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Competition for Cellulose among Three Predominant Ruminal Cellulolytic Bacteria under Substrate-Excess and Substrate-Limited Conditions

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Three predominant ruminal cellulolytic bacteria (*Fibrobacter succinogenes* S85, *Ruminococcus flavefaciens* FD-1, and *Ruminococcus albus* 7) were grown in different binary combinations to determine the outcome of competition in either cellulose-excess batch culture or in cellulose-limited continuous culture. Relative populations of each species were estimated by using signature membrane-associated fatty acids and/or 16S rRNA-targeted oligonucleotide probes. Both *F. succinogenes* and *R. flavefaciens* coexisted in cellulose-excess batch culture with similar population sizes (58 and 42%, respectively; standard error, 12%). By contrast, under cellulose limitation *R. flavefaciens* predominated (>96% of total cell mass) in coculture with *F. succinogenes*, regardless of whether the two strains were inoculated simultaneously or whether *R. flavefaciens* was inoculated into an established culture of *F. succinogenes*. The predominance of *R. flavefaciens* over *F. succinogenes* under cellulose limitation is in accord with the former's more rapid adherence to cellulose and its higher affinity for cellodextrin products of cellulose hydrolysis. In batch cocultures of *F. succinogenes* and *R. albus*, the populations of the two species were similar. However, under cellulose limitation, *F. succinogenes* was the predominant strain (~80% of cell mass) in cultures simultaneously coinoculated with *R. albus*. The results from batch cocultures of *R. flavefaciens* and *R. albus* were not consistent within or among trials: some experiments yielded monocultures of *R. albus* (suggesting production of an inhibitory agent by *R. albus*), while others contained substantial populations of both species. Under cellulose limitation, *R. flavefaciens* predominated over *R. albus* (85 and 15%, respectively), as would be expected by the former's greater adherence to cellulose. The retention of *R. albus* in the cellulose-limited coculture may result from a combination of its ability to utilize glucose (which is not utilizable by *R. flavefaciens*), its demonstrated ability to adapt under selective pressure in the chemostat to utilization of lower concentrations of cellobiose, a major product of cellulose hydrolysis, and its possible production of an inhibitory agent.

Cellulose is the major component of forages, and its digestion and subsequent fermentation by ruminal microbes provide much of the energy for forage-fed ruminants (30). Ruminal degradation of cellulose is mediated primarily by cell-associated enzymes produced by a few predominant cellulolytic bacteria (32). The rate and extent of fiber digestion in the rumen in large measure are dependent on the population size of these cellulolytic bacteria. Although microorganisms inhabiting the rumen have been selected by this specific environment for millions of years, several cellulolytic species have gained prominence through natural selection. Each of these must have some distinct strategies for surviving in the rumen, because the optimal substrate is not always available. The form of interactions among these cellulolytic species (competition or synergism) is difficult to determine in vivo because the rumen environment is so complex.

Cellulolytic species have been reported to display both competition and synergism in the utilization of cellulose in batch-type cocultures (4, 15). Digestion of cellulose by ruminal microbes has been shown to follow first-order kinetics with respect to cellulose concentration (i.e., its rate of degradation is limited by the amount of available substrate rather than by the inherent cellulolytic capabilities of the microflora [29, 31, 34]). Thus, continuous coculture under substrate limitation has the potential to provide more information on strategies of survival and cellulose utilization by these cellulolytic bacteria, because competition should be more intense under substrate limitation than in batch culture (26). Moreover, we would expect that the interaction between bacterial species is probably more complex under cellulose limitation in a chemostat than under limitation for soluble nutrients, because successful competition for growth on cellulose is likely to result from a variety of factors, including rate and extent of adherence to cellulose particles, ability to compete for the soluble products (cellodextrins) of cellulose depolymerization, and production of substances that inhibit other species.

The following series of experiments was conducted to compare cellulose utilization and the relative populations of *Fibrobacter succinogenes* S85, *Ruminococcus flavefaciens* FD-1, and *Ruminococcus albus* 7 during growth in coculture on excess or limiting amounts of cellulose. The outcomes of these competitions, along with the mechanisms underlying these outcomes, should provide a more complete understanding of fiber utilization and interactions among cellulolytic bacteria in the rumen.

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MATERIALS AND METHODS

Culture conditions. *R. flavefaciens* FD-1, *R. albus* 7, and *F. succinogenes* S85 were maintained by syringe transfer of 3% (vol/vol) inocula in cellulose-contain-

ing media at 30- to 48-h intervals. For coculture experiments, strains were grown in different binary combinations in batch culture with excess cellulose and in cellulose-limited chemostats. The culture medium was a chemically defined medium used for previous continuous-culture studies (35) supplemented with 4.2 to 5.6 g of Sigmacell 20 microcrystalline cellulose (Sigma, St. Louis, Mo.) (sieved to particle size of <45 μm) and 0.05 mg each of 3-phenylpropionic acid and phenylacetic acid per liter of medium.

Batch cultures were carried out in Balch-type culture tubes fitted with butyl stoppers and aluminum crimp seals (1). Each tube contained 100 ± 2 mg of Sigmacell 20 microcrystalline cellulose in 10 ml of medium under a CO_2 gas phase. All tubes were incubated on a shaker (100 rpm) at 39°C. The continuous-culture system has been described previously (35). All experiments were performed in an 875-ml working-volume fermentor at 39°C. The medium slurry in the reservoir was homogenized by stirring and diffusive gas sparging with CO_2 and was delivered to the culture vessel by CO_2 segmentation by using a peristaltic pump. Absence of microbial contamination was regularly checked by microscopic observation and by analysis of fermentation end products. Two types of chemostat experiments were conducted: (i) coinoculation experiments in which two species were mixed in equal volumes (3 to 5 ml each) in a sterile vial and then inoculated into the reactor and (ii) challenge experiments in which a 3- to 8-ml culture of one species was added to a steady-state chemostat culture of another species.

Analysis of substrates and products. Batch cultures were sacrificed by removing their entire contents at the end of 36 h of incubation. For each continuous-culture experiment, samples (28 ml) were removed from the reactors with a sterile hypodermic syringe at 6- to 20-h intervals over a 2- to 13-day period that included a steady state (maintained for at least 3 dilutions of reactor volume) in which residual cellulose concentration and bacterial cell N were basically constant over time. Concentrations of cellulose in the reactor or medium reservoir were determined from well-stirred subsamples (~20 ml, weighed to 0.001 g) by a modified neutral detergent method (34). The remainder of the sample was centrifuged in four 1.5-ml portions for 5 min at 12,500 × *g* in a microcentrifuge. The resulting supernatants were analyzed for soluble sugars by a phenol-sulfuric acid method (5) and for fermentation acids and ethanol by high-performance liquid chromatography following treatment with $Ca(OH)_2$ and $CuSO_4$ as described previously (35).

Cell pellets from the above centrifugations were washed twice with 0.9% (wt/vol) NaCl and analyzed for nitrogen content with a Carlo Erba NA 1500 nitrogen analyzer (Fisons Instruments, Saddle Brook, N.J.) as described previously (35). Total cell mass was calculated according to an assumed biomass formula of $C_5H_7O_2N$ for the organic component of biomass and under the assumption that cells contained 90% organic material (16). Expressed in this manner, 1 mM cells corresponds to 125.6 mg (dry weight)/liter.

Relative populations of individual species in cocultures. The relative percentage of each individual strain in the cocultures was estimated by using either signature membrane-associated fatty acids (MFA assay) or 16S rRNA targeted oligonucleotide probe hybridization (RNA assay).

MFA assay. By using pure cultures grown on cellulose or cellobiose, one MFA characteristic for each species was identified: pentadecanoic acid (15:0) for *F. succinogenes* S85, 13-methyltetradecanoic acid (*i*15:0) for *R. flavefaciens* FD-1, and hexadecanoic acid (16:0) for *R. albus* 7. These MFAs were somewhat different from those used by Saluzzi et al. (22). It was shown in separate experiments that the relative proportions of these MFAs did not change significantly within each species as dilution rate or pH varied, as long as growth temperature was maintained at 39°C.

The extraction method for the MFAs was based on a procedure for direct transesterification of all classes of lipids (11). Cell pellets from 20 ml of culture, withdrawn from the chemostat by a syringe, were washed twice with 10 ml of phosphate-buffered saline buffer (0.9% NaCl, 0.2 M Na_2HPO_4, 0.2 M NaH_2PO_4, pH 6.8) and the supernatant was discarded, with care taken to remove all liquid. Cell pellets were resuspended in 2 ml of methanol-toluene (4:1, vol/vol) containing 0.5 mg of eicosanoic acid (20:0) per ml as an internal standard. Cell suspensions were transferred to borosilicate glass tubes (16 by 100 mm) fitted with Teflon-lined screw caps and containing a magnetic stir bar (1.5 by 10 mm). Acetyl chloride (0.2 ml) was added slowly, and the tubes were tightly closed and immediately mixed on a stir plate. The tubes were heated in a boiling water bath for 1 h with continuous stirring. After the tubes had cooled to room temperature, 5 ml of a 6% K_2CO_3 (wt/vol) solution was slowly added to stop the reaction and neutralize the mixture. The tubes were mixed by vortexing and then centrifuged at 3,000 × *g* for 10 min, and a portion of the toluene (upper) phase was transferred into vials and stored at 4°C for gas chromatographic analysis.

For identification of the MFAs, the extracted MFA methyl esters (2 μl) were chromatographed on a 60-m-by-0.25-mm DB-1 fused silica capillary column (J&W Scientific, Folsom, Calif.) with a model 5890A gas chromatograph equipped with a model 5970 mass-selective detector (Hewlett-Packard, Palo Alto, Calif.). Chromatographic conditions were as follows: carrier gas, He at 10 ml/min; injection temperature, 150°C; and oven temperature program, 175°C for 4 min, increased at 4°C/min to 250°C, and held for 3 min. MFAs were identified by comparing fragmentation patterns of peaks in unknown samples with those of authentic standards (obtained from Matreya, Pleasant Gap, Pa.). Quantitative analysis of each MFA extracted from the cocultures was performed under the same chromatographic conditions on a Perkin-Elmer 8500 gas-liquid chromatograph (Norwalk, Conn.) equipped with a flame ionization detector (detector temperature, 300°C). To quantify the MFAs, a relative ratio of peak area of characteristic MFA to the peak area of the internal standard was calculated. The mean of the relative ratios from monoculture samples (at least four replicates) was assumed to be 100% of the population for each species. The relative population of each species in the coculture samples was then calculated as a percentage. For example:

$$\%\text{FD-1} = \frac{\text{relative ratio of } (i15{:}0)/\text{internal standard}}{\text{mean value of } i15{:}0/\text{internal standard in pure culture}} \times 100\%$$

where *i*15:0 is the characteristic MFA for FD-1. The lower detectable limit for the subordinate strain in a binary culture was calculated after correction of the relative ratio for the subordinate strain's characteristic MFA to compensate for the small amount of that MFA produced by the dominant strain.

RNA assay. Four oligonucleotide probes were used for hybridization experiments: RFL196 (5′ AGGATGCCCTTTAATTAT 3′) for *R. flavefaciens* FD-1 (14), RAL196 (5′ GTCATGCGGCTTCGTTAT 3′) for *R. albus* 7 (14), SUB1 (5′ CCATACCGATAAATCTCTAGT 3′) for *F. succinogenes* S85 (27), and the eubacterial probe EUB338 (5′ GCTGCCTCCCGTAGGAGT 3′) (27), used as a positive control. All probes were synthesized and labeled with digoxigenin by National Biosciences Inc. (Plymouth, Minn.), except for the SUB1, which was obtained from Genosys Biotechnologies (The Woodlands, Tex.).

Extraction of total RNA was performed by a modification of the phenol-chloroform method of Odenyo et al. (14). Total RNA was isolated from cell pellets harvested by centrifugation (12,750 × *g*) of 5- to 10-ml cultures for 10 min at room temperature. Cell pellets were suspended in 0.7 ml of 50 mM sodium acetate–10 mM EDTA (pH 5.1) in 2-ml screw-cap conical tubes and combined with zirconium beads (1.2 g, 75- to 100-μm diameter; Biospec Products, Bartlesville, Okla.), 0.7 ml of saturated phenol (pH 5.1), and 0.05 ml of 20% sodium dodecyl sulfate. The tubes were shaken twice for 1 min in a Mini-Bead Beater (Biospec Products), and the mixture was then incubated in a hot water bath (60 to 70°C) for 10 to 15 min. Samples were immediately placed in ice and shaken twice for another 1 min each in the bead beater and then centrifuged at 4°C for 5 min. The aqueous phase was transferred to a clean microcentrifuge tube and extracted with 0.5 ml of saturated phenol (pH 5.1). This was followed by two extractions with phenol-chloroform (1:1) (vol/vol) and two extractions with chloroform alone. Nucleic acids were precipitated from the upper phase by adding 2 volumes of absolute ethanol and 1/10 volume of 3 M sodium acetate and placing samples in a −80°C freezer for 0.5 h. Samples were then centrifuged at 4°C for 5 min. The supernatant was discarded, and the pellet was then washed with 70% ethanol and dissolved in 0.05 to 0.1 ml of water (previously treated with diethylpyrocarbonate [DEPC]) and stored at −80°C.

Total RNA samples (0.1 ml) were denatured with an equal volume of denaturant solution (DEPC-treated H_2O-formaldehyde-5× SSC in a ratio of 5:3:2 [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0]) and incubated for 10 min in a water bath (70 to 80°C). A slot blotter (Schleicher & Schuell, Dassel, Germany) was used to immobilize RNA (usually 10 ng, but in some cases up to 100 ng) from each sample onto MagnaGraph nylon membranes (Schleicher & Schuell). RNAs were cross-linked to the membrane by UV radiation for 0.5 min (Stratagene-1800, Stratagene, La Jolla, Calif.) in 10× SSC. Membranes were air-dried after UV cross-linking and prehybridized with DigEase prehybridization solution (Boehringer-Mannheim, Indianapolis, Ind.) at dissociation temperature (45°C) for at least 2 h. For a 7.6-by-7.6-cm membrane, 10 pM (10^{-3} nM) 5′-digoxigenin-labeled oligo-DNA probes was added to 10 ml of prehybridized solution in a 50-ml Corning centrifuge tube and hybridized overnight at 45°C in a rotating hybridization oven (Stovall Life Sciences, Greensboro, N.C.). After hybridization, membranes were washed twice, 15 min per wash, in 2× SSC at room temperature, followed by two additional washes in either 0.5× SSC (for RFL196-, RAL196-, and SUB1-hybridized membranes) or 0.1× SSC (for EUB338-hybridized membranes).

Membranes were treated with blocking reagents (Boehringer-Mannheim) (DEPC-treated 2% blocking reagents were diluted 5× in 0.1 M sodium maleate buffer, pH 7.5) to prevent nonspecific binding of antibodies to the membrane and then were incubated with alkaline phosphatase-conjugated anti-digoxigenin (750 U/ml) (Boehringer Mannheim) for 1 h at room temperature. The membranes containing hybridized probe and bound conjugated antibody were reacted with LumiPhos 530 chemiluminescent substrate (Boehringer Mannheim) and exposed to X-OMat-AR film (Kodak, Rochester, N.Y.) for 3 h to record the light emission. After developing, the films were scanned with a laser densitometer (Molecular Dynamics, Sunnyvale, Calif.) and the bands were quantified by using IPLab gel software (Molecular Dynamics).

The fraction of each strain present in the binary cultures was reported on the basis of the sum of the hybridized RNAs detected for the two individual strains. The lower detectable limit of the RNA assay was estimated as the ratio of the minimum number of pixels above background that could be detected to the number of pixels detected for a slot blot containing an equivalent amount of RNA from the homologous monoculture.

Adherence assays. Adherence of each strain was assayed by using the same media, temperature, and cellulose substrate as was used in the coculture experiments. Cells were pregrown in cellobiose-containing media in the presence (labeled cells) or absence (unlabeled cells) of ^{14}C-2-methylbutyrate (21). Cells

TABLE 1. Outcomes for binary cocultures of *F. succinogenes* S85, *R. flavefaciens* FD-1, and *R. albus* 7 in cellulose-limited chemostats[a]

Inoculation order	D (h^{-1})[b]	Residual cellulose (g/liter)			Culture composition[c]			Assay method (lower detection limit)[d]
		pH	Initial	Steady state	S85	FD-1	7	
Coinoculation								
S85 + FD-1	0.049	6.44	4.50	1.23	<3.5	>96.5		MFA (3.5)
7 + FD-1	0.016	6.79	7.08	6.55		85.1	14.9	RNA (0.7)
7 + S85	0.024	6.27	4.44	2.39	89.9		10.1	RNA (0.7)
7 + S85	0.020	6.10	5.59	3.53	78.1		21.9	RNA (0.7)
Challenged								
S85 then FD-1	0.030	6.48	4.35	1.42	<3.5	>96.5		MFA (3.5)
FD-1 then 7	0.029	6.52	5.22	1.78		89.2	10.7	RNA (0.7)
S85 then 7	0.031	6.50	5.66	1.40	90.7		9.3	RNA (0.7)

[a] Steady-state data only. Complete time course data not shown.
[b] Dilution rate in reciprocal hours.
[c] Estimated composition at end of incubation, calculated from characteristic biomarkers indicated in the rightmost column.
[d] Values in parentheses indicate minimum percentage of the population of the subordinate strain detectable by the method.

were added to cellulose-containing media and incubated for 1 to 32 min. The adherent and nonadherent populations were determined as the fraction of cell material retained or passed through (respectively) 3-μm-pore-size polycarbonate membranes. For labeled cells, ^{14}C was determined in 10 ml of Opti-fluor counting solution (Packard, Meriden, Conn.) with a 1600 TSR liquid scintillation spectrometer (Packard Instruments, Downers Grove, Ill.). For unlabeled cells, the nonadherent cells were concentrated from the filtrate by centrifugation, and cell nitrogen in both the adherent and nonadherent fractions was determined with a combustion nitrogen analyzer as described above. For both assay methods, the fraction of adherent and nonadherent cell material was calculated relative to the total amount of cell material added to the assay mix; recovery of cells (calculated as the sum of adherent and nonadherent cell material) ranged from 87 to 102% (mean, 96%).

Inhibition assays. To examine the potential inhibition of a subordinate species by end products of a dominant species, growth experiments were performed in culture tubes containing 10 ml of medium with and without a concentrated spent medium obtained from a pure culture of the predominant species. Two methods of concentration were used: (i) rotary evaporation under reduced pressure at 40°C to yield 10×-condensed supernatants and (ii) precipitation of cell-free crude proteins from 10 ml of culture by $(NH_4)_2SO_4$ at 80% of saturation (~0.8 mg of protein per ml). These concentrated solutions (1 ml) were added to 9 ml of culture medium containing 4 g of cellobiose per liter, and growth was determined by measuring the turbidity (optical density at 600 nm) of each culture after a 24-h incubation. Similar cultures amended with 1 ml of water (instead of the concentrated spent culture solutions) were used as controls for growth in the absence of inhibitor.

RESULTS AND DISCUSSION

Adherence to cellulose. Because digestion of cellulose by the most actively cellulolytic ruminal bacteria requires contact with the cellulose particles, the rate and extent of adherence to cellulose particles by each strain are likely to be important factors in determining the outcome of competition. Numerous studies (2, 12, 18, 20) have indicated that adherence of these species to cellulose varies under different experimental conditions, making difficult any direct comparison of the capabilities of these species to adhere to fiber. Consequently, we compared adherence by pure cultures using the same medium and cellulose type used in batch and continuous-coculture experiments. The results indicated that within 30 min of incubation, 70 to 80% of the added *R. flavefaciens* FD-1 cells adhered to the microcrystalline cellulose particles, while only 30 to 40% of the added *F. succinogenes* S85 or *R. albus* 7 cells adhered under the same conditions. This greater extent of adherence of *R. flavefaciens* relative to the other two species is consistent with the observations of Morris (12) and Bhat et al. (2). More than 70% of the total extent of adherence that was observed for each strain occurred during the first minute of incubation. The data indicate a clear superiority of *R. flavefaciens* FD-1 in adhering to cellulose, although for all three strains the process was too rapid to permit a determination of the kinetic order of the process or a measurement of the rate constant of adherence.

Competition among ruminal cellulolytic species in coculture. The culture conditions and outcomes of cellulose-limited cocultures are summarized in Table 1.

(i) *F. succinogenes* S85 and *R. flavefaciens* FD-1. No significant interaction was observed between *F. succinogenes* S85 and *R. flavefaciens* FD-1 when substrate was excess. Both strains were present in approximately equal quantities (58% *F. succinogenes* versus 42% *R. flavefaciens*; standard error [SE], 12%; quantified by the proportion of 16S rRNA). Odenyo et al. (15) previously showed a slight predominance of *R. flavefaciens* FD-1 over *F. succinogenes* S85 in batch culture; the differences in results may lie in their use of acid-swollen cellulose and a complex growth medium.

In contrast, *R. flavefaciens* FD-1 readily outcompeted *F. succinogenes* S85 in cellulose-limited chemostats (as indicated by lack of the *F. succinogenes*-characteristic MFA 15:0 in cell pellets). This domination by *R. flavefaciens* was observed regardless of whether both species were coinoculated into the chemostat or whether *R. flavefaciens* was added to an established steady-state monoculture of *F. succinogenes* (Table 1; Fig. 1B and D). In the latter case, the *F. succinogenes* population declined dramatically after *R. flavefaciens* was introduced into the chemostat. After 6 days of coculture (4.5 turnovers), the *F. succinogenes*-characteristic MFA (15:0) was undetectable, indicating that *F. succinogenes* represented <3.5% of the population (Fig. 1D). The takeover of the *F. succinogenes* monoculture by *R. flavefaciens* was also accompanied by a shift in the relative ratio of major fermentation end products, acetate and succinate: the *F. succinogenes* monoculture produced 8.7 mM acetate and 16.5 mM succinate, while the *R. flavefaciens*-dominated coculture produced 15.5 mM acetate and 10.1 mM succinate, a product distribution similar to that observed in *R. flavefaciens* monocultures grown under similar conditions (23). The replacement of *F. succinogenes* by *R. flavefaciens* in the continuous-culture vessel also increased the extent of cellulose consumption slightly and decreased the concentration of soluble sugars (Fig. 1E). These observations are in agreement with previous studies which showed that *R. flavefaciens* monocultures grown in continuous culture showed slightly higher extents of cellulose consumption and slightly lower soluble sugar concentrations than did *F. succinogenes* monocultures grown under similar conditions (23, 33). Overall, the takeover of the culture by *R. flavefaciens* is in accord with this strain's more rapid and more extensive adherence to cellulose and its

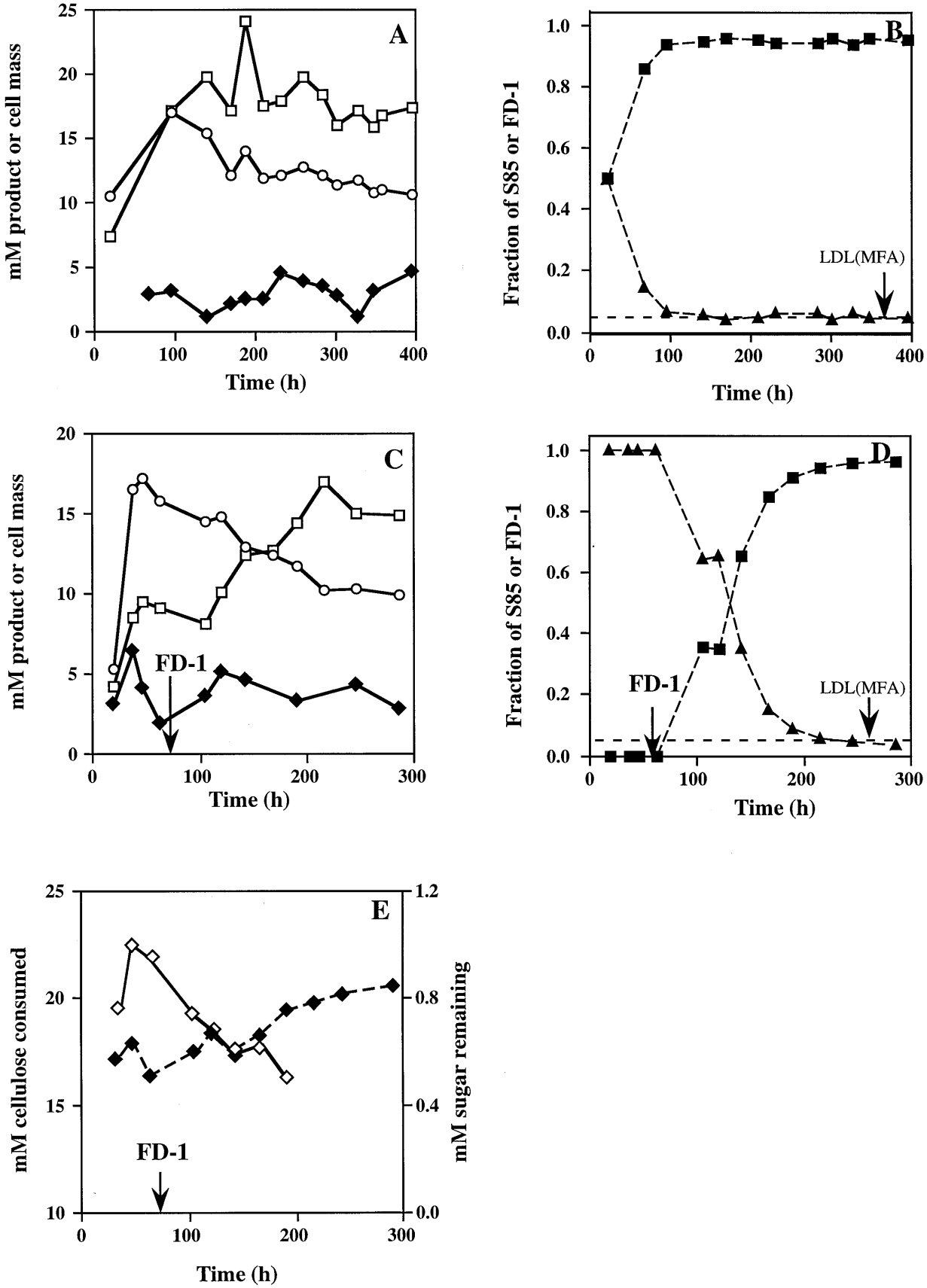

FIG. 1. Time course of competition between *R. flavefaciens* FD-1 and *F. succinogenes* S85 in cellulose-limited chemostats. (A and B) Both species coinoculated (D = 0.049 h^{-1}); (C through E) established culture of *F. succinogenes* S85 (D = 0.030 h^{-1}) challenged with *R. flavefaciens* FD-1 at 63 h. Symbols: ◆, total cell mass; ○, succinate; □, acetate; ---◆---, cellulose consumed; ◇, sugar remaining; ---■---, *R. flavefaciens* FD-1 from MFA assay; ---▲---, *F. succinogenes* S85 from MFA assay. The horizontal dashed line indicates the lower detection limit (LDL) of MFA assay for the subordinate species.

higher affinities for cellodextrins (24). The greater capability of *R. flavefaciens* to compete for cellulose probably provides a selective advantage for this species in the rumen, even if it only attaches to certain plant cell types (10) and cannot use glucose, a minor product of cellulose hydrolysis usable by *F. succinogenes* and *R. albus.*

(ii) *F. succinogenes* S85 and *R. albus* 7. There were no significant differences between *F. succinogenes* S85 and *R. albus* 7 with regard to the extent and the rate of adherence to cellulose. Results from analysis of fermentation end products (succinate versus ethanol) and 16S rRNA assays indicated that in cellulose-excess batch coculture both species coexisted at similar population sizes (56% *F. succinogenes* and 44% *R. albus*; SE, 4.2%). The coexistence of these two strains in batch coculture was consistent with the results reported for *F. succinogenes* and another strain of *R. albus* (strain 8) in batch cocultures grown on cellulose or wheat straw treated with alkaline hydrogen peroxide (15).

The data from fermentation end product, MFA, and RNA assays all demonstrated that both *F. succinogenes* and *R. albus* coexisted in the cellulose-limited coculture, regardless of whether both species were coinoculated or if an established *F. succinogenes* monoculture was challenged with *R. albus* (Table 1; Fig. 2). The relative population of *R. albus* ($\leq$21%) was much lower than that of *F. succinogenes* ($\geq$79%); while the RNA and MFA methods yielded quantitatively different results, the former method should be more definitive owing to its greater specificity. Synergism was not observed between these two strains in the cellulose-limited coculture, in that cellulose consumption by the coculture was slightly lower than that by a *F. succinogenes* monoculture (Fig. 2E). Since both strains coexisted in cellulose-excess batch culture, the decrease of cellulose consumption in a cellulose-limited coculture of *F. succinogenes* and *R. albus* was not likely due to the effect of inhibitors produced by *R. albus*. Furthermore, no growth inhibition effects were found when *F. succinogenes* was grown in batch culture with medium containing high concentrations (180 mM) of ethanol, the major fermentation end product of *R. albus.*

Fredrickson and Stephanopoulos (6) point out that, if competition between two populations in the cultures is pure but not simple, the competitors can coexist under some conditions in a steady state in a homogeneous system with constant inputs. The coexistence of both *F. succinogenes* and *R. albus* in cellulose-limited coculture could be due to a combination of factors which contribute differentially to the competitiveness of each strain. In general, *F. succinogenes* has a higher cell yield, lower maintenance requirement, and lower affinity constant (K_s) for glucose and cellobiose than *R. albus*, while sugar utilization by *R. albus* is more complete than that by *F. succinogenes* (16, 17, 33). *R. albus* 7 also appears to have a greater ability to adapt to selective pressure in a chemostat. The K_s (0.23 mM) for cellobiose of a population of *R. albus* 7 selected in the cellobiose-limited chemostat was even lower than the calculated K_s of *F. succinogenes* for glucose and cellobiose (25). The lower concentration of soluble sugar remaining in the coculture relative to that in the *F. succinogenes* monoculture (Fig. 2E) was consistent with previous reports of more complete sugar utilization by *R. albus*. The small population of *R. albus* 7 that persisted in the cellulose-limited coculture could be due to adaptation by this strain to growth on lower concentrations of soluble sugar (25). The predominance of *F. succinogenes* S85 in cellulose-limited coculture suggested that this strain has greater potential to compete for cellulose than does *R. albus* 7.

(iii) *R. flavefaciens* FD-1 and *R. albus* 7. In batch cocultures of *R. flavefaciens* FD-1 and *R. albus* 7, results were not consistent within or among trials. In some experiments only *R. albus* was present after 24 h of incubation, but in other experiments both species coexisted (data not shown). No inhibition of growth of *R. flavefaciens* was observed when the medium was supplemented with (i) a high concentration (180 mM) of ethanol, the major fermentation end product of monoculture of *R. albus* 7, (ii) supernatant from a culture of *R. albus* 7, or (iii) cell-free protein from a culture of *R. albus* 7.

Although *R. flavefaciens* had a significantly lower $S_{0.5\mu max}$ (24) for cellobiose and several other cellodextrins and displayed a much greater extent of adherence to cellulose particles than did *R. albus*, *R. albus* 7 maintained itself in the cellulose-limited chemostat during the 6-day (4.2-dilution) coculture period (Table 1) regardless of whether two strains were coinoculated or if *R. albus* was introduced into a steady-state monoculture of *R. flavefaciens* (Fig. 3). Detection of ethanol (produced by *R. albus* but not by *R. flavefaciens*) in the coculture supernatant and results of 16S rRNA-targeted hybridization assays indicated that about 10% of the total cell mass was *R. albus* 7 (Table 1; Fig. 3B, D, and F).

There are several possible explanations for the coexistence of *R. flavefaciens* and *R. albus* in the cellulose-limited chemostat. First, because *R. flavefaciens* FD-1 cannot utilize glucose (7) and because the soluble sugar concentration decreased after *R. albus* was introduced into the steady-state monoculture of *R. flavefaciens*, the small population of *R. albus* that persisted in the chemostat may have been utilizing glucose for growth. Second, the presence of this small population of *R. albus* in cellulose-limited coculture may be due to the adaptation of *R. albus* to compete successfully for other soluble cellodextrins. We have demonstrated the ability of *R. albus* 7 to adapt in this fashion in a cellobiose-limited coculture (25), an experiment that resulted in the displacement of *R. flavefaciens* FD-1 by *R. albus* 7. The adaptability of *R. albus* may also be illustrated from the observations of Odenyo et al. These workers reported that the monosaccharide composition of alkaline hydrogen peroxide-treated wheat straw remaining after batch coculture with *R. flavefaciens* FD-1 and *R. albus* 8 was similar to that of the monocultures of the former but not the latter species (13), but they later reported that *R. flavefaciens* FD-1 could not grow in the presence of *R. albus* 8 in the same medium (15). Third, the persistence of *R. albus* 7 may be due in part to production of a factor that inhibits the growth of *R. flavefaciens*. Odenyo and coworkers (14, 15) have reported that *R. albus* 8 batch cocultured with *R. flavefaciens* FD-1 on cellulose or cellobiose produces a bacteriocin-like agent. A similar mechanism might also be involved in our cocultures. Production of such a factor under some but not all growth conditions may explain the inconsistent results observed in batch coculture of *R. flavefaciens* and *R. albus* grown on cellobiose where monocultures of *R. albus* were sometimes, but not always, obtained (25). This interference by *R. albus* could in principle provide a selective advantage for *R. albus* to maintain its population at low cell densities in the cellulose-limited chemostat. It should be noted, however, that we were unable to demonstrate inhibition of *R. flavefaciens* even when using culture supernatants of cocultures that had initially contained *R. flavefaciens* but had subsequently been taken over by *R. albus*; these cultures should have been the most likely to contain the putative inhibitor.

Comparison of interactions under different growth conditions. This study is the first examination of the interactions occurring between paired cultures of ruminal cellulolytic bacteria under substrate-limited conditions. The data presented here indicate that continuous culture under cellulose limitation magnifies the competitive interaction between individual cel-

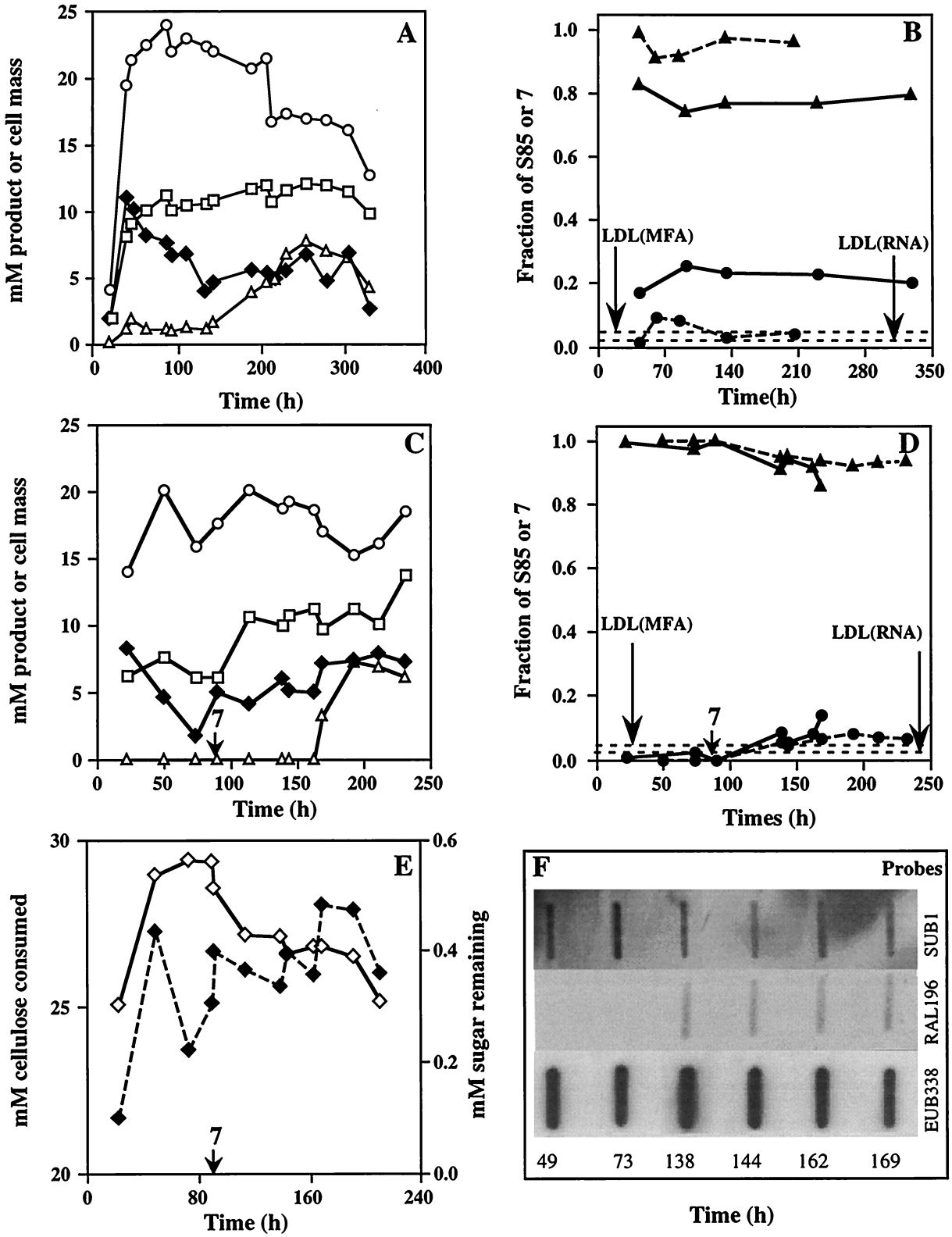

FIG. 2. Time course of competition between *F. succinogenes* S85 and *R. albus* 7 in cellulose-limited chemostats. (A and B) Two species coinoculated (D = 0.020 h^{-1}); (C through F) *R. albus* 7 (8 ml) added to an established culture of *F. succinogenes* S85 (D = 0.031 h^{-1}) at 90 h. Symbols: ◆, total cell mass; ○, succinate; □, acetate; △, ethanol; ---◆---, cellulose consumed; ◇, sugar remaining; ---▲--- and ▲, *F. succinogenes* S85 from MFA and RNA assays, respectively; ---●--- and ●, *R. albus* 7 from MFA and RNA assays, respectively. The horizontal dashed lines indicate the lower detection limits (LDL) of MFA or RNA assay for the subordinate species. Probes: SUB1 for *F. succinogenes* S85, RAL196 for *R. albus* 7, and EUB338 for all bacteria.

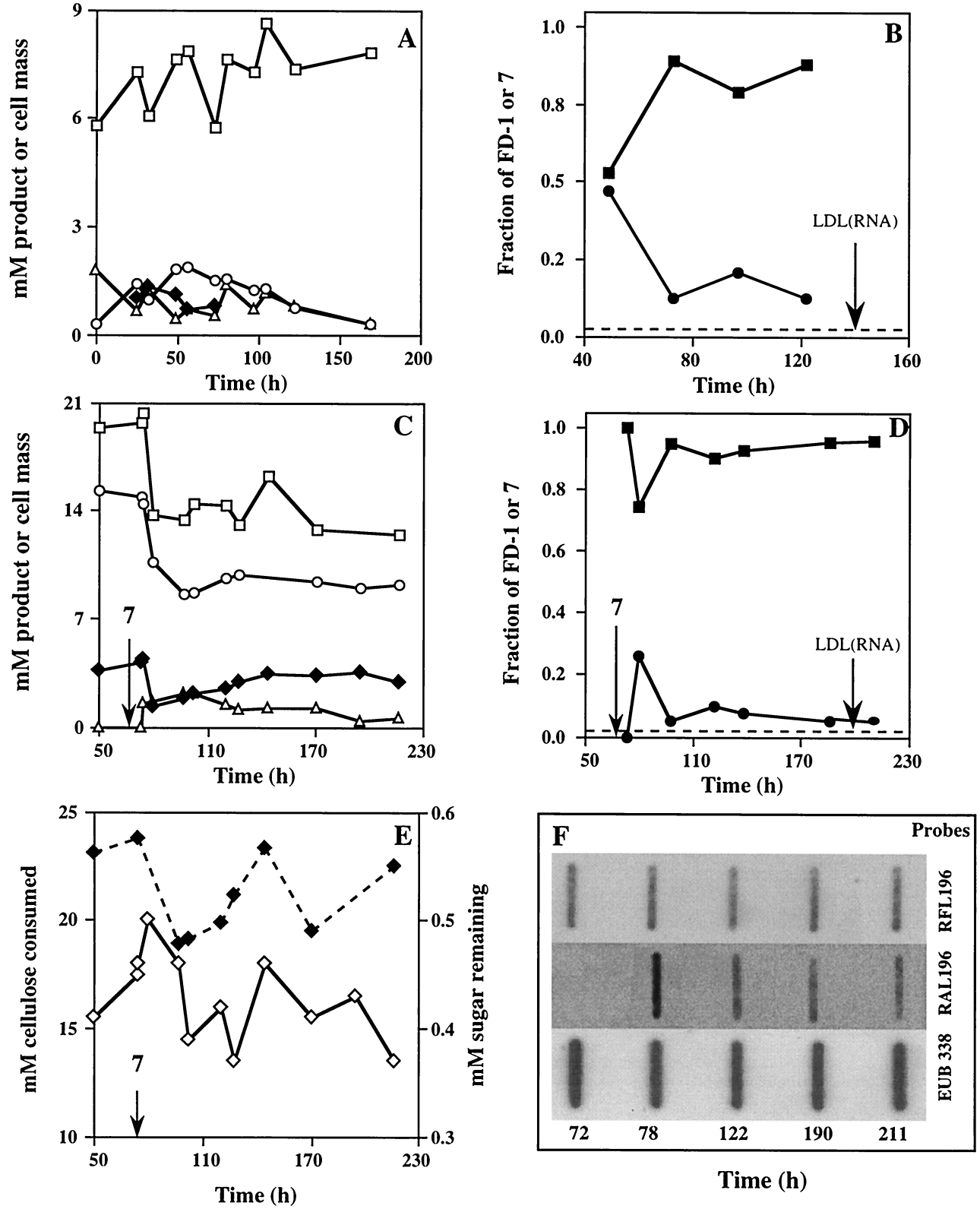

FIG. 3. Time course of competition between *R. flavefaciens* FD-1 and *R. albus* 7 in cellulose-limited chemostats. (A and B) Both species coinoculated into chemostat (D = 0.016 h^{-1}); (C through F) established culture of *R. flavefaciens* FD-1 (D = 0.029 h^{-1}) challenged after 3 days with 200 ml of *R. albus* 7. Symbols: ◆, total cell mass; ○, succinate; □, acetate; △, ethanol; ---◆---, cellulose consumed; ◇, sugar remaining; ---■---, *R. flavefaciens* FD-1 from RNA assay, ---●---, *R. albus* 7 from RNA assay. The horizontal dashed line indicates the lower detection limit (LDL) of RNA assay for the subordinate species. Probes: RFL196 for *R. flavefaciens* FD-1, RAL196 for *R. albus* 7, and EUB338 for all bacteria.

lulolytic species. Although batch cultures usually contained similar populations of each species, coculture under cellulose limitation usually contained a dominant species that represented ≈80% or more of the population.

Outcomes of binary culture experiments conducted under cellulose-limited conditions also differ from those observed under cellobiose-limited conditions. Under cellobiose limitation, one species completely eliminated the other, apparently based on pure and simple competition for a single soluble nutrient (25). By contrast, under cellulose limitation, stable cocultures were usually observed (e.g., between *F. succinogenes* and *R. albus* or between *R. flavefaciens* and *R. albus*). This is likely because several factors are involved in the competition for cellulose (e.g., adherence to cellulose, rate of cellulose hydrolysis, and utilization of hydrolytic products). Two species could coexist in a cellulose-limited chemostat if each were superior to the other in one or more different adaptive features.

Implications for the rumen fermentation. Interpretation of the interactions among these cellulolytic species in vivo based on the results from chemostat data requires caution, because the rumen does not operate as a chemostat (i.e., it is never in a steady state) and contains complex mixtures of feed materials containing a variety of fermentation substrates, as well as many species of noncellulolytic microorganisms. Moreover, the competition among these cellulolytic species may be altered by intraspecific differences among strains. However, our observations that cellulose-limited binary cultures of *R. flavefaciens* FD-1 and *F. succinogenes* S85 were dominated by the former species are in accord with the observations of Saluzzi et al. (22) that batch cultures of two *R. flavefaciens* strains (FD-1 and 17) and two *F. succinogenes* strains (S85 and BL2) grown on clover or alfalfa were also dominated by *R. flavefaciens*. If the trends observed thus far in continuous- and batch culture experiments are representative of those for other strains of these species, they would suggest that *R. flavefaciens*, and to a lesser extent *F. succinogenes*, may be particularly competitive for cellulose in the rumen. *R. flavefaciens* might predominate over *F. succinogenes* in the rumen on some forage diets because of its more rapid and complete adherence. *F. succinogenes* may be an abundant member of the microflora under most dietary conditions because (i) the growth of this species appears not to be inhibited by compounds produced by the other cellulolytic competitors (14, 15; this work), (ii) it has the ability to adhere to the surface of most kinds of feed particles (10), (iii) it has a relatively high cell yield (33), and (iv) it has an ability to store polysaccharide energy reserves (36). Although the population size of *R. albus* 7 was quite small in coculture with either *F. succinogenes* and *R. flavefaciens*, this species has a number of growth strategies that may permit its favorable competition in the rumen. Among these are its ability to adapt to rapid growth on low concentrations of cellobiose (25), its greater ability to degrade hemicellulose (3) and ferment some pentoses (28), and, in some strains, its ability to produce agents that inhibit other cellulolytic bacteria (14, 15). Additional studies incorporating different feed materials and different strains of cellulolytic species, as well as noncellulolytic microbial species, will aid in unraveling the complex factors determining competition among ruminal cellulolytic bacteria.

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