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 ($pHi$) to decrease as the extracellular pH ($pHe$) drops, $pHi$ is usually higher than $pHe$, and the pH gradient across the membrane ($\Delta pHi$) increases as $pHe$ declines (26, 29). This implies that $H^{+}$ is always extruded out of cells, and the extrusion increases with the drop in $pHe$. Even these bacteria appear to need to prevent their $pHi$ from dropping too much. At the lowest $pHe$ permitting growth, the $\Delta pHi$ 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 $pHi$ 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 $pHi$ 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 $pHi$ within favorable or allowable ranges. In this context, the activity of membrane $H^{+}$-ATPase may be important; for the regulation of $pHi$, $H^{+}$-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 $pHe$, maintain a small $\Delta pHi$, and prevent an intracellular accumulation of volatile fatty acid (VFA) anion and that cellulolytic bacteria cannot grow with a low $pHe$ and an increase in $\Delta pHi$ leads to anion toxicity (30). Therefore, the critical question is, Which exerts a greater adverse effect on cells, an increase in $\Delta pHi$ or a drop in $pHe$? Unless the extracellular VFA concentration is very high and $\Delta pHi$ is very large, the adverse effect due to intracellular accumulation of VFA can be smaller than the effect due to the drop in $pHe$.

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 NIAH188 (25), and F. succinogenes ATCC 19699 (25) 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 $pHi$ values. Each bacterium species was grown at pH 7.0 and the lowest $pHi$ 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 pellet 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
−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 subsequently incubated at 39°C for 1 h with gentle shaking.

Preparation of cell membranes. Cells for membrane isolation collected from 800-ml cultures were resuspended in 75 mM Tris-HCl buffer (pH 7.5, 0°C) with 10 mM MgSO4 and disrupted by passage through a French press (1,800 kg/cm2, 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) until more than 90% of the cells were broken (five to seven times). The cells pelleted by centrifugation (15,000 × g, 10 min) were resuspended in 50 mM Tris-HCl buffer (pH 7.5, 0°C) with 10 mM MgSO4 and immediately subjected to the ATPase activity assay.

Assay for enzyme activity and nitrogen. ATPase activity was assayed in terms of the release of inorganic phosphate (Pi) 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 MgSO4 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 Pi 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 Pi released per minute per microbial dry weight.

Acid phosphatase activity was determined by modifying the method of Dvorak et al. (10). The assay mixture (1 ml) contained 10 mM p-nitrophenyl phosphate, 10 mM MgCl2, 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 Pi 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 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 Pi-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 F₁,F₀ 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 1.

<table>
<thead>
<tr>
<th>Species</th>
<th>Enzyme activity (μmol of Pi·min−1·mg of N−1)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>ATPase</td>
</tr>
<tr>
<td>R. albus</td>
<td>0.35 ± 0.02</td>
</tr>
<tr>
<td>F. succinogenes</td>
<td>0.43 ± 0.03</td>
</tr>
<tr>
<td>M. elsdenii</td>
<td>1.52 ± 0.08</td>
</tr>
<tr>
<td>S. bovis</td>
<td>1.84 ± 0.10</td>
</tr>
</tbody>
</table>

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).

<table>
<thead>
<tr>
<th>Species</th>
<th>Inhibition of ATPase activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NaN₃</td>
</tr>
<tr>
<td>R. albus</td>
<td>0.2 mM</td>
</tr>
<tr>
<td></td>
<td>51 ± 2</td>
</tr>
<tr>
<td>F. succinogenes</td>
<td>0.2 mM</td>
</tr>
<tr>
<td></td>
<td>46 ± 4</td>
</tr>
<tr>
<td>M. elsdenii</td>
<td>0.2 mM</td>
</tr>
<tr>
<td></td>
<td>43 ± 2</td>
</tr>
<tr>
<td>S. bovis</td>
<td>0.2 mM</td>
</tr>
<tr>
<td></td>
<td>44 ± 2</td>
</tr>
</tbody>
</table>

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.
3. The permeabilized cells of acid-tolerant bacteria contained four- to fivefold more $\text{H}^+\text{-ATPase}$ 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 $\text{H}^+\text{-ATPase}$ may have been released from membranes by disrupting cells with a French press, which appeared to be comparable to the loss of $\text{H}^+\text{-ATPase}$ activity during the incubation of cells to lyse the cell wall with enzymes.

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

**DISCUSSION**

The amounts of $\text{H}^+\text{-ATPase}$ in acid-intolerant bacteria grown in batch culture were dramatically smaller than those in 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-$\text{H}^+\text{-ATPases}$ to the determined values for $\text{H}^+\text{-ATPase}$ activity could be negligible, as indicated for *Streptococcus faecalis* (20). However, the values of $\text{H}^+\text{-ATPase}$ in the acid-intolerant bacteria might be somewhat underestimated, because during the treatment to assay enzyme activity the $\text{H}^+\text{-ATPase}$ 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 $\text{H}^+\text{-ATPase}$ 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 $\text{H}^+\text{-ATPase}$ 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 $\text{H}^+\text{-ATPase}$ as acid-intolerant bacteria despite the fact that there was little necessity to extrude intracellular $\text{H}^+$ at this pH (21, 23, 25, 29). Russell and colleagues have emphasized the importance of $\text{H}^+\text{-ATPase}$ as an energy-spilling reaction in *S. bovis* and presumed that the $\text{H}^+\text{-ATPase}$ 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 $\text{H}^+\text{-ATPase}$ 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 $\text{H}^+\text{-ATPase}$ and the basal levels of $\text{H}^+\text{-ATPase}$ are high in bacteria with a high ability of fermentation. Moreover, such bacteria have a higher capacity to enhance the biosynthesis of $\text{H}^+\text{-ATPase}$ when it is needed; the biosynthesis of $\text{H}^+\text{-ATPase}$ is probably stimulated by some conditions such as low pH, and high concentrations of intracellular ATP.

There was no difference in the optimal pH of $\text{H}^+\text{-ATPase}$ 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 $\text{H}^+\text{-ATPase}$ activity with the decrease in pH were greater in acid-intolerant bacteria than in tolerant bacteria (Fig. 1). This indicates that the $\text{H}^+\text{-ATPase}$ in acid-intolerant bacteria is more sensitive to low pH than that in acid-tolerant bacteria.

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

**REFERENCES**


