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

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The ruminal cellulolytic bacteria *Ruminococcus flavefaciens* FD-1 and *Fibrobacter succinogenes* S85 coexisted in substrate-excess coculture with about equal population size, but *R. flavefaciens* outcompeted *F. succinogenes* for cellobiose in the substrate-limited cocultures whether the two strains were coinoculated or a steady-state culture of *F. succinogenes* was challenged by *R. flavefaciens*. This outcome of competition between these two strains is due to a classical pure and simple competition mechanism based on affinity for cellobiose. Although the population size of *F. succinogenes* was much higher (>70%) than that of another cellulolytic species, *Ruminococcus albus* 7 in substrate-excess coculture, *F. succinogenes* was replaced by a population of *R. albus* in the substrate-limited coculture in both coinoculation and challenge experiments. *R. albus* outcompeted *F. succinogenes*, apparently due to selection in the chemostat of a population of *R. albus* with a higher affinity for cellobiose. *R. albus* also outcompeted *R. flavefaciens* under substrate-limited conditions.

Numerous ruminal microbes have been reported to digest cellulose, but it is generally agreed that three bacterial species, *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, and *Ruminococcus albus*, are the most active cellulolytic species both in vitro and in vivo (2). These species appear to digest cellulose only when adhering to the substrate (8). Cellulose in laboratory cultures and forage particles in the rumen are often fully colonized by bacteria (7). However, a substantial portion of the cellulolytic population in axenic, cellulose-limited continuous cultures are nonadherent (planktonic) cells, and in at least one case (23), these nonadherent cells were actively dividing. These growing planktonic cells are dependent upon the adherent population to generate soluble products of fiber digestion as growth substrates. One of the most important of these soluble products is cellobiose. This disaccharide is one of the major products of cellulose hydrolysis in the ruminal cellulolytic systems (6, 13) and is able to support the growth of many different species of ruminal microbes, both cellulolytic and noncellulolytic (7, 13). Recently, Pinder et al. (12) showed that concentrations of cellobiose in the rumen (~0.06 mM) far exceed those of both glucose (~0.01 mM) and longer cellodextrins (<0.01 mM).

Several studies have characterized the utilization of cellobiose by ruminal bacteria, but little information is available on the competition among these species for this substrate (9), particularly under substrate-limited conditions which more truly represent most natural environments (18). The purpose of this study was to compare the competition for cellobiose among three predominant ruminal cellulolytic species under conditions of both cellobiose excess and cellobiose limitation.

Culture conditions and analysis of coculture. Coculture experiments were conducted at 39°C with binary combinations of strains either in batch culture containing excess cellobiose or in cellobiose-limited continuous culture. Batch cocultures were incubated with 10 ml of a modified Dehority medium lacking casein and supplemented with 4 g of cellobiose per liter (21) under a CO_2 atmosphere in anaerobic culture tubes fitted with flanged butyl stoppers and aluminum crimp seals (1). Continuous cultures were performed in a chemostat mode under CO_2 sparging in a stirred reactor (working volume, 139 ml) continuously fed modified Dehority medium supplemented with cellobiose (1 to 4 g/liter; 3 to 11 mM) and yeast extract (1 g/liter). Dilution rates (range, 0.024 to 0.166 h^{-1}) and pH (range, 6.24 to 6.73) at steady state for each binary combination were within the range of physiological conditions in the rumen (7). Two types of chemostat experiments were conducted: (i) coinoculation experiments in which two species were mixed 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. Culture samples (7 ml) were removed from the reactor or anaerobic culture tube with a sterile hypodermic syringe. After centrifugation, supernatants were analyzed for residual soluble sugars (3) and fermentation products (21), and cell mass was estimated from the nitrogen content of cell pellets (21). The relative percentage of cell mass for each individual species in the cocultures was estimated by either signature membrane-associated fatty acid (MFA) assay or 16S rRNA targeted oligonucleotide probe hybridization (RNA assay), as described previously (17), except that samples were collected on ice in the chemostat effluent rather than removed directly from the chemostat.

Outcomes expected based on affinity for cellobiose. If the interaction between two strains of bacteria is solely a pure and simple competition for a single growth rate-limiting nutrient, the outcome of such competition in a binary culture chemostat can be predicted from calculation of the J parameter for each strain (4): $J = K_S \cdot D/(\mu_{max} - D)$, where K_S is the Monod saturation constant, D is the dilution rate ($hour^{-1}$), and μ_{max} is the maximum specific growth rate constant ($hour^{-1}$). The strain having the lowest J value will predominate in the che-

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

Inoculation order	D (h^{-1})[b]	pH	Cellobiose (mM glucose equivalent)			Dominant strain	Assay method (lower detection limit [%])[e]
			Initial	1st st. st.[c]	2nd st. st.[d]		
Coinoculation							
S85 + FD-1	0.067	6.24	17.85	2.28		FD-1	MFA (3.5), RNA (1.7)
7 + FD-1	0.024	6.36	24.51	0.24		7	MFA (4.3), RNA (1.4)
7 + S85	0.070	6.40	23.12	0.76		7	RNA (1.0)
Challenged							
S85, then FD-1	0.088	6.73	6.09	0.39	0.13	FD-1	MFA (3.5)
FD-1, then S85	0.169	6.62	16.98	1.09	1.09	FD-1	MFA (3.5)
FD-1, then 7	0.067	6.49	14.26	0.96	1.36	7	MFA (4.3), RNA (1.4)
7, then FD-1	0.167	6.71	12.89	0.09	0.09	7	MFA (4.3), RNA (1.4)
S85, then 7	0.070	6.36	23.12	0.63	0.49	7	RNA (1.7)
7, then S85	0.166	6.54	12.89	0.08	0.08	7	RNA (1.7)

[a] Steady-state data only. Mean values of all time points.
[b] Dilution rate in reciprocal hours.
[c] Steady state (st. st.) before challenge with second strain.
[d] Steady state (st. st.) after challenge with second strain.
[e] Values in parentheses indicate minimum percentage of the population of the subordinate strain detectable; the detection limit for the RNA assay assumes the use of 10 ng in a slot blot, although 100 ng was used in some assays, increasing sensitivity 10-fold.

mostat, eventually displacing the subordinate strain (4). Calculation of J for each strain, based on previously published values for K_s and μ_{max} determined in pure cultures grown on cellobiose (16), revealed that at all dilution rates *R. flavefaciens* FD-1 should outcompete both *F. succinogenes* S85 and *R. albus* 7, and *F. succinogenes* S85 should outcompete *R. albus* 7.

Growth conditions and outcomes of nine cellobiose-limited competition coculture trials are summarized in Table 1. In all cases, the outcome of the competition between each pair of strains was independent of the order of inoculation of the strains.

Competition between *R. flavefaciens* FD-1 and *F. succinogenes* S85. The RNA data from batch cocultures containing excess cellobiose showed that both species coexisted in equal proportions (50.1% for *F. succinogenes* and 49.9% for *R. flavefaciens*; standard error [SE], 5.9%). No inhibition of growth of *F. succinogenes* was observed in culture media supplemented with either culture supernatant of *R. flavefaciens* (at concentrations equivalent to the original *R. flavefaciens* culture) or 80% $(NH_4)_2SO_4$ precipitated cell-free crude proteins (0.08 mg of protein per ml of medium) from an *R. flavefaciens* culture. The relative proportions differ slightly from those reported by Odenyo et al. (9), perhaps due to our use of a different growth medium.

In contrast, cellobiose-limited chemostats were dominated by *R. flavefaciens*. *F. succinogenes*-specific RNA was not detected in steady-state chemostat samples that had been coinoculated with both strains (Fig. 1A and B), even when the amount of total RNA loaded onto the membranes was increased to 100 ng. Introduction of *R. flavefaciens* into an established *F. succinogenes* monoculture resulted in a dramatic decrease in 15:0 (the *F. succinogenes*-characteristic MFA), until within 2 days 15:0 was undetectable (Fig. 1C and D). Dominance by *R. flavefaciens* was also indicated by a shift in the ratio of fermentation end products (millimolar acetate/millimolar succinate) from 0.68 (characteristic of *F. succinogenes* monocultures [20]) to 1.50 (a ratio similar to that of *R. flavefaciens* monocultures [15]) and the reduction in the steady-state concentrations of soluble sugars from 0.39 to 0.13 mM (Table 1). These data are in accord with our previous observations that, although *R. flavefaciens* FD-1 cannot grow on glucose (6), total soluble sugar concentrations in cellulose-limited chemostat culture of *R. flavefaciens* were lower than in those of *F. succinogenes* (15, 20). The observed takeover of the chemostat by *R. flavefaciens* FD-1 was expected on the basis of *R. flavefaciens*' lower J value and higher affinity for cellobiose (16).

Competition between *R. albus* 7 and *F. succinogenes* S85. Data from fermentation end products (succinate versus ethanol), MFA assay, and RNA analyses of cellobiose-limited cocultures were consistent. *R. albus* outcompeted *F. succinogenes* for cellobiose, regardless of whether both strains were coinoculated (Table 1; Fig. 2A and B) or if an established *F. succinogenes* culture was challenged with *R. albus* (Table 1; Fig. 2C and D). Succinate (a major fermentation product of *F. succinogenes*), the *F. succinogenes*-characteristic MFA (15:0), and RNA specific for *F. succinogenes* were not detected in the chemostat 3 days after coinoculation (Fig. 2A and B).

Introduction of *R. albus* into an established culture of *F. succinogenes* resulted in the gradual disappearance of succinate as a fermentation end product, along with the disappearance of both the *F. succinogenes*-characteristic MFA (15:0) and *F. succinogenes*-specific RNA. These changes were coincident with the appearance of ethanol as a fermentation end product, 16:0 as a characteristic MFA, and RNA that hybridized to the specific probe for *R. albus* (Fig. 2B, C, and D). Thus, *R. albus* 7 outcompeted *F. succinogenes* S85, despite its reportedly poorer affinity for cellobiose, demonstrated for this species in batch and continuous-culture experiments (16), and its higher calculated J value.

The success of *R. albus* against *F. succinogenes* was apparently not due to production of an inhibitor, as both strains grew when coinoculated into cellobiose-excess batch cultures (72 and 28% for *F. succinogenes* and *R. albus*, respectively; SE, 11.3%; from the RNA assay). Growth of *F. succinogenes* in pure culture was not inhibited by high concentrations (180

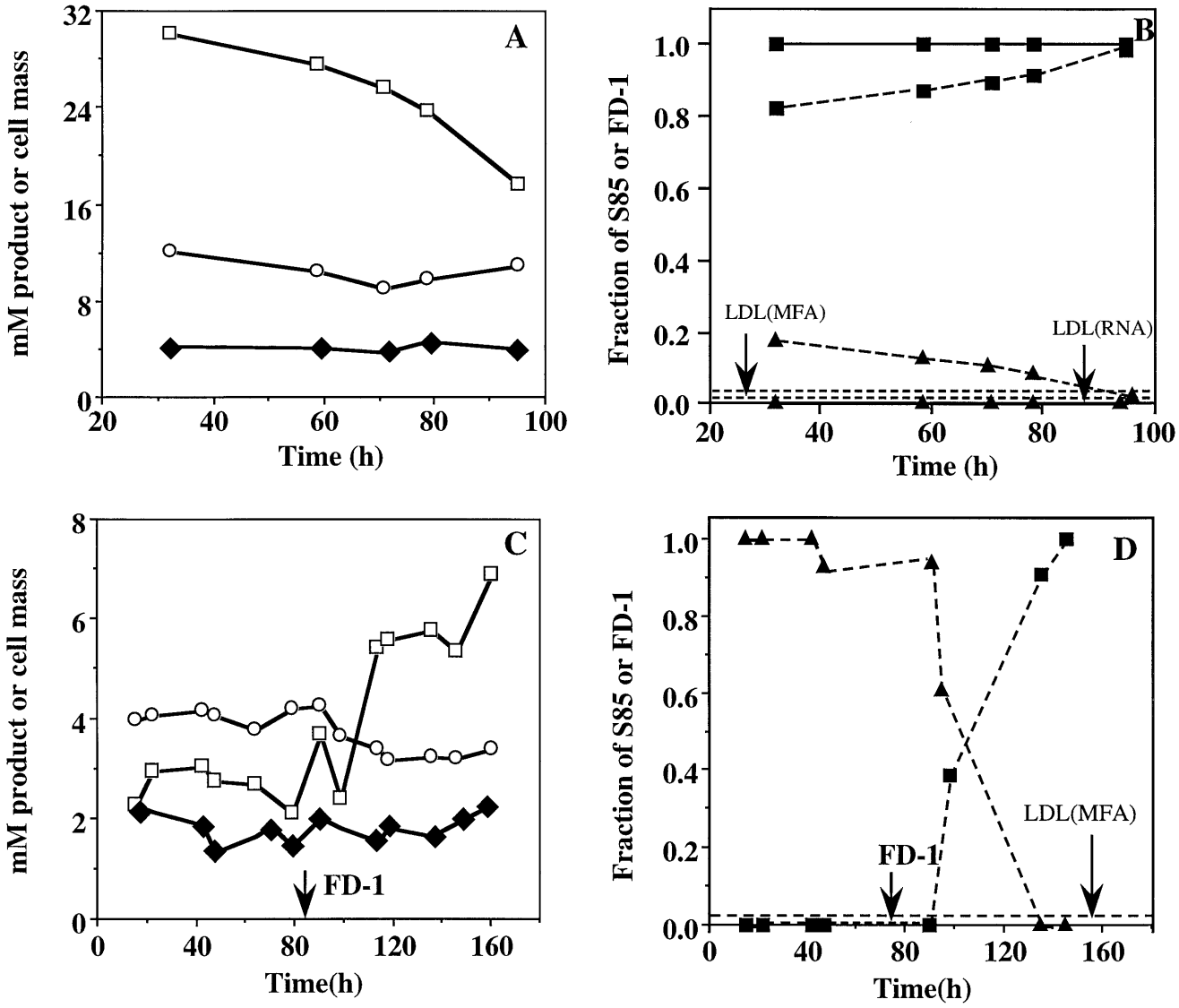

FIG. 1. Time course of competition between *F. succinogenes* S85 and *R. flavefaciens* FD-1 in cellobiose-limited chemostats after coinoculation ($D = 0.067\ h^{-1}$) (A and B) or after an established S85 monoculture ($D = 0.088\ h^{-1}$) was challenged at 83 h with *R. flavefaciens* FD-1 (C and D). Symbols: ◆, total cell mass; ○, succinate; □, acetate; ---▲--- and ▲, *F. succinogenes* S85 from MFA and RNA assays, respectively; ---■--- and ■, *R. flavefaciens* FD-1 from MFA and RNA assays, respectively. The horizontal dashed lines indicate the lower detection limits (LDL) from MFA or RNA assay for the subordinate species.

mM) of ethanol, a major fermentation product of *R. albus*. In addition, growth inhibition of *F. succinogenes* was not observed by including either supernatant or cell-free crude proteins obtained from the culture of *R. albus* 7 into the culture medium.

The takeover of a steady-state culture of *F. succinogenes* by *R. albus* was accompanied by a reduction in the steady-state concentrations of soluble sugars from 0.63 to 0.49 mM (glucose equivalents) at steady state in the *R. albus*-dominated culture. Since the chemostat is a powerful tool for microbial selection (5), growth parameters (μ_{max} and K_S) were remeasured (as described previously [16]) with the 136-h-old chemostat culture. The selected population retained the characteristics of *R. albus*, including growth on and adherence to cellulose with the production of lemon-yellow pigment; formation of ethanol, acetate, and H_2 as fermentation products; and hybridization to the RAL196 probe. The selected population was found to have a μ_{max} for cellobiose similar to that of the original *R. albus* 7 culture ($0.48\ h^{-1}$), but its K_S value (0.23 mM) was fivefold lower than that of the original *R. albus* 7 culture (1.21 mM [16]). Calculations using the Monod equation (18) with this lower K_S value indicate that *R. albus* 7 would outcompete *F. succinogenes* S85 for cellobiose at all physiologically relevant concentrations of cellobiose. The ability of *R. albus* 7 to adapt under selective pressure in the chemostat to give more rapid growth at lower cellobiose concentrations was not observed in the other two species.

Competition between *R. albus* 7 and *R. flavefaciens* FD-1. *R. albus* 7 also outcompeted *R. flavefaciens* FD-1 under cellobiose limitation, as indicated by the presence of ethanol and lack of succinate in the culture supernatant in the coinoculation experiment (Fig. 3A). *R. flavefaciens*-specific RNA could not be detected even after loading 100 ng of total RNA on the slot blot, indicating that *R. flavefaciens* comprised less than 0.14% of the total population.

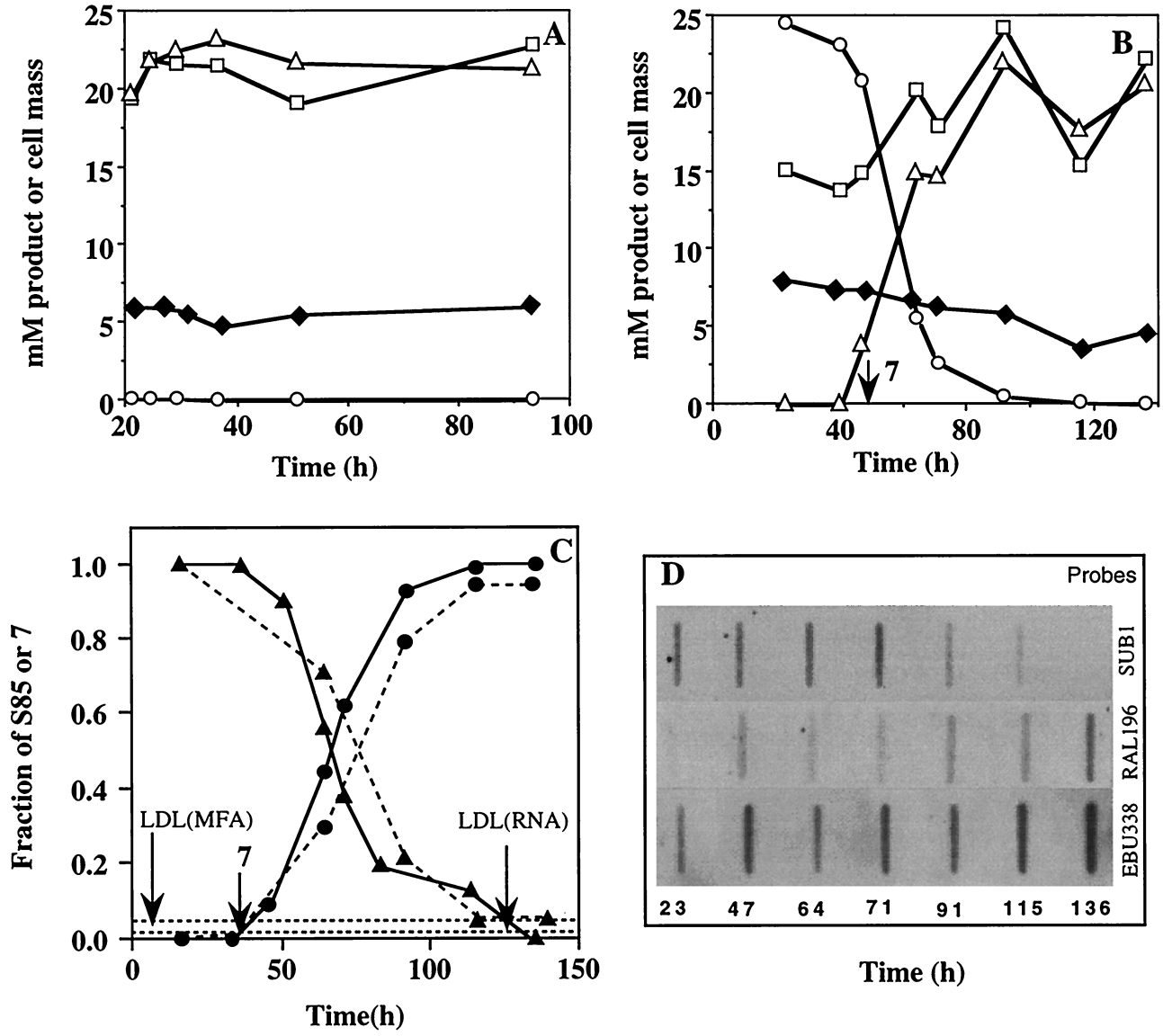

FIG. 2. Time course of competition between *F. succinogenes* S85 and *R. albus* 7 in cellobiose-limited chemostats after coinoculation ($D = 0.07\ h^{-1}$) (A) or after an established S85 monoculture ($D = 0.07\ h^{-1}$) was challenged with *R. albus* 7 at 45 h (B, C, and D). Symbols: ◆, total cell mass; ○, succinate; □, acetate; △, ethanol; ---▲--- and ▲, *F. succinogenes* S85 from MFA assay and RNA assay, respectively; ---●--- and ●, *R. albus* 7 from MFA assay and RNA assay, 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 eubacteria.

In the challenge experiment, the disappearance of succinate (a major fermentation product of FD-1) and the appearance of ethanol (a major fermentation product of *R. albus*) were observed after *R. albus* was introduced (Fig. 3B). The rapid disappearance of *R. flavefaciens*-characteristic MFA (*i*15:0) and *R. flavefaciens* FD-1-specific RNA indicated that the cell population of *R. flavefaciens* dramatically declined after *R. albus* was introduced into the chemostat (Fig. 3C and D). The characteristic MFA and specific RNA of *R. flavefaciens* could not be detected 3 days after *R. albus* was introduced into the chemostat.

The mechanism underlying the success of *R. albus* against *R. flavefaciens* remains to be elucidated. Odenyo et al. (9) have reported that another *R. albus* strain (strain 8) produces a bacteriocin-like compound that inhibited the growth of *R. flavefaciens* FD-1, and the success of *R. albus* 7 against *R. flavefaciens* FD-1 in the present study could, in principle, be due to a similar effect (i.e., production of an inhibitor). However, coinoculation of *R. albus* and *R. flavefaciens* into vials containing an excess of cellobiose produced inconsistent results within or between trials. Batch cultures coinoculated with both strains and incubated for 24 h sometimes produced monocultures of *R. albus* 7 and at other times produced binary cultures in which both species were present in substantial quantities; these results were confirmed by both measurement of fermentation end products and by use of species-specific probes directed toward 16S rRNA (data not shown). However, growth of *R. flavefaciens* in pure culture was not inhibited by supernatants or cell-free crude proteins obtained from the culture of *R. albus* 7 nor high concentrations (180 mM) of ethanol, the major fermentation product of *R. albus* monocultures. Based on the ability of *R. albus* to adapt to growth on low concen-

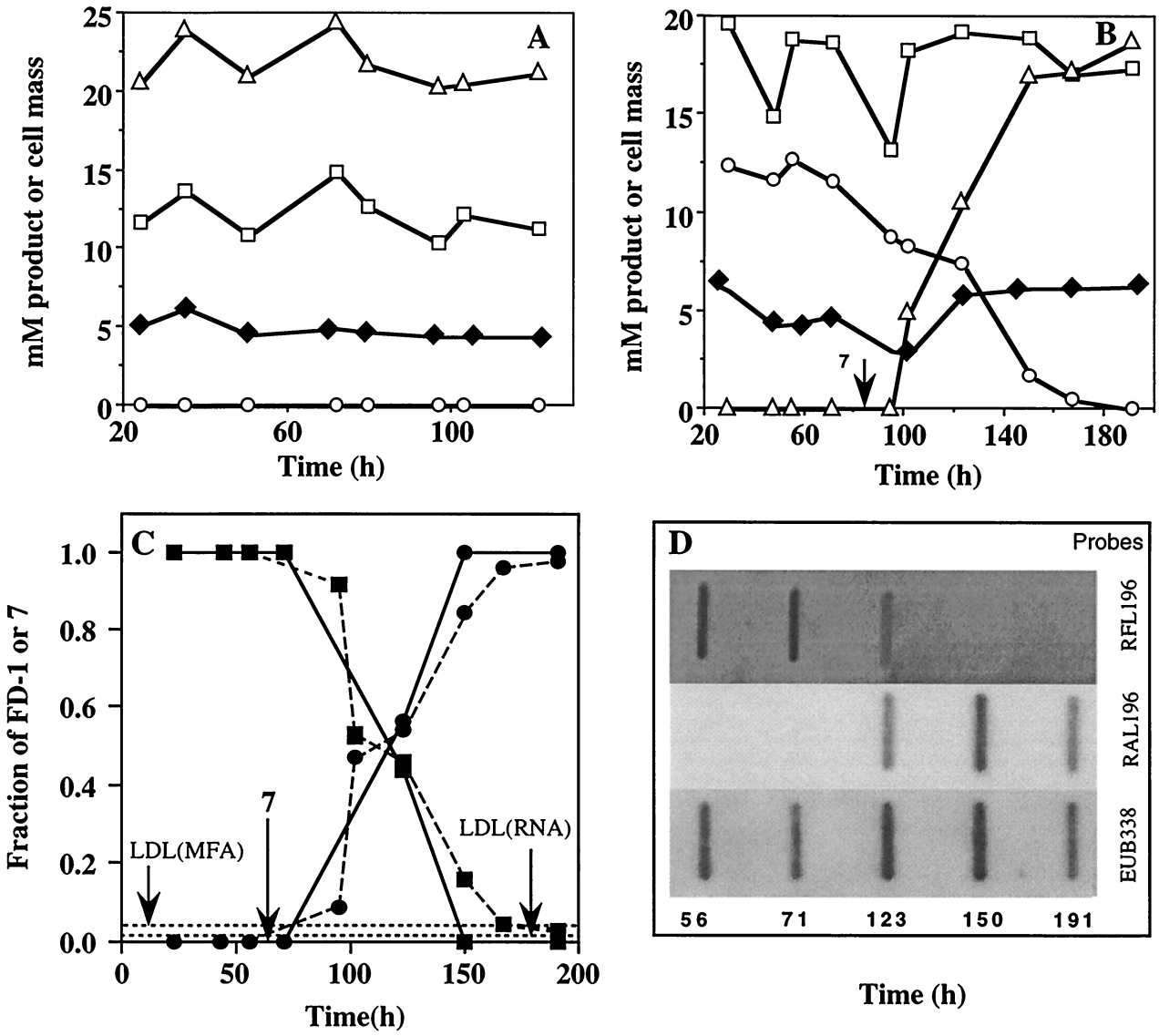

FIG. 3. Time course of competition between *R. flavefaciens* FD-1 and *R. albus* 7 in cellobiose-limited chemostats after coinoculation ($D = 0.024\ h^{-1}$) (A) or after an established FD-1 monoculture ($D = 0.067\ h^{-1}$) was challenged at 78 h with *R. albus* 7 (B and C). Symbols: ◆, total cell mass; ○, succinate; □, acetate; △, ethanol; ---■--- and ■, *R. flavefaciens* FD-1 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: RFL196 for *R. flavefaciens* FD-1, RAL196 for *R. albus* 7, and EUB338 for eubacteria.

trations of the cellobiose in a cellobiose-limited chemostat, the takeover of *R. flavefaciens* culture by *R. albus* may also have been due to adaptation by the latter strain, but the K_S values of these chemostat populations were not tested.

Comparison of interactions among three ruminal cellulolytic bacteria. Although coexistence of both *R. flavefaciens* and *F. succinogenes* has been reported in cellobiose-grown batch cocultures (9; this work), *R. flavefaciens* FD-1 outcompeted *F. succinogenes* S85 for cellobiose in the substrate-limited chemostats. This displacement of *F. succinogenes* by *R. flavefaciens* was due to a classical pure and simple competition mechanism based on substrate affinity. Although cellobiose is the predominant sugar in the rumen under both high- and low-fiber diets (12), its reported concentration (0.06 mM) is 4- to 20-fold lower than the K_S for cellobiose for these three species. If our data are representative of other strains of these species, it would suggest that planktonic cells of *R. flavefaciens*, by virtue of their lower K_S, may possess a greater potential to compete for cellobiose in the rumen than the other two species. This strategy could be a key factor for the survival of this species through natural selection, because most strains of this species cannot grow on other mono- or disaccharides, including glucose. In the case of *F. succinogenes*, other factors, such as storage of polysaccharides and their use during starvation, could be an important strategy for survival during the planktonic phase (22).

Interactions between *F. succinogenes* and *R. albus* in batch-type coculture on cellobiose appear to be weak because these two species coexist (9; this work). However, our observations clearly show that *R. albus* 7 can displace *F. succinogenes* under cellobiose-limiting conditions. The takeover of the culture by *R. albus* was apparently due to selection in the chemostat of a

population of *R. albus* with a higher affinity for cellobiose. It has been reported that *R. albus* has a poorer adherence to cellulose (8) and lower growth yield on cellulose than do the other two species of predominant cellulolytic bacteria (10, 11, 15, 20). However, the ability of this species to degrade hemicellulose and to ferment pentose is much greater than those of the other two species (2, 19). The availability of other monosaccharides in the rumen may reduce greatly the pressure of competition between *R. albus* and *F. succinogenes*. Also, the adaptability of *R. albus* may be an important characteristic that has favored its persistence in the rumen environments and might help *R. albus* to survive and grow in the planktonic phase.

The interaction between *R. albus* and *R. flavefaciens* has varied from study to study. These two species can reportedly coexist in vitro in batch culture (2), and both have been found in the same rumen sample. A bacteriocin-like compound produced by *R. albus* 8 has been reported by Odenyo and coworkers (9), who showed that growth of *R. flavefaciens* FD-1 was inhibited completely in coculture with this strain. However, the growth inhibition of *R. flavefaciens* FD-1 by *R. albus* 7 could not be consistently demonstrated in our study. One possible explanation for these inconsistent observations is that production of an inhibitor by *R. albus* 7 may not be constitutive or may occur only under certain, currently undefined growth conditions or physiological stages. However, the displacement of *R. flavefaciens* by *R. albus* in cellobiose-limited chemostats could also be due to adaptation or to a combination of adaptation and inhibition. Further investigation is needed to clarify the interaction between these two species.

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