Influence of Temperature on Acid-Stress Adaptation in *Listeria monocytogenes*

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Abstract

Our findings show that temperature plays a significant role in the induction of acid-stress adaptation in *Listeria monocytogenes*, and two distinct patterns were observed: (1) Presence of sublethal acid at 37°C or 22°C significantly induced acid-stress adaptation; and (2) Presence of sublethal acid at 4°C did not induce any acid-stress adaptation. Both patterns were confirmed by two experimental models: (1) *L. monocytogenes* cells were first grown at 37°C and then exposed to sublethal acid at 37°C, 22°C, and 4°C prior to lethal acid challenge; (2) Alternatively, *L. monocytogenes* cells were first grown at 4°C for 20 days before pre-exposure to sublethal acid and then challenged with lethal acid. Regardless of whether *L. monocytogenes* cells were simultaneously exposed with both cold stress and sublethal acid stress, or subjected to cold growth first before exposure to sublethal acid, no acid-stress adaptation was induced at 4°C. We also found that acid-stress adaptation in *L. monocytogenes* did not occur in acidic whey at 4°C. Bead beating treatment prior to mild acid pre-exposure at 4°C partially induced acid adaptation in *L. monocytogenes*. Our findings suggest that cold temperature can prevent the risk of acid-stress adaptation in *L. monocytogenes*.

Introduction

IN THE INDUSTRIALIZED COUNTRIES, foodborne listeriosis is responsible for a nearly 25%–30% mortality rate among infected individuals (Siegman-Igra *et al.,* 2002; Ramaswamy *et al.,* 2007). These outbreaks are frequently associated with contamination of *Listeria monocytogenes* in ready-to-eat (RTE) food products such as deli meat, Mexican-style cheese, cold smoked fish, salads, and fresh fruits (Ryser and Marth, 2007; CDC, 2011; Cartwright *et al.,* 2013). The U.S. Food and Drug Administration (FDA) and U.S. Department of Agriculture–Food Safety and Inspection Service have a "zero tolerance" policy for *L. monocytogenes* in RTE food products (Klima and Montville, 1995).

L. monocytogenes exhibits sophisticated adaptive mechanisms to counteract higher levels of lethal acid, heat, salt, or oxidative stresses after pre-exposure to sublethal concentrations of the same stress or different stress environments (O'Driscoll *et al.,* 1996; Lou and Yousef, 1997; Bolton and Frank, 1999). Of all the stress adaptations, acid-stress adaptation in *L. monocytogenes* has received extensive attention. Acid-stress adaptation in *L. monocytogenes*, where cells exhibit enhanced acid tolerance at a lethal pH of 3.5 after pre-exposure to mild acid stress (pH 5.0), was first reported by Hill (1995). Further studies revealed that the activation of acid-stress adaptation required protein *de novo* synthesis (Davis *et al.,* 1996; O'Driscoll *et al.,* 1996). Two enzymatic systems, glutamate decarboxylase (GAD) system and arginine deiminase (ADI) systems, are critical in conferring acid-stress adaptation to *L. monocytogenes* (Cotter and Hill, 2003; Ryan *et al.,* 2009).

Several factors play critical roles in controlling the induction of acid-stress adaptation in *L. monocytogenes*. Extensive studies have determined the influence of sublethal acid concentration, exposure time, type of acidulant, and bacterial growth stage on acid-stress adaptation in *L. monocytogenes*. Davis *et al*. (1996) determined that pre-exposure at pH 5.0 for 1 h was optimal to confer *L. monocytogenes* with acid-stress adaptation. With respect to growth stage, it is well known that stationary phase cells of *L. monocytogenes* have better acid tolerance compared to log phase cells (O'Driscoll *et al.,* 1996; Datta and Benjamin, 1997). All these studies were conducted at the optimum growth temperatures. Under food processing conditions, *L. monocytogenes* cells may be exposed to suboptimal temperatures (4°C–22°C), which are much lower than that of laboratory conditions. The effect of refrigeration temperatures on acid-stress adaptation in *L. monocytogenes* is not known. Therefore, the objective of our study is to determine the effect of temperature on acid-stress adaptation in *L. monocytogenes* serotypes 4b and 1/2a.

Materials and Methods

Bacterial strains

The bacterial strains used in this study included *L. monocytogenes* Scott A (serotype 4b, human clinical, FDA) and

L. monocytogenes EGD strain (BUG 600) (serotype 1/2a, clinical, Institute Pasteur, France). Working stock cultures were prepared by streaking frozen stock culture (stored at $-80^{\circ}C$) onto tryptic soy agar (TSA) slants and maintained at $4^{\circ}C$. Overnight cultures were prepared by inoculating one loop of working stock cultures in 10 mL tryptic soy broth supplemented with 0.6% yeast extract (TSB-YE) and incubating at $37^{\circ}C$ for 18–20 h. Early log-phase culture was prepared by transferring 200 μL overnight culture into 20 mL TSB-YE and incubating at $37^{\circ}C$ until OD_{600nm} reached ~ 0.15 (Thermon Electron Corporation). Experiments were performed using $37^{\circ}C$ grown early log-phase cells unless otherwise noted.

Effect of temperature on induction of acid-stress adaptation

The temperature-dependent acid adaptation was evaluated by initially pre-exposing the *L. monocytogenes* cells to pH 5.0 (1 M lactic acid) or 7.2 TSB-YE at three different temperatures ($37^{\circ}C$, $22^{\circ}C$, and $4^{\circ}C$) for 1 h and subsequently performing postlethal acid challenge in pH 3.5 TSB-YE (3M lactic acid) at $37^{\circ}C$.

FIG. 2. Survival of *Listeria monocytogenes* Scott A **(A)** and BUG 600 **(B)** cold-grown ($4^{\circ}C$) log-phase cells in lethal pH 3.5 tryptic soy broth supplemented with 0.6% yeast extract at $22^{\circ}C$ after 1 h pre-exposure to sublethal pH 5.0 (■) or pH 7.2 (□) at $37^{\circ}C$ or $4^{\circ}C$. Bars marked with an asterisk indicate significant survival differences between pH 5.0 (■) and pH 7.2 (□) pre-exposed cells. CFU, colony-forming units.

One milliliter of $37^{\circ}C$ grown early log phase cultures were transferred into 9 mL pre-acidified (mild acid-stressed cells) or neutral TSB-YE (nonstressed cells) for pre-exposure at $37^{\circ}C$, $22^{\circ}C$, or $4^{\circ}C$. Tubes containing 9 mL pre-acidified or neutral broth were previously equilibrated to $37^{\circ}C$, $22^{\circ}C$, or $4^{\circ}C$. After 1 h pre-exposure, mild acid-stressed and non-stressed samples were centrifuged (Sorvall Biofuge Fresco, $16,060 \times g$). While centrifuging, samples pre-exposed at $4^{\circ}C$ were centrifuged at $4^{\circ}C$ whereas samples pre-exposed at $37^{\circ}C$ or $22^{\circ}C$ were centrifuged at room temperature. After centrifugation, cell pellets were resuspended in pH 3.5 TSB-YE at $37^{\circ}C$ for up to 180 min. *L. monocytogenes* survivors were spread plated at different time intervals on TSA-EF (TSA containing 1.0% esculin and 0.05% ferric ammonium citrate) and incubated at $37^{\circ}C$ for 48 h (Fig. 1).

Similar experiments were also performed using early log-phase cold ($4^{\circ}C$) grown cells ($OD_{600nm} \sim 0.15$), but only two pre-exposure temperatures ($37^{\circ}C$ and $4^{\circ}C$) were used and survivors following $22^{\circ}C$ postlethal acid treatments were enumerated at 90 min for Scott A and at 180 min for BUG 600 (Fig. 2).

Determining factors affecting lack of acid adaptation at $4^{\circ}C$

Increase of mild acid pre-exposure time, various mild acid concentrations, change of acidulants, and addition of salt were analyzed to determine the acid adaptation of $4^{\circ}C$ mild
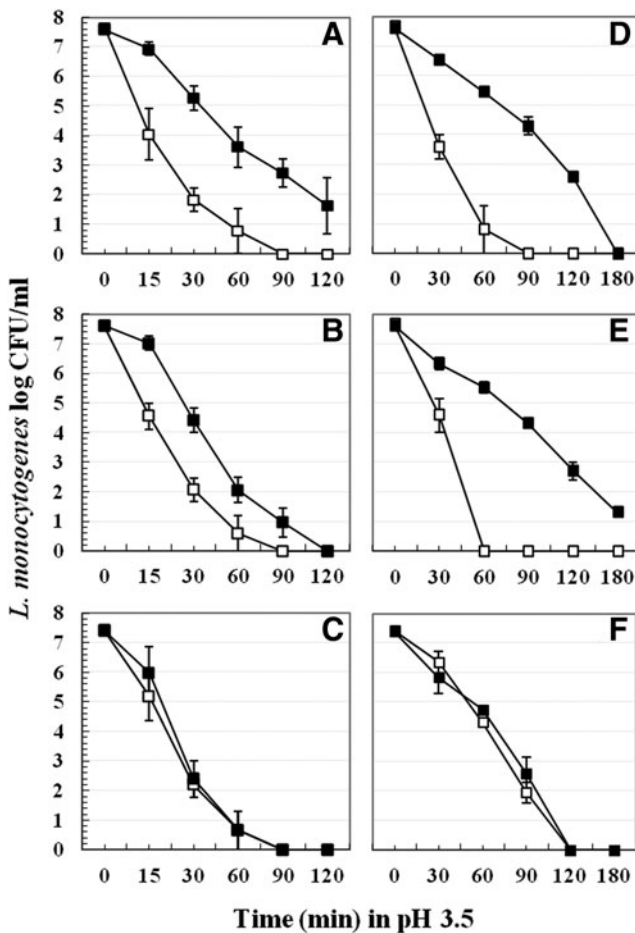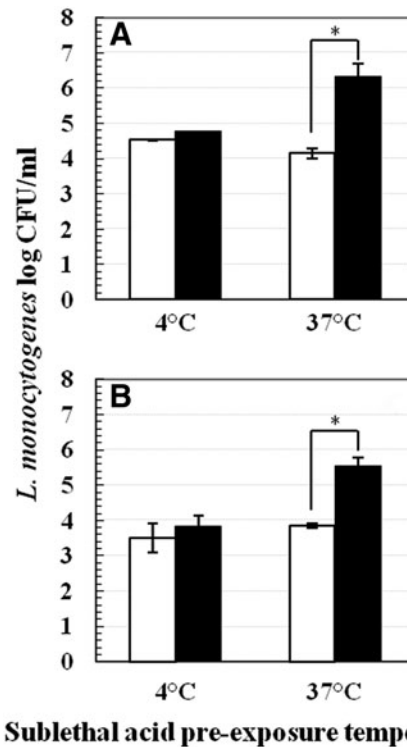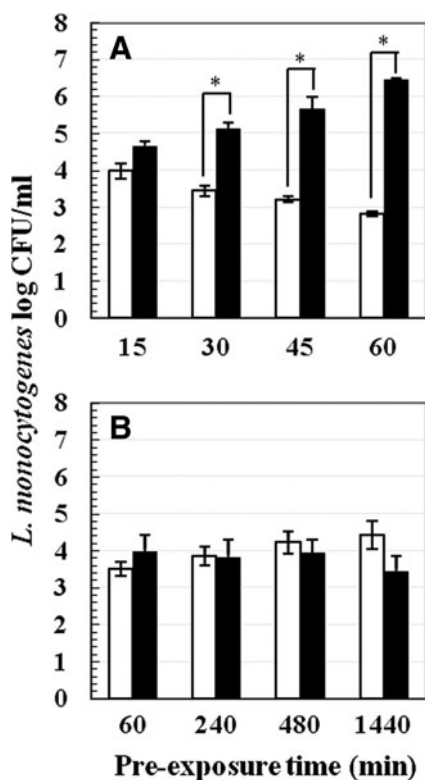

FIG. 1. Survival of *Listeria monocytogenes* Scott A (serotype 4b) **(A, B, C)** and BUG 600 (serotype 1/2a) **(D, E, F)** in lethal pH 3.5 tryptic soy broth supplemented with 0.6% yeast extract at $37^{\circ}C$ after 1 h pre-exposure to sublethal pH 5.0 (■) or pH 7.2 (□) at three temperatures: (A, D) $37^{\circ}C$; (B, E) $22^{\circ}C$; and (C, F) $4^{\circ}C$. CFU, colony-forming units.

FIG. 3. Survival of *Listeria monocytogenes* Scott A in lethal pH 3.5 tryptic soy broth supplemented with 0.6% yeast extract at 22°C for 60 min after pre-exposure to sublethal pH 5.0 (■) or pH 7.2 (□) at two temperatures: **(A)** 37°C for 1 h; and **(B)** 4°C for 24 h. Bars marked with an asterisk indicate significant survival differences between pH 5.0 (■) and pH 7.2 (□) pre-exposed cells. CFU, colony-forming units.
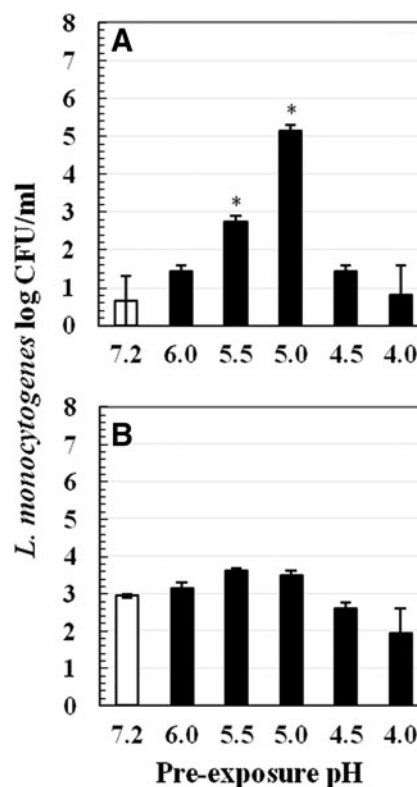

FIG. 4. Survival of *Listeria monocytogenes* Scott A in lethal pH 3.5 tryptic soy broth supplemented with 0.6% yeast extract at 22°C for 90 min after 1 h pre-exposure to different sublethal pH at two temperatures: **(A)** 37°C; and **(B)** 4°C. Bars marked with an asterisk indicate significant survival differences between mild acid-stressed (■) and nonstressed (□) cells. CFU, colony-forming units.

acid-stressed cells. As a control, 37°C mild acid-stressed and nonstressed cells were used in all experiments. All experiments were performed using *L. monocytogenes* Scott A grown to early log phase at 37°C. Postlethal acid inactivation treatment was performed in pH 3.5 TSB-YE (3M lactic acid) at 22°C for 60 or 90 min and survivors were enumerated on TSA-EF. Throughout all experiments, cells pre-exposed at 4°C were centrifuged at 4°C while 37°C pre-exposed cells were centrifuged at room temperature. Lactic acid (1M) was used for mild-acid pre-exposure unless otherwise noted.

The following series of experiments were conducted for the proof of concept at 4°C:

1. Increasing mild-acid pre-exposure time. Pre-exposure was conducted in pH 5.0 or 7.2 TSB-YE at 37°C or 4°C. Pre-exposure time periods tested at 37°C were 15, 30, 45, and 60 min, whereas time periods tested at 4°C were 1, 4, 8, and 24 h. At each time point, mild acid-stressed and nonstressed cells were centrifuged and subjected to lethal inactivation (Fig. 3).
2. Varying mild-acid concentrations. Cells were pre-exposed to different pHs (4.0, 4.5, 5.0, 5.5, 6.0, and 7.2) for 1 h at 37°C or 4°C. After pre-exposure, samples were centrifuged and cell pellets were subjected to lethal acid inactivation (Fig. 4).
3. Effect of various pre-exposure acidulants. Instead of lactic acid, 1M of hydrochloric acid and acetic acid were

used to acidify TSB-YE to pH 5.0. Pre-exposure was performed in pH 5.0 or 7.2 TSB-YE for 1 h at 37°C or 4°C. Thereafter, cell pellets were obtained and subjected to lethal inactivation (Fig. 5).
4. Addition of sodium chloride. TSB-YE used in this study contained 0.5% sodium chloride. Additional salt concentrations tested include 2%, 4%, 6%, 8%, and 10%. Mild acid-stressed and nonstressed cells were prepared by pre-exposure to pH 5.0 or 7.2 TSB-YE supplemented with various sodium chloride concentrations (0.5%, 2%, 4%, 6%, 8%, and 10%) at 37°C or 4°C for 1 h. After pre-exposure, cells were pelleted and subjected to lethal acid inactivation (Fig. 6).

Acid-stress adaptation in acidic whey at different temperatures

Fat reduced milk was heated to 85°C and coagulated by 3M lactic acid. Coagulant was strained and acidic whey (pH ~5.1) was collected. *L. monocytogenes* Scott A early log-phase cells were diluted in TSB-YE with 1:10 and centrifuged to collect cell pellets. Thereafter, cell pellets were resuspended in temperature equilibrated acidic whey for 1 h at 37°C, 22°C, or 4°C. After pre-exposure, calculated 3M lactic acid quantity was directly added into those whey samples to attain final pH of 3.5. Control cells were directly inactivated in pH 3.5 acidic whey. The lethal acid challenge was performed at 22°C and
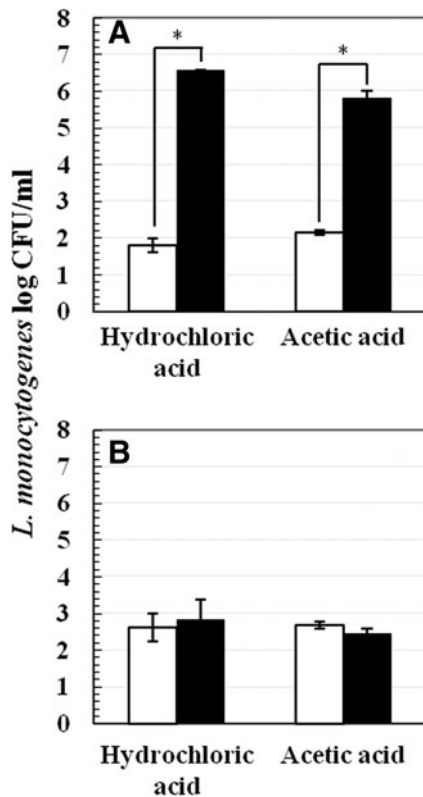
FIG. 5. Survival of *Listeria monocytogenes* Scott A in lethal pH 3.5 tryptic soy broth supplemented with 0.6% yeast extract at 22°C for 90 min after 1 h pre-exposure to sublethal pH 5.0 (■) or pH 7.2 (□) at two temperatures: **(A)** 37°C; and **(B)** 4°C. Pre-exposure with pH 5.0 was adjusted by hydrochloric acid or acetic acid. Bars marked with an asterisk indicate significant survival differences between pH 5.0 (■) and pH 7.2 (□) pre-exposed cells. CFU, colony-forming units.

FIG. 6. Survival of *Listeria monocytogenes* Scott A in lethal pH 3.5 tryptic soy broth supplemented with 0.6% yeast extract at 22°C for 90 min after 1 h pre-exposure to sublethal pH 5.0 (■) or pH 7.2 (□) in the presence of different concentrations of sodium chloride at two temperatures: **(A)** 37°C; and **(B)** 4°C. Bars marked with an asterisk indicate significant survival differences between pH 5.0 (■) and pH 7.2 (□) pre-exposed cells. CFU, colony-forming units.
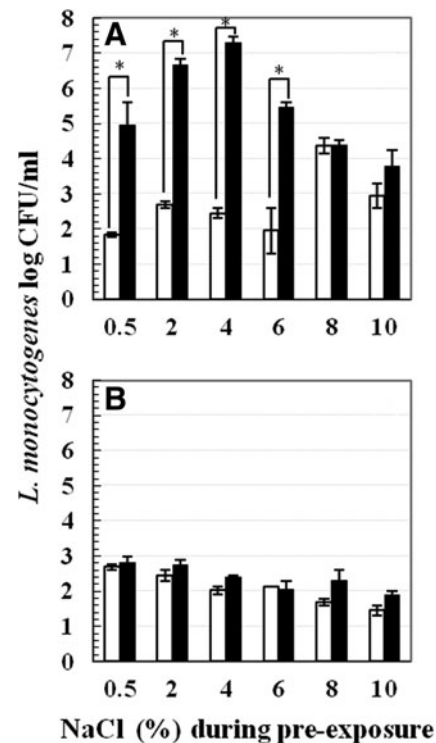
survivors were measured on PALCAM agar plates supplemented with 6 mg/L ceftazidime (Difco, Detroit, MI) every 30 min up to 2 h (Fig. 7).

Effect of bead beating on the induction of acid-stress adaptation at 4°C

Early log phase culture of *L. monocytogenes* Scott A (1.2 mL) was transferred into screw-cap tubes containing 2 g of 0.1 mm zirconium (BioSpec Products, Inc., OK) beads. After 2 min of bead beating (4800 oscillations/min) at room temperature, 100 μL cultures were transferred into 900 μL pre-acidified (mild acid-stress) or neutral broth (nonstress) that was initially kept at 37°C or 4°C for temperature equilibration. Pre-exposure was allowed for 1 h at 37°C or 4°C, after which pellets were recovered and resuspended in pH 3.5 TSB-YE (3M lactic acid) at 22°C. Survivors were measured every 30 min up to 90 min (Fig. 8).

Statistical analysis

All experiments were performed in three replicates with a completely randomized design structure. Data shown here are mean average (±SE) of three individual trials. Student *t*-test ($p < 0.05$) was performed to determine significant mean

separation between survival of mild acid-stressed and non-stressed cells (SPSS version 12.0, Chicago, IL).

Results

Mild acid-stress at 4°C did not induce acid-stress adaptation in L. monocytogenes

Figure 1 shows the acid adaptation of 37°C grown cells of *L. monocytogenes* Scott A and BUG 600, respectively, after 1 h mild acid pre-exposure at 37°C, 22°C, and 4°C. Two distinct patterns of acid adaptation were observed: (1) conditions where acid adaptation occurred; and (2) conditions where acid adaptation did not occur. For both strains, cells pre-exposed to mild acid stress at 22°C or 37°C for 1 h exhibited about 2–5 log colony-forming units (CFU)/mL better survival than nonstressed cells (Fig. 1A, 1B, 1D, 1E). In contrast, no difference in survival between mild acid-stressed and non-stressed cells was observed if pre-exposure was performed at 4°C (Fig. 1C, 1F). For Scott A, 4°C mild acid-stressed had 2–3 log CFU/mL lower survival compared to 22°C or 37°C mild acid-stressed cells after 90 min in pH 3.5 TSB-YE. For BUG 600, this difference was about 2 log CFU/mL. This conceptual finding was also true if the lethal acid challenge was performed at 4°C or 22°C (data not shown).

Lack of mild acid adaptation at 4°C was also observed in *L. monocytogenes* cells grown at 4°C (Fig. 2A and 2B). For both strains, no difference existed between survival of mild

acid-stressed and non-stressed cells if mild acid pre-exposure was performed at 4°C. However, at 37°C, mild acid-stressed cells had about 2 log CFU/mL higher survival compared to nonstressed cells.

Increase in pre-exposure time, varying pH of mild acid exposure, change of acidulants, and addition of salt did not induce acid-stress adaptation in L. monocytogenes *at 4°C*

Increasing mild acid pre-exposure (pH 5.0) time from 1 h to 4, 8, or 24 h also failed to induce acid adaptation at 4°C (Fig. 3B). Survival of both mild acid-stressed and non-stressed cells was approximately 4 log CFU/mL. In contrast, 30 min of pre-exposure to pH 5.0 at 37°C induced acid adaptation as mild acid-stressed cells exhibited 1.5 log CFU/mL higher survival than nonstressed cells (Fig. 3A).

Change of mild acid pre-exposure pH also did not induce acid adaptation at 4°C. Survival of pH 4.0, 4.5, 5.0, 5.5, and 6.0 mild acid-stressed cells were 2–3.5 log CFU/mL, which was not higher than that of nonstressed cells (3 log CFU/mL) (Fig. 4B). However, pH 5.0 and pH 5.5 pre-exposure at 37°C conferred *L. monocytogenes* with 2–4 log CFU/mL greater survival than nonstressed cells (Fig. 4A).

No acid adaptation was observed after pre-exposure to hydrochloric acid or acetic acid at pH 5.0 at 4°C. There was about 3 log CFU/mL survival for 4°C mild acid-stressed cells, which was similar to that of 4°C nonstressed cells (Fig. 5B). However, at 37°C, mild acid-stressed cells exhibited approximately 4 log CFU/mL higher survival than nonstressed cells (Fig. 5A).

Addition of sodium chloride during pre-exposure did not induce acid adaptation at 4°C, as survival of non-acid-stressed cells was similar to that of mild acid-stressed cells (Fig. 6B). In contrast, pronounced acid adaptation was observed when the cells were pre-exposed to pH 5.0 supplemented with sodium chloride at 37°C. In the presence of low concentrations (0.5%–6%) of sodium chloride, mild acid-stressed cells exhibited at least 3 log CFU/mL greater survival than non-acid-stressed cells (Fig. 6A).
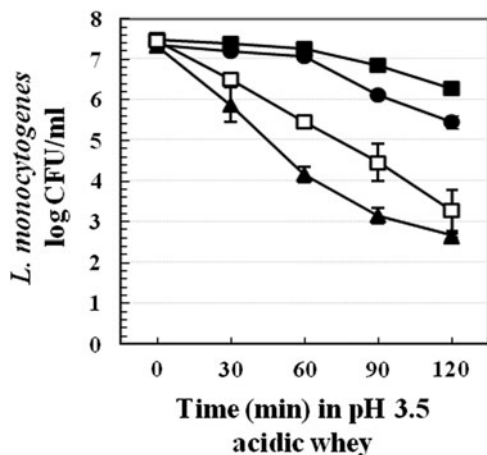
FIG. 7. Survival of *Listeria monocytogenes* Scott A in lethal pH 3.5 acidic whey at 22°C. Cells were pre-exposed to acidic whey (pH 5.1) for 1 h at 37°C (■), 22°C (●), or 4°C (■). Control cells (□) were added to acidic whey and immediately inactivated by lethal pH 3.5 at 22°C. CFU, colony-forming units.
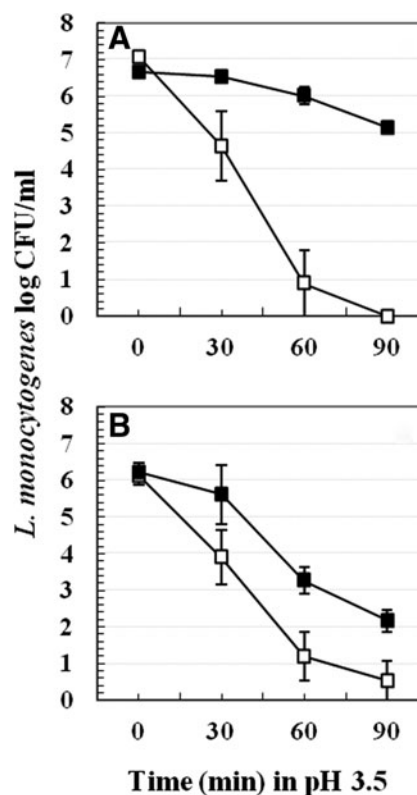
FIG. 8. Survival of 2-min bead-beated *Listeria monocytogenes* Scott A cells in lethal pH 3.5 tryptic soy broth supplemented with 0.6% yeast extract at 22°C after 1 h pre-exposure to sublethal pH 5.0 (■) or pH 7.2 (□) at two temperatures: **(A)** 37°C; and **(B)** 4°C. CFU, colony-forming units.

No acid-stress adaptation of L. monocytogenes *in acidic whey at 4°C*

Cells pre-exposed to acidic whey at 22°C or 37°C showed at least 2 log CFU/mL greater survival compared to control cells. In contrast, cells pre-exposed in acidic whey at 4°C exhibited somewhat impaired survival compared to non-stressed cells (Fig. 7). This indicated that *L. monocytogenes* acid adaptation occurred in acidic whey at 37°C or 22°C but not at 4°C.

Bead-beating of L. monocytogenes *cells induced partial acid-stress adaptation at 4°C*

In this assay, we limited the bead-beating time for 2 min, which reduced the cell viability from the initial 7.2 to 6.2 log CFU/mL. Bead-beated cells induced acid adaptation after pre-exposure to pH 5.0 TSB-YE at 4°C since about 2 log CFU/mL survival difference between mild acid-stressed and nonstressed cells was observed (Fig. 8B). However, at 37°C, 4–5 log CFU/mL greater survival was observed in mild acid-stressed cells compared to nonstressed cells (Fig. 8A).

Discussion

In the present study, we used log-phase *L. monocytogenes* cells instead of stationary-phase cells. One reason is that stationary-phase cells become partially acid adapted due to the

drop of pH during their growth into stationary stage (Davis *et al.,* 1996). Another reason not to work with stationary-phase cells is that after entering stationary phase, cells become naturally resistant because of the activation of stringent response (mediated by (p)ppGpp) and general stress response (mediated by σ^B) (Hecker and Volker, 1998; Ferreira *et al.,* 2001; Godfrey *et al.,* 2002). Such responses occurring in stationary phase can make *L. monocytogenes* resistant to various types of physiological stresses including lethal acid stress.

Ivy *et al.* (2012) and Samelis *et al.* (2003) reported that *L. monocytogenes* stationary-phase cells propagated at 7°C or 10°C showed lower acid tolerance than optimal-temperature-grown stationary-phase cells. Both studies used *L. monocytogenes* cells that were grown to stationary phase in which growth medium pH drops to 5.6–6.0 due to glucose metabolism (Davis *et al.,* 1996). Such drop of media pH can simulate the condition of mild acid pre-exposure in stationary phase. Therefore, such reduced acid tolerance in cold-grown stationary cells observed in these studies could be partially due to repressed acid adaptation under cold temperatures.

Several authors have proposed that the activation of bacterial acid adaptation requires *de novo* proteins synthesis (Davis *et al.,* 1996; O'Driscoll *et al.,* 1996). It is possible that acid shock proteins (i.e., GAD and ADI systems) are not synthesized during mild acid pre-exposure at 4°C. There are currently no specific studies that evaluate mild acid treatment modulated genes as temperature-dependent effect. In two relevant studies, Chan *et al.* (2007) and Ivy *et al.* (2012) investigated the differential expression of 4°C, 7°C, and 37°C grown stationary phase *L. monocytogenes* cells. Since stationary-phase cells can cause some inherent acid adaptation due to decrease in growth medium pH, changes in gene expression profile in such physiological state may provide some additional relevant clues of temperature-dependent behavior of acid shock genes/proteins. Interestingly, these studies suggested down-regulation of GAD and ADI genes in cold-grown stationary-phase cells (Chan *et al.,* 2007; Ivy *et al.,* 2012). In another study, it was observed that GAD and ADI genes were also not induced when cells were exposed to organic acids at 7°C (Stasiewicz *et al.,* 2011). Therefore, there may be a lack of synthesis of some critical acid shock proteins when cells are exposed to mild acidic condition under cold temperatures.

Being exposed to pH 5.0 at 4°C, *L. monocytogenes* encounters two different types of physiological stress, namely, acid stress and cold stress. *L. monocytogenes* can adapt to a cold environment by expressing different protein patterns that contribute to its modulated metabolism pathway, nutrient uptake, protein folding, and lipid biosynthesis (Cacace *et al.,* 2010). Hence, it is likely that between acid and cold adaptation, cold adaptation becomes the priority task for the bacterium to deal with. We hypothesized that *L. monocytogenes* could start to respond to acid stress after cells were fully cold adapted. We therefore tested whether cold-grown log phase cells that are fully cold acclimatized can show acid adaptation at 4°C. However, cold-grown log cells still failed to activate acid adaptation at 4°C (Fig. 2). Therefore, the actual cold adaptation event may not be the sole factor for repressed acid adaptation at 4°C.

Cold stress environment may block the penetration of acid into cytoplasm and subsequently fails to trigger intracellular responses to acid stress. To test this, we used a simple bead-beating approach as an alternative for sonication. Bead beating is routinely applied in proteomic or microarray work to break the bacterial cell envelope to yield more protein or mRNA extraction. Our data demonstrated that bead beating of *L. monocytogenes* cells could partially induce acid adaptation at 4°C (Fig. 8B), whereas the magnitude of acid adaptation at 4°C was still lower than what occurred at 37°C (Fig. 8A). It is possible that blockage of acid entry into cytoplasm of *L. monocytogenes* cells with an intact cell envelope could be one of the factors for repressed acid adaptation at 4°C. A proteomic analysis on cold-adapted *L. monocytogenes* showed that cell wall synthesis was up-regulated at 4°C (Cacace *et al.,* 2010). Another study on *Staphylococcus* spp. revealed that 4°C cold-stressed phenotypes possessed a thicker cell wall compared to normal cells (Onyango *et al.,* 2012). Such cell morphological modifications could build the physiological barriers to prevent acid from entering cytoplasm under cold environment and eventually impede the process of acid adaptation. On the other hand, since cell envelope blockage partially prevents the induction of acid adaptation, it is necessary to investigate other possible mechanisms to have a comprehensive understanding of the cold temperature effect on acid adaptation in *L. monocytogenes*.

Conclusions

Acid-stress adaptation that typically occurs in *L. monocytogenes* at 22°C or 37°C was not induced when cells were pre-exposed to sublethal acid at 4°C. Our findings suggest that cold processing or cold storage temperature can prevent the risk of creating acid-stress resistant phenotypes of *L. monocytogenes*.

Acknowledgments

Disclosure Statement

No competing financial interests exist.

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