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# Intracellular pH Is a Major Factor in the Induction of Tolerance to Acid and Other Stresses in *Lactococcus lactis*

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**This study demonstrates that exposure of log-phase *Lactococcus lactis* subsp. *cremoris* 712 cells to mildly acid conditions induces resistance to normally lethal intensities of environmental stresses such as acid, heat, NaCl, $H_2O_2$, and ethanol. The intracellular pH ($pH_i$) played a major role in the induction of this multistress resistance response. The $pH_i$ was dependent on the extracellular pH ($pH_o$) and on the specific acid used to reduce the $pH_o$. When resuspended in fresh medium, cells were able to maintain a pH gradient even at $pH_o$ values that resulted in cell death. Induction of an acid tolerance response (ATR) coincided with an increase in the ability of cells to resist change to an unfavorable $pH_i$; nevertheless, a more favorable $pH_i$ was not the sole reason for the increased survival at acid $pH_o$. Cells with an induced ATR survived exposure to a lethal $pH_o$ much better than did uninduced cells with a $pH_i$ identical to that of the induced cells. Survival following lethal acid shock was dependent on the $pH_i$ during induction of the ATR, and the highest survival was observed following induction at a $pH_i$ of 5.9, which was the lowest $pH_i$ at which growth occurred. Increased acid tolerance and the ability to maintain a higher $pH_i$ during lethal acid stress were not acquired if protein synthesis was inhibited by chloramphenicol during adaptation.**

The extracellular pH ($pH_o$) range over which microorganisms survive and grow is approximately 1.0 to 11.0 (33). Based on the $pH_o$ values at which they grow, bacteria are divided into the following three groups: neutralophiles, for example, *Escherichia coli*, which grow best at pH values near neutrality; acidophiles, which grow optimally at more acidic pH values; and alkalophiles, which have their pH optimum in the alkaline pH range (2, 33). Most lactic acid bacteria encountered in dairy products are regarded as neutralophiles, but their intracellular pH ($pH_i$) is not as tightly regulated as that of *E. coli* (21). Lactococci (22, 38) and lactobacilli (28), as well as other fermentative bacteria, such as clostridia (1), allow their $pH_i$ to decrease as the $pH_o$ decreases due to the build up of acidic end products. The detrimental effect of low pH on the growth of *Lactococcus* cells has long been recognized. Optimal growth has been reported to occur in the $pH_o$ range of 6.3 to 6.9 (15). During growth, lactococci generally reduce the pH of their growth media to approximately 4.5 due to the accumulation of organic acids (21). To reduce the risk of poor-quality starters resulting from acid damage to cells, the cheese industry has devoted much time and expense to the development of methods to prevent the $pH_o$ of the starter growth medium from dropping to harmful levels (41).

Induced acid tolerance defines a condition whereby, during exposure to mildly acidic conditions, bacteria acquire the ability to survive lethal acid concentrations. This inducible mechanism is referred to as the acid tolerance response (ATR) and has been observed in a variety of bacteria. It was first reported in 1989, when Goodson and Rowbury (10) demonstrated habituation of *E. coli* to normally lethal acidity by prior growth at a sublethal pH. Since then, it has also been observed in *Leuconostoc mesenteroides*, *Lactobacillus plantarum* (28), *Listeria monocytogenes* (24, 31), and a wide variety of potentially pathogenic gram-negative bacteria, such as *Salmonella typhimurium* (8), *S. enteritidis* (16), *Aeromonas hydrophila* (19), and *E. coli* O157:H7 (27). We have previously reported the possession of an ATR by *Lactococcus lactis* subsp. *cremoris* 712 (32), which has since been confirmed by Rallu et al. (35), who observed an ATR in a plasmid-free derivative of the same strain.

A number of other stress responses besides the ATR have been reported for *L. lactis*. A heat shock response similar to the well-documented heat shock response of *E. coli* (40) and an oxidative stress response induced on exposure to hydrogen peroxide have been observed in lactococci (3). $H_2O_2$-induced cells were also more resistant to lethal high temperatures than were uninduced cells. This indicates an overlap between the protective mechanisms induced by heat shock and sublethal oxidative stress and suggests cross-protective ability in *L. lactis*. There are other reports of cross-protection mechanisms in *L. lactis* in the literature. Carbohydrate-starved stationary-phase lactococcal cells showed enhanced resistance to acid, heat, ethanol, and osmotic and oxidative stresses (13), and UV-irradiated cells were better able to survive lethal acid, heat, ethanol, and oxidative challenges than were unirradiated cells (14). This paper is concerned with the role of $pH_i$ in the induction of protective systems against several environmental stresses in exponentially growing cells of *L. lactis* subsp. *cremoris* 712.

## MATERIALS AND METHODS

**Bacterial strain and growth conditions.** The culture used in this study was *L. lactis* subsp. *cremoris* NCDO 712 (5). This strain was recently transferred to the subspecies *cremoris* from the subspecies *lactis* on the basis of DNA homology (9). Cultures were grown routinely at 30°C in TYG medium, which contained 17 g of tryptone per liter, 3 g of yeast extract per liter, 10 g of glucose per liter, 3.27 g of $KH_2PO_4$ per liter, and 2.28 g of $Na_2HPO_4$ per liter and was solidified, when required, with 1.5% agar. The initial pH of the medium was 6.7 unless otherwise stated. The pH was adjusted with 2 N NaOH or 2 N acetic acid, lactic acid, or HCl when appropriate. All medium constituents were obtained from Difco, Detroit, Mich., or Merck, Darmstadt, Germany.

*L. lactis* NCDO 712 was also grown in batch culture in a Biolab fermentor (Braun Biotech U.K., Aylesbury Bucks, United Kingdom) with a 1.5-liter working volume. The pH was maintained at the required value by automatic addition of 2 N NaOH. The temperature was maintained at 30°C; agitation was at 200 rpm, and anaerobic conditions were maintained by sparging of the medium with sterile $N_2$ gas.

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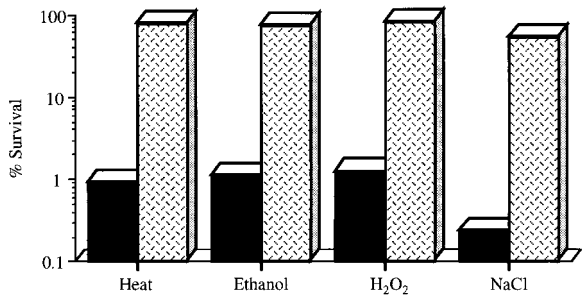


FIG. 1. Cross protection against various environmental stresses by ATR induction. Survival of early-log-phase *L. lactis* subsp. *cremoris* 712 cells growing at $pH_o$ 7.0 (■) or acid adapted at $pH_o$ 5.0 for 2 h (▨) following a 2-h challenge with exposure to 42°C, 15% ethanol, 1.15 mM hydrogen peroxide, or 20% sodium chloride. The data presented are from one of at least three similar experiments.

**Induction of ATR.** Cells grown at a constant pH of 7.0 were harvested by centrifugation early in the exponential phase (optical density at 580 nm [$OD_{580}$] of about 0.2; 32 μg of protein per ml) and resuspended in fresh TYG medium adjusted to the inducing $pH_o$ value with acid (acetic acid unless otherwise stated). Cells were routinely induced for 1 h at 30°C.

**Measurement of acid tolerance.** Cells were centrifuged, resuspended in fresh TYG medium preadjusted with acetic acid to $pH_o$ 4.0, and incubated at 30°C. Cell numbers were estimated as CFU immediately after resuspension (time zero) and after 1, 2, or 3 h, as stated in Results. Numbers of surviving organisms were calculated as a percentage of the cell numbers at time zero.

**Measurement of tolerance to other environmental stresses.** Initially, the effects of various stresses on log-phase cells were studied to identify a suitable lethal challenge for each stress. A stress level that reduced the numbers of uninduced log-phase cells by approximately 99% in 2 h was considered an appropriate lethal challenge. Cells growing exponentially in batch culture at a constant $pH_o$ of 7.0 were centrifuged and then resuspended in fresh TYG medium ($pH_o$ 7.0) containing (i) 15% ethanol, (ii) 1.15 mM hydrogen peroxide, or (iii) 20% sodium chloride or (iv) in fresh TYG medium at 42°C. Samples i, ii, and iii were incubated at 30°C for 2 h, and sample iv was incubated at 42°C for 2 h. Cell numbers were estimated as CFU immediately after resuspension (time zero) and at hourly intervals. Numbers of surviving organisms were calculated as a percentage of the cell numbers at time zero.

**Induction of acid tolerance by other environmental stresses.** Log-phase cells growing at a constant $pH_o$ of 7.0 were resuspended in fresh TYG medium ($pH_o$ 7.0) containing (i) 5% ethanol, (ii) 0.5 mM hydrogen peroxide, or (iii) 4% sodium chloride or (iv) in fresh TYG medium at 37°C. Samples i, ii, and iii were incubated at 30°C for 1 h, and sample iv was incubated at 37°C for 1 h. For comparison, cells were also induced in fresh TYG adjusted to $pH_o$ 5.0 with acetic acid (0.05 M) for 1 h at 30°C. The acid tolerance of each of these cultures was measured as previously described.

**CFU.** Cell numbers were estimated by a spot plating technique. Five-microliter volumes of serially diluted samples were spotted in triplicate onto TYG agar plates. Plates were incubated overnight at 30°C, spots containing between 5 and 50 CFU were counted, and the average was used to calculate the number of CFU per milliliter.

**Measurement of $pH_i$.** The cytoplasmic volume was determined by measuring the difference in accumulation of the cytoplasmic impermeable marker D-[U-$^{14}$C] sorbitol and the permeable marker $^3H_2O$ following rapid separation of the cells from the medium by centrifugation through 1-bromodecane (34). The $pH_i$ of cells removed from culture vessels and resuspended in fresh medium was determined by measurement of [$^{14}$C]benzoic acid accumulation by a modification of the method of Kroll and Booth (23). The method was modified slightly, in that aeration of the cells was kept to a minimum, as oxygen can seriously alter the metabolism of *L. lactis*. In addition, the extracellular water marker [$^3$H]inulin was replaced with [$^3$H]sorbitol. Sorbitol permeates the cell wall, which is impermeable to inulin, and therefore gives a more accurate measurement of cytoplasmic volume (34). [$^{14}$C]benzoic acid and [$^3$H]sorbitol were added (each to a concentration of 1 μCi/ml) to cells from a culture at an $OD_{580}$ of about 0.8 (0.13 mg of protein per ml). One-milliliter volumes of suspensions containing labelled cells were separated from the medium by centrifugation through bromodecane and resuspended in fresh medium. The $^{14}$C and $^3$H labels in 100 μl of the cell suspensions were counted. The ratio of $^3$H to $^{14}$C in the labelled supernatant was used to estimate the $^{14}$C count in the liquid outside the cytoplasmic membrane of the cells in the pellet, and this allowed the cytoplasmic $^{14}$C count to be estimated. The ΔpH was calculated by using the following equation:

$$\Delta pH = \log\{[^{14}C]_i/[^{14}C]_o\,(1 + 10^{pKa - pH_o}) - (10^{pKa - pH_o})\}$$

where pKa is the pKa of the labelled benzoic acid (4.2).

**Estimation of cell protein.** When appropriate, $OD_{580}$ values were converted to protein concentrations, which were assayed by using a commerical protein kit (Bio-Rad Laboratories GmbH, München, Germany).

**Measurement of growth and death rates.** Growth at constant pH was monitored by measuring the $OD_{580}$ of a fermentor culture at regular intervals with a Beckman DU-600 spectrophotometer. Death was determined by measurement of the number of survivors as CFU per milliliter at regular time intervals following exposure to lethal acid concentrations. The specific growth or death rate was calculated as follows: $(\ln x_2 - \ln x_1)/(t_2 - t_1)$, where $x_1$ and $x_2$ are $OD_{580}$ readings taken in the exponential phase of growth or numbers of CFU per milliliter measured during exposure to lethal acid concentrations at times $t_1$ and $t_2$ in hours, respectively.

**Statistical analysis.** The data presented are either from single experiments which were done at least three times or in the form of means accompanied by standard deviations. The data in both tables were analyzed by analysis of variance. Statistical significance was accepted at the $P < 0.05$ level of probability by using Student's *t* test.

## RESULTS

**Acid-induced multistress tolerance.** Induction of the ATR in *L. lactis* subsp. *cremoris* 712 also conferred cross protection against a number of other stresses. Early-log-phase cells were adapted for 1 h with acetic acid at $pH_o$ 5.0, which promoted development of the greatest level of acid tolerance. The abilities of acid-adapted and unadapted cells to survive the effects of potentially lethal challenges with acid, heat, ethanol, sodium chloride, and hydrogen peroxide were compared. Cells exposed to $pH_o$ 5.0 for 1 h acquired almost complete resistance to all of the stresses investigated, whereas ≤1% of the unadapted cells survived the challenges (Fig. 1).

**Acid tolerance induced by other environmental stresses.** The ability of sublethal stresses, other than acid pH, to induce tolerance to potentially lethal acid concentrations in *L. lactis* subsp. *cremoris* 712 was also explored (Fig. 2). The levels of acid tolerance induced by heat, ethanol, hydrogen peroxide, and sodium chloride were compared to that induced by acetic acid at $pH_o$ 5.0, which conferred 100% resistance to the acid stress challenge at $pH_o$ 4.0. Cells exposed to 4% NaCl or 0.5 mM $H_2O_2$ were as susceptible as uninduced cells to the lethal effects of acid; 6 to 8% of the cells survived for 2 h at $pH_o$ 4.0. Exposure to 5% ethanol resulted in increased sensitivity to acid; cells were approximately five times less tolerant than unadapted cells. On the other hand, 1 h of incubation at 37°C (7°C above the optimum for growth) prior to the acid challenge induced the same ATR level as that displayed by acid-induced cells. Therefore, of the stresses investigated, the only sublethal stress that caused increased tolerance to acid was high temperature.

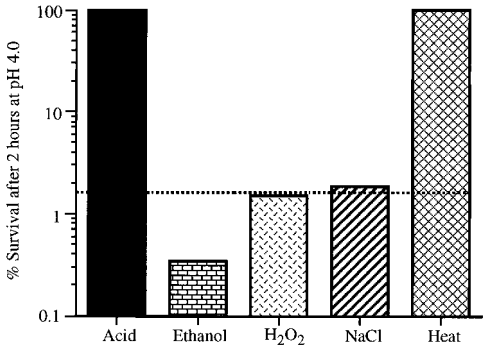


FIG. 2. Acid tolerance induced by other lethal stresses. Early-log-phase *L. lactis* subsp. *cremoris* 712 cells growing at $pH_o$ 7.0 and 30°C (uninduced) were exposed to pH 5.0, 5% ethanol, 0.5 mM hydrogen peroxide, 4% sodium chloride, or 37°C for 1 h prior to acid challenge ($pH_o$ 4.0). The survival level of uninduced cells is indicated by the dotted line. The data presented are from one of at least three similar experiments.

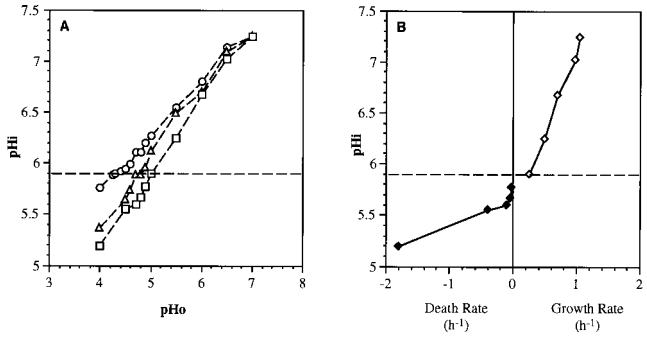


FIG. 3. (A) $pH_i$ of *L. lactis* subsp. *cremoris* 712 cells growing in TYG at $pH_o$ 7.0 and resuspended in fresh medium acidified with acetic acid (□), lactic acid (△), or hydrochloric acid (○). (B) Influence of $pH_i$ on cell growth (◇) or death (◆) in TYG acidified with acetic acid. The $pH_i$ value below which cell death occurs is indicated by the dashed line. Similar data were obtained with lactic acid or HCl. The data presented are from one of at least three similar experiments.

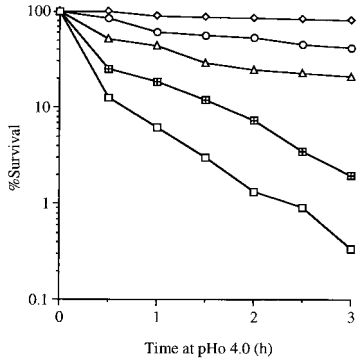


FIG. 4. Influence of the $pH_o$ during the induction period on the ability of *L. lactis* subsp. *cremoris* 712 cells to survive a challenge with $pH_o$ of 4.0. Exponential-phase cells growing at $pH_o$ 7.0 were either unexposed (□) or exposed for 1 h to medium containing acetic acid at $pH_o$ 6.5 (⊞), 6.0 (△), 5.5 (○), or 5.0 (◇) prior to the acid challenge. The data presented are from one of at least three similar experiments.

**Correlation of $pH_o$, $pH_i$, and ATR induction.** The $pH_i$ of *L. lactis* subsp. *cremoris* 712 was measured to investigate whether $pH_i$ has a role in the ATR. Early-log-phase *L. lactis* subsp. *cremoris* 712 cells grown at a constant $pH_o$ of 7.0 in TYG were acidified with acetic acid to $pH_o$ values ranging from 7.0 to 4.0, and the corresponding $pH_i$ values were measured. The $pH_i$ decreased from 7.24 ± 0.15 to 5.19 ± 0.05 as the $pH_o$ was shifted from 7.0 to 4.0. The straight-line graph of $pH_i$ against $pH_o$ indicated a direct relationship between $pH_o$ and $pH_i$ (Fig. 3A). The difference between $pH_i$ and $pH_o$ (ΔpH) increased from 0.24 at $pH_o$ 7.0 to 1.19 at $pH_o$ 4.0. The $pH_i$ of cells acidified with lactic acid or HCl also decreased directly as the external pH was decreased (Fig. 3A). At a $pH_o$ of 7.0, no acid was added to the medium and the $pH_i$ was 7.24. At lower $pH_o$ values, cells exposed to lactic acid established a slightly higher $pH_i$ than did cells exposed to acetic acid, and cells acidified with HCl established the highest of the three $pH_i$ values.

Cells of *L. lactis* subsp. *cremoris* 712 with a $pH_i$ value between 7.24 and 5.9 were capable of growth (Fig. 3B); however, the rate of growth decreased as the $pH_i$ decreased (and the ΔpH increased). At $pH_i$ values of less than 5.9, cells no longer remained viable and the rate of killing increased as the $pH_i$ decreased. At $pH_i$ values lower than 5.9, the ΔpH continued to increase gradually with decreasing $pH_o$; i.e., there was no sudden change in $pH_i$ or ΔpH at the $pH_o$ value which caused a loss of viability as determined by the plate count method.

The ability of *L. lactis* subsp. *cremoris* 712 to survive a lethal acid challenge increased as the inducing $pH_o$ was decreased from 7.0 to 5.0 (Fig. 4). A 2-log reduction in cell numbers occurred when uninduced cells were exposed to an acetic acid challenge at $pH_o$ 4.0 for 2 h. Cells induced at $pH_o$ 5.0 ($pH_i$ 5.9) were completely resistant to the acid challenge. Intermediate levels of survival were observed when intermediate $pH_o$ values were used to induce acid tolerance (Fig. 4).

Early-log-phase cells grown at a constant $pH_o$ of 7.0 were incubated at $pH_o$ 5.0 ($pH_i$ 5.9), and samples taken at intervals were tested for the ability to survive acetic acid at $pH_o$ 4.0 for 2.0 h. Increased resistance was detectable at the first sample point, 10 min after transfer to $pH_o$ 5.0, and the ATR was fully induced within 30 min (Fig. 5).

**Inhibition of protein synthesis prevents ATR induction.** Protein synthesis during ATR induction was investigated by using the protein synthesis inhibitor chloramphenicol. Concentrations as high as 100 μg/ml added at the same time as the cells were exposed to the inducing $pH_o$ of 5.0 did not fully inhibit induction of the ATR (data not shown); however, complete inhibition of induction was achieved when chloramphenicol was added 30 min before exposure to pH 5.0 (Fig. 6). Concentrations as low as 25 μg/ml were sufficient to fully prevent ATR induction. Treatment of cells with 25 μg of chloramphenicol per ml at $pH_o$ 7.0 prior to exposure to $pH_o$ 5.0 (with 25 μg of chloramphenicol per ml present) required at least 30 min for complete inhibition of the ATR (Fig. 6). The incubation of *L. lactis* subsp. *cremoris* 712 cells with chloramphenicol at $pH_o$ 7.0 prior to induction at $pH_o$ 5.0 did not play a direct role in the prevention of induction of the ATR. Whether this step was omitted or included, the same level of acid tolerance was induced in cells subsequently adapted to $pH_o$ 5.0 in the absence of chloramphenicol (data not shown). This demonstrates that proteins synthesized prior to exposure to the sublethal $pH_o$ of 5.0 were not directly responsible for the ATR.

**Relationship between $pH_i$ during a lethal acid challenge and survival of induced and uninduced cells.** Acid-induced ($pH_o$ 5.0) and uninduced cells were exposed to challenge $pH_o$ values of 4.5, 4.25, and 4.0, and survival after 3 h and the $pH_i$ during the acid challenge were measured. At any particular $pH_o$ challenge, induced cells had a slightly higher $pH_i$ and survived better than uninduced cells (Table 1). Even though the differ-

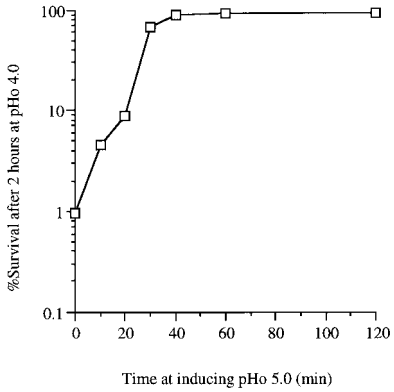


FIG. 5. Time required for ATR induction at $pH_o$ 5.0. Exponentially growing *L. lactis* subsp. *cremoris* 712 cells were acidified to $pH_o$ 5.0 at time zero. The number of survivors of a challenge with $pH_o$ 4.0 for 2 h was determined following adaptation at $pH_o$ 5.0 for increasing time periods. The data presented are from one of at least three similar experiments.

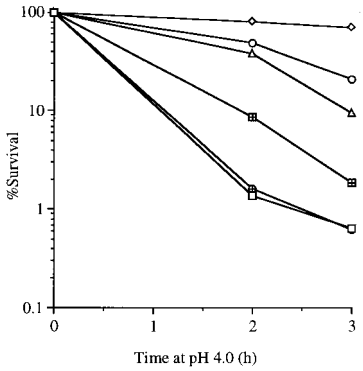


FIG. 6. Determination of the length of time of exposure to chloramphenicol needed to completely prevent induction of the ATR. Log-phase *L. lactis* subsp. *cremoris* 712 cells growing at $pH_o$ 7.0 were incubated with chloramphenicol at 25 μg/ml for 0 (○), 10 (△), 20 (⊞), or 30 (⊕) min and then induced at $pH_o$ 5.0 with the chloramphenicol present prior to a $pH_o$ 4.0 challenge for determination of percent survival. Cells growing without chloramphenicol at $pH_o$ 7.0 (□) and induced at $pH_o$ 5.0 (◇) were included as controls. The data presented are from one of at least three similar experiments.

ences between the $pH_i$ values of adapted and unadapted cells were small, statistical analysis of data obtained from 20 separate trials using cells challenged at $pH_o$ 4.0 confirmed that the $pH_i$ values of the induced and uninduced cells were significantly different.

Cells exposed to chloramphenicol during induction of the ATR subsequently had the same $pH_i$ as uninduced cells during an acid challenge; elimination of the small increase in $pH_i$ coincided with elimination of enhanced resistance to acid (data not shown).

The enhanced ability to maintain a higher $pH_i$ may be important to cell survival; however, it is clear that cells with the same (or almost identical) $pH_i$ value survived better if the ATR was induced than did uninduced cells (Table 1). For example, three times as many induced cells with a $pH_i$ of 5.44 survived as uninduced cells with a $pH_i$ of 5.45.

**Influence of $pH_i$ during ATR induction.** Early-log-phase cells of *L. lactis* subsp. *cremoris* 712 induced at $pH_o$ 5.0 with acetic acid were almost completely resistant to acetic acid at $pH_o$ 4.0 for 2 h, whereas cells induced at $pH_o$ 5.0 with lactic acid or HCl showed a reduced ability to survive exposure to the same $pH_o$ 4.0 challenge. The ability of the cells to survive the lethal acid correlated with the $pH_i$ value during the induction period. The lower the $pH_i$ during induction, the better the cells survived (Table 2). Slight differences in $pH_i$ values during the challenge at $pH_o$ 4.0 were also noted, but the magnitude of the standard deviations indicated that these were unlikely to be significant. This result suggested that the degree of ATR induction was influenced by the $pH_i$ value at the induction step rather than the $pH_o$ value.

TABLE 1. Relationship between $pH_i$ and survival of an acid $pH_o$ challenge by induced and uninduced cells of *L. lactis* subsp. *cremoris* 712

| Induction at $pH_o$ 5.0 for 1 h | $pH_o$ during acid challenge | $pH_i$ at $pH_o$ challenge (mean ± SD) | % Survival after 3 h at challenge $pH_o$ (mean ± SD) |
|---|---|---|---|
| − | 4.50 | 5.45 ± 0.12 | 33 ± 4.5 |
| + | 4.50 | 5.65 ± 0.15 | 100 ± 2.2 |
| − | 4.25 | 5.30 ± 0.15 | 15 ± 3.6 |
| + | 4.25 | 5.44 ± 0.16 | 99 ± 1.4 |
| − | 4.00 | 5.18 ± 0.05 | 0.6 ± 0.3 |
| + | 4.00 | 5.36 ± 0.03 | 73 ± 9.1 |

TABLE 2. Influence of $pH_i$ during induction and on $pH_i$ and survival of *L. lactis* subsp. *cremoris* 712 during acetic acid challenge at $pH_o$ 4.0

| Inducing acid | $pH_o$ during induction | $pH_i$ during induction (mean ± SD) | $pH_i$ during acetic acid $pH_o$ 4.0 challenge (mean ± SD) | % Survival after 2-h $pH_o$ 4.0 challenge (mean ± SD) |
|---|---|---|---|---|
| None | NA[a] | NA | 5.19 ± 0.03 | 2.3 ± 2.0 |
| Acetic | 5.0 | 5.9 ± 0.12 | 5.37 ± 0.05 | 98 ± 2.2 |
| Lactic | 5.0 | 6.12 ± 0.16 | 5.31 ± 0.13 | 56 ± 6.0 |
| Hydrochloric | 5.0 | 6.27 ± 0.13 | 5.26 ± 0.09 | 31 ± 2.2 |
| Lactic | 4.8 | 5.9 ± 0.18 | 5.36 ± 0.09 | 94 ± 6.2 |
| Hydrochloric | 4.4 | 5.9 ± 0.21 | 5.37 ± 0.14 | 99 ± 0.9 |

[a] NA, not applicable.

Stronger evidence in support of this proposal was obtained by exposing early-log-phase cells (for 1 h) to different concentrations of acetic, lactic, and hydrochloric acids. This allowed identification of $pH_o$ values for each of the three acids which gave the same $pH_i$ of 5.9 during ATR induction. Cells at different $pH_o$ values but with a $pH_i$ of 5.9 during induction had the same ability to survive the acetic acid $pH_o$ 4.0 challenge and were equally able to maintain a slightly elevated $pH_i$ at the lethal $pH_o$ challenge (Table 2). This result rules out both $pH_o$ and ΔpH as the primary inducing signal; the same level of survival corresponded to ΔpH values of 0.9, 1.1, and 1.5 for acetic acid, lactic acid, and HCl, respectively.

In another experiment, when *L. lactis* subsp. *cremoris* 712 was induced at $pH_o$ 5.5 with acetic acid and at $pH_o$ 5.0 with HCl, the inducing $pH_i$ values were similar (6.24 and 6.27, respectively). The rates of survival of a subsequent acid challenge by both cultures were similar (Fig. 7). In the same experiment, cells were also induced at $pH_o$ 5.5 with HCl and at $pH_o$ 5.0 with acetic acid and had $pH_i$ values of 6.55 and 5.9, respectively. Cells with the same inducing $pH_o$ value, 5.5 or 5.0, but different $pH_i$ values had very different rates of survival (Fig. 7).

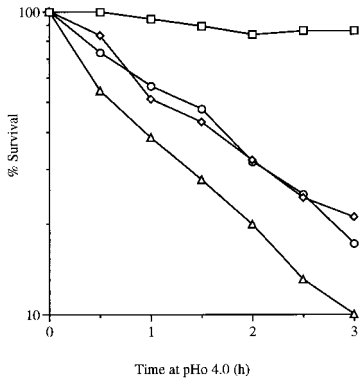


FIG. 7. Survival of an acid challenge (acetic acid, $pH_o$ 4.0, for 2 h) by *L. lactis* subsp. *cremoris* cells induced at $pH_o$ 5.0 or 5.5 with acetic acid or HCl. Acetic acid: $pH_o$ 5.0 and $pH_i$ 5.9, □; $pH_o$ 5.5 and $pH_i$ 6.24, ◇. Hydrochloric acid: $pH_o$ 5.0 and $pH_i$ 6.27, ○; $pH_o$ 5.5 and $pH_i$ 6.55, △. The data presented are from one of at least three similar experiments.

## DISCUSSION

The existence of an ATR in *L. lactis* was previously demonstrated in a preliminary report by us (32) and confirmed by Rallu et al. (35). In this report, we have shown that exposure of exponentially growing *L. lactis* cells to a mild acid pH induced the synthesis of proteins which confer protection not only against lethal acid concentrations but also against other lethal environmental stresses such as heat, ethanol, sodium chloride, and hydrogen peroxide. Cross protection induced by acid pH has previously been reported for *S. typhimurium* (25, 26) and *Listeria monocytogenes* (31). Cross protection in carbohydrate-starved stationary-phase *E. coli* cells has also been reported (18).

In contrast to the broad specificity of tolerance mechanisms induced by exposure of lactococcal cells to mild acid pH, mild intensities of the other environmental stresses tested, with the exception of heat, did not induce tolerance to acid. In general, the response of *L. lactis* to sublethal stresses was similar to that of *S. typhimurium* in that induction of an ATR provided protection against heat and osmotic and oxidative stresses but exposure to sublethal levels of each of these stresses did not confer significant tolerance to acid challenge (25). The only difference between the response of *S. typhimurium* and that of *L. lactis* was that sublethal temperatures did not induce an ATR in salmonella. This, however, may be due to a difference in the time allowed for induction; the salmonella culture was exposed to the inducing temperature for only 10 min, whereas 1 h of elevated temperature was used to adapt the lactococcal strain. It appears that acid pH serves as an important signal for the induction of mechanisms that protect *S. typhimurium* and *L. lactis* against several environmental stresses.

Acidification of TYG growth medium with acetic acid to a $pH_o$ of 5.0 ($pH_i$ 5.9) caused cessation of growth. At $pH_o$ values of less than 5.0, cell viability decreased at a rate dependent on the $pH_o$. The $pH_i$ decreased linearly as the $pH_o$ of TYG decreased and the $\Delta pH$ increased; this relationship was not altered, even at $pH_o$ values below 5.0, at which viable cell numbers were decreasing. A similar observation with another *L. lactis* subsp. *cremoris* strain was reported by Cook and Russell (4). Nannen and Hutkins (30) observed a sudden drop in $\Delta pH$ in a growing culture of *L. lactis* when the $pH_o$ reached 5.0 and the $pH_i$ reached 5.5 and concluded that the sudden decrease in $\Delta pH$ was due to loss of viability. No sudden decrease in $pH_i$ or $\Delta pH$ was observed in our experiments at $pH_o$ 5.0 ($pH_i$ 5.9), below which viability was shown to gradually decrease.

Depending on the specific acid used (acetic, lactic, or hydrochloric) a different $pH_i$ value was obtained for a single $pH_o$. The pKa of acetic acid is 4.76, and in the undissociated form acetic acid passes freely through the cell membrane, acidifying the cytoplasm (20). Lactic acid has a lower pKa of 3.86; therefore, at any $pH_o$ it is more dissociated than acetic acid. This, coupled with the fact that the undissociated form of lactic acid enters the cell by a carrier-mediated electroneutral process (12), might explain the reduced effectiveness of lactic acid at acidifying the cytoplasm. Hydrochloric acid is a strong acid and is therefore completely dissociated at all $pH_o$ values; hence, it is the least effective at reducing the $pH_i$. These data agree with previous observations that the $pH_i$ is influenced by the pKa of the acid to which the cells are exposed (36).

The $pH_i$ of log-phase *S. typhimurium* cells also decreased with the $pH_o$ (8). In minimal medium acidified with HCl, the minimum $pH_o$ at which these grew was 5.0 (6), which corresponds to a $pH_i$ of about 6.6 (8). Cells did not, however, lose viability until the $pH_o$ was reduced below 4.0 ($pH_i$ 5.8). Therefore, *S. typhimurium* survived without growing at $pH_i$ values between 6.6 and 5.8. This indicates that unlike *S. typhimurium*, *L. lactis* subsp. *cremoris* has no range of $pH_i$ values which could be considered bacteriostatic (i.e., cell numbers neither increasing nor decreasing).

The ATR in *L. lactis* subsp. *cremoris* 712 is the product of a classical induction mechanism involving protein synthesis, in that it was inhibited by chloramphenicol and the response level increased sharply within minutes of exposure of log-phase cells to an acid pH. The magnitude of the ATR was dependent on the degree of acidification of the growth medium as indicated by its $pH_o$. However, it was clearly established that the $pH_o$ was participating in the ATR through its effect on the pH of the cell cytoplasm. Cells with identical $pH_o$ values had different ATR levels when the $pH_i$ values were not identical. On the other hand, cells with identical $pH_i$ values established by different species of acid at different $pH_o$ values had very similar levels of acid tolerance (Table 2) and similar survival rates at acid challenge (Fig. 7). On the basis of these results, it is reasonable to conclude that the cytoplasmic concentration of $H^+$ or some metabolite whose concentration is directly dependent on the intracellular $H^+$ concentration stimulates the synthesis of proteins which protect *L. lactis* subsp. *cremoris* 712 against otherwise lethal challenges with acid and other environmental stresses. A number of acid-inducible gene loci have been identified in *E. coli* and *S. typhimurium* (37). For a number of these genes, the level of induction appeared to be related to the pKa of the inducing acid; therefore, it was proposed that expression might be induced by a reduction in $pH_i$. There have been very few studies on the direct involvement of pH in gene expression in lactic acid bacteria. Recently, however, expression of an unidentified chromosomal promoter (17) and an integral membrane protein thought to be an ATP-dependent protease (39) in *L. lactis* was found to be induced by low pH but a direct dependence on $pH_i$ was not determined.

A consequence of induction of ATR in *L. lactis* subsp. *cremoris* 712 was a small but reproducible increase in $pH_i$ when cells were exposed to a lethal acid challenge. The presence of chloramphenicol at the induction step prevented this $pH_i$ increase. Similar results were previously reported for *S. typhimurium* (8). Although small, the induced $pH_i$ increase in *L. lactis* subsp. *cremoris* 712 may have a role to play in protecting the cells from otherwise lethal acidification. However, it is unlikely that the ability of induced cells to maintain a higher $pH_i$ is the sole reason for their resistance to acid challenge. If survival depended solely on $pH_i$ during an acid challenge, then cells with the same $pH_i$, irrespective of how it was established, should have the same probability of surviving an acid challenge. This was not observed; induced cells always survived an acid challenge better than uninduced cells with the same $pH_i$. Other possible protective mechanisms include enhanced DNA repair (7, 11), chaperon proteins (29) which may function in the protection of existing essential proteins from acid damage, and the replacement of acid-sensitive cell constituents with acid-resistant homologs (7).

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