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Changes in Viability, Cell Composition, and Enzyme Levels During Starvation of Continuously Cultured (Ammonia-Limited) *Selenomonas ruminantium*

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Under nitrogen (ammonia)-limited continuous culture conditions, the ruminal anaerobe *Selenomonas ruminantium* was grown at various dilution rates (D). The proportion of the population that was viable increased with D, being 91% at D = 0.5 h^{-1}. Washed cell suspensions were subjected to long-term nutrient starvation at 39°C. All populations exhibited logarithmic linear declines in viability that were related to the growth rate. Cells grown at D = 0.05, 0.20, and 0.50 lost about 50% viability after 8.1, 4.6, and 3.6 h, respectively. The linear rates of decline in total cell numbers were dramatically less and constant regardless of dilution rate. All major cell constituents declined during starvation, with the rates of decline being greatest with RNA, followed by DNA, carbohydrate, cell dry weight, and protein. The rates of RNA loss increased with cells grown at higher D values, whereas the opposite was observed for rates of carbohydrate losses. The majority of the degraded RNA was not catabolized but was excreted into the suspending buffer. At all D values, *S. ruminantium* produced mainly lactate and lesser amounts of acetate, propionate, and succinate during growth. With starvation, only small amounts of acetate were produced. Addition of glucose, vitamins, or both to the suspending buffer or starvation in the spent culture medium resulted in greater losses of viability than in buffer alone. Examination of extracts made from starving cells indicated that fructose diphosphate aldolase and lactate dehydrogenase activities remained relatively constant. Both urease and glutamate dehydrogenase activities declined gradually during starvation, whereas glutamine synthetase activity increased slightly. The data indicate that nitrogen (ammonia)-limited *S. ruminantium* cells have limited survival capacity, but this capacity is greater than that found previously with energy (glucose)-limited cells. Apparently no one cellular constituent serves as a catabolic substrate for endogenous metabolism. Relative to losses in viability, cellular enzymes are stable, indicating that nonviable cells maintain potential metabolic activity and that generalized, nonspecific enzyme degradation is not a major factor contributing to viability loss.

A number of factors, both environmental and intrinsic to the microorganism in question, can affect microbial survival under conditions of total nutrient starvation (11, 12, 36, 46). One factor of particular importance is the nature of the nutrient which limits growth before starvation. Postgate and Hunter have shown that nitrogen-limited cells of *Aerobacter* (*Enterobacter*) *aerogenes* exhibited better survival capabilities than did carbon-limited populations (36). Unfortunately, there have been few similar investigations with other microorganisms because of the tendency of most researchers to study starvation of microorganisms using batch cultures, which are not growth limited until the late exponential phase, and often the limiting nutrient(s) is unknown. In addition, there have been numerous studies on the survival of aerobes, but relatively few on strictly anaerobic bacteria, particularly those which are found in the rumen, cecum, or large intestine of mammals. Within the rumen, the levels of many nutrients are constantly changing due to the discontinuous ingestion of feedstuffs and microbial degradation of these materials. Analyses for organic or inorganic nitrogen (27, 32, 34, 53) and for carbohydrates (23, 45; J. A. Z. Leedle, Ph.D. thesis, University of Illinois, Urbana, 1981) have shown that large variations can occur in rumen contents, depending upon the time after feeding, the type of diet fed, and the composition of the microbial population present. These variations not only affect the growth of various rumen bacterial

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species, but ultimately affect the abilities of the microorganisms to survive, as reflected in the large changes seen in the viable to direct cell counts in rumen contents (J. A. Z. Leedle, Ph.D. thesis, University of Illinois, Urbana, 1981).

In previous studies, we examined the survival abilities of the ruminal anaerobes *Megasphaera elsdenii* (29) and *Selenomonas ruminantium* (30) that had been grown under energy (glucose) limitation using steady state continuous culture conditions. In the current study, we examined with continuous culture conditions the effects of nitrogen (ammonia) limitation and growth rate on the viability and cell composition of *S. ruminantium* during nutrient starvation. In addition, the stability of selected glycolytic and ammonia-assimilating enzymes in these cells was examined.

MATERIALS AND METHODS

Organism and culture conditions. *Selenomonas ruminantium* strain D (22) was obtained from the culture collection of the Department of Dairy Science, University of Illinois, Urbana. Stock cultures were maintained on carbohydrate maintenance agar slants (6) and transferred twice a month. All medium preparation and experimental manipulations were carried out anaerobically as previously described (30). Continuous cultures were grown in an anaerobic chemostat maintained at 39 ± 0.5°C (20). Steady state conditions were verified by constant absorbance measurements (660 nm) and constant cell RNA levels measured as described below. Verification of ammonia-limited growth was made by measurement of both glucose and ammonia levels in spent culture media using the glucose oxidase (Sigma Chemical Co., bulletin 510) and indophenol methods (9, 19), respectively.

The growth medium contained (amount per 100 ml): NH_4Cl, 10 mg; NaCl, 60 mg; KCl, 60 mg; $MgSO_4$, 26 mg; $CaCl_2 \cdot 2H_2O$, 20 mg; hemin, 20 μg; salts and trace elements, 0.1 ml; resazurin, 100 μg; sodium acetate (anhydrous), 246 mg; glucose (1 M), 5.0 ml; potassium phosphate (0.5 M), 1.4 ml; *S. ruminantium* vitamins, 0.5 ml; $Na_2S \cdot 9H_2O$, 50 mg; and Na_2CO_3, 400 mg. Salts and trace elements, glucose, potassium phosphate, vitamins, and sodium carbonate were prepared and added as previously described (30). Hemin was added as an aqueous solution of 0.01% (wt/vol) hemin–25% (vol/vol) ethanol–0.28% (wt/vol) KOH. Sodium sulfide was added as an anaerobic aqueous 2.5% (wt/vol) solution.

Starvation buffer and cell collection and washing. The starvation buffer (S buffer) was a mineral salts buffer containing 1.5 mM dithiothreitol as the reducing agent and was prepared anaerobically as previously described (25). Cell culture samples were collected by centrifugation (8,800 × *g*, 10 min, 15°C). The cells were washed twice at room temperature by centrifugation using S buffer followed by a final resuspension in S buffer to the same cell concentration as the original growth medium. Suspensions (1.5 liters) of *S. ruminantium* were starved in 2-liter serum flasks and incubated with shaking (39°C, N_2 atmosphere).

Total and viable cell counts. Total cell counts were determined by a direct microscopic count using Petroff-Hausser chambers with samples of cultures diluted 1:10 or 1:20 in anaerobic dilution solution. Viable cell counts were determined by serial 10-fold dilution in anaerobic dilution solution followed by plating various dilutions on recovery medium. The plates were incubated (under CO_2, 39°C, 5 days) in anaerobic incubation vessels (25), and the colonies on each plate were counted. Anaerobic dilution solution had the same composition as the growth medium used, but lacked the glucose, vitamins, and sodium carbonate. Recovery medium composition was identical to growth medium but also contained 1.5 g of agar per 100 ml.

Cell analyses. Absorbance of cultures was measured at 660 nm using cuvettes with a 1-cm light path. Dry weights were determined by drying (110°C, 15 h) the pellet of an anaerobically centrifuged (8,800 × *g*, 10 min, 4°C) cell culture or suspension of known volume. Cell components were extracted as previously described (18) and assayed by the diphenylamine method of Burton (8) for DNA, the orcinol method of Schneider (41) for RNA, the method of Lowry et al. (26) for protein, and the phenol-sulfuric acid method for carbohydrate (1).

Analyses of growth media and suspending buffers. Fermentation acids were measured by gas-liquid chromatography using either of two methods: (i) preparation and analysis of butyl esters (39) or (ii) analysis of free acids (30). The starvation buffer was also analyzed for material absorbing at 260 and 280 nm to measure levels of nucleotide bases or nucleic acids or both (52). Ribose or RNA or both in the buffer were measured by the orcinol method described above.

Enzyme activities. Cell suspensions were centrifuged (10,000 × *g*, 10 min, 4°C) and washed twice in 50 mM Tris-hydrochloride–1% (wt/vol) KCl–1 mM dithiothreitol (TKD) buffer (pH 7.4) and resuspended in TKD buffer under argon to 1% (vol/vol) of their original volume. Cell-free extracts were prepared by passing cell suspensions twice through a French pressure cell (20,000 lb/in^2) at 4°C under argon. After centrifugation (15,000 × *g*, 20 min, 4°C), the cell-free extract supernatant was stored under argon on ice until used (1 to 2 h). Protein in the cell-free extracts was measured as described above. Fructose diphosphate aldolase (FDA) activity was measured by the coupled NAD reduction reaction of glyceraldehyde-3-phosphate dehydrogenase as described by Hespell and Canale-Parola (16). One unit of FDA activity was defined as the amount of enzyme required to reduce one nanomole of NAD per minute. Lactate dehydrogenase (LDH) activity was determined as the rate of NADH oxidation. The reaction mixture was prepared in 3-ml cuvettes by mixing 1.8 ml of 28 mM Tris-hydrochloride (pH 7.0), 0.1 ml of 4 mM NADH, and 0.025 to 0.05 ml of a 1:10 dilution (in TKD buffer) of cell-free extract (5 to 50 mg of protein per ml). The reaction was initiated by the addition of 0.1 ml of 0.1 M sodium pyruvate.

One unit of LDH was defined as the amount of enzyme required to oxidize one nanomole of NADH per minute. The urease assay was carried out using Warburg-type incubation vessels with a center well containing 0.05 ml of 2.0 M KOH (CO_2-free). The reaction chamber contained 0.5 ml of 25 mM KH_2PO_4

(pH 7.2), 0.1 ml of 250 mM tetrasodium EDTA (pH 7.2), 0.2 ml of water, and 0.1 ml of [^{14}C]urea (10 mM, 150,000 dpm/μmol). The side arm contained 0.05 to 0.1 ml of cell suspensions (50 to 500 mg of protein). The assay was begun by mixing the cells in the side arm with the contents of the reaction chamber. After 20 min at 30°C, 0.1 ml of 3.0 N H_2SO_4 was added to the side arm and mixed with the contents of the reaction chamber to stop the assay. The KOH in the center well was assayed for $^{14}CO_2$ using a Beckman scintillation counter (model LS230) with Aquasol-2 (New England Nuclear Corp.) as the scintillation cocktail. One unit of urease was defined as the amount of enzyme required to hydrolyze one nanomole of urea per minute. Glutamate dehydrogenase (GDH) activity was measured as the rate of NADPH oxidation by the procedure of Meers et al. (28) as modified by Smith et al. (42). One unit of GDH activity was defined as the amount of enzyme required to oxidize one nanomole of NADPH per minute. Glutamine synthetase (GS) activity was measured in whole-cell suspensions by the forward reaction assay described by Bender et al. (2) as modified by Smith et al. (42). One unit of GS activity was defined as the amount of enzyme required to form one nanomole of γ-glutamyl hydroxamate per minute.

Statistical analyses. Cell composition data were subjected to linear or nonlinear regression (least squares) analysis or both as described previously (30).

RESULTS

Cell composition and viability before starvation. For experiments to determine the effects of starvation on ammonia-limited continuously cultured *S. ruminantium* strain D, the organism was grown at three different dilution rates (D = 0.05, 0.20, and 0.50 h^{-1}). There were no large differences in total cell numbers per milliliter between dilution rates. However, the proportion of the population that was viable was directly related to the dilution rate and ranged from 30.1% for cells grown at D = 0.05 h^{-1} to 91.1% for cells grown at D = 0.50 h^{-1} (Table 1). Analyses of cellular components indicated that there were only small differences between dilution rates for cellular dry weight and levels of protein and DNA. In contrast, faster-grown cells tended to contain greater levels of RNA and lesser levels of carbohydrate than slower-grown cells. Analyses of cell suspensions before and after washing in the S buffer (but before starvation) indicated no significant loss of total or viable cell numbers, cell integrity, or any particular cellular component.

Changes in cell numbers during starvation. The survival of cell suspensions subjected to starvation was measured in terms of both total and viable cell numbers (Fig. 1). In general, total cell number declined modestly, about 30% after 48 h of starvation, while viability declined much more rapidly, to less than 1% of the initial level during the same period. Populations grown at each of the three dilution rates exhibited a

TABLE 1. Cell composition and numbers of ammonia-limited *S. ruminantium* strain D before starvation[a]

D (h^{-1})	Cell population[b]	No. of cells[c]		Cell constituents (μg/ml)			
		Total	Viable	Dry wt	DNA	RNA	Protein	Carbohydrate
0.05	Prewash	2.46 ± 0.26	0.74 ± 0.06	552 ± 42	2.61 ± 0.08	67.1 ± 6.2	321 ± 28	36.5 ± 30.8
	0 h	2.58 ± 0.45	0.61 ± 0.11	635 ± 35	2.61 ± 0.25	64.9 ± 15.8	318 ± 36	32.5 ± 27.4
0.20	Prewash	3.16 ± 0.45	1.68 ± 0.02	673 ± 47	2.72 ± 0.69	83.5 ± 6.3	311 ± 29	17.0 ± 5.2
	0 h	3.15 ± 0.33	1.49 ± 0.19	620 ± 0	2.68 ± 0.08	70.6 ± 5.8	307 ± 25	17.2 ± 6.4
0.50	Prewash	2.58 ± 0.81	2.35 ± 0.31	633 ± 45	1.85 ± 0.11	93.0 ± 15.5	311 ± 10	12.7 ± 6.5
	0 h	2.72 ± 0.41	2.94 ± 0.20	650 ± 141	1.65 ± 0.62	84.9 ± 1.0	296 ± 14	10.3 ± 4.6
$\bar{X}$[d]	Prewash	2.73 ± 0.54	1.59 ± 0.74	619 ± 65	2.39 ± 0.52	81.2 ± 14.2	315 ± 19	22.1 ± 18.2
	0 h	2.82 ± 0.41	1.68 ± 1.06	635 ± 67	2.31 ± 0.60	73.5 ± 11.9	307 ± 23	20.0 ± 16.3

[a] The organism was grown in continuous culture at the dilution rate (D) indicated, washed, and resuspended in S buffer as specified in the text. The data are the means ± standard deviations of two experiments at each D.

[b] Prewash denotes unwashed cells from the growing culture; 0 h denotes cells washed and resuspended in S buffer and at the initiation of starvation.

[c] Cell number ($\times 10^9$) per milliliter of culture or suspension.

[d] Grand mean of all of the data from the above D values.

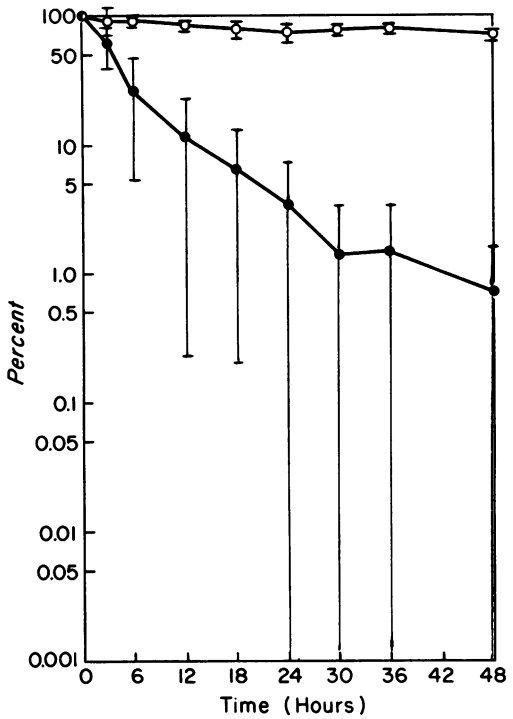

FIG. 1. Changes in the viable (●) and total (○) cell numbers of ammonia-limited *S. ruminantium* during starvation. The data are the grand means of two experiments each of populations grown at D = 0.05, 0.20, and 0.50 h^{-1}. The data are expressed as the percentage of the values at 0 h (Table 1).

steady decline in viability. The populations grown at D = 0.05 h^{-1} consistently exhibited the best survival, and those grown at D = 0.50 h^{-1} exhibited the poorest. Regression analysis of the cell number data indicated that the loss in viability was log-linear for all three populations and that the 50% survival times (ST_{50}) were 8.07, 4.61, and 3.56 h for populations grown at D = 0.05, 0.20, and 0.50 h^{-1}, respectively (Table 2). However, no major differences in the rates of loss of total cell numbers were observed between populations grown at different dilution rates.

Changes in cell composition. The decreases in cell dry weight during starvation were more rapid than decreases in total cell numbers, indicating an overall decline in individual cell mass (Table 2). Total cell numbers remained consistently high, and relatively little cell debris was seen in the suspending buffer until quite late into the starvation time. Of the cellular constituents, DNA was generally lost relatively quickly, although those losses were still at far slower rates than the declines in viability (Table 2 and Fig. 2). Protein levels showed no indication of selective degradation, as their losses closely paralleled those of dry weight. Losses in carbohydrate were relatively low, but appeared to be related to the growth rate at which the organism was grown, with the most rapid losses observed in the cells grown at the lowest dilution rate (D = 0.05 h^{-1}). Of all of the cellular constituents, RNA losses were the greatest for each starving population and were directly related to the growth rate before starvation. An analysis of the starvation buffer indicated that most, if not all, of the lost RNA was apparently expelled outside of the cell, based on the measurement of orcinol-reactive material and material absorbing at 260 nm found in the buffer (data not shown). Similar results were found with starvation of glucose-limited cells (30). However, the levels of orcinol-reactive material in the buffer of cell suspensions grown at D = 0.20 h^{-1} did show a subsequent decline after 24 h of starvation (data not shown), which could not be accounted for in terms of increase in mass or in any component of the cell suspension.

Fermentation acids produced. *S. ruminantium* during ammonia-limited growth produced primarily lactate, regardless of dilution rate (Table 3). The tendency for higher acid levels at lower dilution rates was primarily a reflection of increased glucose utilization rather than differences in molar ratios of acids produced per mole of glucose. The exception to this observation

TABLE 2. Rates of change in population parameters of *S. ruminantium* strain D (ammonia-limited) during starvation

D (h^{-1})	Rate of change in no. of cells[a]		ST_{50}[b] (h)	Rate of change of cellular components[a]				
	Total	Viable		Dry wt	DNA	RNA	Protein	Carbohydrate
0.05	−0.0051	−0.0858	8.07	−0.0099	−0.0198	−0.0492	−0.0097	−0.0205
0.20	−0.0069	−0.1502	4.61	−0.0133	−0.0504	−0.0637	−0.0106	−0.0094
0.50	−0.0060	−0.1944	3.56	−0.0117	−0.0501	−0.0729	−0.0069	−0.0048

[a] The data were subjected to linear regression analysis (see the text), and the rates are the fractional change per hour as determined from the slopes of the regression lines. All regression lines were significant at $P < 0.005$ or better.

[b] ST_{50} is the time for 50% of the initial viable population to become nonviable, calculated from the fractional turnover rate (m) of the regression line of viable cell numbers where $ST_{50} = \ln(2/m)$.

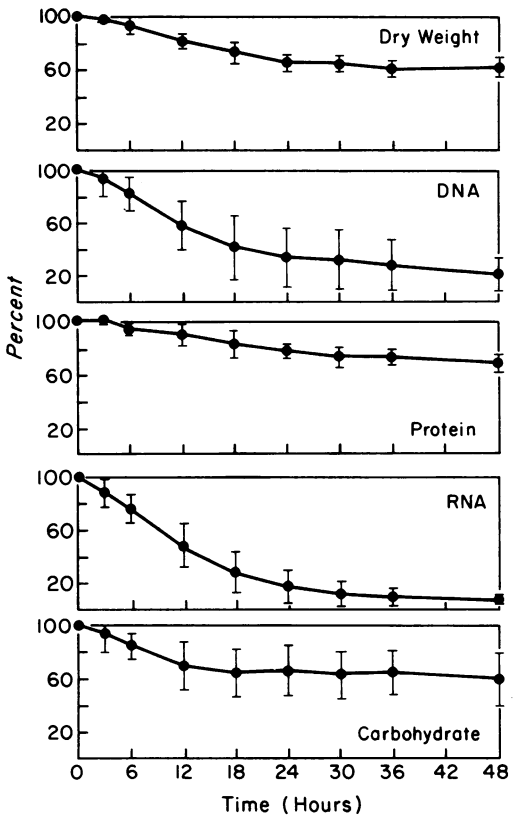

FIG. 2. Changes in cellular dry weight, DNA, RNA, protein, and carbohydrate of *S. ruminantium* during starvation. The data are the grand means ± the standard deviation of two experiments each of populations grown at D = 0.05, 0.20, and 0.50 h^{-1}. The data are expressed as a percentage of the levels at 0 h (Table 1).

occurred with succinate, which was produced at rates of 0.30, 0.18, and 0.13 mol per mol of glucose degraded for D = 0.05, 0.20, and 0.50 h^{-1}, respectively. During starvation, fermentation acids were monitored, and only acetate was found in the suspending buffer. The levels of acetate present increased during starvation, with acetate levels being 90 to 140 nmol/ml after 36 h of starvation. Although differences in acetate levels between starving populations grown at different dilution rates were observed, they were not significant. The acetate present in the buffer could not be attributed to contamination from the high levels of acetate present in the spent growth medium, since 0-h suspensions had no significant levels of lactate present despite the fact that spent growth medium contained as much or more lactate than acetate.

Enzyme activities during starvation. In an effort to determine if premature loss of catabolic or biosynthetic enzyme activities may have contributed to losses in viability, selected enzymes were assayed in starving suspensions of *S. ruminantium*. The glycolytic enzymes, FDA and LDH, exhibited essentially no significant changes in activity during the entire 48 h of the experiment, although whole-cell protein levels exhibited a slight decline (Fig. 3). The nitrogen metabolism enzymes, urease, GDH, and GS, were also analyzed, and although changes in activity were observed (Fig. 4), those changes were not great in comparison to viability changes (Fig. 1). Total activities of both urease and GDH decreased, but specific activities of these enzymes did not. GS, on the other hand, exhibited a slight increase in total activity, which would reflect a significant increase in specific activity. Similar responses were observed with all populations regardless of the dilution rate at which the cells had been previously grown.

Effect of nutrients on survival. To determine the sparing effects, if any, of selected nutrients on survival during starvation, *S. ruminantium* was grown at D = 0.20 h^{-1} and suspended in S buffer alone or supplemented with vitamins or glucose or both or resuspended in the spent growth medium after washing. The results (Table 4) indicated that none of the treatments had any positive survival effects over starvation in S buffer alone, and in fact, they may have hastened death in some cases. The initial death rate for cells resuspended in buffer supplemented with both glucose and vitamins appeared to be more than three times greater (56.5% loss in

TABLE 3. Levels of fermentation acids in the medium during ammonia-limited growth of *S. ruminantium* strain D[a]

Fermentation acid	Level of acid (μmol/ml) for the following dilution rates:		
	0.05 h^{-1}	0.20 h^{-1}	0.50 h^{-1}
Acetate	7.65 ± 1.08	3.81 ± 2.69	4.23 ± 0.97
Propionate	5.28 ± 2.08	3.17 ± 1.39	1.47 ± 0.66
Lactate	43.30 ± 1.03	36.20 ± 5.25	25.60 ± 0.41
Succinate	12.20 ± 1.14	5.32 ± 2.87	3.00 ± 1.33

[a] Cells were grown in continuous culture at the indicated dilution rate as described in the text. The data are the means ± standard deviation of two experiments at each D value. Glucose utilization was 41.0, 29.8, and 23.8 μmol/ml for cells grown at D = 0.05, 0.20, and 0.50 h^{-1}.

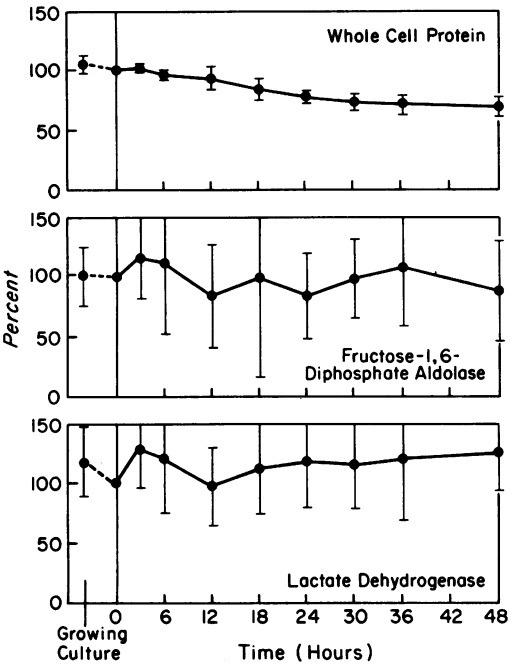

FIG. 3. Changes in whole-cell protein and activities of FDA (fructose-1,6-diphosphate aldolase) and LDH (lactate dehydrogenase) of an *S. ruminantium* strain (ammonia limited) during starvation. The data are expressed as the percentage of the values at 0 h. The 0-h values are as follows (per ml of cell suspension): whole-cell protein, 299 μg; FDA, 12.1 U; and LDH, 680 U. All data are the means ± the standard deviation of seven experiments (two replicates of D = 0.05, 0.20, 0.50, and one at D = 0.35).

viability) than for cells starved in S buffer alone (15.8% loss in viability).

DISCUSSION

When compared with the other bacteria (Table 5), the survival of ammonia-limited, continuously cultured *S. ruminantium* was relatively poor, having ST_{50} values of 3.5 to 8 h. Yet, the survival of *S. ruminantium* was significantly better (ST_{50} = 0.5 to 2.5 h) than when it was grown under glucose limitation before starvation (30). With the ammonia-limited populations, the fastest-grown cells (D = 0.50 h^{-1}) had the highest ratio of viable to total cells in the culture, but also had the poorest survival. This may simply have reflected decreased availability of dead cell materials available for cryptic growth. This explanation alone would seem unlikely, however, as cells starved in spent growth medium, which was likely to contain an abundance of products from lysed or dying cells, exhibited no greater survival ability than those starved in S buffer (Table 4). The likelihood of a role of cryptic growth is further diminished by the observation that both total cell numbers and dry weight declined slowly (Table 2). In addition, these losses in cell mass and numbers were slower than those which were observed with glucose-limited cells, which survive even more poorly and have greater cell lysis (30). An alternate explanation of short survival times of *S. ruminantium* could be that the slower-grown cells survived better because they were better adapted to starvation by their slow growth rate. Yet, just the opposite results were obtained with starvation studies of *Aerobacter aerogenes* (36) and *Megasphaera elsdenii* where faster-grown cells were found to survive better (29).

A relatively rapid loss of cellular DNA compared with dry weight occurred during the initial 12 h of starvation (Table 2 and Fig. 2). Since only a minor portion of the DNA loss could be attributed to cell lysis, it is probable that the DNA loss was indicative of the degradation of excess genome copies. However, such degradation probably played an insignificant role in providing the cell with endogenous substrates to withstand starvation, as the DNA represented a relatively small amount of organic material available to the cell (Table 1). Nevertheless, the possibility exists that such degradation, if uncontrolled, could have caused cell death via lethal lesions in the DNA.

The cellular component which exhibited the most rapid decline among all populations was RNA (Table 2 and Fig. 2). Although this suggests that RNA is a preferred substrate for endogenous metabolism, the analysis of the starvation buffer indicated that most of the RNA appears to be partially broken down and released into the starvation buffer without being completely catabolized. Similar results were observed previously with the starvation of glucose-limited *S. ruminantium* cells (30). However, since the latter had lower initial levels of RNA, the RNA was depleted in a shorter time despite the fact that both types of cells degraded RNA at similar rates on an absolute scale. Thus, it appears likely that glucose-limited cells would begin degradation of essential RNA sooner than the cells in this study and that this may account, in part, for their poorer survival. RNA degradation is a common phenomenon among bacteria during starvation and has been reported for *Peptococcus prevotii* (37), *Aerobacter aerogenes* (44), *Pseudomonas aeruginosa* (15), *Streptococcus lactis* (47), *Escherichia coli* (21), *Megasphaera elsdenii* (29), and *Bdellovibrio bacteriovorus* (17), to name a few examples. The primary site of RNA degradation is generally attributed to ribosomes due to the minimal need for protein synthesis under starvation conditions (24). In contrast to RNA, protein degradation in starving *S. ruminantium* was at a rate less than

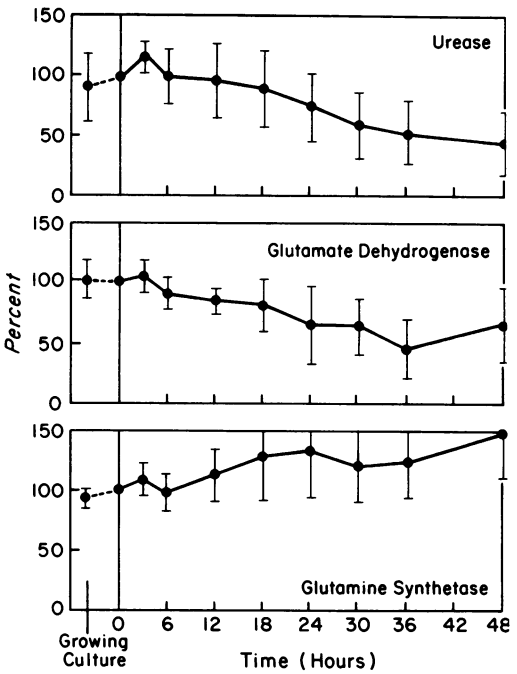

FIG. 4. Changes in urease, GDH, and GS of *S. ruminantium* (ammonia limited) during starvation. The data are expressed as the percentage of the values at 0 h. The 0-h values were: whole-cell protein, 299 μg; urease, 24.0 U; GDH, 15.2 U; and GS, 81.6 U. All data are the means ± the standard deviation of seven experiments (two replicates of D = 0.05, 0.20, 0.50, and one at D = 0.35).

or equal to that of dry weight loss, indicating little preferential degradation of this component (Table 2).

Carbohydrate loss in starving *S. ruminantium* varied inversely with the dilution rate at which the population was grown. At the higher dilution rates, the loss was less rapid than that of dry weight, suggesting that the degradation was not preferential. On the other hand, cells grown at D = 0.05 h^{-1} displayed a carbohydrate degradation rate double that of dry weight loss (Table 2). Often, bacterial species under ammonia limitation accumulate carbohydrate reserves such as glycogen, and this is also the case with *S. ruminantium* (51). Starvation studies of glycogen-rich versus glycogen-poor populations of other organisms have revealed that the former exhibit better survival overall (13, 43). The carbohydrate levels present in this particular *Selenomonas* strain are lower regardless of growth rate than in some other strains of *Selenomonas* (R. Mink, unpublished data; 51) and are lower than that of *M. elsdenii* (29) or other large rumen bacteria or protozoa (10). Nevertheless, this carbohydrate pool present in *Selenomonas* may well play a critical role in its survival, as the slower-grown cells survived best and contained the highest levels of carbohydrate.

During growth, considerable energy uncoupling occurred due to the presence of excess glucose and having ammonia as the limiting factor for growth. In going from lower to higher dilution rates, the cell yields were slightly higher, while both glucose utilization and acid production decreased (Table 3). This suggests that at higher dilution rates, growth was more efficient and there was less energetic uncoupling. In contrast, the environment drastically changed during starvation, and the cells most likely became energy deficient. Acid production shifted to the more energy-efficient acetogenesis and away from inefficient lactate production. Similar results were seen with the starvation of glucose-limited *S. ruminantium* (30) and, to a lesser extent, in *M. elsdenii* (29). However, with the starvation of glucose-limited *S. ruminantium*, acetate production was higher than was observed in this study. This may reflect a more controlled, energetically coupled endogenous metabolism in the ammonia-limited cells during starvation.

Peptococcus prevotii, another anaerobe, produces a combination of acids plus CO_2, H_2, and ammonia during starvation as well as with growth during which the organism ferments nucleosides, purines, and ribose (37). Thus, this organism is already substantially prepared to, and does, ferment RNA as an endogenous substrate during starvation. *P. prevotii*, therefore, does not have to make a major metabolic change at the onset of starvation. This may account for its better survival (Table 5) over *S. ruminantium*. The failure of *S. ruminantium* to form succinate or propionate during starvation may have been due to the inability of the cells to incorporate CO_2. Propionate formation is derived by decarboxylation of succinate which is formed by incorporation of CO_2 into phosphoenolpyruvate to form oxaloacetate (33, 40). Succinate is then ultimately formed by reduction reactions. However, we utilized starvation conditions with a CO_2-free atmosphere, and the amount of CO_2 contributed by the cell in the production of acetate may have been insufficient to drive the formation of oxaloacetate (and therefore succinate and propionate) significantly. Indeed, the addition of CO_2 to the starvation environment might have improved survival by virtue of the possibility that propionate production has been suggested to produce up to three ATPs per propionate in addition to the ATPs formed during glycolysis (19). If so, then survival of *S. ruminantium* would be enhanced in the rumen environment which contains substantial levels of bicarbonate derived from microbial fermentation and saliva input.

TABLE 4. Effect of nutrients on survival of *S. ruminantium*[a]

Starvation condition	% Viability	
	3 h	12 h
Buffer	84.2	6.4
Buffer plus glucose	75.9	6.1
Buffer plus vitamins	59.1	4.2
Buffer plus glucose plus vitamins	43.5	6.8
Spent growth medium	62.8	8.8

[a] The organism was grown in an ammonia-limited continuous culture at D = 0.20 h^{-1} and then washed, resuspended, and starved in S buffer, with or without additions, or in spent growth medium. Glucose and vitamins, when added, were present at 11.1 mM and 0.5% (vol/vol), respectively. The data are expressed as the percentage of viable cell numbers at 0 h (1.66×10^9/ml) and are the means of two experiments.

A possible explanation for the rapid death of *S. ruminantium* during starvation could be the loss of essential enzyme activities. The apparent level of LDH remained high during starvation, but its activity is controlled by pyruvate which acts as a homotropic activator (50). One would expect pyruvate to be low in starving cells in view of its need in biosynthetic reactions of fatty acid and amino acid synthesis, as well as the catabolism to acetate for energy. The high activity observed may have been an artifact of the assay, which contained added pyruvate. Thus, in the cells, LDH may have been inactive, and this is consistent with the lack of lactate production by starving cells. The apparent increase of GS during starvation could be expected, since GS tends to be more important under low-nitrogen conditions than is GDH (42). Yet, urease, which usually parallels GS activity, inexplicably showed no increase in activity. These particular enzymes were by no means all of the enzymes needed by the cells to maintain viability, but they could be considered representative of the cells' enzymes. The fact that none were preferentially degraded implicates a cause other than nonspecific enzyme breakdown in the loss of viability during starvation.

The addition of glucose or vitamins or both to starving cell suspensions did not aid survival (Table 4). When added, substantial amounts of glucose were fermented by the starving cells (data not shown). This suggests that neither a shortage of energy nor the inability to get at endogenous substrates due to a lack of cofactors was the cause of lost viability. Similar results were seen with glucose-limited *S. ruminantium* (29); however, unlike their ammonia-limited counterparts, the glucose-limited cells survived better in spent growth medium.

Why do microorganisms exhibit such marked differences (Table 5) in survival? We and others have alluded to the roles of such environmental variables as growth rate, limiting nutrient, and composition or complexity of the growth medium, but apparent intrinsic differences between microorganisms may be more important. Some organisms may be intrinsically better equipped to efficiently turn over cellular constituents and to scavenge for needed materials both intracellularly and extracellularly. Various bacteria may have different ATP requirements to maintain essential cell functions (35, 48). The specific type of energy metabolism an organism possesses may affect its survival ability. For example, microorganisms that rely on oxidative phosphorylation may obtain energy more efficiently from catabolism of cell materials than those whose sole source is substrate-level phosphorylation. Thus, aerobes appear to survive much better than anaerobes (Table 5). Photosynthetics can survive in an anaerobic nonorganic environment much longer if a source of light for energy generation is available (Table 5). *S. ruminantium* has high rates of loss in dry weight (Table 2) that reflect a high loss of carbon, and the organism is relegated to substrate phosphorylation for energy. Both factors probably contribute greatly to its short survival, whereas extremely low carbon losses and aerobic metabolism probably form the basis of the very long survival of *Arthrobacter crystallopoietes* (Table 5) (3).

S. ruminantium tends to be more numerous in

TABLE 5. Survival times of various bacterial species[a]

Organism	ST_{50} (h)[b]	Reference
Selenomonas ruminantium (N limited)	3.6–8.1	Current study
S. ruminantium (C limited)	0.5–2.5	30
Megasphaera elsdenii (C limited)	3–5	29
Bdellovibrio bacteriovorus	10	18
Peptococcus prevotii	10–12	31
Methanospirillum hungatii	ca. 25	4
Escherichia coli	36	14
Sarcina lutea	65	7
Chromatium vinosum	120 (dark)	49
Rhodospirillum rubrum	344 (light)	5
	12 (dark)	
Nocardia corallina	480	38
Arthrobacter crystallopoietes	2,400	3

[a] Starvations were at physiological temperatures (30 to 37°C) in nonnutrient buffers.

[b] Time required for half of the initial population to die.

the rumens of animals that are fed high-energy diets containing much grain. Because these diets contain substantial amounts of soluble carbohydrates and are more frequently fed to the animals (usually lactating cows or beef lot steers) at high intake levels, more continuous levels of soluble sugars are available for the growth of *S. ruminantium*. Thus, increased *S. ruminantium* numbers may result, in part because the organism is not significantly subjected to nutrient starvation which would lead to rapid death as our studies have shown. On the other hand, high-forage diets are often fed to animals less frequently and contain less soluble carbohydrates. With these latter diets, *S. ruminantium* is less numerous, probably because of several factors, including increased viability losses due to nutrient starvation resulting from lack of soluble sugars in the rumen fluid. Although potential nutrients would be associated with the particulate plant matter with high-forage diets, *S. ruminantium* apparently does not attach to these materials and does not degrade either cellulose or xylans. However, regardless of the type of diet fed, one would expect that if dietary nitrogen limited microbial growth, greater viable cell numbers of *S. ruminantium* (and other ruminal bacteria?) would be sustained as ammonia-limited cells survive better. Finally, it may be noted that during starvation, *S. ruminantium* rapidly loses viability, but apparently maintains enzyme stability and catabolic potential. If a similar situation exists for most other ruminal bacteria, then in the rumen, fermentative capacity may be more related to total cell numbers than to actual viable cell numbers.

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