Applied and Environmental Microbiology, Dec. 1985, p. 1361–1367 Vol. 50, No. 6
0099-2240/85/121361-07$02.00/0
Copyright © 1985, American Society for Microbiology

Responses of *Ruminococcus flavefaciens*, a Ruminal Cellulolytic Species, to Nutrient Starvation

DANIEL E. WACHENHEIM† AND ROBERT B. HESPELL‡*
Department of Dairy Science, University of Illinois, Urbana, Illinois 61801

Received 20 May 1985/Accepted 30 August 1985

Ruminococcus flavefaciens **strain C94, a strictly anaerobic, cellulolytic ruminal bacterial species, was grown either in batch or continuous cultures (cellobiose limited or nitrogen limited) at various dilution rates. Washed cell suspensions were incubated anaerobically at 39°C without nutrients for various times up to 24 h. The effects of starvation on direct and viable cell counts, cell composition (DNA, RNA, protein, and carbohydrate), and endogenous production of volatile fatty acids by the cell suspensions were determined. In addition, the effect of the pH of the starvation buffer on direct and viable cell counts was determined. Survival of batch-grown cells during starvation was variable, with an average time for one-half the cells to lose viability (ST_{50}) of 10.9 h. We found with continuous cultures that viable cell counts declined faster when the initial cell suspensions had been grown at faster dilution rates; this effect was more pronounced for suspensions that had been limited by cellobiose (ST_{50} = 6.6 h at a dilution rate of 0.33 h^{-1}) than for suspensions that had been limited by nitrogen (ST_{50} = 9.5 h at a dilution rate of 0.33 h^{-1}). With continuous cultures, viable cell counts in all cases declined faster than direct cell counts did. The rates of disappearance of specific cell components during starvation varied with the initial growth conditions, but could not be correlated with the loss of viability. Volatile fatty acid production by starving cells was very low, and acetate was the main product. Starved cells survived longer at pH 7.0 than they did at pH 5.5, and this effect of pH was greater for cellobiose-limited cells (mean ST_{50} = 7.1 h) than for nitrogen-limited cells (mean ST_{50} = 12 h). Although it has relatively low ST_{50} values, *R. flavefaciens* has sufficient survival abilities to maintain reasonable numbers in domestic animals having maintenance or greater feed intake.**

Fluctuations of carbohydrate concentrations in the rumen can influence both the relative proportions and the absolute concentrations of the specific carbohydrate-utilizing subpopulations of the ruminal microflora (6, 14). Depending upon the diet of an animal, soluble carbohydrate concentrations can become insufficient to support microbial growth within a few hours after the animal has been fed (12, 31). The results of investigations with selected pure cultures of soluble carbohydrate-fermenting rumen bacteria indicate that these bacteria have less capacity to endure periods of low nutrient availability than bacteria from other ecosystems do (18–20). Thus, cycles of bacterial growth and nonviability may exert an important influence on the overall ruminal microbial populations and energy economy.

Among the carbohydrate-specific ruminal subpopulations, the cellulolytic bacteria are especially important. These bacteria convert the major plant polysaccharide (cellulose) and nitrogenous compounds to hydrolytic products of cellulose, volatile fatty acids, and microbial cells which can be used by other microbes and the host animal. The cellulolytic ruminal bacteria are more sensitive than other ruminal bacteria to certain conditions of stress, such as low pH (26, 30). In investigations of ruminal diurnal variation, the cellulolytic group also exhibits the most dramatic decline in cellular viability when overall bacterial viability is also on the decline (14).

The intent of this investigation was to quantitate the survival capacity of a cellulolytic ruminal species, *Ruminococcus flavefaciens*. Factors which could influence the capacity of *Ruminococcus flavefaciens* to endure starvation, such as the growth rate or the limiting nutrient prior to the onset of starvation or the pH of the environment during starvation, were examined. These studies are the first to shed light on the relative survival capacities of a fiber-digesting, gastrointestinal tract bacterial species.

MATERIALS AND METHODS

Bacterial strain. *Ruminococcus flavefaciens* strain C94 was obtained from the culture collection of the Department of Dairy Science, University of Illinois, Urbana. *Ruminococcus flavefaciens* C94 was chosen because it is a typical strain which has been thoroughly characterized and its nutritional requirements are well known (4, 28).

Growth conditions, media, and buffers. Throughout this investigation, the principles of anaerobiosis as outlined by Hungate (11) and modified by Bryant (3) were used in the preparation of media and buffers and treatment of bacteria. The basal medium used for *Ruminococcus flavefaciens* was RFB medium (Table 1), a liquid medium developed from the media of Bryant and Robinson (4, 5), Latham et al. (13), and Schaefer et al. (28). For batch cultures, a 100-ml inoculum of a fresh overnight culture of *Ruminococcus flavefaciens* was injected into 3 liters of prewarmed RFB medium and incubated at 39°C until the culture reached an optical density at 660 nm of 0.70, as determined with a Bausch & Lomb Spectronic 70 colorimeter. The culture was stirred continuously with a magnetic stirring bar until it was harvested. For chemostat cultures, RFB medium was modified as follows. For cellobiose-limited cultures, the final medium concentrations of the affected nutrients were 0.1% (wt/vol) cellobiose,

* Corresponding author.
† Present address: Eastman Kodak Co., Rochester, NY 14650.
‡ Present address: Northern Regional Research Center, Agriculture Research Service, U.S. Department of Agriculture, Peoria, IL 61604.

TABLE 1. RFB medium used for growth of *Ruminococcus flavefaciens* strain C94

Stock solution	% (vol/vol) in medium
Basal solution[a]	
Mineral solution[b]	5.0
Trace metals[c]	1.0
Volatile fatty acids[d]	0.5
Resazurin (0.1%, wt/vol)	0.075
Secondary solutions[e]	
Cellobiose (6%, wt/vol)	5.0
Yeast extract (3%, wt/vol)[f]	3.25
Na_2CO_3 (8%, wt/vol)	5.0
$Na_2S \cdot 9H_2O$ (2.5%, wt/vol)[e]	1.0

[a] Solutions were added to the main vessel, and the volume was brought up to the final volume, minus postautoclave additions, after the pH was adjusted to 6.8 with KOH.

[b] The mineral solution contained the following (per liter): KH_2PO_4, 18 g; $CaCl_2$, 0.4 g; and $MgCl_2 \cdot 6H_2O$, 0.4 g. (See reference 28).

[c] The trace metal solution contained the following (per liter): tetrasodium EDTA, 500 mg; $FeSO_4 \cdot 7H_2O$, 200 mg; $MnCl_2 \cdot 4H_2O$, 200 mg; $ZnSO_4 \cdot 7H_2O$, 10 mg; H_3BO_3, 30 mg; $CoCl_2 \cdot 6H_2O$, 12 mg; $CuCl_2 \cdot 2H_2O$, 1 mg; $NiCl_2 \cdot 6H_2O$, 2 mg; $NaMoO_4 \cdot 2H_2O$, 30 mg; and Na_2SeO_3, 50 mg. This solution was modified from that of Schaefer et al. (28).

[d] The volatile fatty acid solution contained the following (per liter): isobutyric acid, 18 ml; valeric acid, 20 ml; 2-methyl butyric acid, 20 ml; and isovaleric acid, 20 ml. The pH of the solution was adjusted with NaOH to 7.0 before the final volume adjustment was made. This solution was modified from that of Latham et al. (13).

[e] Solutions prepared as described in the text and added to cooled basal solution.

[f] Yeast extract was the sole source of nitrogen for nitrogen-limited cultures. For cellobiose-limited cultures, 0.1% (wt/vol) ammonium sulfate was added.

0.1% (wt/vol) yeast extract, and 0.1% (wt/vol) ammonium sulfate. For nitrogen-limited cultures, the final concentrations were 0.3% (wt/vol) cellobiose, and 0.1% (wt/vol) yeast extract; the ammonium sulfate was deleted. All other components were added at the same concentrations as given for the batch culture medium (Table 1). A BioFlo C30 benchtop fermentor (New Brunswick Scientific Co., Inc., Edison, N.J.) was modified for anaerobic continuous culture (Wachenheim, M. S. thesis, University of Illinois, Urbana, 1982). The chemostat was inoculated with about 1% (vol/vol) from an overnight culture of *Ruminococcus flavefaciens* and was maintained at 39°C. Subsequent growth and equilibrium in continuous culture were monitored by measuring the optical densities of samples removed from the culture vessel. At least 9 volumes of medium were allowed to pass through the culture vessel before equilibrium was considered to have been reached, even if the optical density stabilized earlier. Nutrient limitation was determined by measuring nutrients in the culture vessel medium (see below) and by diluting cultures 1:1 in cellobiose- or ammonia-free medium, followed by observation of optical density to measure growth. Culture purity was monitored by microscopic observation.

For enumeration of viable bacteria, the CDM medium of Leedle and Hespell (15) was modified for *Ruminococcus flavefaciens* (CDM-C94 medium) (Table 2). After the basal medium was autoclaved in sealed flasks and cooled to 50°C, the flasks of medium were transferred into an anaerobic glove box (95% argon, 5% hydrogen) for subsequent manipulations. The separately prepared, sterile, anaerobic solutions were added to CDM-C94 medium. The medium was poured into sterile plastic petri dishes which had been inside the glove box for at least 12 h for equilibration with the glove box atmosphere (as was done with all plastic ware used). The poured plates were allowed to stand for at least 1 day before they were either used or stacked into plastic food storage containers for storage in the glove box until they were needed.

For starvation of cell suspensions, the S buffer described by Leedle and Hespell (15) was used, with the following modifications. For starvation at pH 6.0, 6.5, and 7.0, PIPES [piperazine-*N*,*N'*-bis(2-ethanesulfonic acid); Sigma Chemical Co., St. Louis, Mo.] was used as the major hydrogen ion buffer. For starvation at pH 5.5 and 6.0, MES [2-(*N*-morpholino)ethanesulfonic acid; Sigma] was used because of its pKa (10). At all pH values, the pHs of both the basal S buffer solution and the 0.5 M sodium phosphate solution (which was added after sterilization) were adjusted with sodium hydroxide.

Starvation conditions. Cultures were transferred into a sterile CO_2-filled vacuum flask which was then sealed and taken into an anaerobic glove box for subsequent manipulations. Culture samples were poured into sterile polycarbonate centrifuge bottles with air-tight seals (Du Pont/Sorvall, Wilmington, Del.), removed from the glove box, and centrifuged for 5 min at 5,000 × g and 4°C. The centrifuge bottles were then returned to the glove box, and the supernatant was decanted. The cell pellets were then suspended in a volume of S buffer equal to the original culture volume and washed as described above. The cells were suspended to their original culture volume (except for batch cultures, which were suspended to one-half of their initial volume) in S buffer. The pH of the S buffer used for the wash was the same as the pH of the final suspension.

Cells were starved by transferring 72-ml portions of the final cell suspensions into 100-ml serum bottles (Bellco Glass, Inc., Vineland, N.J.) containing the same atmosphere (95% argon, 5% hydrogen) that was in the glove box. The bottles were sealed with rubber stoppers, removed from the glove box, and incubated in a prewarmed (39°C) rotary shaking water bath. When samples were removed for analysis at specified time intervals, the entire contents of each bottle were used.

TABLE 2. Composition of CDM-C94 medium used for viable cell counts of *Ruminococcus flavefaciens* C94[a]

Component	Amt per 100 ml
Basal medium[b]	
Agar	2.0 g
$(NH_4)_2SO_4$	0.1 g
Hemin solution (0.01%, wt/vol)[c]	1.0 ml
Mineral solution[d]	5.0 ml
Trace metals[e]	1.0 ml
Resazurin (0.1%, wt/vol)	0.1 ml
Volatile fatty acids[f]	0.50 ml
Incubated rumen fluid[c]	10.0 ml
Trypticase	0.3 g
Yeast extract	0.2 g
Secondary solutions[g]	
Cellobiose (6% wt/vol)	5.0 ml
Na_2CO_3 (8%, wt/vol)	5.0 ml
Cysteine hydrochloride (2.5%, wt/vol)	1.0 ml
$Na_2S \cdot 9H_2O$ (2.5%, wt/vol)	1.0 ml

[a] Modified from CDM medium of Leedle and Hespell (15).

[b] Basal medium was prepared as described in the text.

[c] Prepared as described by Leedle and Hespell (15).

[d] See Table 1, footnote *b*.

[e] See Table 1, footnote *c*.

[f] See Table 1, footnote *d*.

[g] Secondary solutions were prepared and added to the basal medium as described in the text.

Analyses and determinations. At each time interval, the serum bottles were brought into the anaerobic glove box and opened, and the volumes used to determine direct cell counts were removed. After 1:5 dilution in a formal saline solution (10% [vol/vol] formaldehyde in a 0.85% [wt/vol] sodium chloride solution) as described by Warner (32), the preserved samples were stored at 4°C until they were analyzed. Before counting, samples were mixed vigorously by using a rotary type of test tube mixer; this was followed by passing the sample through a Pasteur pipette five times to reduce the size of any bacterial aggregates. After an additional 1:4 dilution of the preserved samples in S buffer, cell counts were determined by using a Petroff-Hausser counting chamber.

For viable cell counts, samples were removed from the starved suspensions and serially diluted in S buffer, and portions of the dilutions (0.05 ml) were spread onto CDM-C94 medium in petri dishes. The plates were then stacked inside anaerobic culture vessels (2) modified as described by Leedle and Hespell (15), the vessels were flushed three times with anaerobic CO_2, and the vessels were incubated for 96 h at 39°C before colonies were counted. Only plates with 10 to 100 colonies were used for enumeration.

After removal of samples for the enumerative procedures described above, the remaining suspensions were divided into one 30-ml portion and two 20-ml portions which were separately centrifuged for 10 min at 8,000 × *g*, and 4°C. Both the resulting cell pellets and the supernatants were frozen at 20°C until subsequent analysis.

The cell pellets obtained from the 30-ml samples were suspended in glass-distilled water and quantitatively transferred into tared weighing planchets. The samples were then dried for 12 h at 50°C or until dry weight determinations made at least 2 h apart gave similar values. After weighing, the dried samples were then suspended in 1 ml of glass-distilled water for protein determinations. After hydrolysis (0.1 N NaOH, 10 min, 70°C), protein was determined by the method of Lowry et al. (17), using cytochrome *c* as the standard. The cell pellets from one set of 20-ml samples were hydrolyzed (12 N HCl, 6 h, 100°C) and then neutralized with 10 N NaOH, and the carbohydrate contents of the hydrolysates were determined by the phenol-sulfuric acid method of Ashwell (1), using D-glucose as the standard. The cell pellets from the remaining 20-ml samples were hydrolyzed (0.5 $NaClO_4$, 30 min, 70°C) and centrifuged at 22,000 × *g*, for 20 min at 4°C, and the supernatants were analyzed for nucleic acid content. The diphenylamine reaction described by Burton (7) was used for DNA determinations; deoxyribose was used as the standard, and the values were adjusted to DNA equivalents by using a correction factor of 2.44. The orcinol reaction described by Schneider (29) was used for RNA determinations; ribose was used as the standard, and the values were adjusted to RNA equivalents by using a factor of 4.90.

Phosphate contents were determined for several cell pellets that were not required for other analytical procedures. The colorimetric reaction of phosphate with ammonium molybdate and ascorbic acid (9) was employed. Monobasic potassium phosphate was used as the standard.

Supernatants were analyzed for ammonia by the iodophenol method described by Chaney and Marbach (8), using ammonium chloride as the standard. For volatile fatty acid analysis, supernatants were lyophilized, and this was followed by butyl esterification and determination of the resulting butyl esters by gas-liquid chromatography as described by Salinitro and Muirhead (27).

TABLE 3. Effect of starvation of mid-exponential batch-grown *Ruminococcus flavefaciens* cells on direct and viable cell counts

Time starved (h)	Direct cell counts ($\times 10^8$, cells per ml)[a]	Viable cell counts ($\times 10^8$, cells per ml)[b]
0	13.4 ± 8.5	3.33 ± 1.15
3	ND[c]	7.17 ± 2.42
6	ND	5.60 ± 0.41
9	ND	6.23 ± 1.15
12	8.25 ± 0.71	4.60 ± 0.14
24	7.45 ± 0.32	1.02 ± 0.19

[a] Data are expressed as mean direct cell counts ± standard deviation from two experiments, each with triplicate samples.
[b] Data are expressed as mean viable cell counts ± standard deviation from two experiments, each with triplicate samples.
[c] ND, Not determined.

Statistical analysis of data. All viable cell counts were subjected to a least-squares linear regression analysis. The line equations, the correlation coefficients (r_{xy}^2), and the significance of regression (variance ratio) were calculated as described by Poole and Borchers (24). The time required for 50% of the population to lose viability (ST_{50}) was determined by two methods. In the first method, the viable cell counts were plotted as a function of length of starvation, and the ST_{50} was determined graphically by drawing a line from the point at which viability decreased by 50% to the independent axis (hours of starvation). In the second method, the data were subjected to a least-squares linear regression analysis. Either colony-forming units per milliliter or log (colony-forming units per milliliter) was used as the dependent variable, and time of starvation was used as the independent variable. The *y* intercept was used as the zero-time value for colony-forming units per milliliter or log (colony-forming units per milliliter), as appropriate. The ST_{50} was then determined from the line equation by using one-half of the *y* intercept as the *y* variable. The analysis of variance methods used were those described by Ott (22).

RESULTS

Growth of *Ruminococcus flavefaciens* in experimental media. In an attempt to use a chemically defined medium, the yeast extract in RFB medium was replaced with 14 mM ammonium sulfate plus a filter-sterilized solution containing the following vitamins (final concentrations, micrograms per liter); biotin, 12.5; *p*-aminobenzoic acid, 200; pyridoxine-hydrochloride, 200; and calcium D-pantothenic acid, 200. In the chemically defined medium, 36 h was required for the culture to reach its maximum optical density of 0.26. With 0.1% (wt/vol) yeast extract in the medium, the culture reached a maximum optical density of 0.76 in 17 h and, with successive subculturing, reached a maximum optical density of 1.2 in 20 h. The maximum growth rate was found to be $0.38\ h^{-1}$ (as determined by the plate count method) or 0.42 h^{-1} (as determined by optical density). Since subculturing did not improve growth in the chemically defined medium, yeast extract-containing RFB medium was used for this investigation.

Foaming and wall growth sometimes occurred with chemostat cultures. Reduction of the gas flow rate decreased the foaming. Wall growth was especially severe at dilution rates greater than $0.33\ h^{-1}$; thus, faster dilution rates were not used for this investigation. The cellobiose-limited cultures removed 97.7% of the cellobiose from the medium, and the nitrogen-limited cultures removed 85.1% of the cellobiose from the medium. The fermentation patterns for strain C94

TABLE 4. Rates of change in cellular constituents of *Ruminococcus flavefaciens* cells during starvation

Method	Limiting nutrient	Dilution rate (h^{-1})	Cell component[a]				DWRA[b]
			Protein	Carbohydrate	RNA	DNA	
Batch	None		−0.15	−0.16	−0.40	−0.85	−0.46
Chemostat	Cellobiose	0.13	−0.93	−0.90	−1.73	−1.51	−1.13
		0.23	−0.08	−1.18	−1.60	−1.31	−1.12
		0.33	−1.04	−1.30	−1.83	−1.43	−1.28
	Nitrogen	0.13	−0.58	−2.04	−0.68	−0.87	−0.86
		0.23	−0.17	−1.08	−1.20	−0.25	−0.43
		0.33	−1.23	−0.78	−0.40	−0.48	−0.75

[a] Data are expressed as the slopes determined from a linear regression analysis of cell composition data after conversion of the data to percentages of the original (zero-time) concentrations.

[b] DWRA expressed as a percentage of the original (zero-time) DWRA was used to determine slopes. DWRA is the sum of the DNA, RNA, carbohydrate, and protein dry weights.

were similar to those reported previously, for *Ruminococcus flavefaciens* strain 67 (23). Acetate was quantitatively the most significant fermentation acid produced (57 and 30 μmol/100 μmol of glucose equivalents fermented in the cellobiose-limited and nitrogen-limited cultures, respectively; values are means for all three dilution rates examined). Succinate and lactate were far less significant products (less than 10 μmol/100 μmol of glucose equivalents fermented). Formate levels were not measured. In all cases, the pH in the culture vessel was 6.8 ± 0.1. Direct cell counts decreased with increasing dilution rates for the cellobiose-limited cells, but not for the nitrogen-limited cells. Viable cell counts increased with increasing dilution rates for both cellobiose- and nitrogen-limited cells.

Starvation of batch-grown *Ruminococcus flavefaciens*. After initiation of starvation, there were apparent increases in viable cell counts (Table 3), which may have been partly due to the presence and gradual breakup of small cell aggregates. After the initial increases, however, the viable cell counts decreased during the period of starvation. The viable cell counts decreased faster than the total cell counts indicating that some cells that lost viability remained intact. The concentrations of all measured cell constituents decreased; the greatest decline was in nucleic acids (Table 4). Dry weight recovery varied erratically among samples. We found that the source of variation was due to phosphates that had coprecipitated along with the bacterial cells. Therefore, the dry weight recovered by assay (DWRA), which was calculated as the sum of protein, carbohydrate, and nucleic acid dry weights, was used as a measure of biomass. The nucleic acids of the batch-grown cells were lost at a rate that was greater than the rate of loss of DWRA, indicating that the nucleic acids, particularly RNA, may have served as substrates for endogenous metabolism.

Starvation of cellobiose-limited, chemostat-grown *Ruminococcus flavefaciens*. In general, the viable cell counts decreased faster for cells that were grown under cellobiose limitation at the higher dilution rates than for cells that were grown under cellobiose limitation at the lower dilution rates (Tables 5 and 6). Direct cell counts decreased slightly or not at all, regardless of the initial dilution rate. Almost all of the cell components decreased in concentration during starvation, with RNA disappearing the fastest (Table 4). At all dilution rates, nucleic acids were lost at rates similar to or greater than the rate of loss for DWRA, as also occurred for batch-grown cells. The rate of loss of carbohydrates increased with increasing dilution rate, while there was little variation in nucleic acid loss and loss of protein was variable. The production of fermentation acids by the starving cells was negligible; acetate (200 pM) was the major acid detected after the onset of starvation. Only traces of succinate, formate, and lactate were detected during starvation.

Starvation of nitrogen-limited, chemostat-grown *Ruminococcus flavefaciens* C94. The viable cell counts for cells that were grown under nitrogen limitation at higher dilution rates declined faster than the viable cell counts for cells that were grown under nitrogen limitation at lower dilution rates (Table 6), but the differences were smaller than those observed with the cellobiose-limited cells. Direct cell counts decreased only slightly during starvation. The levels of all cell constituents decreased during starvation, but the priority in the loss of cell constituents was different for each dilution rate (Table 4). In contrast to the observations made with cellobiose-limited cell suspensions, carbohydrates were lost at lower rates with cells from the higher dilution rates in the nitrogen-limited chemostats. These losses were at rates equal to or greater than those observed for DWRA. No similar patterns were observed for the remaining cell constituents. Both lactate acid (272 pM) and acetic acid (205 pM) were present in the starving suspensions after the onset of starvation. Only traces of succinate and formate were detected during the period of starvation.

Analysis of viability losses. The ST_{50} values were calculated by both graphic and regression methods (see Materials and Methods). The ST_{50} values determined by regression (Table

TABLE 5. Effect of starvation of chemostat-grown, cellobiose-limited *Ruminococcus flavefaciens* cells on direct and viable cell counts

Time starved (h)	Cell counts (× 10^8, cells per ml)					
	Dilution rate of 0.13 h^{-1}		Dilution rate of 0.23 h^{-1}		Dilution rate of 0.33 h^{-1}	
	Direct counts[a]	Viable counts[b]	Direct counts[a]	Viable counts[b]	Direct counts[a]	Viable counts[b]
0	16.9 ± 0.9	1.07 ± 0.10	13.1 ± 0.5	1.44 ± 0.10	11.6 ± 0.2	2.54 ± 0.55
3	ND[c]	1.38 ± 0.24	ND	1.10 ± 0.54	ND	0.95 ± 0.19
6	ND	1.09 ± 0.09	ND	0.83 ± 0.05	ND	1.41 ± 0.33
9	ND	1.29 ± 0.61	ND	1.14 ± 0.37	ND	0.77 ± 0.17
12	13.8 ± 1.1	1.35 ± 0.14	11.7 ± 0.3	1.02 ± 0.06	12.6 ± 2.5	0.73 ± 0.16
24	14.4 ± 1.5	0.58 ± 0.21	9.08 ± 1.0	0.39 ± 0.20	12.6 ± 5.3	0.16 ± 0.01

[a] Data are expressed as mean direct cell counts ± standard deviation from two experiments, each with triplicate samples.

[b] Data are expressed as mean viable cell counts ± standard deviation from two experiments, each with triplicate samples.

[c] ND, Not determined.

TABLE 6. Effect of starvation of chemostat-grown, nitrogen-limited *Ruminococcus flavefaciens* cells on direct and viable cells counts

Time starved (h)	Cell counts (× 10^8, cells per ml)					
	Dilution rate of 0.13 h^{-1}		Dilution rate of 0.23 h^{-1}		Dilution rate of 0.33 h^{-1}	
	Direct counts[a]	Viable counts[b]	Direct counts[a]	Viable counts[b]	Direct counts[a]	Viable counts[b]
0	11.8 ± 1.5	1.98 ± 0.04	12.2 ± 1.4	2.18 ± 0.10	12.6 ± 1.6	2.39 ± 0.21
3	ND[c]	2.16 ± 0.01	ND	1.64 ± 0.10	ND	1.80 ± 0.25
6	ND	1.51 ± 0.09	ND	1.30 ± 0.92	ND	2.41 ± 0.61
9	ND	1.60 ± 0.36	ND	1.13 ± 0.43	ND	1.42 ± 0.10
12	10.8 ± 0.8	1.38 ± 0.12	10.2 ± 2.2	0.88 ± 0.27	10.2 ± 0.9	0.87 ± 0.22
24	10.9 ± 1.2	0.77 ± 0.08	10.4 ± 0.60	0.65 ± 0.10	9.96 ± 0.01	0.45 ± 0.19

[a] Data are expressed as mean direct cell counts ± standard deviation from two experiments, each with triplicate samples.
[b] Data are expressed as mean viable cell counts ± standard deviation from two experiments, each with triplicate samples.
[c] ND, Not determined.

7) were probably more accurate measures of the ability of the cells to survive because the graphic method used the actual zero-time viable count values, which may have underestimated the original viable population due to cell aggregation effects. However, the differences in the ST_{50} values calculated by the two methods were not large in most cases. When the least-squares linear regression analysis procedures were used (24), the regressions of viable cell counts (dependent variable) versus time of starvation showed that the data followed a log-linear function (in most cases, $P < 0.05$) for cells grown at each dilution rate under either cellobiose- or nitrogen-limited conditions. When analysis of variance methods were used (22), the slopes of all of these regressions were found to be different from one another ($P < 0.05$) when they were compared between dilution rates under the same nutrient-limited growth conditions or between different nutrient-limited growth conditions at the same dilution rate. A regression of the ST_{50} values against the dilution rate was a linear function for both the cellobiose-limited cells ($r^2 = 0.89$; $P < 0.05$) and the nitrogen-limited cells ($r^2 = 0.79$; $P < 0.05$). In addition, the slopes of these ST_{50} regressions against dilution rate were different ($P < 0.05$) for cellobiose- and nitrogen-limited cells when they were examined by analysis of variance methods. Similar results were obtained for the last two relationships when the slopes of the regressions for viable counts against dilution rates were used in place of the ST_{50} values.

Starvation of chemostat-grown *Ruminococcus flavefaciens* at different pH values. Previous studies have shown that low pH has negative effects on the growth of cellulolytic rumen bacteria (26). To ascertain whether pH affected the viability of nongrowing *Ruminococcus flavefaciens*, suspensions of cells grown under either cellobiose- or nitrogen-limited conditions were starved in buffers at different pH values. Our data showed that there was a rapid loss of viability, particularly after 12 h of starvation, regardless of the pH of the starvation buffer (Table 8). When these data were statistically examined by using a least-squares regression analysis, the declines in viability followed log-linear functions (Table 9). The slopes of the regressions or the ST_{50} values were found to be different from one another ($P < 0.05$) by using analysis of variance methods when compared between cells starved at different pH values for either cellobiose- or nitrogen-growth limited cells. With cells from cellobiose-limited chemostats, the regression of ST_{50} values versus pH of starvation resulted in a linear function ($r^2 = 0.59$; $P < 0.05$), but with cells from nitrogen-limited chemostats a similar regression resulted in a nonsignificant, nonlinear function ($r^2 = 0.06$; $P > 0.05$).

DISCUSSION

Chemostat-grown *Ruminococcus flavefaciens* C94 cells grown under either cellobiose or nitrogen limitation appeared to survive longer when they were grown at lower dilution rates than when they were grown at higher dilution rates (Table 7). The effect of dilution rate was greater for cells that had been grown under cellobiose limitation than for cells that had been grown under nitrogen limitation. Since the cells that had been grown under cellobiose limitation also showed greater disparities between total and viable cell counts, the size of the viable population may have influenced the ability to survive. One way in which this could occur is by utilization by viable cells of cell constituents released by nonviable cells in the suspensions. The presence of cell aggregates in the zero-time suspensions may have partially contributed to the differences in survival due to the close proximity of the cells to one another, because in the aggregates the cell constituents of the nonviable cells would be less likely to be diluted in the buffer and therefore be more available to viable cells. It was obvious that the starving cells were degrading various cell macromolecules (Table 4), and presumably, some of the degradation products were released from the cells. However, except for the decreased rate of carbohydrate loss with nitrogen-limited cells from higher dilution rates, there were no consistent degradation patterns for cell materials. Comparisons of the losses in direct cell counts and dry weights (Table 4 versus Table 5 or 6) suggest that much of the losses in cell materials might be attributed to cell lysis.

For cells grown under either cellobiose or nitrogen limitation, a lower pH during starvation resulted in a poorer ability to survive (Tables 8 and 9). This effect was more apparent

TABLE 7. Analysis of viability losses with batch- or chemostat-grown *Ruminococcus flavefaciens* cell suspensions

Growth conditions	Limiting nutrient	Dilution rate (h^{-1})	Slope of regression	r^2	ST_{50}[a]
Batch	None		−0.0276	0.57	10.9
Chemostat	Cellobiose	0.13	−0.0128	0.75	24.1
		0.23	−0.0216	0.75	14.1
		0.33	−0.0455	0.88	6.6
	Nitrogen	0.13	−0.0184	0.95	16.5
		0.23	−0.0209	0.93	14.5
		0.33	−0.0323	0.89	9.5

[a] Determined by regression analysis of log(viable cell count) versus time starved, with the *y* intercept used for the zero-time viable cell count value.

TABLE 8. Changes in viability of chemostat-grown *Ruminococcus flavefaciens* cells during starvation under various pH conditions[a]

Limiting nutrient	Time starved (h)	Viable cell counts (× 10^8, CFU/ml)[b]				
		MES buffer		PIPES buffer		
		pH 5.5	pH 6.0	pH 6.0	pH 6.5	pH 7.0
Cellobiose	0	2.32 ± 0.24	2.87 ± 0.81	2.10 ± 0.17	1.85 ± 0.72	1.44 ± 1.04
	6	1.21 ± 0.31	1.28 ± 0.48	1.27 ± 0.05	1.66 ± 0.12	1.44 ± 0.05
	12	1.02 ± 0.02	1.22 ± 0.14	0.91 ± 0.12	1.31 ± 0.20	1.02 ± 0.06
	24	0.14 ± 0.16	0.27 ± 0.28	0.18 ± 0.75	0.21 ± 0.01	0.39 ± 0.20
Nitrogen	0	1.97 ± 0.60	1.61 ± 0.12	2.08 ± 0.64	2.97 ± 0.40	2.18 ± 0.10
	6	1.38 ± 0.84	1.26 ± 0.06	0.98 ± 0.59	0.85 ± 0.49	1.30 ± 0.92
	12	1.04 ± 0.17	0.96 ± 0.21	0.77 ± 0.17	1.43 ± 0.04	0.88 ± 0.27
	24	0.47 ± 0.04	0.49 ± 0.09	0.47 ± 0.10	0.51 ± 0.11	0.65 ± 0.10

[a] Cultures were grown at a dilution rate of 0.23 h^{-1} and a pH of 6.8 ± 0.1.
[b] Viable cell counts are expressed as means ± standard deviations from two experiments, each with triplicate samples.

for the cellobiose-limited cells than for the nitrogen-limited cells. The latter cells contained more carbohydrate in their cell material, and their better survival may be related to usage of this carbohydrate to generate energy for membrane potentials. Overall, our data were also consistent with the observation that ruminococci are also sensitive to pH during nutrient-limited growth in pure culture (26) or in mixed rumen type fermentors (30). Although there have been few investigations into interactions between pH and starvation, Lowendorf et al. (16) demonstrated a similar sensitivity of *Rhizobium melotii* to pH during starvation.

There are considerable advantages for bacterial species present in habitats that display frequent and extended periods of very low nutrient availability, such as soil or marine environments, to have the capacity to survive long periods of nutrient deprivation. The abilities of bacteria from these environments to survive during long periods of starvation have been demonstrated previously (16, 21, 25). However, for rumen bacteria like *Ruminococcus flavefaciens*, it may not be necessary to have the ability to survive for extended periods of starvation. For animals that are fed once or more per day, any period of bacterial starvation must be considerably shorter than 24 h. The ruminococci often adhere to the feed particles and are in close proximity to their nutrient source until the forage particle is largely degraded. When the old, nutrient-poor forage material with adherent ruminococci is mixed by rumen motility and rumination with freshly ingested forage material, the transit time for cells to detach from old forage and attach to fresh forage particles may be relatively short. These factors suggest that there is little selective advantage for ruminococci to be able to survive more than a few hours of starvation, and their ST_{50} values (Tables 7 and 9) seem to be adequate in this regard.

TABLE 9. ST_{50} analysis of chemostat-grown *Ruminococcus flavefaciens* cells starved at various pH values

Limiting nutrient	pH during starvation	Buffer	Slope of regression	r^2	ST_{50}[a]
Cellobiose	5.5	MES	−0.0574	0.95[b]	5.8
	6.0	MES	−0.0458	0.95[b]	7.3
	6.0	PIPES	−0.0456	0.97[b]	6.8
	6.5	PIPES	−0.0418	0.88[c]	7.2
	7.0	PIPES	−0.018	0.91[c]	14.1
Nitrogen	5.5	MES	−0.0259	0.99[b]	12.2
	6.0	MES	−0.0214	0.99[b]	14.1
	6.0	PIPES	−0.0293	0.91[b]	10.9
	6.5	PIPES	−0.0256	0.62[c]	11.8
	7.0	PIPES	−0.0212	0.92[c]	14.2

[a] Determined by regression analysis of $\log_{10}$(viable cell count) versus time starved, with the y intercept used for the zero-time viable cell count value.
[b] Regression was significant ($P < 0.05$).
[c] Regression was significant ($P < 0.10$).

Other species of rumen bacteria that have also been shown to have poor abilities to survive during starvation include *Megasphaera elsdenii* (19), *Selenomonas ruminantium* (18, 21), and mixed rumen bacteria (J. A. Z. Leedle and R. B. Hespell, Ill. Res. 21:14–15, 1979). The basis for this decreased survival capacity of rumen bacteria (and possibly gastrointestinal tract anaerobes in general) is not yet known. It may involve impaired regulatory factors for endogenous metabolism or loss of certain (starvation?) genes or both. However, studies with *S. ruminantium* have shown that starved cells do not lose a number of enzyme activities, suggesting that random proteolytic attack of internal enzymes is not a major causative factor.

LITERATURE CITED

1. **Ashwell, G.** 1966. The phenol-sulfuric acid reaction for carbohydrates. Methods Enzymol. **8**:93–95.
2. **Balch, W. E., G. E. Fox, L. J. Magrum, C. R. Woess, and R. S. Wolfe.** 1979. Methanogens: reevaluation of a unique biological group. Microbiol. Rev. **43**:260–296.
3. **Bryant, M. P.** 1972. Commentary on the Hungate technique for culture of anaerobic bacteria. Am. J. Clin. Nutr. **25**:1324–1328.
4. **Bryant, M. P., and I. M. Robinson.** 1961. Some nutritional requirements of the genus *Ruminococcus*. Appl. Microbiol. **9**:91–95.
5. **Bryant, M. P., and I. M. Robinson.** 1962. Some nutritional characteristics of predominant culturable ruminal bacteria. J. Bacteriol. **84**:605–613.
6. **Bryant, M. P., and I. M. Robinson.** 1968. Effects of diet, time after feeding, and position sampled on numbers of viable bacteria in the bovine rumen. J. Dairy Sci. **51**:1950–1955.
7. **Burton, K.** 1968. Determination of DNA content with diphenylamine. Methods Enzymol. **12**:163–166.
8. **Chaney, A. L., and E. P. Marbach.** 1962. Modified reagents for determination of urea and ammonia. Clin. Chem. **8**:130–132.
9. **Chen, P. S.** 1956. Microdetermination of phosphorus. Anal. Chem. **28**:1756.
10. **Good, H. E., G. D. Winget, W. W. Winter, T. Connolly, S. Izawa, and R. M. M. Singh.** 1966. Hydrogen ion buffers for biological research. Biochemistry **5**:467–477.
11. **Hungate, R. E.** 1950. The anaerobic mesophilic cellulolytic bacteria. Bacteriol. Rev. **14**:1–49.
12. **Johnson, R. R.** 1976. Influence of carbohydrate solubility on non-protein nitrogen utilization in the ruminant. J. Anim. Sci. **43**:184–191.
13. **Latham, M. J., B. E. Brooker, G. L. Pettipher, and P. J. Harris.** 1978. *Ruminococcus flavefaciens* cell coat and adhesion to

cotton cellulose and to cell walls in leaves of perennial ryegrass (*Lolium perenne*). Appl. Environ. Microbiol. **35**:156–165.

14. **Leedle, J. A. Z., M. P. Bryant, and R. B. Hespell.** 1982. Diurnal variations in bacterial numbers and fluid parameters in ruminal contents of animals fed low- or high-forage diets. Appl. Environ. Microbiol. **44**:402–412.
15. **Leedle, J. A. Z., and R. B. Hespell.** 1981. Differential carbohydrate media and anaerobic replica plating techniques in delineating carbohydrate-utilizing subgroups in rumen bacterial populations. Appl. Environ. Microbiol. **39**:709–719.
16. **Lowendorf, H. S., A. M. Baya, and M. Alexander.** 1981. Survival of *Rhizobium* in acid soils. Appl. Environ. Microbiol. **42**:915–957.
17. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
18. **Mink, R. W., and R. B. Hespell.** 1981. Long-term nutrient starvation of continuously cultured (glucose-limited) *Selenomonas ruminanatium*. J. Bacteriol. **148**:541–550.
19. **Mink, R. W., and R. B. Hespell.** 1981. Survival of *Megasphaera elsdenii* during starvation. Curr. Microbiol. **5**:51–56.
20. **Mink, R. W., J. A. Patterson, and R. B. Hespell.** 1981. Changes in viability, cell composition, and enzyme levels during starvation of continuously cultured (ammonia-limited) *Selenomonas ruminantium*. Appl. Environ. Microbiol. **44**:913–922.
21. **Novitsky, J. A., and R. Y. Morita.** 1977. Survival of a psychrophilic marine vibrio under long-term nutrient starvation. Appl. Environ. Microbiol. **33**:635–641.
22. **Ott, L.** 1977. An introduction to statistical methods and analysis, p. 487–493. Duxbury Press, Belmont, Calif.
23. **Pettipher, G. L., and M. J. Latham.** 1979. Production of enzymes degrading plant cell walls and fermentation of cellobiose by *Ruminococcus flavefaciens* in batch and continuous culture. J. Gen. Microbiol. **110**:29–38.
24. **Poole, L., and M. Borchers.** 1977. Some common Basic programs. Adam Osborne and Associates, Berkeley, Calif.
25. **Postgate, J. R., and J. R. Hunter.** 1963. The survival of starved bacteria. J. Appl. Bacteriol. **26**:295–306.
26. **Russell, J. B., and D. B. Dombrowski.** 1980. Effect of pH on the growth of rumen bacteria in continuous culture. Appl. Environ. Microbiol. **39**:604–610.
27. **Salinitro, J. P., and P. A. Muirhead.** 1975. Quantitative method for the gas chromatographic analysis of short-chain monocarboxylic and dicarboxylic acids in fermentation media. Appl. Microbiol. **29**:374–384.
28. **Schaefer, D. M., C. L. Davis, and M. P. Bryant.** 1980. Ammonia saturation constants for predominant species of rumen bacteria. J. Dairy Sci. **63**:1248–1263.
29. **Schneider, W. C.** 1957. Determination of nucleic acids in tissues by pentose analysis. Methods Enzymol. **3**:680–684.
30. **Slyter, L. L., M. P. Bryant, and M. J. Wolin.** 1966. Effect of pH on population and fermentation in a continuously cultured rumen ecosystem. Appl. Microbiol. **14**:573–578.
31. **Takahashi, R., and K. Nakamura.** 1969. Concentrations of free sugars in sheep rumen fluids. Agric. Biol. Chem. **33**:619–621.
32. **Warner, A. C. I.** 1962. Enumeration of rumen microorganisms. J. Gen. Microbiol. **28**:119–128.