

Dynamic Changes of Intracellular pH in Individual Lactic Acid Bacterium Cells in Response to a Rapid Drop in Extracellular pH

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Received 15 November 1999/Accepted 8 March 2000

We describe the dynamics of changes in the intracellular pH (pH_i) values of a number of lactic acid bacteria in response to a rapid drop in the extracellular pH (pH_{ex}). Strains of *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Streptococcus thermophilus*, and *Lactococcus lactis* were investigated. *Listeria innocua*, a gram-positive, non-lactic acid bacterium, was included for comparison. The method which we used was based on fluorescence ratio imaging of single cells, and it was therefore possible to describe variations in pH_i within a population. The bacteria were immobilized on a membrane filter, placed in a closed perfusion chamber, and analyzed during a rapid decrease in the pH_{ex} from 7.0 to 5.0. Under these conditions, the pH_i of *L. innocua* remained neutral (between 7 and 8). In contrast, the pH_i values of all of the strains of lactic acid bacteria investigated decreased to approximately 5.5 as the pH_{ex} was decreased. No pronounced differences were observed between cells of the same strain harvested from the exponential and stationary phases. Small differences between species were observed with regard to the initial pH_i at pH_{ex} 7.0, while different kinetics of pH_i regulation were observed in different species and also in different strains of *S. thermophilus*.

Bacteria have developed different ways to withstand stressful situations, such as a decrease in the pH_{ex} . Neutrophilic bacteria like *Escherichia coli* maintain a pH_i that is close to neutral when the pH_{ex} is decreased and therefore generate large proton gradients (28). Among the gram-positive bacteria, strains of *Enterococcus hirae* which were originally identified as *Streptococcus faecalis* (12) have been studied extensively in order to examine pH homeostasis (14–16). These bacteria also grow at alkaline pH values, and they are considered neutrophiles (31), although they are phylogenetically related to streptococci and lactococci.

Many acid-tolerant fermentative bacteria have developed another strategy; in these organisms the pH_i decreases as the pH_{ex} decreases during growth (4, 23) in order to maintain a constant pH gradient rather than a constant pH_i . Generating a large proton gradient is disadvantageous for fermentative lactic acid bacteria, because proton translocation consumes energy (16), and anaerobic organisms gain significantly less energy from sugar metabolism than aerobes gain. Furthermore, a large proton gradient results in accumulation of organic acid anions in the cytosol (33).

Food fermentations are often carried out by sequential microbial populations; this occurs in dairy fermentations, such as yogurt fermentation (32), as well as in indigenous spontaneous fermentations of cereals and vegetables (7, 10, 20). Lactic acid bacteria, particularly lactobacilli, which are considered the most acid-tolerant bacteria, are often dominant at the end of these fermentations (13, 34). The acid tolerance of these organisms is advantageous, as they have a competitive advantage over known pathogens and other undesirable bacteria when the concentration of organic acids is high (34). A mixture of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus*

thermophilus is used for yogurt fermentation. *S. thermophilus* grows faster in the beginning of a fermentation, whereas *L. delbrueckii* subsp. *bulgaricus* finishes the fermentation due to the more pronounced acid tolerance of this species. Another very important lactic acid bacterium from a dairy viewpoint is *Lactococcus lactis*, whose pH_i has been more extensively investigated (3, 4, 22, 23).

The study described here was undertaken in order to investigate the dynamics of pH regulation in individual bacterial cells. Carboxyfluorescein, which was used throughout this study, is a ratiometric pH probe that exhibits no pH sensitivity when it is excited at 435 nm and maximal sensitivity when it is excited at 490 nm. After we obtained a fluorescent signal at each excitation wavelength, a concentration-independent ratio between pH-sensitive and pH-insensitive signals was calculated. The ratio measurements precluded potential artifacts due to variations in dye concentration. This method has been used successfully to measure pH_i values in populations of bacteria (3, 22). In FRIM, the technique described above is combined with a microscope equipped with a charge-coupled device camera, which allows measurements for single cells to be obtained. As bacterial cells are small, the fluorescence intensity of an individual cell is low, which provides a significant experimental challenge. Although this technique has many advantages, pH_i examinations of bacteria in which FRIM has been used have been limited to studies of developing *Bacillus subtilis* forespores (17, 18) and investigations of a mixture of *L. delbrueckii* subsp. *bulgaricus* and *Listeria innocua* (35).

In this study, we used FRIM combined with a perfusion system, which allowed us to determine the dynamics of pH_i regulation during a change in pH_{ex} , as well as the heterogeneity in pH_i in a population. We investigated a number of strains of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* and thus examined variations within species. Finally, *L. innocua* was included as model pathogenic organism. Previously, we found that pH_i regulation in *L. innocua* was very different from pH_i regulation in *L. delbrueckii* subsp. *bulgaricus* (35).

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TABLE 1. Bacterial strains used and growth conditions

Species or subspecies	Medium	Growth temp (°C)	Strain(s) ^a
<i>Streptococcus thermophilus</i>	M17	37	50, 61, 63, 68
<i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>	MRS	42	NCFB 2772, 01, 08
<i>Lactococcus lactis</i> subsp. <i>lactis</i>	M17	30	02
<i>Listeria innocua</i>	BHI ^b	30	AJL-1

^a NCFB 2772 was kindly provided by G. Grobben, Wageningen University, Wageningen, The Netherlands. AJL-1 was provided by the Alfred Jørgensen Laboratory Ltd., Copenhagen, Denmark. All other strains were commercial starter cultures obtained from the culture collection at MD Foods R & D, Aarhus, Denmark.

^b BHI, brain heart infusion broth.

MATERIALS AND METHODS

Abbreviations. FRIM, fluorescence ratio imaging; pH_i , intracellular pH; pH_{ex} , extracellular pH; ΔpH , pH gradient ($pH_i - pH_{ex}$); OD_{600} , optical density at 600 nm.

Bacterial strains and growth conditions. The bacterial strains, media, and growth conditions used in this study are shown in Table 1. MRS and brain heart infusion broth were purchased from Difco, and M17 broth was obtained from Oxoid. Stationary cultures were grown overnight (OD_{600} for the lactic acid bacteria, approximately 4 to 5; OD_{600} for *L. innocua*, 1.3), and exponential-phase cultures were harvested from mid-exponential growth (OD_{600} for the lactic acid bacteria, approximately 1; OD_{600} for *L. innocua*, 0.4).

Buffers and solutions. The pH values of citrate-potassium phosphate buffers were adjusted by mixing citric acid (25 mM) and K_2HPO_4 (50 mM). A 1 M glucose stock solution was added to all buffers to obtain a final glucose concentration of 10 mM prior to each experiment in order to supply energy to the cells. Solutions containing 50 μM 5(6)-carboxyfluorescein (Sigma) in buffer were prepared from a concentrated stock solution (3 mM in dimethyl sulfoxide) by dilution in buffer at pH 7.0 and 5.0. All chemicals were analytical grade and were obtained from Merck, unless indicated otherwise.

Staining protocol. Cells were harvested by centrifugation (10,000 $\times g$, 2 min) and were resuspended in buffer (pH 7.0) to an OD_{600} of 0.6. Subsequently, cells were incubated in the presence of 10 μM 5(6)-carboxyfluorescein diacetate succinimidyl ester (Molecular Probes Inc., Eugene, Oreg.) at 37°C for 30 min. When perfusion experiments were performed, cells were analyzed immediately after staining, while the pH-equilibrated cells used for validation of pH_i measurements were stored on ice in the dark for a maximum of 1 h prior to analysis.

The buffers used in this study contained citric acid at a concentration corresponding to a concentration of undissociated citric acid of less than 0.2 mM in the pH 5.0 buffer. We noticed that ΔpH in *L. delbrueckii* subsp. *bulgaricus* NCFB 2772 at pH_{ex} 5.0 was approximately 0.5 pH unit lower when the staining buffer was a citrate phosphate buffer than when a pure potassium phosphate buffer was used (data not shown).

Immobilization of cells for microscopic analysis. Stained cells were immobilized by drawing aliquots of an appropriate dilution through a 0.45- μm -pore-size membrane filter and mounting the part of the filter containing the bacteria in a perfusion chamber as previously described (35).

Fluorescence microscopy. The microscope setup used has been described previously (6) and consisted of a monochromator providing two excitation wavelengths (490 and 435 nm) and an inverted microscope equipped with a $\times 100$ objective. The emitted light (515 to 565 nm) was collected with a cooled charge-coupled device camera. Experiments were controlled by using the software package Metafluor 3.5 (Universal Imaging Corp., West Chester, Pa.), and background subtraction and image analysis were performed with saved experimental data as previously described (35).

The perfusion chamber (model RC-21A; Warner Instrument Corp., Hamden, Conn.) was mounted on the stage of the microscope. A schematic diagram of the chamber has been published previously (21). Solutions were perfused through the inlet of the chamber at a rate of 8.3 $\mu l s^{-1}$ by using a modified Alitea-XV pump (Microlab Aarhus A/S, Aarhus, Denmark). After passage through the chamber, the liquid was continuously removed from the outlet reservoir by another pump. The perfusion pump was calibrated prior to each session.

In each experiment the perfusion chamber was filled with pH 7.0 buffer after the filter was mounted, and perfusion was initiated at 2 min with a pH 5.0 perfusion solution. All experiments were performed at least twice on different occasions, and in general, the average from one experiment was within the standard deviation of the duplicate experiment for every acquisition point. For clarity, the results of a single experiment are presented below.

Equilibration of pH_i with pH_{ex} . Stained cells were suspended in buffers having different pH values. Valinomycin (Sigma) and nigericin (Molecular Probes Inc.)

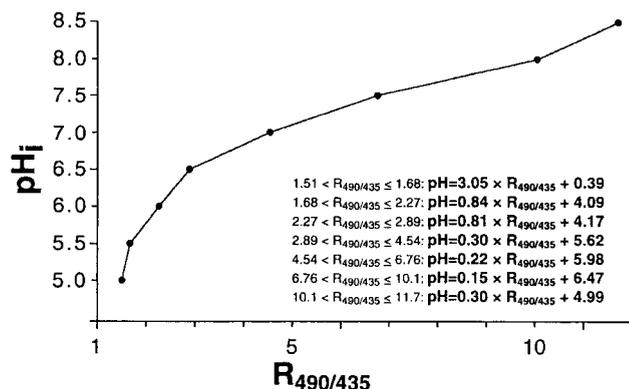


FIG. 1. Correlation between excitation ratio 490 nm/435 nm ($R_{490/435}$) and pH_i in pH-equilibrated cells of *L. delbrueckii* subsp. *bulgaricus* NCFB 2772 in buffers with pH values ranging from 5.0 to 8.5. At least 20 cells were used for every calibration point. Linear equations were determined for adjacent calibration points, which resulted in seven equations describing the relationship between $R_{490/435}$ and pH_i over the pH range investigated.

were each added to a final concentration of 5 μM , and this was followed by incubation at 37°C for 10 min. Valinomycin renders plasma membranes permeable to potassium ions, and nigericin exchanges potassium for protons; thus, the combined actions of these compounds result in equilibration of both potassium ions and protons across the membrane. The cells were immobilized as described above, and the chamber was filled with buffer containing valinomycin and nigericin before ratio images were acquired.

Addition of valinomycin and nigericin had almost no effect on *Lactococcus lactis* subsp. *lactis* 02, and stained cells of this strain were therefore permeabilized by treatment with 70% ethanol for 30 min prior to resuspension in the appropriate buffers to obtain pH-equilibrated cells.

Calculation of pH_i . Calculation of pH_i from the ratio images was based on pH-equilibrated cells of *L. delbrueckii* subsp. *bulgaricus* NCFB 2772. A piecewise linear equation for the ratio value and pH was derived from the equations shown in Fig. 1. Conversion was automatically performed with Microsoft Excel, and the ratio value for every cell at every time point was converted to pH_i before the average and standard deviation were calculated.

RESULTS

Rate of pH change during perfusion. The pH in the chamber during the experiments was estimated by filling the chamber with pH 7.0 buffer containing 50 μM carboxyfluorescein and recording the two excitation images at 15-s intervals. After 60 s, pH 5.0 buffer containing the same concentration of fluorochrome was flushed through the chamber. In two such experiments, ratio images were recorded close to the center of the chamber, where the membrane filter was located. In a third experiment, ratio images were recorded near the outlet of the chamber, and the resulting values are shown in Fig. 2. The data show that the shift from pH 7.0 to 5.0 occurred rapidly. In the center of the chamber, the decrease began almost simultaneously with the perfusion, and the complete change occurred within 30 s after initiation. At the outlet, the response was slightly delayed, but the change was still complete within 1 min. All subsequent analyses were performed close to the center of the chamber. The ratios in Fig. 2 cannot be converted to pH_i values by using the equation described above because the experimental setup was different (i.e., a large volume of fluorescent buffer was used instead of stained cells).

Validation of pH_i calculation from ratios in different bacterial species. The piecewise linear equation described in Fig. 1 was obtained by using *L. delbrueckii* subsp. *bulgaricus* NCFB 2772, and we examined whether this equation could be used to determine pH_i in all of the species investigated. To do this, all strains were pH equilibrated at pH_{ex} 7.0 and 6.0, and the ratios for more than 20 cells in each experiment were recorded on a

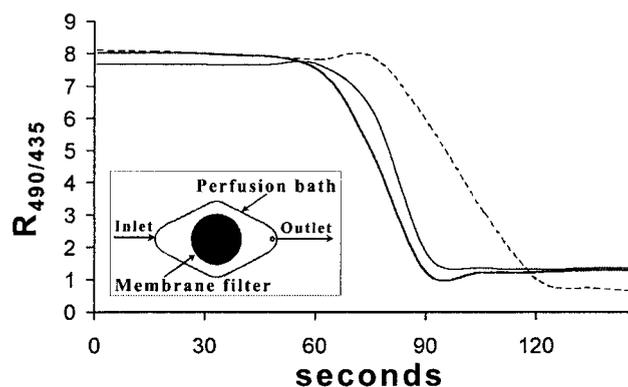


FIG. 2. Rate of $R_{490/435}$ change during perfusion. The chamber was filled with pH 7.0 buffer containing the fluorescent probe carboxyfluorescein, and thus the initial level was pH 7.0. Perfusion was initiated after 60 s with pH 5.0 buffer containing carboxyfluorescein, and the final level corresponded to pH 5.0. The solid lines show the results of two independent perfusion experiments performed in the area covered by the membrane filter, and the dotted line shows the results of an experiment in which the analysis was performed close to the outlet. The inset is a schematic diagram of the perfusion chamber. The membrane filter was located in the center of the diamond-shaped bath. The perfusion liquid flowed from left to right and left the bath through the outlet.

spreadsheet. The equation was subsequently used to convert ratio values to pH_i values, as shown in Table 2. For all strains, the pH_i should have been the same as the pH_{ex} after equilibration. The largest difference between pH_i and pH_{ex} for the strains was 0.2, which is close to the accuracy of the method (35), and the equation was therefore used to convert ratios to pH_i values throughout the experiment.

Change in the pH_i of lactic cocci as a response to decreasing pH_{ex} . Figure 3 shows the changes in the pH_i values of stationary-phase cells of four strains of *S. thermophilus* as the pH_{ex} was decreased from 7.0 to 5.0. At 2 min perfusion was initiated, and at 2.5 min the pH_{ex} was 5.0 in the center of the chamber, where the cells were located. All of the streptococcal strains had initial pH_i values between 7.4 and 7.6 (Fig. 3). The ΔpH at the end of the experiment (>20 min) was close to 0.5 pH unit for all strains. The standard deviations in the starting pH_i values for the streptococci ranged from 0.15 to 0.25 pH unit, which indicated that the populations were homogeneous. *S. thermophilus* 63 maintained a high pH_i for a longer period than the other strains; the pH_i of this strain decreased to 6.5 after 5 min of perfusion (Fig. 3C), while the pH_i values of the other

TABLE 2. Calculated pH_i values for pH-equilibrated cells as determined by the equation derived from *L. delbrueckii* subsp. *bulgaricus* NCFB 2772 data

Strain	pH_i at:	
	pH_{ex} 6.0	pH_{ex} 7.0
<i>S. thermophilus</i> 50	6.1 ± 0.1^a	7.0 ± 0.1
<i>S. thermophilus</i> 61	6.1 ± 0.1	7.0 ± 0.1
<i>S. thermophilus</i> 63	6.2 ± 0.1	7.1 ± 0.1
<i>S. thermophilus</i> 68	6.2 ± 0.1	7.1 ± 0.1
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> NCFB 2772	6.0 ± 0.2	7.0 ± 0.0
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> 01	6.1 ± 0.3	7.2 ± 0.1
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> 08	5.8 ± 0.2	7.1 ± 0.1
<i>L. lactis</i> subsp. <i>lactis</i> 02	6.2 ± 0.1	7.1 ± 0.1
<i>L. innocua</i>	6.2 ± 0.1	7.2 ± 0.1

^a Values are means \pm standard deviations based on the data obtained for at least 20 cells.

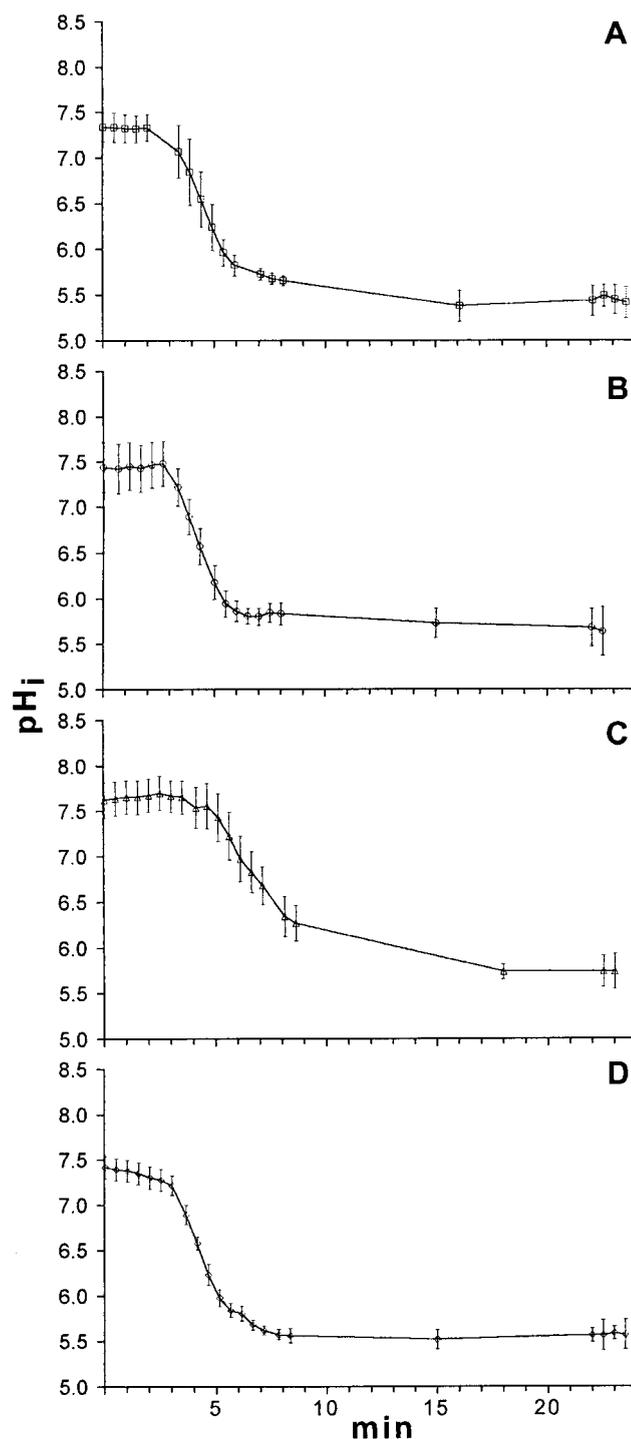


FIG. 3. Change in the pH_i of four strains of *S. thermophilus* as the pH_{ex} was decreased from 7.0 to 5.0. Perfusion was initiated after 2 min. (A) *S. thermophilus* 50. (B) *S. thermophilus* 61. (C) *S. thermophilus* 63. (D) *S. thermophilus* 68. Each line shows the average values for at least 20 individual cells, and the error bars indicate the standard deviations.

strains reached this level within 2 to 2.5 min (Fig. 3A, B, and D). The pH_i profile of stationary-phase cells of *L. lactis* subsp. *lactis* is shown in Fig. 4. The behavior of this bacterium was similar to the behavior of *S. thermophilus* 63 (Fig. 3C), as the

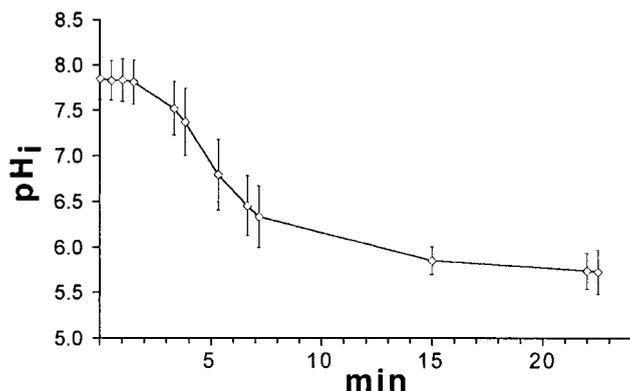


FIG. 4. Change in the pH_i of *L. lactis* subsp. *lactis* 02 as the pH_{ex} was decreased from 7.0 to 5.0. Perfusion was initiated after 2 min. The line shows the average values for at least 20 individual cells, and the error bars indicate the standard deviations.

pH_i decreased slowly. In addition, the ΔpH was relatively high (0.8 pH unit) when the pH_i stabilized at pH_{ex} 7.0 or 5.0.

Change in the pH_i of *L. delbrueckii* subsp. *bulgaricus* in response to a decrease in the pH_{ex} . The pH_i profiles for three strains of *L. delbrueckii* subsp. *bulgaricus* harvested from stationary-phase cultures are shown in Fig. 5. The pH_i values of all of these strains decreased more rapidly than pH_i values of the cocci decreased (Fig. 3 and 4). After 2.5 min of perfusion, the pH_i values of all three strains had decreased to 6.0 or less. The ΔpH was 0.5 pH unit for *L. delbrueckii* subsp. *bulgaricus* NCFB 2772 and 01 (Fig. 5A and B) and as low as 0.3 pH unit for *L. delbrueckii* subsp. *bulgaricus* 08 (Fig. 5C). The initial pH_i values were 7.3 to 7.5, and the standard deviations for all three strains were less than 0.1 pH unit. The heterogeneity in pH_i increased to 0.2 to 0.3 pH unit after perfusion.

Change in the pH_i of *L. innocua* in response to a decrease in the pH_{ex} . *L. innocua* is an example of a homeostatic bacterium (35), and under the same perfusion conditions that were used for the lactic acid bacteria, the pH_i of stationary-phase cells was close to neutral (i.e., between 8.0 and 7.1) when the pH_{ex} was decreased from 7.0 to 5.0 (Fig. 6). The heterogeneity in pH_i values was more pronounced after perfusion, and the heterogeneity reached a level of almost 0.9 pH unit.

Change in the pH_i in response to a lower pH_{ex} in exponentially growing cells. Cells harvested from exponential cultures of *L. delbrueckii* subsp. *bulgaricus* NCFB 2772, *L. lactis* subsp. *lactis*, and *L. innocua* were examined in order to investigate the influence of growth phase on pH_i regulation (Fig. 7). The responses of these cells were comparable to the responses of stationary-phase cells of the same species (Fig. 4, 5A, and 6). The heterogeneities of the populations were also similar, although the standard deviation in the pH_i of *L. innocua* after perfusion was less pronounced than that in the stationary-phase culture (Fig. 6).

DISCUSSION

In most studies of pH_i in lactic acid bacteria the workers have used the ion distribution of radioactively labeled weak acids to measure pH_i (4, 11, 13, 23, 26, 27, 36). This method involves equilibration of a weak acid between the medium and the cytosol, and it is therefore not possible to measure rapid changes in pH_i . Recent studies in which spectrofluorometric determination of pH_i was used included dynamic measurements obtained after various substances were added (3, 19),

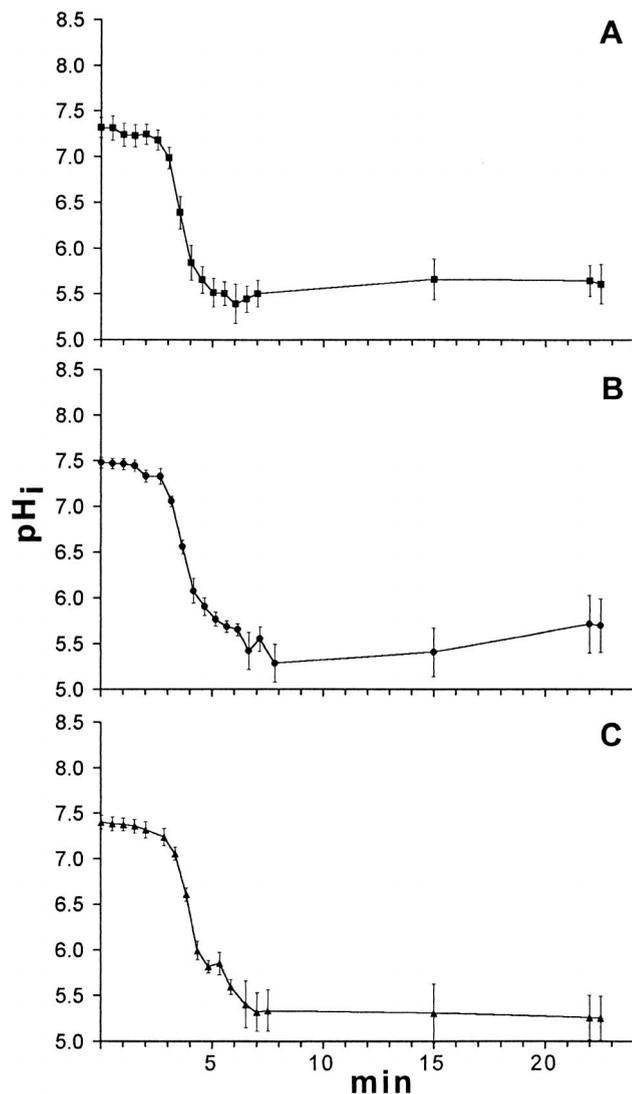


FIG. 5. Change in the pH_i of three strains of *L. delbrueckii* subsp. *bulgaricus* as the pH_{ex} was decreased from 7.0 to 5.0. Perfusion was initiated after 2 min. (A) *L. delbrueckii* subsp. *bulgaricus* NCFB 2772. (B) *L. delbrueckii* subsp. *bulgaricus* 01. (C) *L. delbrueckii* subsp. *bulgaricus* 08. Each line shows the average values for at least 20 individual cells, and the error bars indicate the standard deviations.

but as the measurements were determined in a cuvette, it was not possible to determine the pH_i values for single cells. Recently, we demonstrated that the pH_i values of single cells of *L. delbrueckii* subsp. *bulgaricus* and *L. innocua* could be determined by FRIM at a pH_i range of 5.0 to 8.0 (35). In this study, we used the same method to monitor the dynamic changes in the pH_i values of a number of lactic acid bacteria as the pH_{ex} was rapidly decreased from 7.0 to 5.0. The decrease in pH_{ex} did not constitute a severe acid shock, as lactic acid bacteria naturally acidify the external medium to pH values below 5.0 during growth (11).

It has been suggested that pH homeostasis is best reflected in the ability to restore the pH_i after perturbation (2), including rapid shifts in pH_{ex} . The results of previous studies of lactic acid bacteria have not been entirely consistent with regard to pH regulation at low pH_{ex} values. In *L. lactis* at pH_{ex} 5.0, the ΔpH ranges from 0.4 pH unit (4) to 2 pH units (3, 22, 29), and

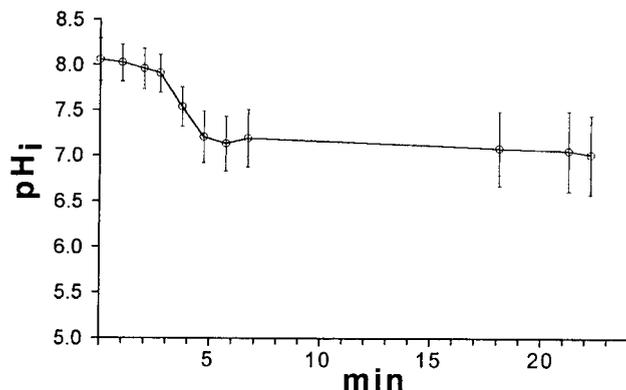


FIG. 6. Change in the pH_i of *L. innocua* as the pH_{ex} was decreased from 7.0 to 5.0. Perfusion was initiated after 2 min. The line shows the average values for at least 20 individual cells, and the error bars indicate the standard deviations.

in *L. plantarum* at pH_{ex} 4.5, the ΔpH ranges from 0.7 pH unit (20) to almost 2 pH units (36). Some of the differences might be attributed to the presence of organic acid anions (34); e.g., low levels of lactate (less than 30 mM) significantly reduce the pH_i of *L. lactis* (4). The concentrations of compensating cations, such as potassium and sodium ions, are also known to influence pH_i values (2, 12), and experimental differences complicate comparisons of the results of different studies. In this study, however, we compared cells under the same experimental conditions for all of the species investigated, and we observed that the pH_i values of all of the lactic acid bacteria investigated decreased, which resulted in ΔpH values of 0.5 to 0.8 pH unit. The pH_i values for the populations of lactic acid bacteria were also quite homogeneous, which indicated that a pH_{ex} of 5.0 is not a pronounced stress for these bacteria. In contrast, the ΔpH for *L. innocua* was more than 2 pH units when the pH_{ex} was 5.0, which confirmed that this bacterium is homeostatic (Fig. 6 and 7). The greater heterogeneity in pH_i at pH_{ex} 5.0 (Fig. 6) may reflect greater stress imposed on *L. innocua* at low pH_{ex} values.

In our experiments, exponentially growing cells of *L. delbrueckii* subsp. *bulgaricus* NCFB 2772, *L. lactis* subsp. *lactis*, and *L. innocua* exhibited the same pattern of pH_i regulation (Fig. 7) as stationary-phase cells exhibited (Fig. 5A and 6). This

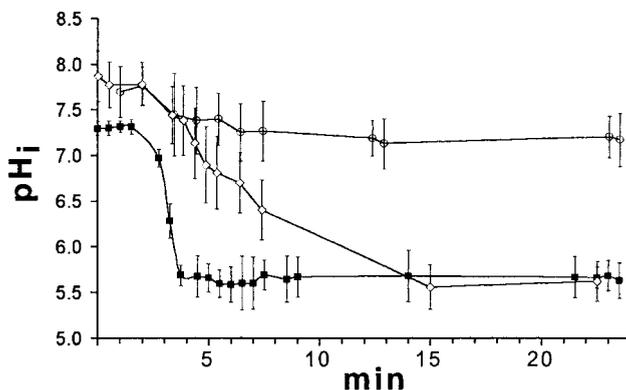


FIG. 7. Changes in the pH_i of exponentially growing cells of *L. innocua* (\circ), *L. lactis* subsp. *lactis* 02 (\diamond), and *L. delbrueckii* subsp. *bulgaricus* NCFB 2772 (\blacksquare) as the pH_{ex} was decreased from 7.0 to 5.0. Perfusion was initiated after 2 min. Each line shows the average values for at least 20 individual cells, and the error bars indicate the standard deviations.

is somewhat surprising, as it is generally accepted that cells entering the stationary phase undergo radical changes which ensure that they can deal with physical stresses (30), and it is known that low pH_{ex} values (such as the pH_{ex} values in stationary phase) induce adaptation mechanisms that increase survival (5, 8, 9, 27). The similarities in pH_i regulation in stationary and exponentially growing cells may reveal a universal characteristic of these bacteria, but the influence of methodological artifacts needs to be investigated to confirm this observation. For example, we cannot eliminate the possibility that incubation in buffer containing glucose and prefluorochrome induces similar physiological changes in the two growth phases.

The rate and pattern of pH_i regulation in the species investigated appear to mirror the acid tolerance of the bacteria. In the very acid-tolerant lactobacilli the pH_i decreases faster than it decreases in the moderately acid-tolerant lactic cocci during a change in pH_{ex} , and the pH_i of *L. innocua* does not decrease below 7.0. The different rates of pH_i decrease observed for the strains of *S. thermophilus* (Fig. 3) may also be correlated with differences in acidification performance. *S. thermophilus* 63, which exhibited the slowest decrease in pH_i during perfusion (Fig. 3C), was investigated because it exhibited poor acidification when standard fermentation tests in milk were performed (unpublished data).

There are several possible mechanisms by which a bacterium can regulate pH_i , but the most important mechanism in fermentative bacteria appears to be the proton-translocating ATPase (11, 16, 24). The pH data for this enzyme isolated from *Lactobacillus casei* and *Lactobacillus plantarum* revealed that the pH optima were 5.0 to 5.5 (1, 10, 24), and these values are markedly lower than the pH optima for strains of *S. thermophilus* and *L. lactis*, which were determined to be 7.0 to 7.5 (24). Another parameter involved in pH_i regulation is the overall proton permeability of the plasma membrane. In *L. casei* and *L. plantarum*, this permeability was minimal at pH 4.0 (1, 10), and in the acid-sensitive organism *Actinomyces viscosus* it was minimal at pH 6.0 (1). These observations could explain the rapid decreases in pH_i values in lactobacilli (Fig. 5), as these bacteria may not actively regulate pH_i until the pH_{ex} is low.

Other factors, such as the cytoplasmic buffering capacity, are thought to have little influence on pH_i regulation (11), and similar values have been found with most bacteria (2). Decarboxylation of amino acids leads to biochemical consumption of protons, and this process may contribute to acid tolerance during growth (25). However, the buffers used in this study did not contain amino acids, and therefore it is unlikely that consumption of amino acids was involved in pH_i regulation to significant extent.

Although the mechanisms behind the observed differences in pH_i regulation cannot be evaluated without further studies, the physiological significance of maintaining a small ΔpH is obvious. The energy requirement for proton translocation and accumulation of organic acid anions is reduced in lactic acid bacteria compared to homeostatic bacteria, and this is probably one of the reasons for the predominance of lactic acid bacteria in food fermentations.

It is conceivable that the differences in the rate of pH_i decrease in the lactic acid bacteria investigated could be used to improve industrial fermentations, as the change in pH_i appears to mirror acid tolerance. We are planning to test this hypothesis in experiments in which we will use *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* as model organisms in mixed-culture fermentations.

ACKNOWLEDGMENTS

The skillful technical assistance of Jan Hansen is gratefully acknowledged. We thank N. Arneborg and A. Gravesen for critically reading the manuscript.

This work was part of the FØTEK2 program supported by the Danish Dairy Research Foundation (Danish Dairy Board) and the Danish government.

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