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# Proton-Translocating Adenosine Triphosphatase Activity in Lactic Acid Bacteria[1]

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## ABSTRACT

*Streptococcus thermophilus* 19258 and 573, *Lactococcus lactis* ssp. *lactis* C2, *Lactococcus lactis* ssp. *cremoris* HP, and *Lactobacillus casei* 685 were grown in various media and assayed for proton-translocating ATPase ($H^+$-ATPase) activity. The $H^+$-ATPase was extracted from the membrane fractions of protoplasted cells using a French pressure cell, and activity was measured by the release of inorganic phosphate from ATP. The pH optima for the $H^+$-ATPase, assayed in vitro, were 7.5 for *S. thermophilus* and *Lactococcus lactis* ssp. *cremoris* HP and 5.0 for *Lactobacillus casei* 685. In contrast, for cells grown in batch culture the $H^+$-ATPase activity was always greatest when the cytoplasmic pH was less than the optimum for the enzyme. The $H^+$-ATPase activity generally increased as the pH decreased until an extracellular pH of 5.0 was reached. Below an extracellular pH of 5.0, activities of this enzyme dropped markedly. The aciduric lactic acid bacterium, *Lactobacillus casei* 685, had higher basal levels of this enzyme than the streptococci and lactococci. Results suggest that this enzyme may be involved in regulation of the intracellular pH in lactic acid bacteria.
(Key words: ATPase, lactic acid bacteria, *Streptococcus thermophilus*)

**Abbreviation key:** $\Delta pH$ = pH gradient (intracellular pH minus extracellular pH), $pH_{in}$ = intracellular pH, $pH_{out}$ = extracellular pH, $P_i$ = inorganic phosphate.

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## INTRODUCTION

Regulation of the cytoplasmic or internal pH ($pH_{in}$) is a fundamental requirement for the survival and viability of lactic acid bacteria. Many microorganisms are able to maintain a fairly constant $pH_{in}$ even when the pH of the external medium ($pH_{out}$) decreases as a result of fermentation end products. Ultimately, however, a point occurs at which the $pH_{in}$ also begins to decline. Eventually, the difference between the $pH_{in}$ and the $pH_{out}$ ($\Delta pH$) will collapse or approach zero, resulting in loss of cell viability (4).

Despite the importance of pH homeostasis in the lactic acid bacteria, relatively little is known about how these microorganisms control their $pH_{in}$. In other fermentative bacteria, such as *Enterococcus faecalis* 9790 (formerly *Streptococcus faecalis* 9790), maintenance of an alkaline cytoplasm has been shown to occur primarily by means of a proton-translocating ATPase ($H^+$-ATPase) (5, 7, 8, 9). This membrane-bound enzyme is believed to expel protons accumulated by the cell as a result of lactic acid production. According to the chemiosmotic theory (11), the $H^+$-ATPase acts as a reversible ion translocating pump, which catalyzes the movement of hydrogen ions ($H^+$) across the cell membrane as a consequence of the hydrolysis or synthesis of ATP (6, 10). Kobayashi et al. (9) have shown that in *E. faecalis* the $H^+$-ATPase was at a basal level when the cytoplasmic pH was equal to or greater than 7.5. However, when the $pH_{in}$ was lowered below 7.5 as a result of organic acid production, $H^+$-ATPase activity increased. When the $pH_{in}$ was artificially lowered by the addition of gramicidin D, the level of $H^+$-ATPase also increased. Kobayashi et al. (9) further showed that $H^+$-ATPase activity also increased when cells of *E. faecalis* were transferred from neutral to acidic medium and that the $pH_{in}$ increased as the $H^+$-ATPase activity

increased. As a result of these studies, these workers suggested that the $H^+$-ATPase is the primary component for pH regulation in fermentative bacteria (9).

We have measured $H^+$-ATPase activity in *Streptococcus thermophilus* and other lactic acid bacteria during batchgrowth and report that this enzyme may also be involved in $pH_{in}$ regulation in these bacteria.

## MATERIALS AND METHODS

### Bacteria and Growth Media

Bacteria used in this study included *Streptococcus thermophilus* 19258 and 573, *Lactobacillus casei* 685, *Lactococcus lactis* ssp. *lactis* C2, and *Lactococcus lactis* ssp. *cremoris* HP. The *S. thermophilus* and lactococci strains were grown in Elliker broth (3) containing 1.5% lactose or M17 broth (14) containing 5.0% lactose and incubated at 42°C or 32°C, respectively. *Lactobacillus casei* was grown in MRS broth (Difco Laboratories, Detroit, MI) at 35°C.

### Membrane Preparation

Preparation of membrane extracts was performed as described by Kobayashi et al. (7) with the following modifications. Cells grown overnight were used to inoculate fresh medium at the rate of 40 ml/2 L (2%). Cells were grown in the appropriate medium and temperature and harvested at various times and various $pH_{out}$ during batch growth. Harvested cells (usually 200 ml) were centrifuged at 10,000 × *g* (model J2-21, Beckman Instruments, Inc., Fullerton, CA) at 4°C and washed twice in 1.0 m*M* $MgCl_2$. Pellets were used immediately or were frozen at –20°C. Cells were resuspended in .5 *M* glycylglycine-KOH buffer, pH 7.2, containing 2 m*M* $MgCl_2$ to give approximately 100 mg/ml of cells (wet weight). Mutanolysin (Sigma Chemical Co., St. Louis, MO) was added to the cell suspension (50 units/ml) and incubated for 2 h at 37°C with gentle shaking (80 rpm). The resulting protoplasts were collected by centrifugation at 10,000 × *g* for 10 min at 4°C. Pelleted protoplasts were then burst by suspension in prewarmed (37°C) 1 m*M* $MgCl_2$ (containing 10 μg/ml, final concentration, of DNase, Sigma) at the rate of 10 mg/ml of cells (wet weight). The suspension was incubated for 30 min at 37°C, with gentle shaking (80 rpm). After the unbroken cells were removed by centrifugation at 5000 × *g* for 5 min at 4°C, membranes were collected by centrifugation at 15,000 × *g* for 3 min at 4°C and resuspended in 1 m*M* $MgCl_2$. The membranes were washed twice and resuspended in 1 m*M* $MgCl_2$ and passed through a precooled French Press (SLM Aminco, Urbana, IL) at 703.66 kg/$cm^2$ (10,000 psi). The enzyme extract was then stored at –70°C.

### Enzyme Assay

The $H^+$-ATPase activity was measured by colorimetric determination of inorganic phosphate ($P_i$) liberated from ATP, as described by Kobayashi and Anraku (6). The enzyme was assayed in a standard reaction mixture consisting of .05 *M* Tris-HCl, pH 7.0, 5.0 m*M* $MgCl_2$, 5.0 m*M* Na-ATP, and 10 μg bovine serum albumin in a total volume of 3.0 ml. In some experiments, a .05 *M* Tris buffer containing .05 *M* maleic acid (13) was used at pH from 5.0 to 8.6. The assay was started by addition of 100 μl membrane extract (approximately 10.0 μg protein), and the mixture was incubated at 37°C for 10 min. The reaction was stopped by the addition of .3 ml of .1*N* HCl, and then 2.1 ml of a stop solution containing .3 ml of 5*N* $H_2SO_4$, .3 ml of 2.5% ammonium molybdate, .3 ml of 3% $NaHSO_3$ – 1% *p*-methylaminophenol sulfate, and 1.2 ml of deionized distilled $H_2O$ was added. The stopped reaction mixture was held for 10 min at 22°C. The absorbance at 660 nm was then measured (DU-64 Spectrophotometer, Beckman) against a background consisting of the reaction mixture minus the membrane extract. One unit of ATPase was defined as the amount of $P_i$ produced per minute per milligram of protein. *Lactobacillus casei* 685 was assayed as above except that the reaction mixture buffer consisted of .05 *M* Trizma containing .05 *M* malic acid, pH 5.5. All enzyme assays were performed in duplicate or triplicate.

### Determination of Protein Concentrations and Intracellular pH

Protein was determined by the Bradford method using bovine serum albumin as the standard (2). The $pH_{in}$ was determined as described previously (12).

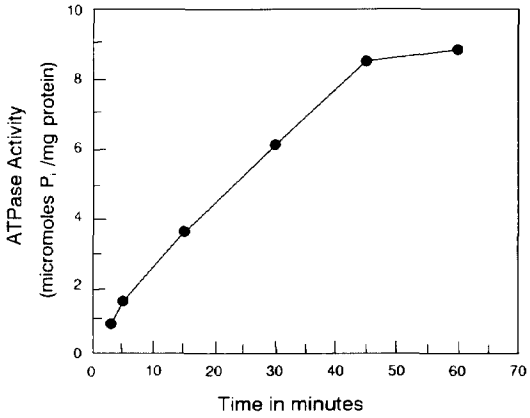


Figure 1. The $H^+$-ATPase activity [micromoles of inorganic phosphate ($P_i$) per milligram of protein] in *Streptococcus thermophilus* 19258.

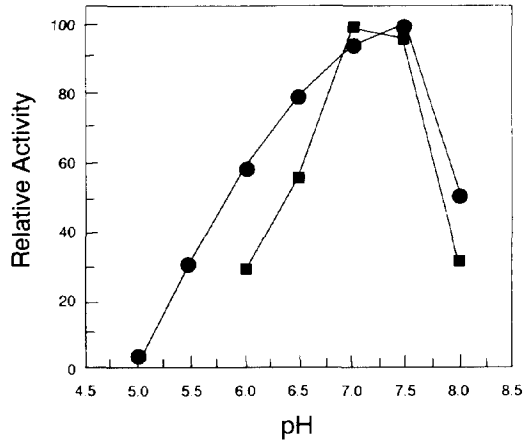


Figure 2. Effect of pH on the relative activity of $H^+$-ATPase from *Streptococcus thermophilus* 573 (■) and *Lactococcus lactis* ssp. *cremoris* HP (●) at 37°C.

## RESULTS

### Measurement of $H^+$-ATPase in Membrane Extracts

Hydrolysis of ATP by membrane extracts of *S. thermophilus* 19258 was measured over a 60 min incubation period at 37°C in a series of reaction mixture tubes. At various times, tubes were removed, and the reaction was stopped. The reaction rate was linear for at least 45 min, under typical assay conditions (Figure 1). Activity was inhibited when the specific $H^+$-ATPase inhibitor, N,N′-dicyclohexylcarbodiimide (.025 m*M*), was added to the reaction mixture; neither an increase nor stimulation of activity was observed when 50 m*M* $Na^+$ or $K^+$ were added (data not shown). These results indicated that the ATP hydrolyzing activity in the membrane extracts of *S. thermophilus* was due to the presence of a $H^+$-ATPase.

### Effect of pH on the Activity of $H^+$-ATPase

The pH optima of the $H^+$-ATPase from the various organisms were determined by varying the pH of the standard reaction mixture. For *S. thermophilus* 573 and *Lactococcus lactis* ssp. *cremoris* HP, the maximum activity of $H^+$-ATPase occurred at pH 7.5 (Figure 2). In contrast, the maximum activity in the more acid tolerant *Lactobacillus casei* 685 occurred at pH 5.5 (data not shown), a considerably lower pH than the other lactic acid bacteria studied. This pH optimum is similar to that described by Bender et al. (1), who reported maximal $H^+$-ATPase activity at pH 5.0 for cell membranes of *Lactobacillus casei* 4646.

### Effect of Temperature on Activity of $H^+$-ATPase

The $H^+$-ATPase isolated from the membrane fraction of *S. thermophilus* 573 was assayed at various incubation temperatures. The standard reaction mixture was prewarmed to 32, 37, 42, 65, 70, or 100°C. The reaction was stopped after 10 min of incubation. The $H^+$-ATPase activities increased with increasing temperature, with a maximum activity of 4.6 μmol/min per mg protein at 65°C (Figure 3). Activity decreased markedly at 100°C to less than 10% of the maximum activity. At this high temperature, significant autohydrolysis of the substrate (ATP) also occurred. The $H^+$-ATPase from *S. thermophilus* 19258 behaved in a similar fashion; maximum activity with this strain occurred at 70°C. The optimum temperature of the lactococcal $H^+$- ATPase was much lower, with maximum activities occurring at 37 to 40°C (data not shown).

### $H^+$-ATPase Activity in Lactic Acid Bacteria During Batch Growth

*Streptococcus thermophilus 573.* The $H^+$-ATPase activity was determined in *S. ther-*

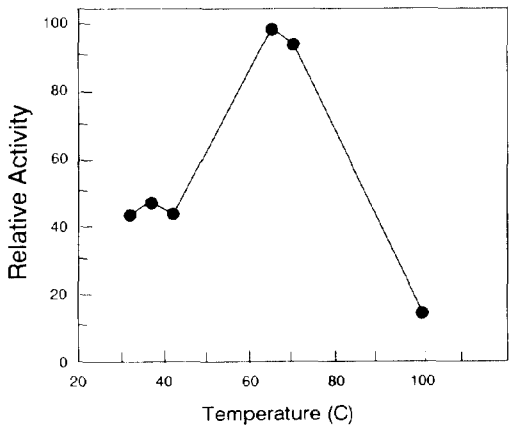


Figure 3. Effect of temperature on the relative activity of $H^+$-ATPase from *Streptococcus thermophilus* 573.

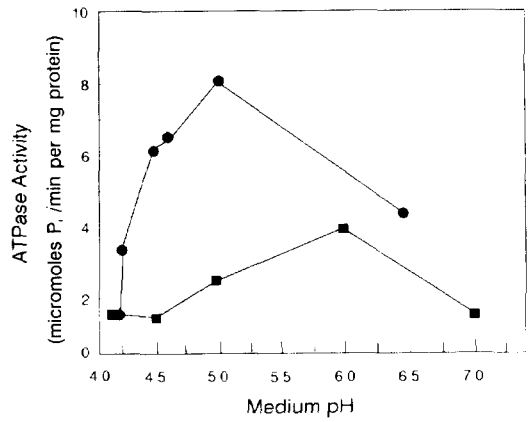


Figure 4. Specific activity of $H^+$-ATPase [micromoles of inorganic phosphate ($P_i$)/min per milligram of protein] from *Streptococcus thermophilus* 573 during batch growth in Elliker broth (■) and M17 broth (●) at 42°C.

*mophilus* 573 during batch growth in various media. For cell grown in Elliker broth, activities ranged from 1.62 μmol $P_i$/min per mg protein at pH 7.0 to a maximum of 3.40 μmol $P_i$/min per mg protein at pH 5.9 (Figure 4). The $H^+$-ATPase activity in *S. thermophilus* 573 grown in M17 medium was somewhat greater, with a maximum activity of 8.1 μmol $P_i$/min per mg protein at pH 5.0. Below pH 5.0, however, activity decreased sharply.

*Streptococcus thermophilus 19258.* For *S. thermophilus* 19258, grown in M17 medium at 42°C, the $H^+$-ATPase activity was found to remain fairly constant, ranging from 2.63 units/mg protein at the start of growth ($pH_{out}$ 6.7) to 2.27 units/mg protein at pH 4.7 (data not shown). The maximum activity (3.01 μmol $P_i$/min per mg protein) occurred at an external pH of 4.9. Similar results were found when *S. thermophilus* 19258 was grown in Elliker medium at 42°C where the activity ranged from .93 to 2.96 μmol $P_i$/min per mg protein ($pH_{out}$ 6.17 to 4.89).

*Lactococcus lactis ssp. lactis C2 and Lactococcus lactis ssp. cremoris HP.* Similarly high activities were also observed for the mesophilic lactococci. Results with *Lactococcus lactis* ssp. *lactis* C2 and *Lactococcus lactis* ssp. *cremoris* HP indicated that $H^+$-ATPase activity increased with decreasing pH until the ΔpH began to collapse. *Lactococcus lactis* ssp. *lactis* C2 grown in Elliker broth had $H^+$-ATPase activities ranging from 2.1 to 5.0 μmol $P_i$/min per mg protein at $pH_{out}$ 6.2 to 4.5 (Figure 5). The greatest level of $H^+$-ATPase activity occurred at $pH_{out}$ 4.9. *Lactococcus lactis* ssp. *cremoris* HP had a maximum activity at pH 5.2, although activities at all stages of growth were generally higher than those found in *Lactococcus lactis* ssp. *lactis* C2. For both strains, $H^+$-ATPase activities decreased below pH 4.9 to 5.2.

*Lactobacillus casei 685. Lactobacillus casei* was able to grow to a $pH_{out}$ of 3.38; the cell density reached more than twice that of the streptococci and lactococci studied (optical density = 5.53 at 16 h of growth). These organisms were found to maintain a ΔpH of greater than one pH unit at $pH_{out}$ values of 3.8 to 5.5 (12). Maintenance of this ΔpH at acidic pH may be explained by the greater basal levels of $H^+$-ATPase activity of 4.22 to 5.88 μmol $P_i$/min per mg protein observed in this organism compared with *S. thermophilus*. In contrast to the lactococci and streptococci, even when the medium pH approached 4.5 the $H^+$-ATPase activity did not decrease.

## DISCUSSION

Kobayashi has suggested that one of the primary mechanisms by which bacteria maintain a neutral or near neutral cytoplasmic pH is via the proton-extruding $H^+$-ATPase (5, 7, 8, 9). When the $pH_{in}$ is high, the activity of the $H^+$-ATPase is at a relatively low basal level. As

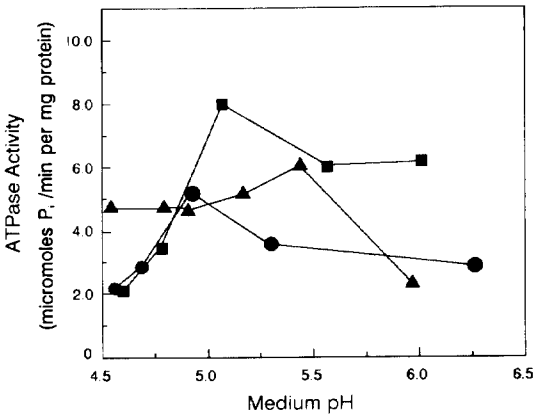


Figure 5. Specific activity of $H^+$-ATPase [micromoles of inorganic phosphate ($P_i$)/min per milligram of protein] from *Lactococcus lactis* ssp. *lactis* C2 (●) and *Lactococcus lactis* ssp. *cremoris* HP (■) during batch growth in Elliker broth at 32°C and *Lactococcus casei* 385 (▲) in MRS broth at 35°C.

the $pH_{in}$ decreases, $H^+$-ATPase activity increases, due in part to an increase in enzyme biosynthesis. In the studies reported here, the greatest level of $H^+$-ATPase activity in lactococci and streptococci occurred in cells harvested from media at a $pH_{out}$ of 4.9 to 5.9 [corresponding to $pH_{in}$ of approximately 6.0 to 6.5 (12)]. Below a pH of about 5.0, however, the $H^+$-ATPase activity decreased sharply, which correlated with a rapid decrease in the $pH_{in}$. In contrast, the aciduric *Lactobacillus casei* 685 had relatively high $H^+$-ATPase activity even at low $pH_{out}$ (< 5.0), which is consistent with the observation that this organism maintains a large $\Delta pH$ at low $pH_{out}$ (12). This correlation between the $pH_{in}$ and the level of $H^+$-ATPase suggests that, like *E. faecalis*, the $H^+$-ATPase may be involved in the pH regulatory system in these lactic acid bacteria.

The maximum activity of the streptococcal and lactococcal $H^+$-ATPase, assayed in vitro, occurred at pH 7.0 to 7.5. However, activities were always greater in cells harvested from acidic pH than from neutral pH. This was also true for *Lactobacillus casei* 685, although the pH optimum for the ATPase from this organism was much lower. Thus, the amplified level of $H^+$-ATPase in cells at an acidic $pH_{out}$ may explain how these bacteria are able to maintain a $\Delta pH$ and a high $pH_{in}$. However, because greater $H^+$-ATPase activities were observed as the cytoplasmic pH decreased and became less than the pH optimum of the enzyme, more enzyme must be synthesized as the pH decreases.

## ACKNOWLEDGMENTS

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