Microbiology (1996), 142, 2975–2982 Printed in Great Britain

Acid tolerance in *Listeria monocytogenes*: the adaptive acid tolerance response (ATR) and growth-phase-dependent acid resistance

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Listeria monocytogenes **acquired increased acid tolerance during exponential growth upon exposure to sublethal acid stress, a response designated the acid tolerance response (ATR). Maximal acid resistance was seen when the organism was exposed to pH 5·0 for 1 h prior to challenge at pH 3·0, although intermediate levels of protection were afforded by exposure to pH values ranging from 4·0 to 6·0. A 60 min adaptive period was required for the development of maximal acid tolerance; during this period the level of acid tolerance increased gradually. Full expression of the ATR required *de novo* protein synthesis; chloramphenicol, a protein synthesis inhibitor, prevented full induction of acid tolerance. Analysis of protein expression during the adaptive period by two-dimensional gel electrophoresis revealed a change in the expression of at least 23 proteins compared to the non-adapted culture. Eleven proteins showed induced expression while 12 were repressed, implying that the ATR is a complex response involving a modulation in the expression of a large number of genes. In addition to the exponential phase ATR, *L. monocytogenes* also developed increased acid resistance upon entry into the stationary phase; this response appeared to be independent of the pH-dependent ATR seen during exponential growth.**

Keywords: *Listeria monocytogenes*, acid tolerance response, ATR, low pH adaptation, stress survival

INTRODUCTION

The ability of bacterial pathogens to withstand environmental stress, both inside and outside the host, plays a critical role in determining their success as pathogens. The low pH of gastric secretions acts as a formidable barrier to successful host invasion. Ability to survive the acidic conditions of the stomach can contribute to virulence by increasing the likelihood of intestinal colonization. Indeed, Gorden & Small (1993) have shown a correlation between low infectious dose and high levels of acid resistance among the enteric bacteria. Expression of acid resistance in enterobacteria is not constitutive; rather it shows considerable fluctuation depending upon a number of environmental factors including pH, nutrient availability and aeration (Lin *et al.*, 1995; Rowbury, 1995). The ability of *Listeria monocytogenes*, a Gram-positive, food-borne pathogen, to grow and survive under acidic conditions has been extensively studied (Cole *et al.*, 1990; Conner *et al.*, 1990; McClure *et al.*, 1989; Young & Foegeding, 1993), but little is known about the influence of environmental factors on the ability of this organism to survive low pH.

A number of neutralophilic bacteria have now been shown to develop acid tolerance under certain conditions. Much of the data relate to the widely studied enterobacteria *Salmonella typhimurium* and *Escherichia coli* (Rowbury, 1995). In *Sal. typhimurium* the acquisition of acid tolerance can occur by one of three apparently distinct mechanisms. In the first, exponential-phase cells acquire acid tolerance upon exposure to mild acid shock (pH 5·8). This response appears to depend on *de novo* protein synthesis and also relies on the magnesium-dependent proton-translocating ATPase (Foster & Hall, 1990, 1991; Foster, 1991). In the second, stationary-phase cells can develop high levels of acid resistance in a pH-dependent manner (Lee *et al.*, 1994). These two responses have been termed the exponential phase ATR (acid tolerance response) and the stationary phase ATR, respectively. A third system requires the stationary-phase-specific sigma factor, RpoS, and is independent of the pH-dependent resistance mechanisms (Lee *et al.*, 1994). The ability of *E.*

Abbreviation: ATR, acid tolerance response.

coli to develop acid resistance upon exposure to mild acid shock (pH 5·0) has also been demonstrated (Goodson & Rowbury, 1989a, b; Raja *et al.*, 1991). In addition, it has recently been shown that RpoS plays an important role in the development of acid tolerance in *E. coli* and *Shigella flexneri*. The *rpoS* gene from *Shi. flexneri* can confer acid resistance on acid-sensitive *E. coli* and an *rpoS* mutant of *Shi. flexneri* is extremely acid-sensitive (Small *et al.*, 1994). Another Gram-negative organism showing the ability to develop acid tolerance in a manner analogous to *Sal. typhimurium* is the gastrointestinal pathogen *Aeromonas hydrophila*; this organism exhibits an exponential phase ATR which is dependent upon *de novo* protein synthesis (Karem *et al.*, 1994).

Less is known about the acquisition of acid tolerance amongst Gram-positive bacteria. Work carried out on the oral streptococci has demonstrated that they are capable of developing acid tolerance after exposure to sublethal acidic conditions (Belli & Marquis, 1991). Furthermore, the membrane proton-translocating ATPase has been implicated as one of the principal determinants of acid resistance in these organisms (Abrams & Jensen, 1984; Bender *et al.*, 1986; Kobayashi *et al.* 1984, 1986). An additional mechanism affording protection against low pH has also been identified in the oral streptococci. This mechanism involves the arginine deiminase pathway, which leads to the production of carbon dioxide and ammonia from the breakdown of arginine. This pathway can function at exceptionally low pH, and supplementation of the growth medium with arginine can confer protection against acid challenge, presumably because of the ability of ammonia to bind protons (Casiano-Colón & Marquis, 1988).

Little is known about the acquisition of acid tolerance by *L. monocytogenes*. One preliminary report demonstrated that *L. monocytogenes* showed increased survival at pH 3·0 when first grown at an acidic pH (Kroll & Patchett, 1992). This study demonstrated that a culture of *L. monocytogenes* grown at pH 5·0 survived well when challenged at pH 3·0 compared to a culture grown at pH 7·0 prior to the challenge. Little else is known about acquired acid tolerance in this important food-borne pathogen. Here, we define some of the parameters associated with the acid tolerance response of this organism and show that, like *Sal. typhimurium*, it possesses a distinct growth-phase-dependent acid resistance mechanism.

METHODS

Bacterial strains and growth conditions. *L. monocytogenes* F4642 (Scott A) was provided by B. Lund (AFRC Institute of Food Research, Norwich Laboratory, Norwich). Cultures of strain Scott A were grown in Brain Heart Infusion broth (BHI; Difco). All cultures were incubated at 30 °C with shaking. Under these conditions cultures entered stationary phase at an OD_{600} of approximately 1·1 and at a cell density of approximately 3×10^9 c.f.u. ml^{-1}. Experiments carried out with exponential-phase cultures were performed on cultures with an OD_{600} of 0·1–0·6. Strains were maintained at 4 °C on BHI agar slopes and stored long-term at −80 °C with 7 % (v/v) dimethyl sulfoxide. Dilution series were performed in 0·1 % (w/v) peptone (Oxoid).

Assay of acid resistance. Cultures were grown to the appropriate phase of growth in BHI and a viable cell count was performed. The pH was then adjusted to 3·0 with HCl and further viable counts were carried out at specified time intervals. In each case, 1 ml of the culture was transferred to 9 ml BHI at room temperature and allowed to recover for 1 h. This enabled the resuscitation and recovery of a high proportion of acid-damaged cells (our unpublished results). The resuscitated suspension was then serially diluted in 0·1 % peptone and 10 µl of each dilution was spread onto BHI agar plates. Colonies were counted after incubation for 24–48 h at 30 °C. The detection limit of this method was 10^3 c.f.u. ml^{-1}. Experiments were performed in triplicate and error bars indicate the standard deviation from the mean. Where results are presented as percentage survival, this was calculated as viable cell counts after acid challenge expressed as a percentage of viable cell counts immediately prior to treatment.

Two-dimensional gel electrophoresis. This was carried out using a Multiphor II electrophoresis unit (Pharmacia Biotech) using the method first described by O'Farrell (1975) with modifications as recommended by the manufacturer. Proteins were resolved using isoelectric focusing in the first dimension and SDS-PAGE in the second dimension. Proteins were prepared for electrophoresis as follows. Cells were grown in 200 ml BHI at 30 °C. The pH of one culture was adjusted to 5·8 with HCl and it was incubated at this adaptive pH for 1 h. Cultures were then harvested (9000 ***g*** for 15 min) in mid-exponential phase. Pellets were resuspended in 10 ml extraction buffer (1 mM EDTA, 1 mM DTT, 25 mM Tris pH 7·0, 10 % (v/v) glycerol, 0·1 % Triton) and total cellular proteins were extracted using an MSE Soniprep sonicator. The sonication was carried out on ice and the cell suspension was pulsed 10 times (12 µm amplitude) for 15 s with 45 s intervals between pulses. Cell debris was removed by centrifugation at 8000 r.p.m. for 10 min. The supernatant was diluted 1 in 4 with IEF sample buffer (13·5 g urea, 0·5 ml 2-mercaptoethanol, 0·5 ml Pharmalyte 3-10, 0·13 ml Triton X-100, 0·05 % bromophenol blue; stored in aliquots at −80 °C) prior to running in the first dimension. The first dimension gel was a precast Immobiline DryStrip (Pharmacia) with a linear pH gradient (3–10) while the second dimension gel was a precast ExcelGel SDS (Pharmacia) with an 8–18 % (w/v) polyacrylamide gradient. These gels were run according to the instructions of the manufacturer. Gels were stained using the Bio-Rad Silver Stain Plus kit. Images of the gels were recorded using a monochrome CCD camera linked to an image analysis software package (GlobalLab Data Translation). Once captured, the images were imported into Freelance Graphics v. 2.0, numbers and symbols were added and the images were then printed on a Kodak XLS 8600PS printer. The analyses were repeated at least three times and only proteins that showed reproducible changes in expression are indicated.

RESULTS

The ATR in *L. monocytogenes* strain Scott A

Cells growing exponentially in BHI were killed rapidly when the pH was reduced to 3·0 with HCl. Within 80 min the cells lost all detectable viability (Fig. 1). Prior to acid challenge the pH of such a culture was typically 6·5–7·0. When an identical culture was exposed to a sublethal pH of 5·8 [this pH was initially chosen based on the pH required to induce an ATR in *Sal. typhimurium* (Foster & Hall, 1990)] for 1 h before challenging with pH 3·0, the rate at which cells lost viability was altered significantly. After 30 min at pH 3·0 there was 1000-fold greater

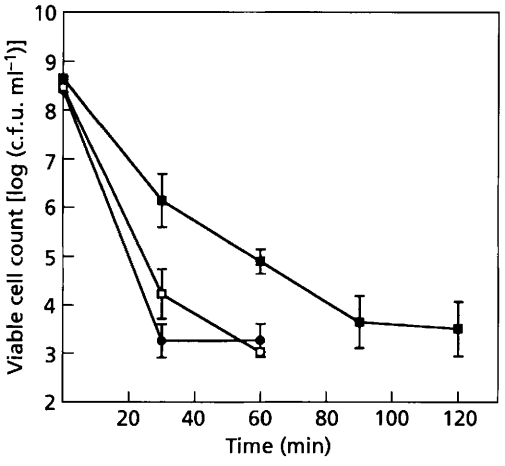

Fig. 1. The ATR of *L. monocytogenes* Scott A. Cultures were grown to exponential phase ($\approx 10^8$ c.f.u. ml^{-1}) and either pre-adapted for 1 h at pH 5·8 (■), left untreated (●) or pre-adapted at pH 5·8 in the presence of 100 μg chloramphenicol ml^{-1} (□). (The chloramphenicol was added 15 min prior to reducing the pH to 5·8.) The pH of each culture was then reduced to pH 3·0 with HCl and cell viability was measured at the time intervals shown.

survival in the culture pre-treated at pH 5·8 compared with the non-treated culture. Even after 2 h at pH 3·0 survivors were still detected in this culture (Fig. 1). Thus, prior exposure of strain Scott A to sublethal pH stress induces a considerable degree of resistance to killing by low pH, a result which confirms the presence of an ATR in *L. monocytogenes*.

To establish more precisely the optimal pH required for the induction of the ATR in *L. monocytogenes*, acid tolerance (defined as survival at pH 3·0 for 90 min) was measured after prior exposure to a range of sublethal pH values, from pH 4·0 to pH 7·0, for 1 h (Fig. 2a). The culture pre-adapted at pH 5·0 showed the greatest level of resistance to acid-killing; virtually no loss in viability was detected after 90 min at pH 3·0 (Fig. 2a). Cultures pre-adapted at pH values lower than 5·0 did not display the same level of resistance, but even at pH 4·0 there was an increase in resistance compared to the pH 7·0-treated culture. It is clear that all acidic pH values between 4·0 and 6·0 can confer enhanced acid tolerance upon *L. monocytogenes* compared to a culture not previously exposed to acidic conditions (i.e. the pH 7·0-treated culture). The data imply that the ATR is optimally induced between pH 4·8 and pH 5·2 (Fig. 2a).

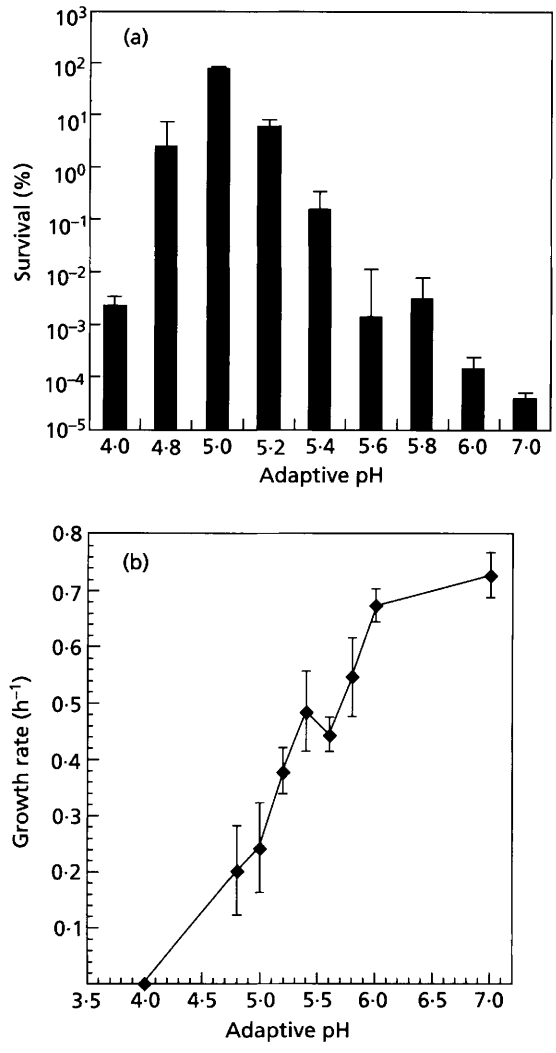

Fig. 2. pH-dependence of the ATR in *L. cytogenes* Scott A. (a) Cultures were grown to exponential phase ($\approx 10^8$ c.f.u. ml^{-1}) and the pH of each was reduced to a value in the range 4·0–7·0 with HCl. (All the pH values are accurate to one decimal place.) Cultures were incubated at this pH for 1 h, after which the pH was reduced to 3·0 with HCl for 90 min. Viable cell counts were carried out immediately before and after the acid challenge and results are presented as percentage survival. (b) Effect of adaptive pH on growth rate. Cultures were grown to mid-exponential phase and the pH of each was adjusted with HCl to a different pH, identical to those tested in (a). The growth rate of each culture was measured by recording changes in OD_{600} over the ensuing hour.

The effect of adaptive pH on growth rate

The lower pH during the adaptive period was likely to have consequences for the growth rate of the culture. It was possible that the pH-dependent changes in acid resistance simply reflected the effects of pH on growth rate. The extent of these effects on growth rate were assessed by directly measuring growth rate during the adaptive period. The pH of a series of mid-exponential-phase cultures ($\approx 10^8$ c.f.u. ml^{-1}) was adjusted to either 7·0, 6·0, 5·8, 5·6, 5·4, 5·2, 5·0, 4·8 or 4·0 with HCl. The growth rate of each was measured by recording OD_{600} over the ensuing hour. Significant changes in growth rate occurred over this pH range; cells grown at pH 7·0 had a growth rate approximately threefold greater than cells grown at pH 5·0, while at pH 4·0 *L. monocytogenes* Scott A showed no detectable growth (Fig. 2b). There did not appear to be a good correlation between acid resistance and growth rate. For example, adaptive pHs of 4·8 and 5·2 induced similar levels of acid resistance (Fig. 2a) yet the

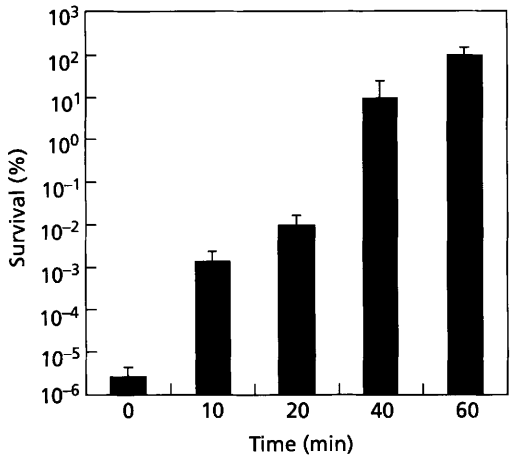

Fig. 3. Time-dependent acquisition of acid tolerance in *L. monocytogenes* Scott A. Cultures were grown to exponential phase ($\approx 10^8$ c.f.u. ml^{-1}) and the pH of each was reduced to 5·0 for either 0, 10, 20, 40 or 60 min with HCl. Viable cell counts were performed immediately before and after an acid challenge at pH 3·0 for 90 min and results are presented as percentage survival.

growth rate at pH 4·8 was approximately half that at pH 5·2 (Fig. 2b). Additionally, there was little change in growth rate between pH 7·0 and 6·0, yet there was a significant increase in acid resistance when cells were pre-adapted at pH 6·0 (Fig. 2). These data indicate that the protective effect of pre-adapting *L. monocytogenes* at sublethal pH is unlikely to be a growth-rate-related phenomenon.

Rate of induction of the ATR

It was important to determine the period of time required for optimal development of acid tolerance during the ATR. A rapid increase in acid resistance (within minutes) might indicate that the response was occurring at the physiological level (increased activity of enzymes already present in the cell), while a more gradual acquisition of acid tolerance would indicate that major changes in cellular composition were required. To this end, cultures were grown to exponential phase ($\approx 10^8$ c.f.u. ml^{-1}) and pre-adapted at pH 5·0 for either 0, 10, 20, 40 or 60 min prior to challenge with pH 3·0. Viable plate counts were performed immediately before acid challenge and after 90 min at pH 3·0 (Fig. 3). The acquisition of acid tolerance occurred gradually over the 60 min adaptive period indicating that in *L. monocytogenes* the ATR is not a rapid response.

The ATR involves *de novo* protein synthesis

To investigate whether protein synthesis was required for the acquisition of acid tolerance during the ATR, chloramphenicol (100 μg ml^{-1}) was included in the growth medium during the exposure to sublethal acid conditions (pH 5·8). This concentration of chloramphenicol was sufficient to achieve greater than 95% inhibition of protein synthesis in Scott A (data not shown). When this protein synthesis inhibitor was included in the growth medium during pre-adaptation the protective effect conferred by the mild acid shock was substantially reduced (Fig. 1). After 60 min at pH 3·0 the culture adapted in the absence of chloramphenicol had approximately 100-fold more survivors than the culture adapted in the presence of the antibiotic (Fig. 1). Chloramphenicol had no detrimental effect on the ability of non-adapted cells to survive the lethal pH of 3·0 (i.e. the rate of killing was not enhanced by the presence of this antibiotic; data not shown). Tetracycline (10 μg ml^{-1}), another inhibitor of protein synthesis, also impaired the induction of the ATR (data not shown). Together, these results demonstrate that in *L. monocytogenes* the ATR is dependent, at least partially, upon *de novo* protein synthesis.

Protein expression during the ATR

The data presented above imply that an alteration in the expression of one or more proteins plays an important role in establishing the ATR of *L. monocytogenes*. To observe more directly the changes taking place at the level of protein synthesis, total cellular proteins were isolated from a non-pre-adapted mid-exponential-phase culture and from a mid-exponential culture pre-adapted at pH 5·8 for 1 h. The proteins extracted from these cultures were analysed by two-dimensional gel electrophoresis (Fig. 4). The expression of at least 23 proteins was altered at pH 5·8 compared with the non-adapted culture. Of these, 11 showed induced expression while 12 were repressed at pH 5·8. Interestingly, most of the proteins which were induced at pH 5·8 were also present in the non-adapted culture, though to a lesser extent. Only one protein has been identified which was exclusively synthesized during the ATR (protein 1; Fig. 4). At least five proteins present at pH 7·0 were completely absent from the pre-adapted culture (proteins 7, 8, 10, 12 and 16). These data indicate that the ATR in *L. monocytogenes* is a complex response to environmental pH involving the induction and repression of a large number of genes.

Growth-phase-dependent acid tolerance

The finding that in some organisms resistance to low pH, and indeed stress resistance in general, increases during stationary phase prompted an examination of acid resistance in *L. monocytogenes* during this phase of growth. The acid resistance of an exponentially growing culture was compared to that of a 24-h-old culture (Fig. 5a). At pH 3·0 the exponential-phase culture was seen to lose viability rapidly (after 60 min there were no detectable survivors), whereas the stationary-phase cells showed a high degree of tolerance to this lethal pH. Even after 3 h at pH 3·0 the stationary-phase culture still contained greater than 10^8 c.f.u. ml^{-1} (Fig. 5a). Thus, *L. monocytogenes* displays the ability to develop acid tolerance in a growth-phase-dependent manner.

To define more precisely the growth-phase-associated changes in acid tolerance, the degree of resistance to acid (i.e. survival at pH 3·0 for 90 min) was measured

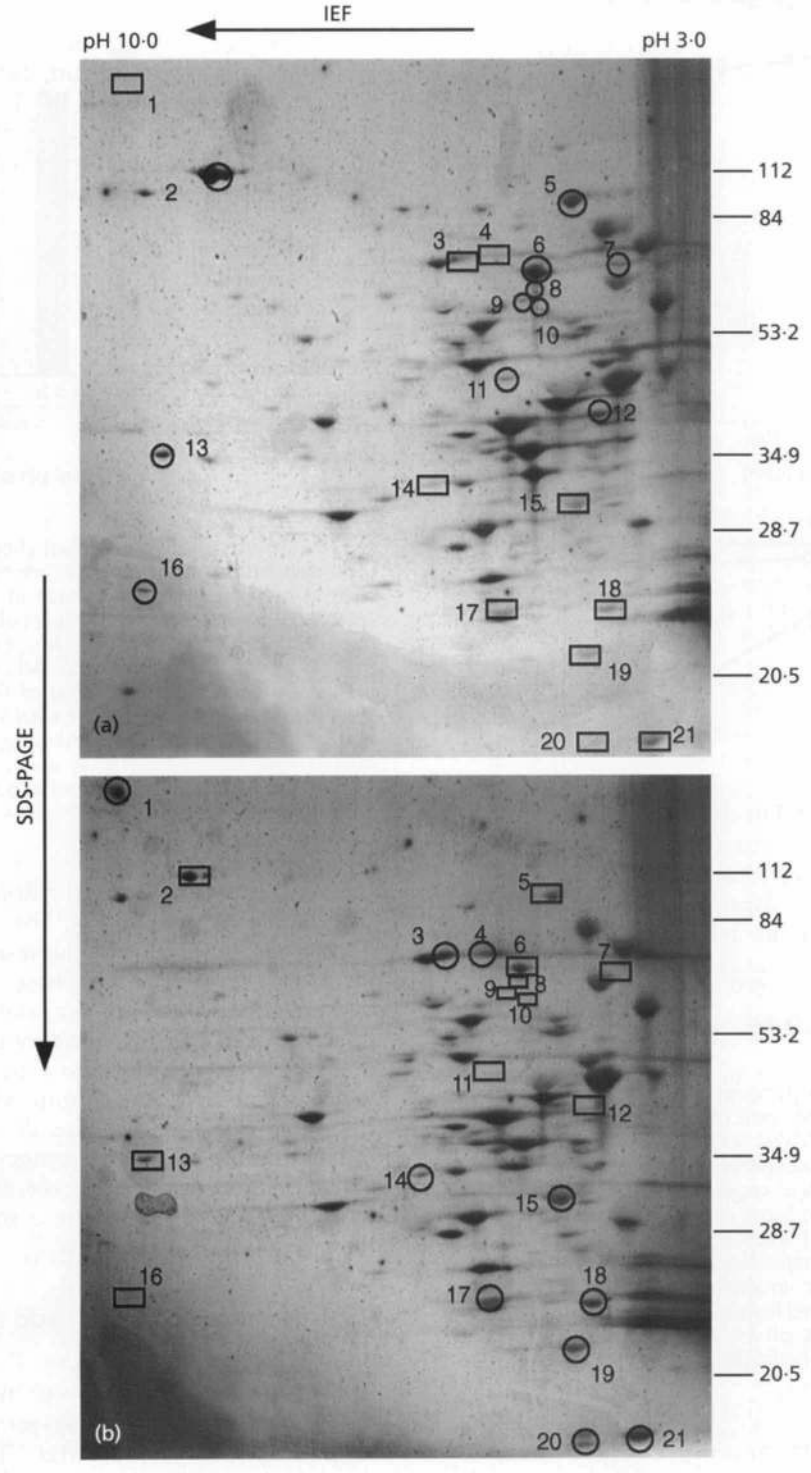

Fig. 4. Two-dimensional electrophoretic analysis of protein expression during the ATR in *L. monocytogenes* Scott A. Cultures were grown to exponential phase and either pre-adapted at pH 5·8 (b) or left untreated (a). Proteins were extracted from both cultures and equal concentrations of protein were separated by two-dimensional gel electrophoresis: isoelectric focusing in the first dimension and SDS-PAGE in the second (indicated by arrows). Gels were silver-stained and the figure shows an enlarged digitally-captured image of these gels (see Methods). Numbered rectangles indicate proteins whose expression is repressed at that pH (compared to the other pH) while numbered circles indicate proteins induced at that pH. The numbers on the upper gel match those on the lower gel. The numbers on the right side of the gels indicate the molecular mass of the standards in kDa.

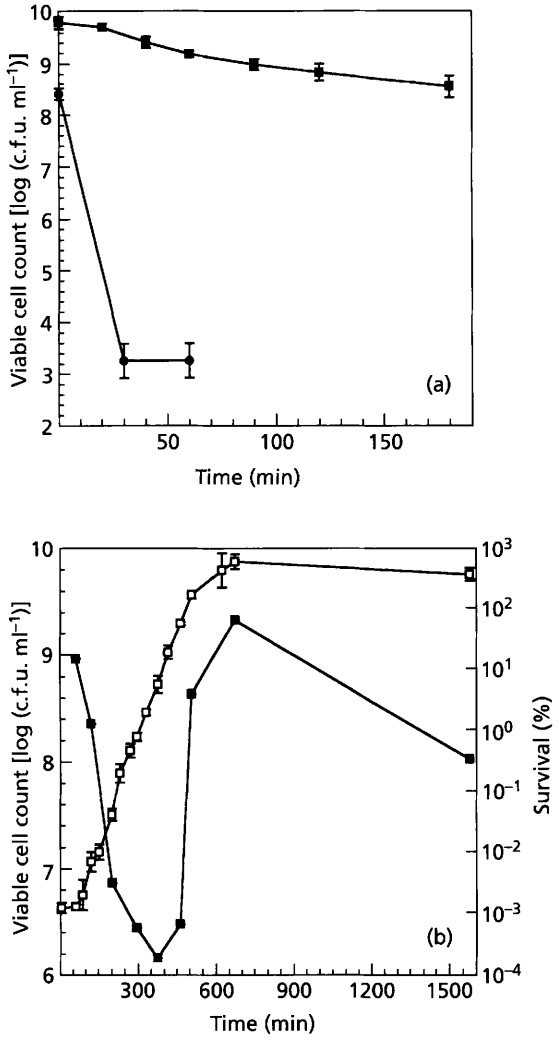

Fig. 5. Growth-phase-dependent acid tolerance in *L. monocytogenes*. (a) Cultures were grown to either exponential (●) or stationary (■; 24 h culture) phase. The pH was then reduced to 3·0 with HCl and cell viability was measured at the time intervals shown. (b) Acid resistance measured throughout growth. A culture of strain Scott A was set up and viable cell counts were performed at appropriate intervals in order to monitor growth (□). Samples were also removed from the culture throughout growth in order to assess acid resistance. Viable cell counts were performed immediately before and after an acid challenge at pH 3·0 for 90 min and results are presented as percentage survival (■).

throughout growth. At appropriate time intervals, samples were removed from a culture of Scott A growing in BHI and the resistance of the cells to acid was measured. Initially, the level of acid tolerance was high (presumably because the inoculum used to set up this culture was itself in stationary phase) but it dropped rapidly during early exponential phase (Fig. 5b). Mid-exponential-phase cells were found to be the most sensitive to low pH. During the transition between exponential phase and stationary phase the level of acid tolerance increased rapidly (Fig. 5b). The highest level of acid tolerance appeared in early stationary phase; cells from this point of the growth curve

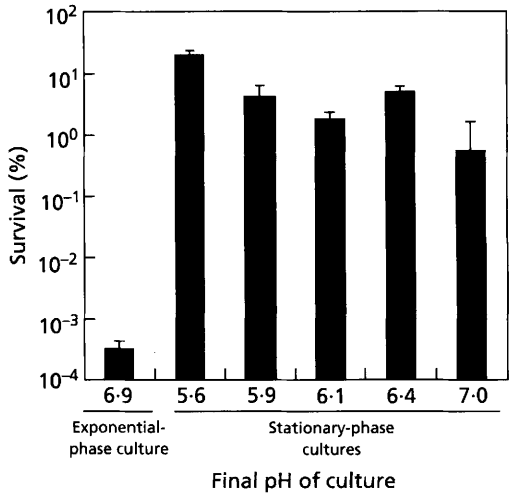

Fig. 6. Growth-phase-dependent changes in acid tolerance are pH-independent. Cultures of *L. monocytogenes* strain Scott A were grown to stationary phase in BHI buffered with either MES, MOPS or Tris buffers. One culture was unbuffered and had a final pH of 5·6. An exponential-phase culture ($\approx 7{\cdot}0 \times 10^8$ c.f.u. ml^{-1}) was also included as a control. The final pH values (shown on the *x*-axis) of the buffered cultures were 5·9 (55 mM MES), 6·1 (55 mM MOPS), 6·4 (55 mM Tris pH 7·5) and 7·0 (55 mM Tris pH 8·0). Viable cell counts were performed immediately before and after an acid challenge at pH 3·0 for 90 min and results are presented as percentage survival.

showed less than a 1 log reduction in the viable cell count after 90 min at pH 3·0 (Fig. 5b). There appeared to be a decline in the level of acid resistance after prolonged incubation in stationary phase. There is an apparent discrepancy between Fig. 5(a) and 5(b) in the level of acid tolerance seen in late stationary phase. This is probably due to the fact that the last time point in Fig. 5(b) was taken 26·6 h after inoculation whereas in Fig. 5(a) the stationary-phase culture was 24 h old. Further work is required to investigate dynamics of acid tolerance during the stationary phase. However, these results do confirm that in *L. monocytogenes* there is strong link between acid tolerance and growth phase.

Growth-phase-dependent acid tolerance is pH-independent

The final pH of a culture of strain Scott A grown to stationary phase in BHI was typically between 5·6 and 6·0, sufficiently low to induce the ATR (see Fig. 2a). It was possible that the high level of acid resistance detected in stationary phase was due to pH changes that take place during growth. To test this possibility, a series of cultures were set up containing buffers capable of preventing pH changes in the medium. Cultures grown to stationary phase were buffered such that their final pH values were 5·9, 6·1, 6·4 and 7·0. The unbuffered control reached a final pH of 5·6. An exponential-phase culture was also grown for comparison. The acid resistance of each culture was measured by exposure to pH 3·0 for 90 min and the results are shown graphically in Fig. 6. As expected, the

exponential-phase cells were killed rapidly at pH 3·0 while the unbuffered stationary-phase culture (final pH of 5·6) displayed a high level of resistance to acid-killing. The four buffered stationary-phase cultures also showed a high level of acid resistance. There was, however, a 60-fold decline in the level of acid resistance of the pH 7·0-buffered culture compared to the unbuffered culture (Fig. 6). This indicated that a decrease in the pH during growth contributed to the high level of acid resistance seen in stationary phase. The contribution of pH changes to acid resistance was small, however, compared to the 1000-fold increase in resistance which occurred in a pH-independent manner (Fig. 6). These data indicate that the increase in acid resistance that occurs upon entry into stationary phase is largely independent of pH changes that occur during growth.

DISCUSSION

The results presented here demonstrate that *L. monocytogenes* develops acid tolerance upon exposure to sublethal acid conditions, a response that has been designated the ATR (acid tolerance response). The effectiveness of this response appears to be critically dependent upon two principal factors: (i) the pH of the adaptive exposure and (ii) the duration of the adaptive period. Maximal acid tolerance is achieved when the adaptation is carried out at pH 5·0 for 60 min. It is interesting to note that the growth rate of *L. monocytogenes* decreases approximately threefold when the pH is lowered from 7·0 to 5·0, but it seems unlikely that this decrease alone could provide the signal for the induction of the ATR. If this was the case, cells exposed to pH 4·0 should also induce a high level of acid resistance (pH 4·0 decreases growth rate to zero but is still a sublethal pH) but this was not observed. Above and below pH 5·0 the level of acid resistance achieved by pre-adaptation declined gradually. This gradual decline implies that the ATR is not an all-or-none response in this organism. A similar result has been demonstrated previously for *Sal. typhimurium*, though in this case it appears that distinct mechanisms are induced as the adaptive pH is lowered from 5·8 to 4·5 (Foster, 1991).

The ATR in *L. monocytogenes* is shown here to depend, at least partially, on the *de novo* synthesis of proteins. This is also true for the acquisition of acid tolerance in a number of other organisms including *Sal. typhimurium* (Foster & Hall, 1990), *E. coli* (Raja *et al.*, 1991), *A. hydrophila* (Karem *et al.*, 1994) and *Streptococcus faecalis* (Kobayashi *et al.*, 1986). In those cases where a two-dimensional electrophoretic analysis of ATR proteins has been undertaken (Hickey & Hirshfield, 1990; Foster, 1991; Karem *et al.*, 1994) it is clear that the response is complex and involves the induction and repression of the synthesis of a large number of proteins. The same appears to hold true for the ATR in *L. monocytogenes*. This finding is consistent with the observation that the ATR is not a rapid response and takes 60 min for full induction of acid tolerance. No data are yet available to indicate possible roles for any of these proteins in acid tolerance. Indeed, it is important to note that an alteration in the level of expression in response to a change in pH does not necessarily imply a role in acid tolerance. For this reason the number of proteins directly contributing to acid tolerance is currently unknown. In streptococci the membrane-bound proton-translocating ATPase appears to play a key role in acid tolerance and its expression is up-regulated under acidic conditions (Kobayashi *et al.*, 1986). It is possible that this enzyme also plays a role in the survival of *L. monocytogenes* at low pH. Other possible mechanisms of acid resistance include acid-induced DNA repair systems, increased cytoplasmic buffering capacity and decreased proton permeability of the cell membrane (perhaps by removing 'leaky' membrane proteins; see for example Rowbury & Goodson, 1993).

The data presented imply that in addition to the pH-dependent ATR, *L. monocytogenes* also possesses a growth-phase-dependent acid tolerance system which is independent of pH. *Sal. typhimurium* is known to possess a similar acid resistance mechanism. In this case, acid resistance is induced in stationary phase in a pH-independent manner and is dependent on the presence of the stationary-phase-specific sigma factor, RpoS (Lee *et al.*, 1994). In stationary phase this sigma factor is responsible for redirecting the transcription of a large number of genes whose products confer increased stress resistance upon the cells (reviewed in Hengge-Aronis, 1993). No such sigma factor has yet been identified in *Listeria*, but it seems plausible to suggest that one may exist in this organism. Both *E. coli* and *Shi. flexneri* have also been shown to develop increased acid tolerance in stationary phase (Arnold & Kasper, 1995; Gorden & Small, 1993) and again the RpoS protein appears to play a role in this response (Small *et al.*, 1994). Thus, the available data indicate that the development of increased acid tolerance in stationary phase may be a conserved phenomenon amongst bacteria. Presumably this response protects stationary-phase cells during encounters with acidic environments; the low metabolic activity of the cells would prevent them from rapidly developing acid tolerance (as seen in exponential-phase cells) and so a stationary-phase-specific system is necessary.

ACKNOWLEDGEMENTS

The authors would like thank to Dr Bizhan Pourkomailian, Dr Martin B. Cole, Professor Bevan Moseley and Professor Ian R. Booth for helpful discussions. This work was partly funded by a research grant from the Ministry for Agriculture, Fisheries and Food.

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Received 16 November 1995; revised 26 April 1996; accepted 28 May 1996.