Activity of H$^+$-ATPase in Ruminal Bacteria with Special Reference to Acid Tolerance

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Batch culture experiments showed that permeabilized cells and membranes of *Ruminococcus albus* and *Fibrobacter succinogenes*, acid-intolerant cellulolytic bacteria, have only one-fourth to one-fifth as much H$^+$-ATPase as *Megasphaera elsdenii* and *Streptococcus bovis*, which are relatively acid tolerant. Even in the cells grown in continuous culture at pH 7.0, the acid-intolerant bacteria contained less than half as much H$^+$-ATPase as the acid-tolerant bacteria. The amounts of H$^+$-ATPase in the acid-tolerant bacteria were increased by more than twofold when the cells were grown at the lowest pH permitting growth, whereas little increase was observed in the case of the acid-intolerant bacteria. These results indicate that the acid-intolerant bacteria not only contain smaller amounts of H$^+$-ATPase at neutral pH but also have a lower capacity to enhance the level of H$^+$-ATPase in response to low pH than the acid-tolerant bacteria. In addition, the H$^+$-ATPases of the acid-intolerant bacteria were more sensitive to low pH than those of the acid-tolerant bacteria, although the optimal pHs were similar.

Feeding high-concentrate diets to ruminants often reduces fiber digestion, which is primarily caused by the lower pH in the rumen (15, 16). Fibrolytic ruminal bacteria are generally sensitive to low pH, and the growth of the main cellulolytic bacteria, *Ruminococcus albus*, *Ruminococcus flavefaciens*, and *Fibrobacter succinogenes*, is greatly suppressed at pH 6.0 to 6.1 and completely inhibited below pH 5.9 even after adaptation to low pH (3, 23, 28).

Generally, bacteria should extrude H$^+$ at acidic pH (7). Even in ruminal bacteria that allow their intracellular pH (pH$_i$) to decrease as the extracellular pH (pH$_e$) drops, pH$_i$ is usually higher than pH$_e$ and the pH gradient across the membrane (ΔpH) increases as pH$_e$ declines (26, 29). This implies that H$^+$ is always extruded out of cells, and the extrusion increases with the drop in pH$_e$. Even these bacteria appear to need to prevent their pH$_i$ from dropping too much. At the lowest pH permitting growth, the ΔpH values in the cellulolytic bacteria described above (ca. 0.5 [23, 25]) are much smaller than those in relatively acid-tolerant ruminal bacteria such as *Streptococcus bovis*, *Megasphaera elsdenii*, and *Selenomonas ruminantium* (1 to 1.2 [22, 26, 29]). This led us to presume that the cellulolytic bacteria are less capable of expelling intracellular H$^+$.

The bacteria that allow their pH$_i$ to decrease appear to keep growing at low pH by changing their fermentation pathway to the pathway producing lactate that is considered to be less sensitive to low pH (3, 29). However, the cellulolytic bacteria mentioned above produce no or little lactate. Moreover, *R. albus*, which can produce small amounts of lactate, failed to increase lactate production even when it was grown by keeping the culture at the lowest pH permitting growth (3). This suggests that the cellulolytic bacteria need to maintain pH$_i$ near neutral.

The intolerance of the cellulolytic bacteria to low pH may be in part ascribed to their incapability to extrude as much H$^+$ as is required to keep their pH$_i$ within favorable or allowable ranges. In this context, the activity of membrane H$^+$-ATPase may be important; for the regulation of pH$_i$, H$^+$-ATPase is

generally considered to play a key role in anaerobic bacteria, especially neutrophilic bacteria (5, 7, 19).

As another aspect of acid tolerance, Russell and Wilson have stated that acid-resistant ruminal bacteria have evolved the capacity to survive a decrease in pH$_i$, maintain a small ΔpH, and prevent an intracellular accumulation of volatile fatty acid (VFA) anion and that cellulolytic bacteria cannot grow with a low pH$_i$ and an increase in ΔpH leads to anion toxicity (30). Therefore, the critical question is, Which exerts a greater adverse effect on cells, an increase in ΔpH or a drop in pH$_i$? Unless the extracellular VFA concentration is very high and ΔpH is very large, the adverse effect due to intracellular accumulation of VFA can be smaller than the effect due to the drop in pH$_i$.

The purpose of this study is to examine whether the activity (total activity per cell mass) of H$^+$-ATPase in *R. albus* and *F. succinogenes* (hereafter referred to as acid-intolerant bacteria) is lower than that in *S. bovis* and *M. elsdenii* (acid-tolerant bacteria). In addition, whether the amount of H$^+$-ATPase per cell is enhanced in response to low pH was examined.

MATERIALS AND METHODS

Bacteria and growth conditions. The sources and conditions of small-scale batch culture of *S. bovis* JB1 (14), *M. elsdenii* NIAH1102 (13), *R. albus* NIAH1188 (23), and *F. succinogenes* ATCC 19699 (23) were as described previously. When large amounts of bacterial cells were collected, 1-liter bottles were used (11). The medium for batch culture was a ruminal fluid medium (23) containing 5 g of either glucose (for *S. bovis* and *M. elsdenii*) or cellobiose (for *R. albus* and *F. succinogenes*) per liter. Cells were harvested at the end of the exponential phase of growth. Batch culture was conducted in triplicate.

Continuous culture was conducted by using an apparatus reported previously: an overflow type with 180-ml culture vessels (12). The medium was similar to that for batch culture except that glucose and cellobiose were reduced to 3 g/liter, which provided a carbohydrate-limited condition. The dilution rate was set at 0.1/h, and the pH of the medium reservoirs was adjusted so that the cultures were kept at desired pH values. Each bacterium species was grown at pH 7.0 and the lowest pH permitting growth. A steady state checked by constant pH and cell density was usually attained in 1 to 2 days, and 7 to 8 h after the establishment of the steady state, cells remaining in the culture vessels were harvested. Continuous culture was performed in duplicate.

Cell permeabilization. To assay the H$^+$-ATPase activity in intact cells, cells were made permeable by the method of Belli and Marquis (4). Samples (50 ml) of cultures were immediately cooled in an ice bath and centrifuged (20,000 × *g*, 10 min, 4°C). The pelleted cells were resuspended in 2.5 ml of 75 mM Tris-HCl buffer (pH 7.0) with 10 mM MgSO$_4$. Toluene (250 µl) was added to each cell suspension, vortexed vigorously, and incubated for 5 min at 39°C. Each cell suspension was then frozen in a Dewar vessel containing ethanol precooled at

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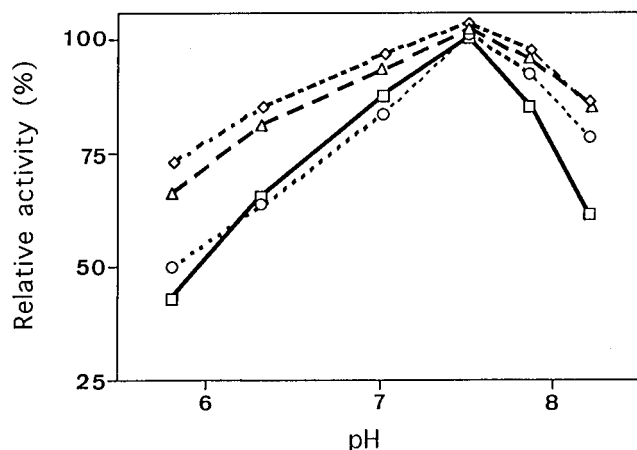
FIG. 1. Effect of pH on the activity of ATPase in permeabilized cells of *R. albus* ($\square$), *F. succinogenes* ($\bigcirc$), *M. elsdenii* ($\triangle$), and *S. bovis* ($\diamondsuit$).

−80°C and thawed at 39°C. This treatment was repeated once more. The resulting permeabilized cells were collected by centrifugation and resuspended in 1.0 ml of the same buffer. The cell suspensions were immediately subjected to the enzyme activity assay.

Preparation of cell membranes. Cells for membrane isolation collected from 800-ml cultures were resuspended in 75 mM Tris-HCl buffer (pH 7.0, 0°C) with 10 mM MgSO$_4$, and disrupted by passage through a French press (1,800 kg/cm^2, 2 to 3°C) until more than 90% of the cells were broken (five to seven times). After unbroken cells were removed by centrifugation (15,000 × g, 10 min, 4°C), the supernatant was ultracentrifuged (300,000 × g, 1 h, 4°C) and the pellet containing membrane vesicles was washed once with the same buffer. The membrane fraction was then resuspended in 50 mM Tris-HCl buffer (pH 7.5, 0°C) with 10 mM MgSO$_4$ and immediately subjected to the ATPase activity assay.

Since it was possible that some H$^+$-ATPase was released from membranes during the disruption of cells with a French press, membranes were isolated by a milder procedure developed by Bender et al. (5) with some modifications. Cells were suspended in 75 mM Tris-HCl buffer (pH 7.5) containing 400 mM sucrose, 2 mM MgSO$_4$, 10 mM 6-aminohexanoate, 5 mM *p*-aminobenzamide, mutanolysin (10 U/ml; Sigma Chemical Co., St. Louis, Mo.), lysozyme (2.5 mg/ml), and *N*-acetylmuramidase SG (10 μg/ml; Seikagaku Corporation, Tokyo, Japan). The cell suspensions were subsequently incubated at 39°C for 1 h with gentle shaking. The cells pelleted by centrifugation (15,000 × g, 10 min) were resuspended in 10 mM Tris-HCl buffer (pH 7.5) containing DNase I (10 U/ml, type II; Sigma) and RNase A (10 μg/ml, type II-A; Sigma) and vortexed at a medium setting for 10 min at room temperature to burst cells. After removal of the unbroken cells by centrifugation (15,000 × g, 10 min), membranes were pelleted by ultracentrifugation (300,000 × g, 1 h).

Assay for enzyme activity and nitrogen. ATPase activity was assessed in terms of the release of inorganic phosphate (P$_i$) from ATP basically by the method of Belli and Marquis (4) except the pH of the reaction mixture was changed. Unless otherwise stated, 50 mM Tris-maleate buffer (pH 7.5) with 10 mM MgSO$_4$ and 5 mM ATP was used. Since the reaction was usually linear for 15 to 20 min, incubation at 39°C was routinely conducted for 15 min. For the P$_i$ assay, the method of Chen et al. (9) was adopted. Enzyme activity was determined by subtracting the values without ATP and expressed as micromoles of P$_i$ released per minute per milligram of nitrogen (N) in the sample (permeabilized cells or membranes). Sample N was determined by digestion by the Kjeldahl method, followed by the quantitation of ammonia by the indophenol method (8).

Acid phosphatase activity was determined by modifying the method of Dvorak et al. (10). The assay mixture (1 ml) contained 5 mM *p*-nitrophenyl phosphate, 10 mM MgCl$_2$, 0.1 mg of bovine serum albumin, and an enzyme sample in 100 mM sodium acetate buffer (pH 5.8). Incubation was performed at 39°C for 20 min, and the reaction was stopped with 0.1 ml of 0.5 N NaOH. The P$_i$ liberated was measured as described above. An essentially similar method was applied to the determination of alkaline phosphatase activity except that 50 mM Tris-HCl (pH 9.0) was used and the reaction was terminated with 0.1 ml of 0.5 N HCl (18). The activity of 5′-nucleotidase was assayed by using 5′-AMP as a substrate (24).

Enzyme activities and N were assayed in duplicate for each sample. Data were evaluated by a Student's *t* test or a Tukey's test (31).

RESULTS

Quantitation of H$^+$-ATPase in cells. As shown in Fig. 1, the optimal pH of ATPase activity in permeabilized cells was found to

TABLE 1. Activities of enzymes releasing P$_i$ in permeabilized cells

Species	Enzyme activity (μmol of P$_i$ · min^{-1} · mg of N^{-1})[a]			
	ATPase	Acid phosphatase	Alkaline phosphatase	5′-Nucleotidase
R. albus	0.35 ± 0.02	0.02 ± 0.01	0.01 ± 0.00	0.01 ± 0.00
F. succinogenes	0.43 ± 0.03	0.01 ± 0.00	0.02 ± 0.01	0.01 ± 0.01
M. elsdenii	1.52 ± 0.08	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.01
S. bovis	1.84 ± 0.10	0.02 ± 0.00	0.03 ± 0.01	0.05 ± 0.01

[a] Mean ± standard error of the mean (*n* = 3). The values obtained for ATPase activity from *R. albus* and *F. succinogenes* were significantly different from the values for ATPase activity from *M. elsdenii* and *S. bovis* (*P* < 0.01).

be approximately 7.5 for all the bacterial species examined. However, the peaks were narrower in acid-intolerant bacteria than acid-tolerant bacteria. Similar results were obtained when membrane fractions were used (data not shown).

Quantities of some P$_i$-releasing enzymes in permeabilized cells are shown in Table 1. The amounts of ATPase in the cells of each bacterium species were much larger than those of acid and alkaline phosphatases and 5′-nucleotidase. Since the activities of the two phosphatases were much lower at pH 7.5 and the activity of 5′-nucleotidase without 5′-AMP was zero (data not shown), the contribution of these three enzymes to the values of ATPase activity determined by this method must be negligible. In other words, the values for ATPase activity shown in Table 1 virtually reflect the activity of ATPase per se.

For all the bacteria examined, ATPase activity was greatly inhibited by 1 mM azide (90% or more) and to a lesser extent by *N*,*N*′-dicyclohexylcarbodiimide (60 to 80% at 1 mM) (Table 2). The results with permeabilized cells were similar to those with membranes. Little inhibition by *N*-ethylmaleimide may indicate that the contribution of Na$^+$-ATPase to the total ATPase reaction was negligible (17, 18). Neither Na$^+$ nor K$^+$ had any effect on ATPase activity (data not shown). These observations are compatible with the assumption that the ATPase activity determined in these experiments represents mostly H$^+$-ATPase activity (1, 5, 18, 32). The sensitivity to inhibitors suggests that all the H$^+$-ATPases of the bacteria examined belong to the F$_1$F$_0$ type (2, 17).

Amounts of H$^+$-ATPase in cells. The activity of H$^+$-ATPase per cellular N determined by three methods is shown in Table

TABLE 2. Effects of inhibitors on ATPase activity

Species	Sample[a]	Inhibition of ATPase activity (%)[b]					
		NaN$_3$		DCCD[c]		NEM[d]	
		0.2 mM	1 mM	0.2 mM	1 mM	0.2 mM	1 mM
R. albus	PC	51 ± 2	89 ± 3	44 ± 3	75 ± 3	0 ± 0	0 ± 0
	FP	64 ± 4	92 ± 4	56 ± 3	81 ± 2	0 ± 0	3 ± 0
F. succinogenes	PC	43 ± 2	93 ± 3	39 ± 1	78 ± 1	0 ± 0	5 ± 1
	FP	47 ± 3	96 ± 2	52 ± 2	75 ± 3	0 ± 0	2 ± 0
M. elsdenii	PC	39 ± 2	90 ± 3	38 ± 1	75 ± 2	0 ± 0	7 ± 1
	FP	43 ± 2	93 ± 2	47 ± 2	77 ± 3	0 ± 0	3 ± 1
S. bovis	PC	55 ± 3	91 ± 4	38 ± 1	62 ± 2	0 ± 0	6 ± 1
	FP	47 ± 2	95 ± 3	43 ± 2	77 ± 4	0 ± 0	4 ± 0

[a] PC, permeabilized cells. FP, membranes prepared with a French press.
[b] Mean ± standard error of the mean (*n* = 3).
[c] DCCD, *N*,*N*′-dicyclohexylcarbodiimide.
[d] NEM, *N*-ethylmaleimide.

TABLE 3. Amounts of H$^+$-ATPase in permeabilized cells and membrane fractions

Species	H$^+$-ATPase activity (μmol of P$_i \cdot$ min$^{-1} \cdot$ mg of N^{-1})[a]		
	PC	Membrane fraction	
		FP	EL
R. albus	0.46 ± 0.03	0.36 ± 0.03	0.28 ± 0.01
F. succinogenes	0.42 ± 0.04	0.33 ± 0.02	0.30 ± 0.02
M. elsdenii	1.84 ± 0.08	1.52 ± 0.04	1.41 ± 0.04
S. bovis	2.30 ± 0.12	1.83 ± 0.09	1.58 ± 0.03

[a] Mean ± standard error of the mean ($n = 3$). PC, permeabilized cells; FP, cells were disrupted by passage through a French press; EL, cell walls were lysed with enzymes. For permeabilized cells and membrane fractions, the values obtained for H$^+$-ATPase activity from R. albus and F. succinogenes were significantly different from the values for M. elsdenii and S. bovis ($P < 0.01$).

3. The permeabilized cells of acid-tolerant bacteria contained four- to fivefold more H$^+$-ATPase than those of acid-intolerant bacteria. Similar results were obtained with membrane fractions. There was little difference between the values obtained with the membrane fractions prepared by two different procedures. A certain amount of H$^+$-ATPase may have been released from membranes by disrupting cells with a French press, which appeared to be comparable to the loss of H$^+$-ATPase activity during the incubation of cells to lyse the cell wall with enzymes.

The amounts of H$^+$-ATPase in cells grown in continuous culture are shown in Table 4. Acid-intolerant bacteria contained almost the same amounts of H$^+$-ATPase irrespective of culture pH. This result was in sharp contrast to that obtained with acid-tolerant bacteria, in which the amounts of H$^+$-ATPase at low pH were more than twice as large as those at pH 7.0. At pH 7.0, acid-tolerant bacteria had more than twice as much H$^+$-ATPase as acid-intolerant bacteria. These results indicate that acid-tolerant bacteria not only contain larger amounts of H$^+$-ATPase at neutral pH but also have a higher capacity to enhance the amount of H$^+$-ATPase at low pH than acid-intolerant bacteria.

DISCUSSION

The amounts of H$^+$-ATPase in acid-intolerant bacteria grown in batch culture were dramatically smaller than those in

TABLE 4. Effect of culture pH on the amount of H$^+$-ATPase in cells grown in continuous culture[a]

Species	pH	H$^+$-ATPase activity (μmol of P$_i \cdot$ min$^{-1} \cdot$ mg of N^{-1})[b]
R. albus	7.0	0.36 ± 0.02
	6.0	0.43 ± 0.04
F. succinogenes	7.0	0.37 ± 0.02
	6.0	0.45 ± 0.03
M. elsdenii	7.0	0.85 ± 0.04
	5.3	1.92 ± 0.09[c]
S. bovis	7.0	0.94 ± 0.05
	4.7	2.45 ± 0.11[c]

[a] Permeabilized cells were used to assay H$^+$-ATPase activity.
[b] Mean ± standard error of the mean ($n = 2$).
[c] Significantly different from the value obtained at pH 7.0 because of the low pH ($P < 0.01$).

acid-tolerant bacteria. This conclusion could be supported by the fact that essentially the same results were obtained by three different methods. Contribution of other membrane-ATPases to the determined values for H$^+$-ATPase activity could be negligible, as indicated for Streptococcus faecalis (20). However, the values of H$^+$-ATPase in the acid-intolerant bacteria might be somewhat underestimated, because during the treatment to assay enzyme activity the H$^+$-ATPase of these bacteria lost activity more rapidly than that of acid-tolerant bacteria (5 to 10% decrease at 4°C in 10 h compared to little loss in acid-tolerant bacteria [unpublished data]).

The difference in the amounts of H$^+$-ATPase between acid-intolerant and -tolerant bacteria in batch culture (four- to fivefold) was comparable to that in continuous culture at low pH (about fourfold). This may be due to the fact that the final pH values in batch culture were low. It appears that the biosynthesis of H$^+$-ATPase is stimulated in response to low pH (21).

Acid-tolerant bacteria grown in continuous culture at pH 7.0 contained more than twice as much H$^+$-ATPase as acid-intolerant bacteria despite the fact that there was little necessity to extrude intracellular H$^+$ at this pH (21, 23, 25, 29). Russell and colleagues have emphasized the importance of H$^+$-ATPase as an energy-spilling reaction in S. bovis and presumed that the H$^+$-ATPase activity is regulated by ATP (6, 27). Since the continuous culture in our study was conducted under an energy-limited condition, the higher amounts of H$^+$-ATPase in acid-tolerant bacteria grown at pH 7.0 could not be explained by energy spilling. Possibly, each bacterium constitutively synthesizes a certain amount of H$^+$-ATPase and the basal levels of H$^+$-ATPase are high in bacteria with a high ability of fermentation. Moreover, such bacteria have a higher capacity to enhance the biosynthesis of H$^+$-ATPase when it is needed; the biosynthesis of H$^+$-ATPase is probably stimulated by some conditions such as low pH$_i$ and high concentrations of intracellular ATP.

There was no difference in the optimal pH of H$^+$-ATPase among the bacteria examined (Fig. 1). This is in contrast to the case in oral streptococci, in which the pH optima reflected acid tolerance (5). However, the decreases in H$^+$-ATPase activity with the decrease in pH were greater in acid-intolerant bacteria than in tolerant bacteria (Fig. 1). This indicates that the H$^+$-ATPase in acid-intolerant bacteria is more sensitive to low pH$_i$ than that in acid-tolerant bacteria.

In conclusion, the acid intolerance of cellulolytic ruminal bacteria appears to be due in part to the low amounts of H$^+$-ATPase and the low capacity to enhance the biosynthesis of H$^+$-ATPase in response to low pH. In addition, H$^+$-ATPase of these bacteria is sensitive to low pH. As described in the introduction, both pH$_i$ and ΔpH affect acid sensitivity. However, if it is possible to manipulate cellulolytic bacteria to synthesize more H$^+$-ATPase and maintain their pH$_i$ above 6.3 at pH$_e$ 5.5, they may keep growing unless VFA concentration is very high. If cellulolytic bacteria could grow at pH 5.5 in the rumen, digestion of fiber should be greatly improved.

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