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ABSTRACT

The objectives of this research were to determine the effect of lactic acid and low pH on the intracellular pH in three species of lactic acid bacteria. A pH gradient (intracellular pH minus the extracellular pH) of .9 to 1.4 pH units was achieved by several strains of lactic acid bacteria, including *Streptococcus thermophilus* 19258 and 573, *Lactococcus lactis* ssp. *lactis* C2, and *Lactococcus lactis* ssp. *cremoris* HP during log phase of growth in various media. A noticeable decline of the pH gradient occurred at an internal pH of 5.5 to 6.0. In late stationary phase, the pH gradient was generally reduced to .5 pH units or less. In contrast, the aciduric *Lactobacillus casei* 685 maintained a large pH gradient (> 1.0 pH units) even when the medium pH was reduced to less than 4.0. Rapid growth of lactococci and streptococci in media containing excess lactose did not occur when the intracellular pH was reduced below a critical pH of 5.0 or at a neutral pH when proton-uncoupling agents were present. (Key words: lactic acid bacteria, intracellular pH, lactic acid)

Abbreviation key: CCCP = carbonyl cyanide m-chlorophenylhydrazone, ΔpH = pH gradient (intracellular pH minus extracellular pH), PEG = [^{3}H]polyethylene glycol, pH_{in} = intracellular pH, pH_{out} = extracellular pH, SMM = simulated milk medium.

INTRODUCTION

It has been generally accepted that optimum growth of bacteria occurs within a specific pH range, depending upon the species of bacterium (1, 7). Although the pH of the medium or extracellular pH (pH_{out}) indirectly influences cell growth and metabolism, it is the intracellular or cytoplasmic pH (pH_{in}) that ultimately has the greatest effect on cellular activity (7). Kashket (7) has suggested that the pH_{in} is in fact the relevant pH influencing the cell's enzymatic machinery. Several metabolic activities in the streptococci, lactococci, and other lactic acid bacteria, for example, have been reported to be regulated by the pH_{in}, including amino acid transport, peptide transport, and DNA uptake (2, 3, 10, 11, 14, 15). Kashket has further proposed that when the pH_{in} is brought below a particular threshold value cells will stop functioning or die (7).

Many neutrophilic bacteria, such as *Escherichia coli*, are unable to grow when the pH_{in} is below 6.6 to 6.8 (1). In contrast, aciduric lactic acid bacteria, such as the lactobacilli, reportedly can grow until the pH_{in} reaches 4.4 (pH_{out} = 3.5) (7). However, the critical or minimum pH_{in} compatible for growth of other lactic acid bacteria, including lactococci and thermophilic streptococci, have not yet been established.

The objectives of this research were to determine the effect of lactic acid and low pH on the pH_{in} in three species of lactic acid bacteria grown in various media and to determine the critical or minimum pH_{in} below which growth cannot occur.

MATERIALS AND METHODS

Bacteria, Culture Media, and Growth Conditions

Strains used in these experiments included *Streptococcus thermophilus* 19258 and 573, *Lactococcus lactis* ssp. *lactis* C2, *Lactococcus lactis* ssp. *cremoris* HP, *Enterococcus faecalis* 9790 (formerly *Streptococcus faecalis* 9790),

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and *Lactobacillus casei* 685. The *S. thermophilus* strains were grown at 42°C, the lactococci and *L. casei* were grown at 32°C, and *E. faecalis* was grown at 37°C. The *S. thermophilus* and the lactococci were grown in various media, including M17 broth (13), Elliker broth (4), and a simulated milk medium (SMM), and *E. faecalis* 9790 was grown in 2KTY medium (5). The *L. casei* 685 was grown in MRS broth (Difco, Detroit, MI).

The SMM was composed of sweet whey (Dodge Dairy, Dodge, NE), 2.5% casamino acids (Difco), and 1.0% yeast extract (Difco). The media was filter sterilized. This medium was used to simulate growth in milk while allowing clear separation of the cells from the medium for pH_{in} determinations.

Each lactic medium contained an excess of carbohydrate (5% lactose for Elliker, M17, and SMM and 2% for MRS) to ensure continued growth. The concentration of lactose remaining in the culture after extended growth was determined by an enzyme-linked UV method (Lactose/D-Galactose kit, Boehringer Mannheim Biochemicals, Indianapolis, IN).

Cell growth was routinely measured by determining the optical density of appropriately diluted samples at 625 nm. To relate optical density of cells to dry cell weight, samples of washed cells of known optical density were placed in tared weighing dishes and dried for 6 h at 57°C. The dry weight was found to be .35 mg/ml of cells at an optical $density_{625nm}$ equal to 1.0.

Measurement of the Cytoplasmic Volume

The cytoplasmic volumes were determined by the method of Kashket et al. (8). The $[^3H]_2O$ (.03μCi/μl) was added to 6 to 8 ml cell suspensions at the rate of 20 μl/ml of cells and incubated (22°C) for 10 min. Six replicate samples (1.0 ml each) were added to 1.5 ml microcentrifuge tubes containing .5 ml of a Dow-Corning (Midland, MI) silicone oil mixture (35% 556 Fluid and 65% 550 Fluid) and centrifuged at maximum speed for 1 min in a microcentrifuge (Model E, Beckman Instruments, Inc., Fullerton, CA). The $[^3H]_2O$ was used to label the total pellet aqueous volume ($volume_{total}$). A parallel portion of the initial cell suspension was also incubated for 10 min with [^{3}H]polyethylene glycol (PEG; 2.0 mCi/g, .05 μCi/μl) at the rate of 12 μl/ml of cells. Cells were subsequently sampled and centrifuged as above. This was used as an external marker to estimate the extracellular volume ($volume_{out}$) of the pelleted cells. Samples (50 μl) of all supernatant fluids and the pellets were dispensed in separate scintillation vials containing 4 ml of scintillation cocktail (Ready Value, Beckman). Vials were then counted [in the counts per minute (CPM) mode] for 10 min each in a liquid scintillation counter (LS 3801, Beckman). The cytoplasmic volume ($volume_{in}$) was calculated from the total pellet volume less the extracellular volume as

$$volume_{in} = volume_{total} - volume_{out}.$$

The cytoplasmic volume of *L. lactis* ssp. *lactis* was found to be 2.34 μl/mg ± .22 (n = 8) dry weight of cells. This value was also used to estimate the cytoplasmic volume of *L. lactis* ssp. *cremoris* and *S. thermophilus*. Cell volumes of *E. faecalis* and *L. casei* were determined to be 1.97 ± .34 and 2.97 ± .22 μl/mg dry weight of cells, respectively.

Measurement of the Intracellular pH

Cells were inoculated at a level of 1% and were periodically harvested for pH_{in} determination. The amount of cell growth was determined by turbidity measurement using a spectrophotometer at 625 nm. The pH_{out} readings were determined using a pH meter. The pH_{in} was determined according to previously described procedures (8) with modifications. The basis for measurement of pH_{in} has recently been reviewed (1, 9).

Cells (usually 6 to 8 ml) were incubated for 10 min with [^{14}C]benzoic acid (7.3 mCi/mmol, .03 μCi/μl) added at a rate of 8 μl/ml of cells (final benzoic acid concentration was approximately 30 μ*M*). Samples (1.0 ml) were centrifuged through silicone oil, and supernatant fluids and pellets were counted as described above. Because the probe can cross the cell membrane only in its undissociated form, it accumulates inside cells based on the probe pK_a and cytoplasmic pH. From the distribution ratio ([in]/[out]) of the [^{14}C]benzoic acid and the pK_a of benzoic acid (4.19), the pH gradient (ΔpH) ($pH_{in} - pH_{out}$) was calculated using the Henderson-Hasselbalch equation as

$$\Delta pH = \log [benzoate]_{in}/[benzoate]_{out}.$$

The $[benzoate]_{in}$ was determined from the CPM in the cell pellets divided by the cell volume ($[CPM]_{in}$), and the $[benzoate]_{out}$ was determined from the CPM in the supernatant ($[CPM]_{out}$). When the pH_{out} was less than the $pK_a + 1.0$ (i.e., less than 5.19), a correction factor was necessary (9). In these cases, the following equation was used:

$$\Delta pH = \log \{([CPM]_{in}/[CPM]_{out}) (10^{pK_a - pH} + 1) - 10^{pK_a - pH}\}.$$

Because the pH_{out} could easily be determined by electrode measurement, the pH_{in} could be calculated as

$$pH_{in} = pH_{out} + \Delta pH.$$

Chemical disruption of the ΔpH was achieved by the addition of proton-uncoupling agents, carbonyl cyanide m-chlorophenylhydrazone (CCCP; 25 μ*M*, final concentration) or gramicidin D (5 μg/ml culture, final concentration). These ionophores, which allow protons to channel freely back and forth through the cell membrane, were incubated with cells for 10 min prior to addition of the [^{14}C]benzoic acid.

Chemicals

Radiolabeled [^{14}C]benzoic acid and [^{3}H]$_2$O were obtained from Sigma Chemical Co. (St. Louis, MO). Gramicidin and CCCP were also purchased from Sigma. Labeled [^{3}H]PEG was purchased from New England Nuclear Corp. (Boston, MA).

RESULTS

Intracellular pH Determinations

The relationship between the pH_{out} and the pH_{in} in *S. thermophilus* 19258 during growth in Elliker-lactose medium is shown in Figure 1. Standard deviations for all ΔpH and pH_{in} determinations were found to be ± .05 or less. A ΔpH of .8 to 1.0 was maintained during log phase, but the gradient decreased to less than .5 pH units (pH_{in} = 5.0) after 10 h of growth. Continued incubation (24 h) led to a collapse of the ΔpH.

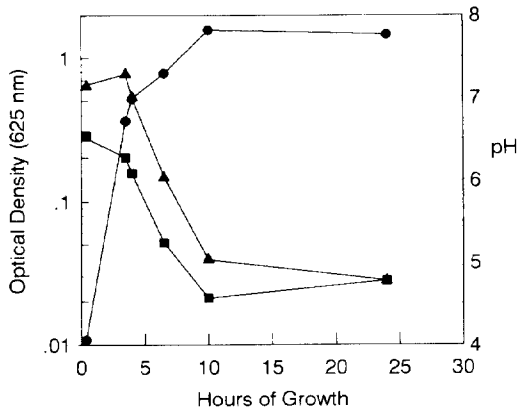

Figure 1. Growth of *Streptococcus thermophilus* 19258 in Elliker-lactose broth at 42°C. Optical density at 625 nm (●); intracellular pH (▲), extracellular pH (■).

Growth of *S. thermophilus* in M17 broth and a SMM gave similar results, with maximum ΔpH of .95 and .97 at pH_{out} of 6.28 (6 h) and 5.95 (4 h), respectively (Figure 2). The ΔpH was decreased to approximately .6 pH units or less in all three media studied when the pH_{out} fell below 4.87 (or after 24 h of growth). Subsequently, the ΔpH in cells held at 42°C for up to 72 h began to decline further and approached zero at pH_{out} of 4.60 to 4.80 (pH_{in} 4.7 to 5.0). Although the buffering capacity of the

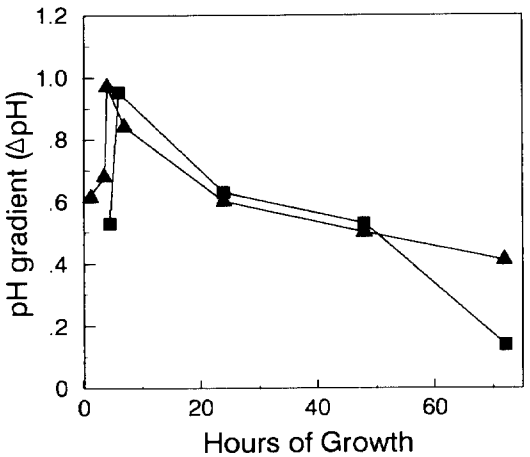

Figure 2. Formation of a pH gradient (intracellular pH – extracellular pH) in *Streptococcus thermophilus* 19258 during growth in M17-lactose broth (■) and simulated milk medium (▲).

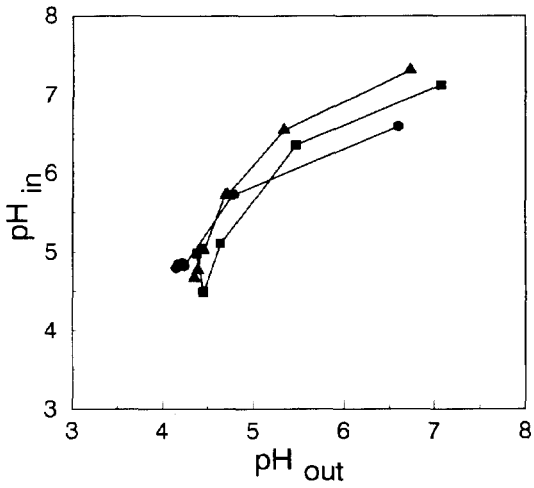

Figure 3. Relationship between the intracellular pH (pH_{in}) and the extracellular pH (pH_{out}) in *Streptococcus thermophilus* 573 during growth in Elliker-lactose broth (●), M17-lactose broth (■), and simulated milk medium (▲).

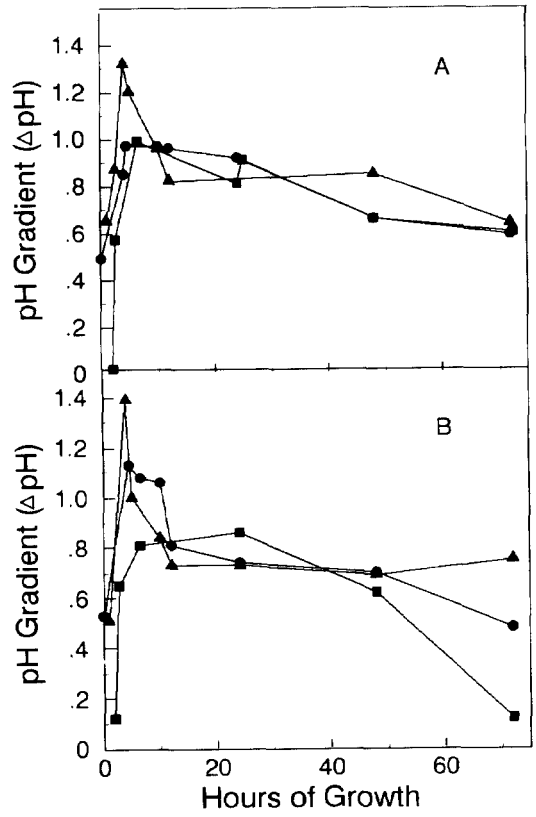

Figure 4. Formation of a pH gradient (intracellular pH – extracellular pH) in *Lactococcus lactis* ssp. *lactis* C2 (A) and *Lactococcus lactis* ssp. *cremoris* HP (B) during growth in Elliker-lactose broth (●), M17-lactose broth (■), and simulated milk medium (▲).

M17 medium was greater than either Elliker medium or the SMM (data not shown), in the presence of excess lactose the rate at which the medium pH decreased (and the pH at which the gradient collapsed) appeared to be the same for all media studied.

A ΔpH was also formed by cells of *S. thermophilus* 573 grown in various media (Figure 3). A maximum ΔpH of .89 to 1.21 was achieved during the log phase of growth in M17 broth, Elliker broth, and SMM. A rapid decline of the ΔpH began to occur at a pH_{out} of 4.5 to 5.0 (optical $density_{625}$ = .8 – 1.2). Dissipation of the ΔpH occurred at a slightly lower pH (4.5) than that found in *S. thermophilus* 19258. Interestingly, *S. thermophilus* 573 grown in Elliker broth was able to maintain a ΔpH (.5 pH units) even at a very low pH_{out} (< 4.2).

As with the streptococci, the *Lactococcus* species also formed large ΔpH during batch growth. A maximum ΔpH of .97 to 1.40 units was formed in *L. lactis* ssp. *lactis* C2 (Figure 4A), whereas *L. lactococcus* ssp. *cremoris* HP (Figure 4B) formed a similar ΔpH of .81 to 1.39 in the three media studied. A rapid decline in the ΔpH generally occurred after about 6 to 8 h of growth (optical $density_{625}$ = 1.0 – 1.2 or at pH_{out} of 4.6 to 5.0 and pH_{in} of 5.0 to 5.5). However, a ΔpH of approximately .6 was maintained for up to 72 h by cells of *L. lactis* ssp. *lactis* C2 in all three media and of *L. lactococcus* ssp. *cremoris* HP in SMM. Comparison of *L. casei* 685, *E. faecalis* 9790, and *S. thermophilus* 19258 demonstrated the differences in the ability of these strains to tolerate low pH (Figure 5). *Lactobacillus casei,* which is reportedly able to grow at lower pH than streptococci (7), maintained the cytoplasmic pH near 6.0 when the pH_{out} was very low (less than 4.5). A large ΔpH (> 1.2 pH units) was sustained by *L. casei* 685 even when the pH_{in} was less than 4.0. In contrast, the ΔpH of *S. thermophilus* and *E. faecalis* 9790 began to collapse below pH_{out} of 5.0 and 6.5, respectively.

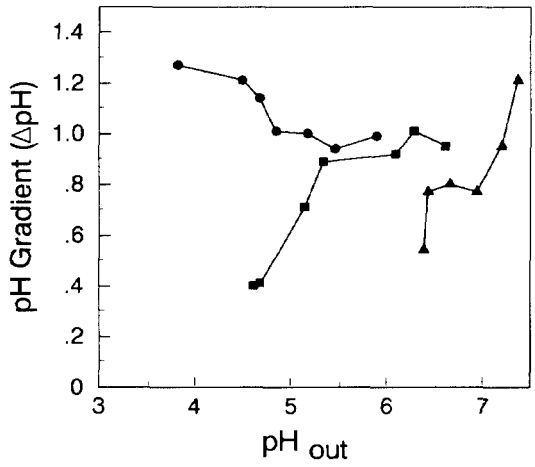

Figure 5. Relationship between the extracellular pH (pH_{out}) and the pH gradient in *Streptococcus thermophilus* 19258 (■), *Lactobacillus casei* 685 (●), and *Enterococcus faecalis* (▲).

Effect of Proton-Uncoupling Agents on the pH Gradient

The addition of gramicidin to growing cells of *S. thermophilus* 19258 caused the ΔpH to collapse (data not shown). Gramicidin-treated cells at a pH_{out} of 7.0 had a ΔpH of 0, whereas control cells maintained a gradient of .51 (pH_{in} = 7.51). Growth of the treated cells did not occur below pH_{in} of 6.75 in the absence of a ΔpH. The protonophore, CCCP, also caused the ΔpH to decrease, but the ΔpH was maintained at about .36 pH units.

DISCUSSION

It has long been recognized that lactic starter cultures grow best when the medium pH is near neutral and that growth rates decline as the extracellular medium becomes acidic (6). Few studies have been performed, however, to explain these effects. In the experiments reported in this research, the optimum pH for growth of the lactic acid bacteria studied occurred at pH_{in} range of approximately 7.5 to 6.0 (pH_{out} > 5.0). In each of the three species of lactic acid bacteria studied, a similar decline in the internal and external pH occurred during log phase due to the production of lactic acid. Similar results were also reported for *L. lactis* ssp. *cremoris* (12), although the magnitude of the ΔpH was somewhat less than reported here. The greatest change in pH seemed to occur at 6 to 12 h of growth in each of the three types of media studied. A gradient of .6 to 1.44 pH units was achieved in early log phase, and a noticeable decline in the ΔpH between the extracellular medium and the cytoplasm occurred during the late log phase of growth, corresponding to pH_{in} of 5.0 to 5.5 or pH_{out} < 5.0.

The results indicate that the critical or minimum pH compatible for cell growth was similar for all three growth media studied, even though the buffering capacities of the media were slightly different (data not shown). The amount of lactose remaining after extended fermentation (72 h) by the lactococci and streptococci was approximately 3.0%. Therefore, cessation of growth below pH_{out} of 5.0 had apparently occurred because the ΔpH had dissipated (resulting in a low pH_{in}) rather than because of carbohydrate depletion. Therefore, growth and metabolic activities are inhibited as a consequence of the deleterious effects of a low pH_{in}. More acid tolerant stains of lactic acid bacteria (e.g., *L. casei*) are able to maintain the cytoplasmic pH between 5.1 and 6.4 even at a low pH_{out} (3.8), indicating possible differences in the mechanisms that control the pH_{in}.

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