  $0.12\text{ J cm}^{-2}$  for both sides of the membrane. Membranes were then air-dried and prehybridized in Rapid-Hyb buffer (Amersham) for 1–2 h at dissociation temperature ( $48^{\circ}\text{C}$  for *R. albus* probe S-S-R.alb-0196-a-A-18 and *F. succinogenes* probe S-Ss-F.s.-suc-0207-a-A-21, and  $41^{\circ}\text{C}$  for *R. flavefaciens* probe S-S-R fla-0196-a-A-17). These temperatures were also used for hybridization. At the end of prehybridization, the Rapid-Hyb buffer was poured out and 14 ml Rapid-Hyb buffer containing 10 pM 5'-digoxigenin-labelled oligo-DNA probes was poured into the tube and the hybridization reaction continued overnight (usually 16 h).

**Detection and quantification.** After hybridization, membranes were washed twice with  $2 \times$  SSC and 0.1% SDS at room temperature (15 min), and twice with  $0.5 \times$  SSC and 0.1% SDS at the specific hybridization temperature (15 min). Membranes were then immersed in blocking solution (10-fold dilution of 10%, v/v, blocking stock solution in maleate buffer (150 mM NaCl, 100 mM maleic acid, pH adjusted to 7.5 with NaOH) and shaken for 30 min. Alkaline-phosphatase-conjugated anti-digoxigenin ( $750\text{ U ml}^{-1}$ ; Roche Molecular Biochemicals) was added at 1  $\mu\text{l}$  blocking solution  $\text{ml}^{-1}$  and the solution was shaken for 1 h. Membranes were washed twice, 15 min per wash with maleate buffer, followed by a 2 min wash in detection buffer (0.1M Tris/HCl, 0.1M NaCl, pH 9.5). Lumiphos 530 (Roche) was used as chemiluminescent substrate for the hybridized RNA and exposure to Lumi-film (Roche) for 3 h was used to record light emission. After developing, the films were scanned on a Molecular Dynamics laser densitometer and the bands were quantified with ImageQuant software (Molecular Dynamics).

The amounts of RNA in each band from the densitometer image were determined from standard curves prepared with purified RNA from pure cultures and slot-blotted on the same membranes as the RNAs from samples. The relative population size of each cellulolytic species among the total cellulolytic population was expressed as the ratio of the amount of each bacterial RNA detected to the sum of the amounts of RNAs detected for the three cellulolytic species. Across all experiments with tricultures of the three cellulolytic strains (in the absence of added non-cellulolytic strains), the sums of the RNA detected using the three species-specific probes averaged 91.3% of the RNA detected using the

bacterial domain probe S-D-Bact-0338-a-A-18 of Amann *et al.* (1990). Differences among the relative population sizes for the cellulolytic strains were contrasted by a two-tailed *t*-test within the general linear model procedure of the software application (SAS Institute, 1985).

Although the RNA isolation procedure was conducted under conditions that minimize DNA extraction, the potential cross-reaction of each of the probes with DNA was tested. For these analyses, DNA was isolated from pure cultures using a Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer's instructions for Gram-positive and Gram-negative bacteria, except that each centrifugation step was extended for an additional 1 min. Although hybridization of the probes to 100 ng purified DNA could not be detected, weak hybridization was sometimes detected when the amount of DNA was increased to 1000 ng. By normalizing the signal to that obtained for the hybridization of 50 ng RNA, the extent of cross-reaction with a probe to the DNA of that species was estimated to range from 0 to 0.59% and the extent of cross-reaction to the DNA of the non-target species was estimated to range from 0 to 0.26%, relative to the extent of hybridization detected using purified RNA and the species-specific, RNA-targeted probe.

**Inhibitor screening.** Two methods were used to test the antagonistic activities between the bacterial strains. In the liquid culture assay, 2 ml supernatant of spent culture of the test strain was added to culture tubes containing 10 ml MDM/cellobiose. The indicator strain was then inoculated into tubes at room temperature, the tubes placed in a  $39^{\circ}\text{C}$  incubator and  $\text{OD}_{600}$  was recorded at intervals to allow determination of maximum growth rate. Sterilized water (2 ml) was supplemented to control tubes when determining growth in the absence of inhibitor.

Inhibitory activity was also measured using a plate assay, modified from that of Tagg *et al.* (1973). A lawn of indicator strain was first seeded by mixing 0.2 ml of an overnight culture with 15.5 ml melted ( $\sim 45^{\circ}\text{C}$ ) solid medium [MDM supplemented with ( $\text{l}^{-1}$ ): 10 g agar, 2 g Trypticase, 1 g yeast extract, 0.25 g each of cysteine/HCl and  $\text{Na}_2\text{S}_2\text{O}_8$ ]. This suspension was plated on sterile 100  $\times$  15 mm Petri dishes in an anaerobic chamber (5%  $\text{H}_2/95\%$   $\text{CO}_2$ , v/v). A drop ( $\sim 50\ \mu\text{l}$ ) of test strain from either exponential-phase or stationary-phase cultures was spotted onto the lawn as it solidified. The plate was examined for zones of inhibition after overnight incubation in the chamber at  $39^{\circ}\text{C}$ .

**Protease sensitivity.** The sensitivities of the inhibitors were tested with two broad-spectrum proteases, *Streptomyces griseus* protease (EC 3.4.24.31; Sigma cat. no. P5147) and porcine pancreatin (Sigma cat. no. P7545). A filter-sterilized stock solution (10 mg protease  $\text{ml}^{-1}$  in 0.01 M phosphate buffer, pH 7.0) was mixed with the overnight culture or culture supernatant of the test strain to give a final concentration of 100  $\mu\text{g}$  enzyme  $\text{ml}^{-1}$  and incubated for 4 h at  $40^{\circ}\text{C}$  (for pancreatin) or  $37^{\circ}\text{C}$  (for *Streptomyces griseus* protease). The mixtures were taken into the anaerobic chamber and allowed to reduce for 1 h before drop testing. Elimination or reduction in diameter of the zones of inhibition was examined by comparison to controls that contained no added enzyme or that contained enzyme but no added test culture or culture supernatant.

**Determination of the molecular mass range of the inhibitor.** Supernatants (3.5 ml) of test strain were loaded onto MICROSEP microconcentrators (Pall-Filtron) of 1, 3, 10 or 30 kDa molecular mass cut-off and centrifuged at 7000 g for 3, 2, 1.5 or 1 h, respectively. The inhibitory activity of both the

concentrated sample and the filtrate were checked by the plate assay described above.

## RESULTS

### Relative population sizes of ruminal cellulolytic species in cellobiose co-cultures

**Batch cultures.** Batch-mode incubation of cultures co-inoculated with the three cellulolytic bacterial species resulted in a triculture that consisted of a similar population size of *F. succinogenes* and *R. albus*, and a smaller population of *R. flavefaciens* (Table 1). Fermentation end products were mainly acetate, formate, succinate (produced primarily by *F. succinogenes*) and ethanol (produced only by *R. albus*) (Table 2).

Batch-mode incubation of the three cellulolytic species co-inoculated with *Sel. ruminantium* D contained *Sel. ruminantium* as the dominant species. Microscopy revealed a predominance of curved rod-shaped cells and large amounts of lactate were detected in fermentation end products. *Sel. ruminantium* is nearly a homolactic fermenter at rapid growth rates (Wallace, 1978) and lactate is not produced in significant amounts by any of the three cellulolytic species (Hungate, 1966). Moreover, succinate (from *F. succinogenes* and *R. flavefaciens*) and ethanol (from *R. albus*) were not detectable. Although the population of *Sel. ruminantium* was not quantified, its dominance was confirmed by the observation that the proportion of RNA attributable to the three cellulolytic species (determined using species-specific probes) repre-

sented only 10.1% of the RNA detected using the bacterial domain probe (data not shown). Among the cellulolytic species, *R. albus* was more abundant than *F. succinogenes* and *R. flavefaciens*, which each accounted for only half the population size of *R. albus*.

Co-cultures of *Str. bovis* JB1 with the cellulolytic triculture were dominated by *Str. bovis*. Lactate was almost the sole fermentation end product (Table 2) and only 11.2% of the RNA detected with the bacterial domain probe was attributable to the three cellulolytic species (data not shown). *R. albus* again was the most abundant of the cellulolytic species, accounting for two-thirds of the cellulolytic population (Table 1).

**Continuous cultures.** Table 1 shows that cellobiose-limited chemostats co-inoculated with the three cellulolytic species produced monocultures of *R. albus* at all dilution rates tested. The population pattern was consistent with the detection of acetate and ethanol as sole non-gaseous fermentation end products (Table 2). Succinate, a major fermentation product of both *F. succinogenes* and *R. flavefaciens* (Hungate, 1966), was not observed. These results are in agreement with data from two-membered continuous cultures in which *R. albus* displaced either *F. succinogenes* or *R. flavefaciens* (Shi & Weimer, 1997).

By comparison, cellobiose-limited continuous cultures initially inoculated with a mixture of four strains of *Sel. ruminantium* along with the three cellulolytic species displayed a dramatic change in the population dis-

**Table 1.** Population distributions in cellobiose co-cultures of the three cellulolytic species [*F. succinogenes* S85 (*F.s.*), *R. albus* 7 (*R.a.*) and *R. flavefaciens* FD-1 (*R.f.*)] in the presence or absence of the non-cellulolytic bacteria *Sel. ruminantium* (*S.r.*) or *Str. bovis* (*S.b.*)

Cultures were grown under batch conditions for 24 h. Batch cultures were supplemented with 1 g yeast extract l<sup>-1</sup>. Cellobiose-limited chemostats were grown to steady-state conditions at the indicated dilution rate, *D*.

Inoculation	<i>D</i> (h <sup>-1</sup> )	Percentage of cellulolytic population*		
		<i>F.s.</i>	<i>R.a.</i>	<i>R.f.</i>
Batch				
<i>F.s.</i> + <i>R.a.</i> + <i>R.f.</i>	–	44.2 <sup>a</sup>	43.7 <sup>d</sup>	12.2 <sup>c</sup>
<i>F.s.</i> + <i>R.a.</i> + <i>R.f.</i> + <i>S.r.</i> †	–	24.5 <sup>b</sup>	49.9 <sup>c</sup>	25.6 <sup>b</sup>
<i>F.s.</i> + <i>R.a.</i> + <i>R.f.</i> + <i>S.b.</i>	–	9.7 <sup>c</sup>	68.5 <sup>b</sup>	21.8 <sup>b</sup>
Continuous				
<i>F.s.</i> + <i>R.a.</i> + <i>R.f.</i>	0.016	< 2.1 <sup>c</sup>	> 97.1 <sup>a</sup>	< 0.8 <sup>d</sup>
	0.026	< 2.1 <sup>c</sup>	> 97.1 <sup>a</sup>	< 0.8 <sup>d</sup>
	0.046	< 2.1 <sup>c</sup>	> 97.1 <sup>a</sup>	< 0.8 <sup>d</sup>
<i>F.s.</i> + <i>R.a.</i> + <i>R.f.</i> + <i>S.r.</i> ‡	0.021	< 2.1 <sup>c</sup>	3.3 <sup>e</sup>	94.6 <sup>a</sup>
	0.034	< 2.1 <sup>c</sup>	5.8 <sup>e</sup>	92.2 <sup>a</sup>
<i>F.s.</i> + <i>R.a.</i> + <i>R.f.</i> + <i>S.b.</i>	0.021	< 1.9 <sup>c</sup>	3.4 <sup>e</sup>	94.7 <sup>a</sup>
	0.045	< 1.9 <sup>c</sup>	93.6 <sup>a</sup>	4.5 <sup>e</sup>

\* Means in the same column with different superscripts differ ( $P < 0.05$ ).

† *Sel. ruminantium* strain D.

‡ *Sel. ruminantium* strains D, GA192, HD4 and H18.

**Table 2.** Fermentation data for cellobiose co-cultures of the three cellulolytic species [*F. succinogenes* S85 (*F.s.*), *R. albus* 7 (*R.a.*) and *R. flavefaciens* FD-1 (*R.f.*)] in the presence or absence of the non-cellulolytic bacteria *Sel. ruminantium* (*S.r.*) or *Str. bovis* (*S.b.*)

Cultures were grown under batch conditions for 24 h. Batch cultures were supplemented with 1 g yeast extract l<sup>-1</sup>. The final culture pH ranged from 5.90 to 5.99 and the fraction of added anhydroglucose that was consumed ranged from 88.7 to 97.7%. Cellobiose-limited chemostats were grown to steady-state conditions at the indicated dilution rate, *D*. Mean pH ranged from 6.49 to 6.87 and the range of mean concentrations of residual anhydroglucose equivalents in the cultures was 0.14–0.39 mM (98.0–99.3% substrate consumption).

Inoculation	<i>D</i> (h <sup>-1</sup> )	Anhydroglucose consumed (mM)	Products produced/anhydroglucose consumed					
			Succinate	Lactate	Formate	Acetate	Propionate	Ethanol
Batch								
<i>F.s.</i> + <i>R.a.</i> + <i>R.f.</i>	–	20.21	0.37	–	0.65	0.62	–	0.28
<i>F.s.</i> + <i>R.a.</i> + <i>R.f.</i> + <i>S.r.</i> *	–	25.61	–	1.05	0.17	0.44	0.45	–
<i>F.s.</i> + <i>R.a.</i> + <i>R.f.</i> + <i>S.b.</i>	–	22.84	–	1.78	0.02	0.12	0.01	–
Continuous								
<i>F.s.</i> + <i>R.a.</i> + <i>R.f.</i>	0.016	18.46	–	–	–	1.06	–	0.62
	0.026	18.14	–	–	–	1.03	–	0.67
	0.046	18.40	–	–	–	1.17	–	0.44
<i>F.s.</i> + <i>R.a.</i> + <i>R.f.</i> + <i>S.r.</i> †	0.021	18.57	–	0.13	0.21	0.73	0.63	0.10
	0.034	18.58	–	0.11	0.44	0.80	0.66	0.06
<i>F.s.</i> + <i>R.a.</i> + <i>R.f.</i> + <i>S.b.</i>	0.021	18.33	0.32	0.10	0.85	0.83	0.01	0.10
	0.045	18.45	–	0.06	–	0.71	0.02	0.51

\* *Sel. ruminantium* strain D.

† *Sel. ruminantium* strains D, GA192, HD4 and H18.

**Table 3.** Population distributions of the three cellulolytic species [*F. succinogenes* S85 (*F.s.*), *R. albus* 7 (*R.a.*) and *R. flavefaciens* FD-1 (*R.f.*)] in cellulose batch and in cellulose-limited chemostats under steady-state conditions in the presence or absence of the non-cellulolytic bacteria *Sel. ruminantium* (*S.r.*) or *Str. bovis* (*S.b.*)

Cultures were grown under batch conditions for 48 h. Batch cultures were supplemented with 1 g yeast extract l<sup>-1</sup>. Cellulose-limited chemostats were grown to steady-state conditions at the indicated dilution rate, *D*.

Inoculation	<i>D</i> (h <sup>-1</sup> )	Percentage adherent cellulolytic population*			Percentage planktonic cellulolytic population*		
		<i>F.s.</i>	<i>R.a.</i>	<i>R.f.</i>	<i>F.s.</i>	<i>R.a.</i>	<i>R.f.</i>
Batch							
<i>F.s.</i> + <i>R.a.</i> + <i>R.f.</i>	–	10.8 <sup>d</sup>	88.9 <sup>b</sup>	< 0.4 <sup>d</sup>	16.3 <sup>e</sup>	83.5 <sup>b</sup>	< 0.3 <sup>e</sup>
<i>F.s.</i> + <i>R.a.</i> + <i>R.f.</i> + <i>S.r.</i> †	–	< 1.9 <sup>e</sup>	> 96.8 <sup>a</sup>	< 1.3 <sup>d</sup>	< 2.1 <sup>f</sup>	> 96.5 <sup>a</sup>	< 1.4 <sup>de</sup>
<i>F.s.</i> + <i>R.a.</i> + <i>R.f.</i> + <i>S.b.</i>	–	9.8 <sup>de</sup>	89.8 <sup>ab</sup>	< 0.4 <sup>d</sup>	13.2 <sup>ef</sup>	86.5 <sup>ab</sup>	< 0.3 <sup>e</sup>
Continuous							
<i>F.s.</i> + <i>R.a.</i> + <i>R.f.</i>	0.016	59.5 <sup>b</sup>	38.3 <sup>d</sup>	2.2 <sup>bed</sup>	52.4 <sup>bc</sup>	44.2 <sup>de</sup>	3.4 <sup>cd</sup>
	0.026	69.0 <sup>a</sup>	26.5 <sup>e</sup>	4.5 <sup>abc</sup>	59.1 <sup>ab</sup>	35.0 <sup>ef</sup>	5.9 <sup>bc</sup>
	0.046	73.5 <sup>a</sup>	21.6 <sup>e</sup>	5.0 <sup>abc</sup>	40.0 <sup>cd</sup>	44.2 <sup>de</sup>	15.8 <sup>a</sup>
<i>F.s.</i> + <i>R.a.</i> + <i>R.f.</i> + <i>S.r.</i> ‡	0.021	30.4 <sup>c</sup>	67.9 <sup>c</sup>	1.7 <sup>cd</sup>	41.5 <sup>cd</sup>	55.4 <sup>cd</sup>	3.0 <sup>de</sup>
	0.034	32.9 <sup>c</sup>	65.8 <sup>c</sup>	1.3 <sup>d</sup>	32.4 <sup>d</sup>	66.1 <sup>c</sup>	1.6 <sup>de</sup>
<i>F.s.</i> + <i>R.a.</i> + <i>R.f.</i> + <i>S.b.</i>	0.021	68.1 <sup>a</sup>	24.7 <sup>e</sup>	7.2 <sup>a</sup>	60.3 <sup>ab</sup>	31.4 <sup>f</sup>	8.3 <sup>b</sup>
	0.045	72.1 <sup>a</sup>	22.0 <sup>e</sup>	5.9 <sup>a</sup>	71.5 <sup>a</sup>	28.3 <sup>f</sup>	< 0.2 <sup>e</sup>

\* Means in the same column with different superscripts differ ( $P < 0.05$ ).

† *Sel. ruminantium* strain D.

‡ *Sel. ruminantium* strains D, GA192, HD4 and H18.

**Table 4.** Fermentation data for cellulose co-cultures of the three cellulolytic species [*F. succinogenes* S85 (*F.s.*), *R. albus* 7 (*R.a.*) and *R. flavefaciens* FD-1 (*R.f.*)] in the presence or absence of the non-cellulolytic bacteria *Sel. ruminantium* (*S.r.*) or *Str. bovis* (*S.b.*)

Batch cultures were grown for 48 h and were supplemented with 1 g yeast extract l<sup>-1</sup>. The final culture pH ranged from 5.89 to 6.23 and the fraction of added anhydroglucose that was consumed ranged from 62.8 to 72.3%. Cellulose-limited chemostats were grown to steady-state conditions at the indicated dilution rate, *D*. Mean pH ranged from 6.49 to 6.87 and the range of mean concentrations of soluble sugars in the cultures was 0.40–3.87 mM anhydroglucose equivalents. The fraction of added cellulose that was consumed was 72.1–89.3%.

Inoculation	<i>D</i> (h <sup>-1</sup> )	Anhydroglucose consumed (mM)	Products produced/anhydroglucose consumed					
			Succinate	Lactate	Formate	Acetate	Propionate	Ethanol
Batch								
<i>F.s.</i> + <i>R.a.</i> + <i>R.f.</i>	–	15.53	0.08	–	–	1.66	–	0.79
<i>F.s.</i> + <i>R.a.</i> + <i>R.f.</i> + <i>S.r.</i> *	–	15.67	–	–	0.02	1.49	0.23	0.62
<i>F.s.</i> + <i>R.a.</i> + <i>R.f.</i> + <i>S.b.</i>	–	17.88	0.09	0.02	0.03	0.58	0.02	0.62
Continuous								
<i>F.s.</i> + <i>R.a.</i> + <i>R.f.</i>	0.016	22.06	1.10	–	0.14	0.89	–	–
	0.026	22.06	0.88	–	0.09	0.63	–	0.15
	0.046	19.72	0.81	–	0.09	0.54	–	–
<i>F.s.</i> + <i>R.a.</i> + <i>R.f.</i> + <i>S.r.</i> †	0.021	21.42	0.47	–	0.05	0.73	0.51	0.19
	0.034	17.80	0.86	–	0.08	0.83	0.35	–
<i>F.s.</i> + <i>R.a.</i> + <i>R.f.</i> + <i>S.b.</i>	0.021	18.00	1.31	–	0.04	0.85	0.03	0.21
	0.045	19.73	0.83	–	0.04	0.69	0.01	0.21

\* *Sel. ruminantium* strain D.

† *Sel. ruminantium* strains D, GA192, HD4 and H18.

tribution pattern in which *R. flavefaciens* became dominant over the other two cellulolytic species. A large amount of propionate, but no succinate, was observed, suggesting a complete conversion of succinate (produced by *R. flavefaciens*) to propionate by a substantial population of *Sel. ruminantium*.

In the presence of *Str. bovis*, the population distribution of cellulolytic species was affected by dilution rate. At higher *D* values (0.045 h<sup>-1</sup>), the pattern was similar to those of the triculture chemostats, with *R. albus* dominating the competition among the cellulolytic species. At lower *D* values (0.021 h<sup>-1</sup>), results were similar to those of chemostats with *Sel. ruminantium*. These population data were in agreement with the fermentation end product data, which showed higher amounts of succinate but lower amounts of ethanol at *D* = 0.021 h<sup>-1</sup>. By contrast, more ethanol (but no succinate) was observed at *D* = 0.045 h<sup>-1</sup>.

#### Relative population sizes of ruminal cellulolytic species in cellulose co-cultures

**Batch cultures.** *R. albus* was much more abundant than *F. succinogenes* and *R. flavefaciens* in both cellulose-adherent and planktonic phases of cellulose batch cultures and this outcome was not affected by the inclusion of *Sel. ruminantium* D or *Str. bovis* JB1 (Table 3). High amounts of ethanol and very low amounts of succinate in the culture supernatants (Table 4) were in accord with the population data. Clearly, there were no

effects on the competition among the three cellulolytic species in co-culture with *Str. bovis*, although there was a slight enhancement ( $P < 0.05$ ) of *R. albus* or inhibition of *F. succinogenes* in the presence of *Sel. ruminantium*.

**Continuous cultures.** Co-existence among the three cellulolytic species was observed in all cellulose-limited continuous co-cultures (Table 3). In tricultures of the three cellulolytic species, a slight dominance of *F. succinogenes* over *R. albus* was observed, along with a small population of *R. flavefaciens*. This was in sharp contrast to batch cultures, which were clearly dominated by *R. albus*. As in batch culture, the presence of *Str. bovis* did not alter the competition among the cellulolytic species. There was, however, a shift of the population pattern between *R. albus* and *F. succinogenes* in tricultures co-inoculated with a mixture of *Sel. ruminantium* strains. Appearance of propionate in fermentation products also indicated the presence of *Sel. ruminantium*, but substantial residual concentrations of succinate suggested that *Sel. ruminantium* populations were low in the co-cultures.

#### Identification of inhibitors involved in competition among the cellulolytic species

Table 5 shows the effect of supernatants from pure cultures on the growth of the three cellulolytic species on cellobiose in batch mode. The growth of *R. flavefaciens* FD-1 was suppressed and its lag time was prolonged by supernatant from the *R. albus* 7 culture. Although

**Table 5.** Effect of supernatants from pure cultures on growth parameters of pure cultures of *R. flavefaciens* FD-1 (*R.f.*), *R. albus* 7 (*R.a.*) and *F. succinogenes* S85 (*F.s.*) in cellobiose batch culture

Means in the same column having different superscripts differ ( $P < 0.05$ ). Results are mean values of triplicate cultures. NT, Not tested.

Supernatant	Final OD <sub>600</sub>			$\mu_{\max}$ (h <sup>-1</sup> )			Lag (h)		
	<i>R.f.</i>	<i>R.a.</i>	<i>F.s.</i>	<i>R.f.</i>	<i>R.a.</i>	<i>F.s.</i>	<i>R.f.</i>	<i>R.a.</i>	<i>F.s.</i>
Control (water)	0.67 <sup>a</sup>	0.80 <sup>a</sup>	1.06 <sup>a</sup>	0.46 <sup>a</sup>	0.35 <sup>a</sup>	0.34 <sup>a</sup>	7.6 <sup>b</sup>	5.6 <sup>a</sup>	11.0 <sup>b</sup>
<i>R.f.</i>	0.70 <sup>a</sup>	0.88 <sup>a</sup>	0.46 <sup>b</sup>	NT <sup>c</sup>	0.37 <sup>a</sup>	0.29 <sup>b</sup>	NT <sup>c</sup>	5.6 <sup>a</sup>	13.8 <sup>a</sup>
<i>R.a.</i>	0.14 <sup>b</sup>	0.87 <sup>a</sup>	1.09 <sup>a</sup>	0.26 <sup>b</sup>	NT <sup>c</sup>	0.35 <sup>a</sup>	19.6 <sup>a</sup>	NT <sup>c</sup>	11.6 <sup>b</sup>
<i>F.s.</i>	0.71 <sup>a</sup>	0.89 <sup>a</sup>	1.08 <sup>a</sup>	0.44 <sup>a</sup>	0.34 <sup>a</sup>	NT <sup>c</sup>	8.6 <sup>b</sup>	5.6 <sup>a</sup>	NT <sup>c</sup>

occasional tubes within the replicated set showed growth, the maximum OD<sub>600</sub> (0.14) was lower than that of controls not containing this supernatant (0.67). Inhibition was also shown in that *R. flavefaciens* FD-1 did not grow in the medium that included half of the volume from supernatant of *R. albus* 7, while the growth of *R. albus* 7 and *F. succinogenes* S85 was not affected under the same conditions (data not shown). Inhibition of *F. succinogenes* S85 by a supernatant of *R. flavefaciens* FD-1 was also observed in terms of both decreased maximum growth rate and increased lag time.

Additional experiments were performed to further demonstrate the inhibition between *R. albus* 7 and *R. flavefaciens* FD-1, and between *R. flavefaciens* FD-1 and *F. succinogenes* S85. Overnight cultures of each test strain were spotted onto agar lawn seeded with an indicator strain. After 16 h incubation, zones of inhibition were observed around the colonies of *R. albus* 7 on *R. flavefaciens* FD-1 lawns and around the colonies of *R. flavefaciens* FD-1 on *F. succinogenes* S85 lawns. Using this method, no other inhibitory activities were observed for any other combination of test culture and culture supernatant from the five strains (three cellulolytic strains, *Sel. ruminantium* D and *Str. bovis* JB1).

The inhibitor from *R. albus* 7 was protease-sensitive. *Streptomyces griseus* protease completely destroyed the inhibitory activity of a supernatant from an overnight culture of *R. albus* 7 and pancreatin reduced the size of the inhibition zone relative to culture supernatants not treated with pancreatin. Thus the inhibitor from *R. albus* 7 is protein in nature, as reported by Odenyo *et al.* (1994a) for a bacteriocin-like substance produced by *R. albus* 8. The molecular mass range of the *R. albus* 7 agent, estimated by ultrafiltration, fell between 10 and 30 kDa.

## DISCUSSION

A number of studies have described interactions between cellulolytic species and non-cellulolytic species of ruminal bacteria (Scheifinger & Wolin, 1973; Stanton & Canale-Parola, 1980; Kudo *et al.*, 1987; Pavlostathis *et al.*, 1990; Debroas & Blanchart, 1993; Williams *et al.*,

1994; Horvan *et al.*, 1996). Several reports have also described interactions between two or among three cellulolytic species of ruminal bacteria (Odenyo *et al.*, 1994a, b; Fondevila & Dehority, 1996; Mosoni *et al.*, 1997; Shi *et al.*, 1997; Shi & Weimer, 1997). Most of these studies have been performed under batch culture conditions. This report is the first to examine interactions of tricultures of ruminal cellulolytic species and the impacts of non-cellulolytic bacteria on these interactions under substrate-limited, continuous culture conditions. Although the experiments involved only a single strain of each cellulolytic species and should be interpreted cautiously, the observed co-existence of several strains at one or more trophic levels suggests a complex pattern of interactions among these strains.

The observed suppression of *R. flavefaciens* FD-1 in both cellobiose and cellulose batch co-cultures (Tables 1 and 3) is consistent with the data of Odenyo *et al.* (1994a, b). The inhibition of *R. flavefaciens* FD-1 by *R. albus* 7 more than compensated for the fact that *R. flavefaciens* FD-1 has a greater  $\mu_{\max}$  than do both *R. albus* 7 and *F. succinogenes* S85 and shorter lag time than does *F. succinogenes* S85 in cellobiose medium (Table 5). Both growth suppression (Table 5) and possible inhibition of adherence to cellulose particles (Mosoni *et al.*, 1997) by *R. albus* 7 could have led to undetectable levels of *R. flavefaciens* FD-1 in cellulose batch co-cultures. By contrast, the ability of *R. albus* 7 to outcompete *F. succinogenes* S85 in cellulose batch co-cultures (Table 3) may have been due to the former's shorter lag time in cellulose medium (as suggested by visual inspection of pure cultures). During growth on cellulose, the distribution patterns of the adherent and planktonic populations of all three cellulolytic species were similar ( $P < 0.05$ ). This result suggests that many of the factors governing the interactions among the cellulolytic bacteria during growth on cellulose particles may be similar to those governing interactions during growth on soluble substrates.

In continuous culture with a single limiting nutrient, pure and simple competition is expected to yield a monoculture of a single strain based on a combination of maximum growth rate and affinity for substrate

(Hansen & Hubbell, 1980). Thus, the dominance by *R. albus* in our cellobiose triculture chemostats (Table 1) is consistent with a previous report that this strain adapted to cellobiose limitation more readily than did the other two species (Shi & Weimer, 1997). However, the suppression of *R. flavefaciens* may also have been due to the production of an inhibitory agent by *R. albus*. According to the definition of pure and simple competition by Fredrickson & Stephanopoulos (1981), competition in our chemostat cultures was not pure because competition for nutrients was not the sole interaction. Furthermore, competition for cellulose was not simple due to the complex profile of substrates (soluble cellodextrins of different chain lengths) available as cellulose degradation proceeded. In theory, populations of up to  $n$  strains can co-exist in a spatially homogeneous system with constant inputs if  $n$  nutrients exert dynamic effects on the system (Fredrickson & Stephanopoulos, 1981). As a result, our cellulose-limited chemostats yielded a triculture of cellulolytic species rather than a monoculture.

In continuous culture on cellobiose (Table 1), but not on cellulose (Table 3), addition of a mixture of *Sel. ruminantium* strains allowed *R. flavefaciens* FD-1 to outcompete the other two cellulolytic species, although *Sel. ruminantium* itself appeared to be the dominant species in the co-culture. The prevalence of *R. flavefaciens* appears to be due to a combination of several factors: its lower  $K_s$  for cellobiose (Shi & Weimer, 1996), its inhibition of *F. succinogenes* and reduced inhibition, resulting from a presumably lower concentration of inhibitor produced by a smaller population of *R. albus*. The predominance of *R. albus* at the higher dilution rate ( $0.045 \text{ h}^{-1}$ ) in the presence of *Str. bovis* (Table 1) is probably due to a higher concentration of inhibitor produced by a greater population size of *R. albus* at the higher growth rate. *R. flavefaciens* outgrew the other two cellulolytic species at  $D = 0.021 \text{ h}^{-1}$ , but *R. albus* outgrew the other two at  $D = 0.045 \text{ h}^{-1}$ , suggesting that there may be a critical growth rate between these values that determines the outcome of the competition. Although the rumen is not a truly continuous culture habitat, the dilution at which this putative switch might occur is within the range of mean dilution rates reported for rumen contents (Hungate, 1966). In our experiments, the dilution rates on cellobiose were kept low because this permitted direct comparison with the cellulose-grown cultures that were grown at these same low dilution rates (i.e. they permitted comparison of interactions for two different substrates, without the confounding effect of growth rate). All of these organisms certainly are capable of much higher growth rates on high concentrations of cellobiose. However, it is unlikely that they encounter such high concentrations in the rumen, where cellobiose is produced from the slow hydrolysis of cellulose, and its concentration is kept low by intense competition among the many species (cellulolytic and non-cellulolytic) that can utilize this substrate (Russell, 1985).

*F. succinogenes* competed much more effectively against

*R. albus* under cellulose limitation than in cellulose batch culture (Table 3), perhaps because of its lower  $K_s$  for cellodextrins (Shi & Weimer, 1996). Moreover, under cellulose limitation a small population of *R. flavefaciens* was detected, presumably due to a reduced concentration of inhibitor produced by the smaller population of *R. albus*. The addition of several strains of *Sel. ruminantium* selectively enhanced the population of *R. albus*. Because *F. succinogenes* (succinate producer) and *Sel. ruminantium* (succinate utilizer) have been shown to grow well in binary culture (Scheifinger & Wolin, 1973), the selective stimulation of *R. albus* suggests that *Sel. ruminantium* may enhance some aspect of cellulose digestion or metabolism by *R. albus*, rather than by suppressing *F. succinogenes*. This stimulation of *R. albus* may be due to enhanced adherence to cellulose, as the enhancement in cellulose chemostats was greater for the adherent population (Table 3) and the effect was not noted in cellobiose chemostats (Table 1).

*Sel. ruminantium* and *Str. bovis* display some similarities in physiological characteristics, including the use of soluble sugars and cellodextrins (but not cellulose) as energy sources, a homolactic fermentation at high growth rates and a heterolactic fermentation at low growth rates (Wallace, 1978; Russell & Hino, 1985). In our study, both species also dominated the cellulolytic species in cellobiose-limited chemostats (Table 1), indicating a superior ability of these non-cellulolytic species to compete for this soluble sugar when it was provided exogenously (rather than it being released during attachment to, and hydrolysis of, cellulose by the cellulolytic species). However, some differences in these two non-cellulolytic species were observed with respect to their effects on competition among the cellulolytic species. The fact that *Sel. ruminantium* altered this competition over a wide range of dilution rates, while *Str. bovis* did not, suggests the existence of specific interactions among these species that are not easily predictable from physiological characteristics alone.

The production of a bacteriocin-like inhibitor by a ruminal cellulolytic bacterium was first reported for *R. albus* 8 by Odenyo *et al.* (1994a). Kalmokoff & Teather (1997) have shown that bacteriocin-like activities are common among isolates of another ruminal species, *Butyrivibrio fibrisolvens*. In our study, *R. albus* 7 was shown to produce a protein active against *R. flavefaciens* FD-1 and a second inhibitory activity of *R. flavefaciens* FD-1 against *F. succinogenes* S85 was also observed (Table 5). These data support suggestions that bacteriocins or other allelochemicals produced by ruminal microbes may represent an important survival and interaction strategy that confers competitive fitness in the ruminal environment (Odenyo *et al.*, 1994a; Kalmokoff & Teather, 1997), although *in situ* data supporting this notion is lacking.

Studies on the interactions among single strains of different ruminal bacterial species must be interpreted cautiously to avoid generalizations that may not hold for other strains of these species. However, the trend of

dominance by *R. albus* suggested in this study is consistent with oligonucleotide probe data that showed *R. albus* was generally far more abundant than either *F. succinogenes* and *R. flavefaciens* in the bovine rumen (Weimer *et al.*, 1999). This suggests that factors affecting the interactions of these species in substrate-limited continuous cultures are likely to be similar to those in the rumen. The overall success of *R. albus* in both *in vivo* and *in vitro* studies may be due to several factors: (i) production of substances that inhibit the growth of *R. flavefaciens* (Odenyo *et al.*, 1994a, this study); (ii) successful competition for adherence to cellulose (Mosoni *et al.*, 1997); (iii) greater adaptability under selective pressure to more rapid growth at low cellobiose concentrations (Shi & Weimer, 1997); (iv) improved adherence through interaction with non-cellulolytic species (this study); and (v) greater ability to degrade hemicellulose and ferment pentoses (Dehority, 1973). Purification and characterization of the proteinaceous inhibitor from *R. albus* 7 is in progress. Further studies on the inhibition of *F. succinogenes* S85 by *R. flavefaciens* FD-1 and the stimulation of *R. albus* by *Sel. ruminantium* during growth on cellulose will provide more information on the mechanisms underlying the interactions between ruminal bacteria.

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