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

EILÍS O’SULLIVAN AND SÉAMUS CONDON*
Department of Microbiology, University College Cork, Cork, Ireland
Received 25 June 1997/Accepted 2 September 1997

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, H₂O₂, and ethanol. The intracellular pH (pHᵢ) played a major role in the induction of this multistress resistance response. The pHᵢ was dependent on the extracellular pH (pHₒ) and on the specific acid used to reduce the pHₒ. When resuspended in fresh medium, cells were able to maintain a pH gradient even at pHᵢ 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 pHᵢ; nevertheless, a more favorable pHᵢ was not the sole reason for the increased survival at acid pHᵢ. Cells with an induced ATR survived exposure to a lethal pHᵢ much better than did uninduced cells with a pHᵢ identical to that of the induced cells. Survival following lethal acid shock was dependent on the pHᵢ during induction of the ATR, and the highest survival was observed following induction at a pHᵢ of 5.9, which was the lowest pHᵢ at which growth occurred. Increased acid tolerance and the ability to maintain a higher pHᵢ during lethal acid stress were not acquired if protein synthesis was inhibited by chloramphenicol during adaptation.

The extracellular pH (pHₒ) range over which microorganisms survive and grow is approximately 1.0 to 11.0 (33). Based on the pHᵢ 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 (pHᵢ) 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 pHᵢ to decrease as the pHₒ 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 pHₒ 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 pHᵢ of the starter growth medium from dropping to harmful levels (41).

Induced acid tolerance defines a condition whereby, during exposure to mildly acid 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 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). H₂O₂-induced cells were also more resistant to lethal high temperatures than were unirradiated 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 pHᵢ 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 KH₂PO₄ per liter, and 2.28 g of Na₂HPO₄ 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, lactate 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 the medium with sterile N₂ gas.
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 [OD580] of about 0.2; 32 μg of protein per ml) and resuspended in fresh TYG medium adjusted to the inducing pH 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 predosed with acetic acid to pH 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 of 7.0 were centrifuged and then resuspended in fresh TYG medium (pH 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 of 7.0 were resuspended in fresh TYG medium (pH 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 5.0 with acetic acid (0.1 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. The cytoplasmic volume was determined by measuring the difference in accumulation of the cytoplasmic impermeable marker [14C]sorbitol and the permeable marker [3H]H2O following rapid separation of the cells from the medium by centrifugation through 1-bromododecane (34). The pH of cells removed from culture vessels and resuspended in fresh medium was determined by measurement of [3H]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 [3H]inulin was replaced with [3H]sorbitol. Sorbitol permeates the cell wall, which is impermeable to inulin, and therefore gives a more accurate measurement of cytoplasmic volume (34). [14C]benzoic acid and [3H]sorbitol were added (each to a concentration of 1 μCi/ml) to cells from a culture at an OD580 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 bromododecane and resuspended in fresh medium. The [14C] and [3H] labels in 100 μl of the cell suspensions were counted. The ratio of [3H] to [14C] in the labelled supernatant was used to estimate the percentage of the labelled cytoplasmic volume of the cells in the pellet, and this allowed the cytoplasmic [14C] count to be estimated. The ΔpH was calculated by using the following equation:

$$\Delta pH = \log\left[\frac{[14C]}{[14C]}\right] (1 + 10^{\frac{-pK_a}{T}} - 1)$$

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

Estimation of cell protein. When appropriate, OD580 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 OD600 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 x1 − ln x2)/(t2 − t1), where x1 and x2 are OD600 readings taken in the exponential phase of growth or numbers of CFU per milliliter measured during exposure to lethal acid concentrations at times t1 and t2.

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 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 3.0 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 5.0, which conferred 100% resistance to the acid stress challenge at pH 4.0. Cells exposed to 4% NaCl or 0.5 mM H2O2 were as susceptible as uninduced cells to the lethal effects of acid; 6 to 8% of the cells survived for 2 h at pH 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.
Correlation of pHo, pHi, and ATR induction. The pHi of L. lactis subsp. cremoris 712 was measured to investigate whether pHi has a role in the ATR. Early-log-phase L. lactis subsp. cremoris 712 cells grown at a constant pHo of 7.0 in TYG were acidified with acetic acid to pHi values ranging from 7.0 to 4.0, and the corresponding pHi values were measured. The pHi decreased from 7.24 ± 0.15 to 5.19 ± 0.05 as the pHo was shifted from 7.0 to 4.0. The straight-line graph of pHi against pHo indicated a direct relationship between pHo and pHi (Fig. 3A). The difference between pHi and pHo (ΔpH) increased from 0.24 at pHo 7.0 to 1.19 at pHo 4.0. The pHi of cells acidified with lactic acid or HCl also decreased directly as the external pH was decreased (Fig. 3A). At a pHi of 7.0, no acid was added to the medium and the pHi was 7.24. At lower pHi values, cells exposed to lactic acid established a slightly higher pHi than did cells exposed to acetic acid, and cells acidified with HCl established the highest of the three pHi values.

Cells of L. lactis subsp. cremoris 712 with a pHi value between 7.24 and 5.9 were capable of growth (Fig. 3B); however, the rate of growth decreased as the pHi decreased (and the ΔpH increased). At pHi values of less than 5.9, cells no longer remained viable and the rate of killing increased as the pHi decreased. At pHi values lower than 5.9, the ΔpH continued to increase gradually with decreasing pHi; i.e., there was no sudden change in pHi or ΔpH at the pHi 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 pHi 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 pHi 4.0 for 2 h. Cells induced at pHi 5.0 (pHi 5.9) were completely resistant to the acid challenge. Intermediate levels of survival were observed when intermediate pHi values were used to induce acid tolerance (Fig. 4).

Early-log-phase cells grown at a constant pHo of 7.0 were incubated at pHi 5.0 (pHi 5.9), and samples taken at intervals were tested for the ability to survive acetic acid at pHi 4.0 for 2.0 h. Increased resistance was detectable at the first sample point, 10 min after transfer to pHi 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 pHi 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 pHi 7.0 prior to exposure to pHi 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 pHi 7.0 prior to induction at pHi 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 pHi 5.0 in the absence of chloramphenicol (data not shown). This demonstrates that proteins synthesized prior to exposure to the sublethal pHi of 5.0 were not directly responsible for the ATR.

Relationship between pHi during a lethal acid challenge and survival of induced and uninduced cells. Acid-induced (pHi 5.0) and uninduced cells were exposed to challenge pHi values of 4.5, 4.25, and 4.0, and survival after 3 h and the pHi during the acid challenge were measured. At any particular pHi challenge, induced cells had a slightly higher pHi and survived better than uninduced cells (Table 1). Even though the differences...
ences between the pH values of adapted and unadapted cells were small, statistical analysis of data obtained from 20 separate trials using cells challenged at pH 4.0 confirmed that the pH values of the induced and uninduced cells were significantly different.

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

The enhanced ability to maintain a higher pH may be important to cell survival; however, it is clear that cells with the same (or almost identical) pH 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 of 5.44 survived as uninduced cells with a pH of 5.45.

**Influence of pH during ATR induction.** Early-log-phase cells of *L. lactis* subsp. cremoris 712 induced at pH 5.0 with acetic acid were almost completely resistant to acetic acid at pH 4.0 for 2 h, whereas cells induced at pH 5.0 with lactic acid or HCl showed a reduced ability to survive exposure to the same pH 4.0 challenge. The ability of the cells to survive the lethal acid correlated with the pH value during the induction period. The lower the pH during induction, the better the cells survived (Table 2). Slight differences in pH values during the challenge at pH 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 value at the induction step rather than the pH value.

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 values for each of the three acids which gave the same pH of 5.9 during ATR induction. Cells at different pH values but with a pH of 5.9 during induction had the same ability to survive the acetic acid pH 4.0 challenge and were equally able to maintain a slightly elevated pH at the lethal pH challenge (Table 2). This result rules out both pH 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, lactic, and HCl, respectively.

In another experiment, when *L. lactis* subsp. cremoris 712 was induced at pH 5.5 with acetic acid and at pH 5.0 with HCl, the inducing pH 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 5.5 with HCl and at pH 5.0 with acetic acid and had pH values of 6.55 and 5.9, respectively. Cells with the same inducing pH value, 5.5 or 5.0, but different pH values had very different rates of survival (Fig. 7).

**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 7.0 were incubated with chloramphenicol at 25 μg/ml for 0 (○), 10 (▲), 20 (●), or 30 (□) min and then induced at pH 5.0 with the chloramphenicol present prior to a pH 4.0 challenge for determination of percent survival. Cells growing without chloramphenicol at pH 7.0 (▲—▲) were almost completely resistant to acetic acid at pH 4.0 for 2 h, whereas cells induced at pH 5.0 with lactic acid were almost completely resistant to acetic acid at pH 4.0 during ATR induction. Cells at different pH values but with a pH of 5.9 during induction had the same ability to survive the acetic acid pH 4.0 challenge and were equally able to maintain a slightly elevated pH at the lethal pH challenge (Table 2). This result rules out both pH 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, lactic, and HCl, respectively.

**FIG. 7.** Survival of an acid challenge (acetic acid, pH 4.0, for 2 h) by *L. lactis* subsp. cremoris cells induced at pH 5.5 or 5.0 with acetic acid or HCl. Acetic acid: pH 5.0 and pH 5.5 and pH 6.24, □; pH 4.5 and pH 4.0. Hydrochloric acid: pH 5.0 and pH 6.27, ○; pH 5.5 and pH 6.55, △. The data presented are from one of at least three similar experiments.

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

<table>
<thead>
<tr>
<th>Induction</th>
<th>pH during acid challenge (mean ± SD)</th>
<th>% Survival after 3 h at challenge pH (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.00</td>
<td>5.18 ± 0.05</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td>4.25</td>
<td>5.44 ± 0.16</td>
<td>99 ± 1.4</td>
</tr>
<tr>
<td>4.50</td>
<td>5.65 ± 0.15</td>
<td>100 ± 2.2</td>
</tr>
<tr>
<td>4.75</td>
<td>5.80 ± 0.21</td>
<td>95 ± 3.6</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Inducing acid</th>
<th>pH&lt;sub&gt;H&lt;/sub&gt;&lt;sub&gt;i&lt;/sub&gt; during induction (mean ± SD)</th>
<th>pH&lt;sub&gt;H&lt;/sub&gt;&lt;sub&gt;i&lt;/sub&gt; during acetic acid pH 4.0 challenge (mean ± SD)</th>
<th>% Survival after 2-h pH 4.0 challenge (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>NA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.19 ± 0.03</td>
<td>2.3 ± 2.0</td>
</tr>
<tr>
<td>Acetic</td>
<td>5.0</td>
<td>5.37 ± 0.05</td>
<td>98 ± 2.2</td>
</tr>
<tr>
<td>Lactic</td>
<td>5.0</td>
<td>5.31 ± 0.13</td>
<td>56 ± 6.0</td>
</tr>
<tr>
<td>Hydrochloric</td>
<td>5.0</td>
<td>5.26 ± 0.09</td>
<td>31 ± 2.2</td>
</tr>
<tr>
<td>Lactic</td>
<td>4.8</td>
<td>5.36 ± 0.09</td>
<td>94 ± 6.2</td>
</tr>
<tr>
<td>Hydrochloric</td>
<td>4.4</td>
<td>5.37 ± 0.14</td>
<td>99 ± 0.9</td>
</tr>
</tbody>
</table>

<sup>a</sup> NA, not applicable.
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 of 5.0 (pH, 5.9) caused cessation of growth. At pH values of less than 5.0, cell viability decreased at a rate dependent on the pH. The pH decreased linearly as the pH of TYG decreased and the ΔpH increased; this relationship was not altered, even at pH 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 ΔpH in a growing culture of *L. lactis* when the pH, reached 5.0 and the pH, reached 5.5 and concluded that the sudden increase in ΔpH was due to loss of viability. No sudden decrease in pH or ΔpH was observed in our experiments at pH 5.0 (pH, 5.9), below which viability was shown to gradually decrease.

Depending on the specific acid used (acetic, lactic, or hydrochloric) a different pH value was obtained for a single pH. 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, 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 values; hence, it is the least effective at reducing the pH. These data agree with previous observations that the pH is influenced by the pKa of the acid to which the cells are exposed (36).

The pH of log-phase *S. typhimurium* cells also decreased with the pH (8). In minimal medium acidified with HCl, the minimum pH, at which these grew was 5.0 (6), which corresponds to a pH of about 6.6 (8). Cells did not, however, lose viability until the pH, was reduced below 4.0 (pH, 5.8). Therefore, *S. typhimurium* survived without growing at pH values between 6.6 and 5.8. This indicates that unlike *S. typhimurium*, *L. lactis* subsp. *cremoris* has no range of pH 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. However, it was clearly established that the pH was participating in the ATR through its effect on the pH of the cell cytoplasm. Cells with identical pH values had different ATR levels when the pH values were not identical. On the other hand, cells with identical pH values established by different species of acid at different pH 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. 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 was not determined.

A consequence of induction of ATR in *L. lactis* subsp. *cremoris* 712 was a small but reproducible increase in pH when cells were exposed to a lethal acid challenge. The presence of chloramphenicol at the induction step prevented this pH increase. Similar results were previously reported for *S. typhimurium* (8). Although small, the induced pH 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 is the sole reason for their resistance to acid challenge. If survival depended solely on pH during an acid challenge, then cells with the same pH, 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. 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).

ACKNOWLEDGMENTS

This work was funded in part by Forbairt (The Irish Science and Technology Agency). We also gratefully acknowledge the AAIR Concerted Action Programme PL920630, which supported a short-term stay at Marischall College, Aberdeen, Scotland. We are indebted to I. R. Booth for providing the opportunity for E. O’Sullivan to work in his laboratory. We also acknowledge the excellent technical assistance of Dan Walsh.

REFERENCES
