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The Effect of Growth and Starvation on the Lysis of the Ruminal Cellulolytic Bacterium *Fibrobacter succinogenes*

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Growing cultures of *Fibrobacter succinogenes* assimilated more ammonia than could be accounted for by cellular protein, RNA, or DNA and released large amounts of nonammonia nitrogen. The difference between net and true growth was most dramatic at low dilution rates, but mathematical derivations indicated that the lysis rate was a growth rate-independent function. The lysis rate was sevenfold greater than the true maintenance rate (0.07 h^{-1} versus 0.01 h^{-1}). Because slowly growing cells had as much proton motive force and ATP as fast-growing cells, lysis was not a starvation response per se. Stationary-phase cells had a lysis rate that was 10-fold less than that of growing cells. Rapidly growing cells were not susceptible to phenylmethylsulfonyl fluoride, but phenylmethylsulfonyl fluoride increased the lysis rate of the cultures when they reached the stationary phase. This latter result indicated that autolysins of stationary-phase cells were being inactivated by a serine proteinase. When growing cells were treated with the glycolytic inhibitor iodoacetate, the proteinase-dependent transition to the stationary phase was circumvented, and the rate of lysis could be increased by as much as 50-fold.

In ruminant animals, microbial protein is the primary source of protein for the animal, and microbial protein turnover is a wasteful process that decreases amino acid availability. Protozoal predation has often been cited as the prime cause of bacterial protein turnover in the rumen (28), but Krebs et al. (18) noted high rates of bacterial turnover in defaunated sheep. Leng and Nolan (19) listed autolysis as a contributing factor, but this process was not described.

Brock and Madigan (2) cited bacterial lysis in their discussion of the stationary phase, but it should be realized that bacteria need autolytic enzymes to expand their cell wall and grow. Because bacterial cells have a very high turgor pressure (22), the action of autolytic enzymes must be carefully orchestrated. If autolytic activity is too low, growth rate will be sacrificed, but excessive autolytic activity could cause cell lysis.

In the surface stress model of Koch (16, 17), peptidoglycan is first deposited at the inner surface, and the older, outer layers are then cut by autolytic enzymes. This continual process of synthesis and degradation allows the stress to be gradually transferred to more recently synthesized portions of the peptidoglycan. Since each new layer of the peptidoglycan is slightly longer than the preceding one, the wall is continually expanded.

Doyle and his colleagues hypothesized that the autolysins of bacilli were regulated by proton motive force (10, 11, 15). In this model of autolytic regulation, proton motive force decreases pH near the cell membrane, and acidic pH prevents the autolysins from completely degrading the peptidoglycan. This model was supported by the observation that uncouplers promoted lysis, but a direct role for proton motive force in the regulation of autolysins was not easily demonstrated.

Fibrobacter succinogenes is a cellulolytic ruminal bacterium that digests even the most crystalline forms of cellulose (3). Because *F. succinogenes* can utilize ammonia but not amino acids as a nitrogen source (4, 5), bacterial turnover could be estimated from ammonia utilization and the accumulation of amino nitrogen in the medium. The following experiments with *F. succinogenes* demonstrated that (i) proton motive force had little role in autolysis, (ii) growing cultures had a higher lysis rate than stationary-phase cells, (iii) autolysis increased the apparent maintenance energy of growing cells, and (iv) the autolytic activity of stationary-phase cells was decreased by a phenylmethylsulfonyl fluoride (PMSF)-sensitive proteinase.

MATERIALS AND METHODS

Growth and lysis. *F. succinogenes* S85 was grown anaerobically (O_2-free atmosphere of CO_2) in a minimal medium that contained (per liter) 292 mg of K_2HPO_4, 292 mg of KH_2PO_4, 240 mg of $(NH_4)_2SO_4$, 480 mg of NaCl, 100 mg of $MgSO_4 \cdot 7H_2O$, 64 mg of $CaCl_2 \cdot 2H_2O$, 4 g of Na_2CO_3, 0.3 g of cysteine hydrochloride, 1 g of cellobiose, and a volatile fatty acid mixture (9). The cultures were incubated (39°C) in 80-ml serum bottles or 18- by 150-mm tubes which were sealed with butyl rubber stoppers. Continuous cultures were grown in a 360-ml vessel, and samples were taken after a 98% turnover of medium as described previously (29). When needed, 10 ml of culture was anaerobically transferred from exponential or continuous cultures into an 18- by 150-mm tube containing iodoacetate (5 μmol) or PMSF (10 μmol). Cultures were maintained at 39°C.

Membrane potential and ATP. Cultures were anaerobically transferred with a hypodermic syringe (2.0 ml) to 13- by 100-mm tubes which contained [^{3}H]tetraphenylphosphonium bromide ([^{3}H]TPP$^+$; 0.5 μCi, 23 μCi/μmol), ^3H_2O (4 μCi), or [U-^{14}C]sucrose (0.4 μCi, 540 μCi/μmol). After 5 min of incubation at 39°C, the cultures were centrifuged (13,000 × *g*, 5 min) through silicon oil (50:50 mixture of Hysol 550 and Hysol 560 [Hysol Co., Olean, N.Y.], incubated overnight in an anaerobic glove box to remove O_2). A supernatant sample (20 μl) was removed, and the remaining sample was frozen (−15°C). The pellet at the bottom of the tube was removed with a pair of dog nail clippers. Supernatant samples and cell pellets were counted by liquid scintillation. The intracellular volume (2.8 to 1.2 μl/mg of protein for exponential- and stationary-phase cells, respectively) was estimated from the relative uptake of ^3H_2O (4 μCi) and [U-^{14}C]sucrose (0.4 μCi, 540 μCi/μmol), a nonmetabolizable sugar, as described previously (26, 29). The membrane potential ($\Delta\Psi$) was calculated from the uptake of [^{3}H]TPP$^+$ by use of the Nernst equation (62 mV × log [in]/[out]), and the nonspecific binding of [^{3}H]TPP$^+$ was estimated from cells which had been treated with nigericin plus valinomycin (10 μM each) or toluene (1% of a 1:9 [vol/vol] toluene-ethanol mixture). Cells for ATP determination were prepared as described previously (26) and assayed with a luminometer (model 1250; LKB Instruments, Inc., Gaithersburg, Md.) to measure light output of a luciferine-luciferase mix (Sigma Chemical Co., St. Louis, Mo.).

Cells. Growth and lysis were monitored for changes in optical density (600 nm, 1-cm cuvette) as well as changes in cell protein. Cells were centrifuged (10,000 ×

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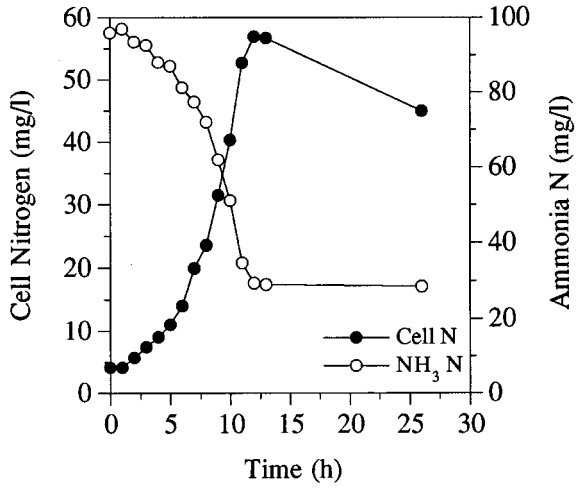

FIG. 1. Cellular N and ammonia utilization of *F. succinogenes* growing exponentially in batch culture.

g, 15 min, 5°C), washed twice in 0.9% NaCl, and stored at −15°C. Cells were treated with 0.2 N NaOH (100°C, 10 min), and protein was determined by the method of Lowry et al. (20). Cellular RNAs and DNAs were determined by the orcinol method (27) and the diphenylamine method (6), respectively. Cell N was calculated from protein, RNA, and DNA, with the N content of protein and nucleic acids being 16 and 12%, respectively. Viability was determined by serially diluting cultures in basal medium (3 g of cellobiose liter^{-1}) as described previously (29). The most probable number was based on four replicates.

Cell-free supernatants. Cell-free supernatants (10,000 × *g*, 15 min, 5°C) were stored at −15°C. Cellobiose and cellular polysaccharide were assayed by an anthrone procedure (13). Soluble protein was measured by the Bradford dye method (1). Ammonia was assayed by the method of Chaney and Marbach (7). Free amino acids were measured by high-pressure liquid chromatography (C_{18} Sep-Pak column; Millipore Corp., Milford, Mass.) (30) following derivatization with phenylisothiocyanate (8). Peptides were hydrolyzed with HCl prior to derivatization and amino acid analysis. Autolysin activities were measured as described previously (14, 15).

Statistics. All measurements were performed in triplicate (unless noted otherwise), and the coefficient of variation was less than 10%.

RESULTS

When *F. succinogenes* was grown in batch culture in a defined medium with cellobiose as an energy source, the maximum specific growth rate was 0.35 h^{-1} (Fig. 1). Batch cultures never utilized all of the ammonia, and approximately 80% of the ammonia utilization could be accounted for by protein, RNA, and DNA nitrogen. Growth ceased when the cellobiose was depleted. During the stationary phase, the viable cell number decreased at a high rate, but the declines in cellular DNA and protein were at least 30-fold slower (Fig. 2). The rate of decline in cellular protein was only 14% higher than the rate of DNA decay.

In continuous culture, the nitrogen recovery was growth rate dependent, and cellular nitrogen accounted for only 40% of the ammonia utilization at a dilution rate of 0.05 h^{-1} (Fig. 3a). The uninoculated medium did not have amino nitrogen, but peptides and amino acids could be detected in the cell-free medium. The cell-free supernatant also had a large amount of non-amino acid, non-ammonia, but phenylisothiocyanate-reactive nitrogen (data not shown). The slowly growing continuous cultures had as high a membrane potential and ATP as cells growing exponentially in batch culture (Fig. 3b).

When the cell yields and growth rates were plotted as double reciprocals, the maintenance coefficient, the slope, was 2.5 mg

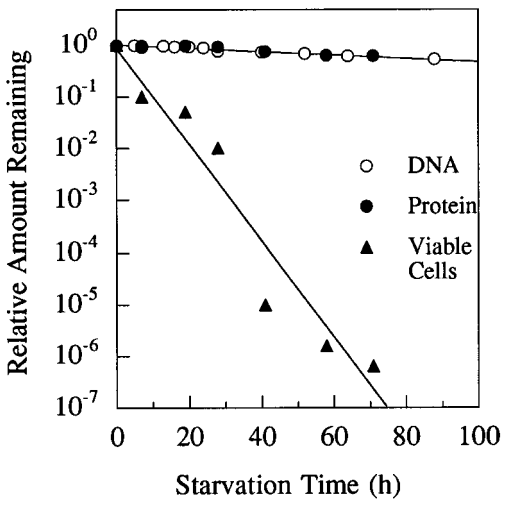

FIG. 2. Effect of time on the relative amounts of DNA, protein, and viable cell numbers remaining in stationary-phase cultures.

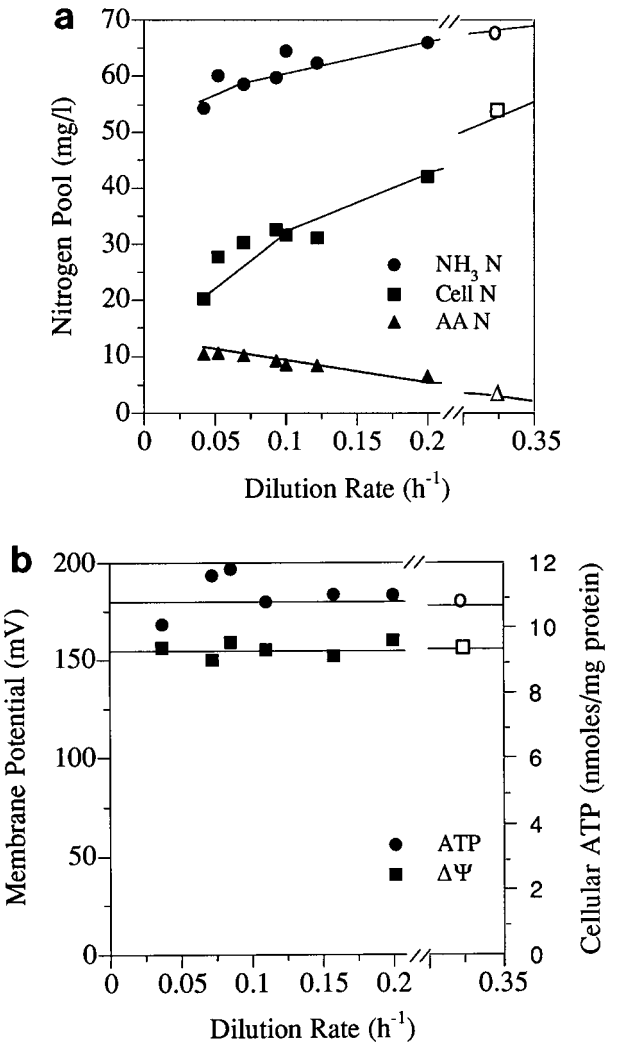

FIG. 3. (a) Ammonia utilization, cellular N, and extracellular amino nitrogen of *F. succinogenes* growing in continuous culture (closed symbols) and in batch culture (open symbols); (b) ATP and membrane potential (ΔΨ) of the cells.

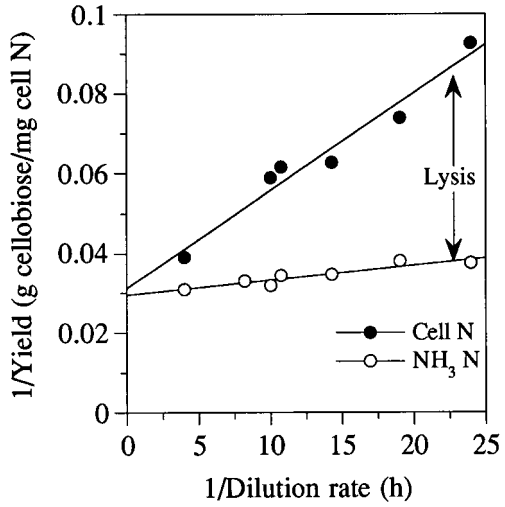

FIG. 4. Maintenance plot of 1/yield versus 1/dilution rate. The closed symbols show yield values calculated with the net accumulation of cell nitrogen N, and this line has a slope (maintenance coefficient) of 2.5 mg of cellobiose mg of cell N^{-1} h^{-1} and an intercept (1/theoretical maximum growth yield) of 33 mg of cell N per g of cellobiose. The open symbols show yield values calculated from ammonia utilization, and this line has a slope (maintenance coefficient) of 0.3 mg of cellobiose mg of cell N^{-1} h^{-1} and an intercept (1/theoretical maximum growth yield) of 33 mg of cell N per g of cellobiose.

of cellobiose per mg of cell N per h, and the theoretical maximum growth yield, the reciprocal of the intercept, was 33 mg of cell N per mg of cellobiose (Fig. 4). These values, however, did not include cells that had lysed. Yield values based on total ammonia utilization had the same theoretical maximum growth yield (intercept), but the maintenance coefficient (slope) was 8.33-fold less.

When exponentially growing cultures were treated with the glycolytic inhibitor iodoacetate, the cells stopped growing, and there was an immediate decrease in cell protein (Fig. 5). The rate of decay of cell protein (lysis) was higher than the growth rate (0.41 versus 0.35 h^{-1}). When the continuous cultures were treated with iodoacetate, the lysis rate decreased as a function

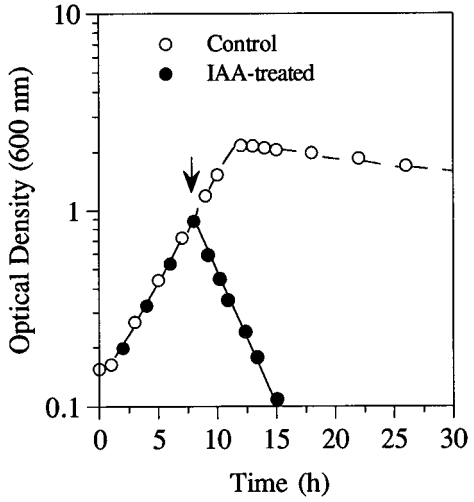

FIG. 5. Effect of iodoacetate (IAA) added at the time indicated by the arrow on the optical density of *F. succinogenes* cells growing in batch culture. Controls without IAA are shown by the dotted line.

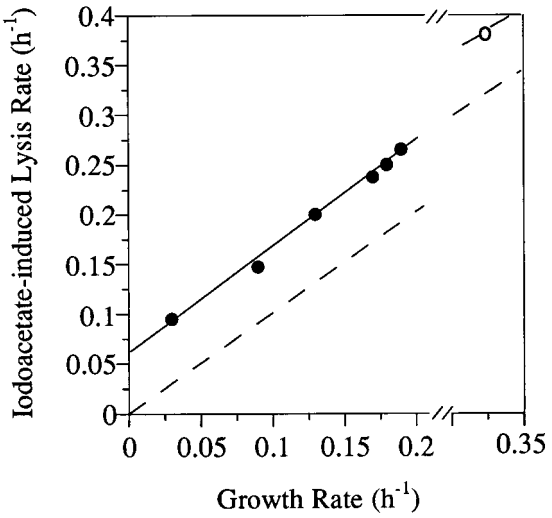

FIG. 6. Effect of iodoacetate (IAA) on the lysis rate of *F. succinogenes* cells growing in continuous culture (closed symbols) and in batch culture (open symbol). The growth rate is shown by the dotted line.

of dilution rate, but the lysis rate was approximately 0.06 h^{-1} greater than the growth rate (Fig. 6).

The proteinase inhibitor PMSF had no effect on the growth rate of exponentially growing cells, but the cultures had a higher rate of lysis when they entered stationary phase (Fig. 7a). The PMSF-induced rate of lysis was similar to the iodoacetate-induced lysis rate (0.40 versus 0.41 h^{-1}). When PMSF was added to stationary cultures, the induced lysis rate declined with the starvation time (Fig. 7b).

DISCUSSION

Previous work indicated that *F. succinogenes* is very susceptible to starvation (29). *F. succinogenes* stores glycogen, but this reserve material is utilized rapidly. Once the glycogen has been depleted, the cells can no longer maintain a membrane potential or transport activity, and viability decreases rapidly. Stationary cells maintained their integrity (cellular DNA and protein), and appeared to have a mechanism of preventing cell lysis. Growing cells, by contrast, assimilated disproportional amounts of ammonia, released non-ammonia nitrogen into the cell-free culture fluid, and lysed at least 10-fold faster than cells in the stationary phase did.

Previous work indicated that *F. succinogenes* cultures had a maintenance coefficient that was 20-fold greater than the endogenous metabolic rate (29), but this estimate of maintenance was not corrected for cell lysis. Maintenance energy can typically be estimated from growth rate-dependent changes in yield, but Pirt (23) emphasized the fact that maintenance equations were developed for "growing cultures" that were not lysing. Lysis is the difference between net and total growth. Because *F. succinogenes* can utilize ammonia but not amino nitrogen (4, 5), the difference between ammonia assimilation and nitrogen retention provided a method for estimating lysis.

Maintenance energy is usually described by a specific coefficient (m) in the equation $1/Y = m/\mu + 1/Y_G$ (where μ is the observed growth rate and Y is the cell yield), but Marr et al. (21) indicated that maintenance could also be expressed by a specific negative growth rate constant (a). The two expressions are related, as shown by the equation $a = m \cdot Y_G$. Because lysis is also a negative growth function, traditional estimates of

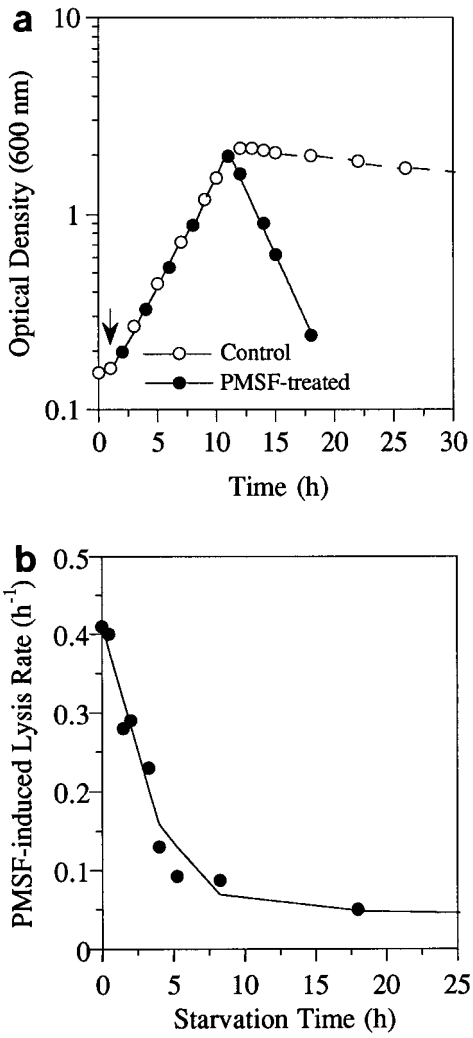

FIG. 7. (a) Effect of PMSF added at the time indicated by the arrow on the optical density of *F. succinogenes* cells growing in batch culture (controls without PMSF are shown by the dotted line); (b) relationship between starvation time and the rate of PMSF-induced lysis.

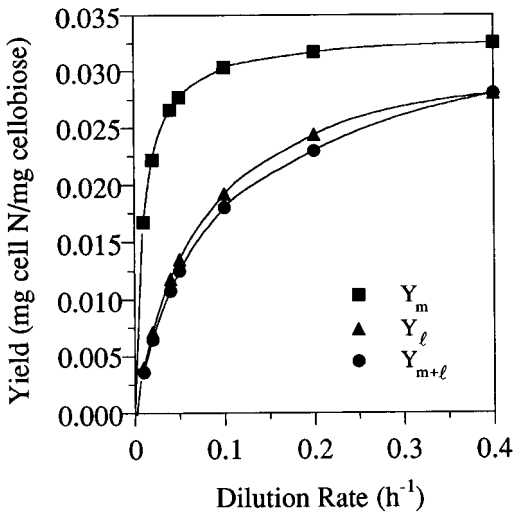

FIG. 8. Effect of maintenance (m), lysis (l), or maintenance plus lysis ($m+l$) on the yield of *F. succinogenes* cells growing in continuous culture.

maintenance (a_{app} or m_{app}) would include a lysis function (l): $a_{app} = a + l$ and $m_{app} = a/Y_G + l/Y_G$. Because the Pirt plot of ammonia assimilation had a slope, m, that was 8.3-fold less than the plot of nitrogen retention, it appeared that lysis was a more important determinant of yield than maintenance per se (Fig. 8). If $m_{app} = a/Y_G + l/Y_G$, $m_{app} = m + l/Y_G$, $m_{app} - m = l/Y_G$, and $(m_{app} - m)/Y_G = l$, the a and l terms would be 0.01 and 0.07 h^{-1}, respectively.

Growth rates are not usually adjusted for maintenance or lysis, but Marr et al. (21) indicated that nongrowth functions would decrease the growth rate of bacteria by the equation $\mu - a_{app} = \mu_{observed}$. The a terms of bacteria are usually low, but the l of *F. succinogenes* was relatively high. If the assumptions of Marr et al. (21) are correct, even exponentially growing cells would have a maximum growth rate (μ_{max}) that was significantly greater than the observed growth rate (μ): $\mu_{max} = \mu + a + l$ and 0.43 h^{-1} = 0.35 h^{-1} + 0.01 h^{-1} + 0.07 h^{-1}. When the growth rate declines, however, l approaches μ, and the impact is even greater.

The idea that growing *F. succinogenes* cultures were continuously lysing was supported by the effect of iodoacetate, a glycolytic inhibitor. When cells were treated with iodoacetate, growth ceased and cell protein decreased. The iodoacetate-induced lysis rate was proportional to the growth rate of the continuous culture, but the iodoacetate-induced lysis rate was always 0.06 h^{-1} greater than the growth rate. This latter estimate of lysis was in close agreement with the mathematically derived l term.

The linearity of maintenance plots indicates that maintenance is a growth rate-independent function that is proportional only to cell mass and time (24, 25). Because the Pirt plots of ammonia assimilation and nitrogen retention were both linear, it appeared that lysis (the difference) was also growth rate independent. However, *F. succinogenes* cells that had entered the stationary phase appeared to have a much lower rate of protein and DNA decay than growing cells (0.07 h^{-1} versus 0.007 or 0.006 h^{-1}, respectively). This result indicated that nongrowing *F. succinogenes* cells had a mechanism for preventing cells lysis.

Jolliffe et al. (14) noted that the proteinase inhibitor PMSF promoted the lysis of *Bacillus subtilis*, and they suggested that the autolysins were being inactivated by a serine proteinase. PMSF had no effect on exponentially growing *F. succinogenes* cultures, but PMSF promoted lysis when the cells reached the stationary phase. On the basis of these results, it appeared that a serine proteinase was inactivating the autolysins when rapid growth was no longer possible. As the cells starved, the autolysin was gradually inactivated, and the cultures were less susceptible to PMSF-induced lysis (Fig. 7b). When exponentially growing *F. succinogenes* cells were treated with iodoacetate, the transition to the stationary phase was circumvented, and the lysis rate was at least 50-fold higher (0.41 versus 0.007 h^{-1}). On the basis of these results, energy supply per se is probably not the critical aspect of autolysin regulation in *F. succinogenes*. The study of autolysin regulation in *F. succinogenes* was confounded by the fact that this gram-negative bacterium never released detectable autolysin into the cell-free medium like bacilli do (10).

One might view the lysis of growing *F. succinogenes* cells as a detrimental phenomenon that would decrease its niche in the rumen, but this assumption does not address the aspect of maximum growth rate and cell division. Jolliffe et al. (14) noted that an autolysin-deficient strain of *B. subtilis* formed long

chains and grew at a lower rate than the wild type. Given these constraints, excessive autolysin activity could be a compromise that increases cell number and maximum growth rate.

Because the autolysis and growth of *F. succinogenes* may be a highly integrated processes, *l* may not be an aspect of ruminal physiology that is easily modified. Harrison et al. (12), however, noted that mineral salts could increase bacterial flow from the rumen. Mineral salts should have no direct effect on growth, but they would increase the ruminal fluid dilution rate. When the fluid dilution rate increases, fluid-phase bacteria have less time to lyse and turnover.

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