Role of *Listeria monocytogenes* σ^B in Survival of Lethal Acidic Conditions and in the Acquired Acid Tolerance Response

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The food-borne pathogen *Listeria monocytogenes* can acquire enhanced resistance to lethal acid conditions through multiple mechanisms. We investigated contributions of the stress-responsive alternative sigma factor, σ^B , which is encoded by *sigB*, to growth phase-dependent acid resistance (AR) and to the adaptive acid tolerance response in *L. monocytogenes*. At various points throughout growth, we compared the relative survival of *L. monocytogenes* wild-type and $\Delta sigB$ strains that had been exposed to either brain heart infusion (pH 2.5) or synthetic gastric fluid (pH 2.5) with and without prior acid adaptation. Under these conditions, survival of the $\Delta sigB$ strain was consistently lower than that of the wild-type strain throughout all phases of growth, ranging from 4 orders of magnitude less in mid-log phase to 2 orders of magnitude less in stationary phase. Survival of both $\Delta sigB$ and wild-type *L. monocytogenes* strains increased by 6 orders of magnitude upon entry into stationary phase, demonstrating that the *L. monocytogenes* growth phase-dependent AR mechanism is σ^B independent. σ^B -mediated contributions to acquired acid tolerance appear to be greatest in early logarithmic growth. Loss of a functional σ^B reduced the survival of *L. monocytogenes* at pH 2.5 to a greater extent in the presence of organic acid (100 mM acetic acid) than in the presence of inorganic acid alone (HCl), suggesting that *L. monocytogenes* protection against organic and inorganic acid may be mediated through different mechanisms. σ^B does not appear to contribute to pH_i homeostasis through regulation of net proton movement across the cell membrane or by regulation of pH_i buffering by the GAD system under the conditions examined in this study. In summary, a functional σ^B protein is necessary for full resistance of *L. monocytogenes* to lethal acid treatments.

Listeria monocytogenes is a food-borne pathogen responsible for approximately 10% of all deaths related to food-borne illnesses in the United States (27). To cause illness, this pathogen must first survive environmental stresses encountered during food processing, distribution, and preparation, and then it must survive and overcome physiological barriers imposed by the host. For example, the low pH of the human stomach is an important host defense barrier against food-borne bacterial pathogens (3). However, through multiple mechanisms, *L. monocytogenes* can acquire enhanced resistance to acid stress. For example, growth phase-dependent acid resistance (AR) is acquired upon entry into the stationary phase (12). The adaptive acid tolerance response (ATR), which enables enhanced resistance to lethal acid exposure, results from preexposure of bacterial cells to milder acidic conditions (ATR) (12).

The adaptive ATR plays an important role in the survival of *L. monocytogenes* in a variety of foods (15) and in the ability of this pathogen to cause illness (26, 28). In *L. monocytogenes*, acid adaptation offers cross protection against heat, ethanol, oxidative, and osmotic stresses and against the bacteriocin nisin (20, 25, 28, 36). Adaptive ATR cross protection may also enhance the ability of *L. monocytogenes* to cause illness by contributing to bacterial survival of a variety of challenges imposed by a host. Such host challenges include exposure to gastric fluid, bile, and competitive intestinal flora; the presence of organic acids found in the small intestine; and the oxidative products in the phagosome (15, 37).

One mechanism that contributes to bacterial survival under changing environmental conditions is transcription redirection through association of different alternative sigma factors with the core RNA polymerase. For example, the alternative sigma factors σ^S (RpoS) (24) and σ^B (21) have been associated with general stress responses in gram-negative and gram-positive bacteria, respectively. In *Bacillus subtilis*, σ^B regulates a large general stress response regulon, contributing to the transcription of more than 100 genes that are induced by exposure to environmental stresses such as heat, acid, ethanol, salt, and oxidative stress (1, 2, 14, 31, 33, 38). In *Staphylococcus aureus*, σ^B activity is induced by mild acid stress (7) and the adaptive ATR in *S. aureus* appears to be σ^B mediated (9). In *L. monocytogenes*, σ^B contributes to cellular survival under several adverse conditions, such as carbon depletion and exposure to acid, oxidative, low temperature, and osmolarity stresses (5, 6, 16). We have found previously that *L. monocytogenes* cells in the stationary phase display two distinct mechanisms of AR: a σ^B -dependent AR mechanism and an ATR mechanism that is at least partially σ^B independent (16). In this study, we investigated the contributions of σ^B to acid tolerance throughout growth in *L. monocytogenes*. To define a possible role for σ^B in additional *L. monocytogenes* AR mechanisms, we also examined the effects of loss of σ^B function on intracellular pH buffering and in net charge flux across the cell membrane at pH 2.5.

MATERIALS AND METHODS

Bacterial strains. *L. monocytogenes* 10403S (serotype 1/2a) and *L. monocytogenes* FSL A1-254 ($\Delta sigB$) were used for acid survival, proton flux, and glutamate decarboxylase assays. *L. monocytogenes* FSL A1-254 (40) has an in-frame 297-bp deletion between nucleotides 1490 and 1788 in the *sigB* operon of the parent

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strain, 10403S. *L. monocytogenes* FSL S1-059 and FSL S1-063 were used for σ^B activity assays. *L. monocytogenes* FSL S1-063 and FSL S1-059 bear transcriptional fusions of the β -glucuronidase (GUS) gene *gus* to the σ^B -dependent promoter upstream of *opuCA* in *L. monocytogenes* 10403S (18) or in *L. monocytogenes* FSL A1-254, respectively.

Construction of *gus* fusion strains. The plasmid pNF580 (19), which bears the *gus* gene, was used to construct *L. monocytogenes* strains containing chromosomal *gus* reporter fusions with the *opuCA* promoter region. Specifically, a 312-bp fragment bearing the predicted *opuCA* promoter region (17) was amplified by using primers *opuCA*-F1 (5' GCG GAT CCC GTG CCA CAA GTA CGA AAT CA 3') and *opuCA*-R1 (5' GCT CTA GAA TCA TCT TCA TTG TTG TCG TT 3'). PCR was performed by using Vent polymerase (New England Biolabs, Beverly, Mass.) and the following cycling conditions: 3 min at 94°C for 1 cycle; 30 s at 94°C, 30 s at 58°C, and 30 s at 72°C for 35 cycles; and a final extension of 5 min at 72°C.

PCR products were purified by using the QiaQuick PCR purification kit (Qiagen, Valencia, Calif.) and digested by using *Xba*I and *Bam*HI for cloning upstream of the promoterless *gus* gene in pNF580. The resulting pDS2 plasmid, which bears an *opuCA*-*gus* fusion, was constructed in OneShot *Escherichia coli* cells (Invitrogen, Carlsbad, Calif.). The plasmid was electroporated into both *L. monocytogenes* 10403S and *L. monocytogenes* FSL A1-254. Transformants were selected on brain heart infusion (BHI; Difco, Sparks, Md.) agar plates containing 10 μ g of chloramphenicol/ml. Isolates were serially passaged at 42°C in BHI broth containing 10 μ g of chloramphenicol/ml to allow for selection of chromosomal integration of the fusion plasmids. Chromosomal integration of the plasmids was confirmed by PCR and sequence analysis. The *sigB* genotype in each strain was verified by PCR.

Growth conditions and cell viability. Single colonies from BHI agar plates were used to inoculate tubes containing 5 ml of BHI broth, which were then incubated overnight. All cultures were grown in BHI and incubated with shaking (250 rpm) at 37°C. The overnight cultures were then subcultured (1:1,000) in 10 ml of BHI and grown to an optical density at 600 nm (OD_{600}) of 0.4 prior to all experiments, except where otherwise indicated. For acid stress assays, a 6 N HCl solution was used to acidify the media to pH 4.5 or 2.5. For all experiments, cell viabilities were assessed by serially diluting 100- μ l culture aliquots in 900 μ l of phosphate-buffered saline, plating 100 μ l of each dilution on BHI agar, incubating them for approximately 18 h at 37°C, and enumerating the cells by standard plate counting procedures.

Acid tolerance during *L. monocytogenes* growth. Cell viabilities were compared after exposure to BHI, pH 2.5, with and without preexposure to a milder acidic environment (BHI, pH 4.5), as previously described (16) but with the following modifications. Cell cultures that had been grown to an OD_{600} of 0.4 as described above were subcultured again (1:200) in 100 ml of fresh BHI. At several time points throughout growth, 1-ml samples were collected and cells were harvested by centrifugation at $13,000 \times g$ for 2 min. The cell pellets were resuspended in 1 ml of BHI (pH 2.5) and incubated at 37°C for 60 min. For adaptation, cell pellets were resuspended in 1 ml of BHI (pH 4.5) and incubated for 60 min before centrifugation and resuspension in BHI (pH 2.5). Cell viability was measured at 0 and 60 min after lethal acid (pH 2.5) exposure. Results are reported as the means of data collected from two independent replications.

Survival in synthetic gastric fluid. Cell cultures at an OD_{600} of 0.4 were subcultured (1:200) in 100 ml of fresh BHI, and the cells were harvested at the early log (OD_{600} = 0.2), mid-log (OD_{600} = 0.4), and stationary (16 h after medium inoculation) phases. Cells were resuspended in 1 ml of synthetic gastric fluid (8.3 g of proteose peptone liter⁻¹, 3.5 g of D-glucose liter⁻¹, 2.05 g of NaCl liter⁻¹, 0.6 g of KH₂PO₄ liter⁻¹, 0.11 g of CaCl₂ liter⁻¹, 0.37 g of KCl liter⁻¹, 0.05 g of bile liter⁻¹, 0.1 g of lysozyme liter⁻¹, and 13.3 mg of pepsin liter⁻¹; adjusted to pH 2.5 with 6 N HCl) (10) and incubated at 37°C for 60 min. For adaptation, cells were resuspended in 1 ml of BHI (pH 4.5) and incubated for 60 min before resuspension in synthetic gastric fluid. Cell viability was measured as described above at 0 and 60 min after exposure to synthetic gastric fluid. Results are reported as the means of three independent experiments, each performed in duplicate.

Survival in organic acid. Cell survival in the presence of acetic acid was compared with cell survival in the presence of inorganic acid (HCl) at pH 2.5. Aliquots (1 ml) of overnight cell cultures were centrifuged at $13,000 \times g$ for 2 min. Harvested cells were resuspended in 1 ml of BHI (pH 2.5) (inorganic acid), 1 ml of BHI (pH 2.5) (10 mM acetic acid), or 1 ml of BHI (pH 2.5) (100 mM acetic acid) and incubated for 60 min at 37°C. Cell viability was measured as described above at 0 and 60 min after exposure to the acidified BHI media. Results are reported as the means of three independent experiments performed in duplicate. The *t* test was used to calculate *P* values for these assay results.

GUS activity assay. GUS activity assays were performed on *L. monocytogenes* strains FSL S1-059 (Δ *sigB*, *opuCA*-*gus* fusion) and FSL S1-063 (wild-type σ^B , *opuCA*-*gus* fusion) collected at several time points during growth. Cell cultures at an OD_{600} of 0.4 (grown from overnight cultures as described previously) of either *L. monocytogenes* FSL S1-059 or FSL S1-063 were used to subculture (1:200) 100 ml of BHI media. Aliquots (1 ml) were harvested at several points throughout growth by centrifugation at $13,000 \times g$ for 2 min and were washed and resuspended in 400 μ l of AB light buffer (0.1 M potassium phosphate [pH 7.0], 60 mM K₂HPO₄, 40 mM KH₂PO₄, 0.1 M NaCl). An aliquot of 100 μ l was removed and serially diluted in phosphate-buffered saline to determine cell numbers. The remaining 300 μ l was frozen in liquid nitrogen and stored at -80°C.

For the GUS activity assay, 50 μ l of AB light buffer with 0.1% Triton X-100 was added to 50 μ l of thawed cell suspensions, and the mixtures were incubated at room temperature for 60 min. The mixtures were incubated again for 60 min at room temperature following the addition of 20 μ l of the GUS substrate, 4-methylumbelliferyl β -D-glucuronide (4-MUG) (Sigma-Aldrich Corp., St. Louis, Mo.). 4-MUG is hydrolyzed by GUS into the fluorescent molecule methylumbelliferone (MU⁻). Relative fluorescence units were measured in a Fusion 96-well plate reader (Packard Instruments, Boston, Mass.) with excitation and emission wavelengths set at 365 and 455 nm, respectively. GUS activity was expressed as picomoles of 4-MUG cleaved per minute per log CFU. Results are reported as the means of two independent experiments, each performed in duplicate.

Net proton flux assay. The net proton flux assay was performed as described by Jordan et al. (22) with the following modifications. Cells from overnight cultures were harvested, washed, and resuspended in 100 mM KCl at a concentration of 30 mg (wet weight) of cells/ml. The pH of a 20-ml cell suspension in 100 mM KCl was adjusted with 0.5 M HCl to pH 2.5. The solution was stirred continuously. Following a 1-min equilibration period, the pH was recorded at 30-s intervals for 10 min. An increase in the medium pH was assumed to represent a net flux of protons across the cell membrane. The Δ pH is calculated as the external pH (pH_e) at a given time minus the initial pH_e . Results are reported as the means of three independent experiments performed in duplicate.

GAD assay. The glutamate decarboxylase (GAD) assay was used to measure the extracellular concentration of gamma aminobutyric acid (GABA) present in the medium under assay conditions. This assay was performed as described by Cotter et al. (10) with the following modifications. Stationary-phase cells were harvested and resuspended in BHI, pH 2.5. After a 60-min incubation at 37°C, 20 μ l of spent cell culture medium was incubated for 30 min at 37°C with 80 μ l of the assay solution (0.3 M Tris buffer [pH 8.9], 10 mM α -ketoglutarate, 2 mM 2-mercaptoethanol, 0.5 mM NADPH, and 1 U of GABase/ml; Sigma-Aldrich Corp.). One unit of GAD was defined as that which catalyzes the formation of 1 μ mol of GABA min⁻¹ mg of total protein⁻¹. The amount of GABA in the spent medium was deduced from the amount of NADPH produced in the assay. The NADPH formed was measured spectrophotometrically at 340 nm. Total protein was calculated using the Bio-Rad (Hercules, Calif.) protein assay. Results are reported as the means of three independent experiments performed in duplicate. The *t* test was used to calculate *P* values for these assay results.

RESULTS

σ^B contributes to AR and to the adaptive ATR in *L. monocytogenes*. To investigate the role of σ^B in regulating growth phase-dependent AR (12), we compared the relative abilities of the 10403S wild-type strain and an otherwise isogenic nonpolar Δ *sigB* strain to survive lethal acid exposure (pH 2.5) at various points throughout growth. The role of σ^B in adaptive ATR regulation was also tested by exposing cells to milder acidic conditions (pH 4.5) prior to exposure to pH 2.5. The wild-type and Δ *sigB* strains show identical growth characteristics in BHI (pH 7.4) under the conditions used in this study (data not shown). Relative survival following lethal acid exposure increased for both the Δ *sigB* and the wild-type strains as each approached the stationary phase (Fig. 1). Specifically, the percent survival for each strain following 60 min of exposure to pH 2.5 increased by 6 orders of magnitude from the early log (i.e., at 3.5 h postinoculation) to stationary phase (i.e., at 8 h postinoculation), suggesting the presence of a σ^B -independent,

FIG. 1. σ^B contributions to AR and to adaptive ATR throughout *L. monocytogenes* growth in BHI. Wild-type 10403S (triangles) and $\Delta sigB$ (squares) cultures at an OD₆₀₀ of 0.4 were inoculated into fresh BHI, and OD measurements were taken throughout subsequent growth. Samples from wild-type 10403S and $\Delta sigB$ cultures collected at several points during growth were exposed to BHI (pH 2.5) for 60 min following adaptation in BHI (pH 4.5) (AD; solid symbols) or without adaptation (NA; open symbols). The percent survival was calculated as described in Materials and Methods. Error bars represent the ranges of variation from the means.

growth phase-dependent AR mechanism(s) in both strains. Survival of the $\Delta sigB$ strain following lethal acid exposure was consistently lower than that of the wild-type strain throughout growth, ranging from 4 orders of magnitude less in the mid-log phase (i.e., at 4.5 h postinoculation) to 2 orders of magnitude less in the stationary phase. Therefore, σ^B contributes to *L. monocytogenes* AR throughout growth.

As shown in Fig. 1, preexposure to mild acid (pH 4.5) also enhanced the survival of both wild-type and $\Delta sigB$ cells to subsequent exposure to lethal acid (pH 2.5). Wild-type strain survival increased by 5 orders of magnitude in early log-phase cells and by 4 orders of magnitude in mid-log phase cells but was not enhanced in stationary-phase cells (Fig. 1). Preexposed $\Delta sigB$ cell survival increased by 2 and 3 orders of magnitude in the early and mid-log phases but also by 1 order of magnitude in the stationary phase. Therefore, while σ^B contributes to adaptive ATR, with the greatest σ^B -specific effects observed in exponential-phase cells, *L. monocytogenes* also appears to have a pH-dependent, σ^B -independent acid tolerance mechanism that can be induced throughout growth.

σ^B activity is induced by entry into stationary phase. σ^B activity was deduced from GUS activity measured in strains FSL S1-059 ($\Delta sigB$) and FSL S1-063 (wild-type σ^B), each of which bears a transcriptional *gus* fusion to the σ^B -dependent *opuCA* promoter. The GUS activity assay was determined by using cells collected throughout growth. As shown in Fig. 2, during early log growth and up to 5 h postinoculation, GUS activity in FSL S1-063 was low (<10 GUS activity units). However, FSL S1-063 GUS activity increased to >30 GUS activity units upon entry into and throughout the stationary phase, whereas, in the absence of an intact *sigB*, GUS activity in FSL S1-059 never exceeded background levels (<3 GUS activity units) throughout growth.

σ^B enhances cell survival in synthetic gastric fluid. To determine if the contribution of σ^B to acid survival in a rich medium also could be extended to conditions mimicking passage through the human stomach, we compared the relative survival of the $\Delta sigB$ and the wild-type strains in synthetic gastric fluid. Wild-type and $\Delta sigB$ cultures at early log, mid-log, and stationary phase were assayed for survival in gastric fluid with and without preexposure to pH 4.5. As shown in Fig. 3, entry into the stationary phase enhanced the survival of non-acid-adapted cells for both strains in synthetic gastric fluid, suggesting that, as with survival to lethal acid exposure, the ability of *L. monocytogenes* to survive in gastric fluid is growth phase dependent. Survival in synthetic gastric fluid was at least partially σ^B dependent, as survival of stationary-phase $\Delta sigB$ cells was over 4 orders of magnitude lower than that observed for the wild-type strain.

In addition to resistance to synthetic gastric fluid throughout growth, σ^B also appears to contribute to the acquisition of enhanced resistance to synthetic gastric fluid following preexposure to milder acidic conditions in log-phase *L. monocytogenes* cells. Specifically, during early log phase, the percent survival of the wild-type strain that had been preexposed to pH 4.5 increased by almost 3 orders of magnitude. Recoveries of both the nonadapted $\Delta sigB$ cells and the preexposed $\Delta sigB$ cells remained below detection limits (10 CFU/ml) for early log-phase cells. Mid-log preexposed wild-type cells had a larger increase in percent survival (5 orders of magnitude) than mid-log $\Delta sigB$ cells (3 orders of magnitude). No additional increment in AR was achieved in preexposed wild-type stationary-phase cells, as nonexposed stationary-phase wild-type cells already displayed approximately 100% recovery after 60 min in gastric fluid. On the other hand, the percent survival of stationary-phase $\Delta sigB$ cells that had been preexposed to pH 4.5

FIG. 2. σ^B activity throughout *L. monocytogenes* growth. Samples were collected from FSL S1-063 (open circles) and FSL S1-059 ($\Delta sigB$) (open triangles) at several points during growth and assayed for GUS activity. GUS activity was calculated as picomoles of hydrolyzed 4-MUG per minute per log CFU for FSL S1-063 (closed circles) and FSL S1-059 ($\Delta sigB$; closed triangles). Error bars represent the standard deviations from the means.

increased over 4 orders of magnitude, suggesting the presence of a σ^B -independent pH-dependent mechanism for enhanced tolerance to stresses imposed by synthetic gastric fluid in stationary-phase *L. monocytogenes* cells.

Role of σ^B in organic acid stress. We also investigated the contribution of σ^B to protection of *L. monocytogenes* from organic acid stress relative to protection from inorganic acid stress. Specifically, we compared the relative abilities of stationary-phase cells of the wild-type and $\Delta sigB$ strains to survive

stress associated with undissociated acetic acid or with HCl alone at an external pH of 2.5.

As shown in Fig. 4, wild-type strain survival did not differ following exposure to organic acid (either 10 or 100 mM acetic acid; $P = 0.41$ or 0.043, respectively) or inorganic acid alone. Specifically, wild-type survival was reduced by ~70% in 100 mM acetic acid and by ~50% in HCl alone under our experimental conditions. On the other hand, $\Delta sigB$ strain survival was more than 1 log lower in the presence of 100 mM acetic

FIG. 3. σ^B contributes to survival of *L. monocytogenes* and to adaptive ATR in synthetic gastric fluid. Wild-type 10403S (gray bars) and $\Delta sigB$ (white bars) cells collected at early log ($OD_{600} = 0.2$), mid-log ($OD_{600} = 0.4$), and stationary phase (16 h after medium inoculation) were exposed to synthetic gastric fluid (pH 2.5) for 60 min without adaptation (NA) and with adaptation in BHI (pH 4.5) (AD). The percent survival was calculated as described in Materials and Methods. Error bars represent the standard deviations from the means. Counts for early log $\Delta sigB$ cells were below the detection limits for the assay (10 CFU/ml).

FIG. 4. σ^B contributes to *L. monocytogenes* survival in both inorganic and organic acid. Stationary-phase (overnight) cells were exposed to either BHI (pH 2.5) or BHI (pH 2.5) containing 10 or 100 mM acetic acid, prepared as described in Materials and Methods. The percent survival is shown on the y axis. Error bars represent the standard deviations from the means.

acid ($P = 0.0074$) than in the presence of either 10 mM acetic acid ($P = 0.044$) or inorganic acid at the same pH. These results suggest that survival of *L. monocytogenes* in organic acid under these conditions is enhanced by the presence of σ^B . σ^B appears to contribute to *L. monocytogenes* survival both under low external pH conditions and in the presence of undissociated acetic acid.

Role of σ^B in net proton flux. To investigate possible σ^B -directed mechanisms contributing to AR in *L. monocytogenes*, we compared net proton fluxes for wild-type and $\Delta sigB$ cells exposed to pH 2.5. Net proton flux was inferred by the Δ pH in the cell suspension (pH_o) observed during a 10-min period following the adjustment of the suspension pH to 2.5 with HCl. No difference was observed in the Δ pH between the $\Delta sigB$ and the wild-type strains during these 10-min periods (data not shown). An increase of approximately 0.40 U in pH_o was observed for both strains. Neither strain demonstrated viability loss during the 10-min assay, as determined by viable plate counts. These results suggest that net proton movement across cell membranes is not dependent on σ^B under these conditions.

σ^B regulation of glutamate decarboxylase. Buffering mechanisms for maintenance of a near-neutral pH (pH_i) can contribute to the survival of neutralophilic bacteria following exposure to low-pH environments (3, 8). Therefore, we also investigated whether σ^B contributes to pH_i buffering in *L. monocytogenes* through regulation of the GAD system. This system has been described previously for *E. coli* (35) and *Shigella flexneri* (39) and has only recently been characterized in *L. monocytogenes* (10). The GAD system encompasses at least 2 proteins: a GABA antiporter at the cell membrane and a cytoplasmic glutamate decarboxylase enzyme. When exposed to low pH, the GAD system is believed to offer protection from reduced pH by consuming an intracellular proton to convert an extracellular glutamate (i.e., a glutamate from the medium that is transported into the cell) into GABA, which is then transported out of the cell. Thus, this reaction may decrease the net

proton concentration in the cytoplasm. To determine if the GAD system is under σ^B regulation in *L. monocytogenes*, we compared GAD activity in the wild-type strain with that of the $\Delta sigB$ strain after exposing cells to pH 2.5 for 60 min. GAD activities of the wild-type (273 ± 46) and the $\Delta sigB$ (338 ± 49) strains did not differ ($P = 0.35$, t test) under the conditions used in this study. These results suggest that GAD activity is not σ^B dependent under these conditions.

DISCUSSION

L. monocytogenes displays growth phase-dependent AR as well as adaptive ATR (12). We have shown previously that *L. monocytogenes* stationary-phase cells have two distinct mechanisms of AR: a σ^B -dependent AR and an acid-inducible ATR mechanism that is at least partially σ^B independent (16). In this study, we found that, although both $\Delta sigB$ and wild-type *L. monocytogenes* strains have increased AR upon entry into the stationary phase, the $\Delta sigB$ strain was consistently less AR than the wild-type strain throughout growth. These results suggest that a functional σ^B protein is necessary for the full level of AR observed in wild-type *L. monocytogenes* cells. Although σ^B activity appears to be growth phase dependent, with the highest levels of σ^B activity occurring as cultures approach the stationary phase, the enhanced AR observed in the $\Delta sigB$ strain upon entry into the stationary phase appears to be due to a σ^B -independent mechanism(s) that regulates growth phase-dependent AR in *L. monocytogenes*.

The relative contributions of σ^B to the adaptive ATR appear to be greater in log-phase *L. monocytogenes* cells than in stationary-phase cells. The regulatory function of σ^B in log-phase adaptive ATR is likely to be due to rapid induction of σ^B activity and, hence, transcription of σ^B -regulated genes following acid exposure, since the level of σ^B activity during log phase and particularly during early log phase is very low under balanced growth conditions (Fig. 2). Becker et al. (5) inferred from primer extension analyses that σ^B activity in log-phase

L. monocytogenes can be induced from nondetectable levels to a level similar to that observed for stationary-phase cells following exposure of cells to mildly acidified media (pH 5.3). Similarly, σ^B activity is induced by mild acid exposure in log-phase *B. subtilis* (23) and *S. aureus* (7), and acid-induced log-phase ATR in *B. subtilis* and *S. aureus* is at least partially σ^B dependent (9, 38). It is possible that the σ^B -independent mechanism that appears to be growth phase dependent, and that appears to contribute to protection against acid stress in late-log- and stationary-phase *L. monocytogenes* cells, cannot be induced during the early phases of growth.

Adaptive ATR can also confer cross protection against other stress conditions. Acid-adapted *L. monocytogenes* shows enhanced resistance against heat, ethanol, osmotic stress, or stress due to crystal violet, a surface-active agent (28), while heat adaptation results in enhanced protection against acid stress (32). At least 37 proteins are induced by mild acid (pH 5.5) exposure in *L. monocytogenes*, while over 47 proteins are induced by exposure to pH 3.5. Between these groups of proteins, 23 overlap (32). These 23 proteins are likely to contribute to enhanced cellular survival in lethal acid conditions following exposure to mild acid conditions.

***L. monocytogenes* resistance to organic acid exposure.** Protonated organic acids cross cell membranes more freely than inorganic acid molecules (11, 13, 30, 41). Under the conditions used in these experiments (pH 2.5) the majority of acetic acid molecules (99.5%) are protonated. Once inside the higher pH environment of the cell, protonated molecules dissociate, which causes the pH_i of the cell to decrease (4). Accordingly, the pH_i of *L. monocytogenes* cells exposed to protonated molecules of acetic acid is lower than that of cells exposed to HCl at a similar external pH (41). However, as equivalent concentrations of different organic acids have different inhibitory effects on *L. monocytogenes* (41), a reduction of the internal pH is unlikely to be the sole stress associated with the presence of organic acids. It has been proposed that the presence of organic acids inhibits bacterial cell growth by decreasing cellular pH_i , by increasing energy consumption for pH homeostasis, and by disrupting substrate transport and macromolecular synthesis (8).

Loss of a functional σ^B reduced survival of *L. monocytogenes* to a greater extent at pH 2.5 in the presence of organic acid (100 mM of acetic acid) than in the presence of inorganic acid (HCl alone). O'Driscoll et al. (29) demonstrated that different protein expression patterns are observed in *L. monocytogenes* cells exposed to organic acids than in cells exposed to inorganic acids. In combination with our findings, these observations suggest that the physiological requirements for the survival of organic and inorganic acid challenges differ and that σ^B may mediate *L. monocytogenes* protection against organic and inorganic acid stresses through different mechanisms.

σ^B -directed mechanisms of AR. We investigated the role of σ^B in pH_i homeostasis through regulation of net proton movement across the cell membrane or by regulation of pH_i buffering by the GAD system. Recently, Shabala et al. (34) investigated proton flux in *L. monocytogenes* at several pH levels in the presence or absence of glucose. Cells were shown to initiate net proton efflux after 5 min at pH 4 or 6 in the presence of 10 mM glucose while under similar pH conditions, but in the absence of glucose, a continuous net proton influx was ob-

served for a period of at least 50 min. As our experiment was conducted in the absence of glucose, the observed $+\Delta 0.4$ pH_o increment in the medium following exposure of cells to pH 2.5 may be due to a net proton influx. Although a loss of viability was not detected for either strain during the time course of this assay, it is also possible that the ΔpH_o is due, in part, to cellular leakage of alkaline materials resulting from membrane damage caused by the extremely low pH. Under the conditions used in these studies, net proton movement across cell membranes and regulation of the GAD system for pH_i buffering both appear to be σ^B independent in *L. monocytogenes*. However, it is possible that σ^B contributes to pH_i buffering in *L. monocytogenes* through regulation of an as yet unknown system.

The results from this study contribute to our understanding of AR mechanisms in *L. monocytogenes*. We have shown that σ^B contributes to cellular survival through at least two mechanisms in *L. monocytogenes*: (i) a general acid tolerance to which σ^B contributes throughout all growth phases and (ii) a pH-inducible ATR mechanism that is at least partially σ^B dependent in exponential-phase cells. Furthermore, our results suggest that σ^B may contribute to the success of *L. monocytogenes* as a pathogen by enhancing its survival during passage through the gastric fluid.

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