

Identification of Sigma Factor σ^B -Controlled Genes and Their Impact on Acid Stress, High Hydrostatic Pressure, and Freeze Survival in *Listeria monocytogenes* EGD-e

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The gene encoding the alternative sigma factor σ^B in *Listeria monocytogenes* is induced upon exposure of cells to several stresses. In this study, we investigated the impact of a *sigB* null mutation on the survival of *L. monocytogenes* EGD-e at low pH, during high-hydrostatic-pressure treatment, and during freezing. The survival of $\Delta sigB$ mutant exponential-phase cells at pH 2.5 was 10,000-fold lower than the survival of EGD-e wild-type cells. Moreover, the $\Delta sigB$ mutant failed to show an acid tolerance response. Upon preexposure for 1 h to pH 4.5, the survival at pH 2.5 was 100,000-fold lower for the $\Delta sigB$ mutant than for the wild type. The glutamate decarboxylase (GAD) acid resistance system is important in survival and adaptation of *L. monocytogenes* in acidic conditions. The σ^B dependence of the *gad* genes (*gadA*, *gadB*, *gadC*, *gadD*, and *gadE*) was analyzed in silico. Putative σ^B -dependent promoter sites were found upstream of the *gadCB* operon (encoding a glutamate/ γ -aminobutyrate antiporter and a glutamate decarboxylase, respectively) and the *lmo2434* gene (*gadD*, encoding a putative glutamate decarboxylase). Reverse transcriptase PCR revealed that expression of the *gadCB* operon and expression of *gadD* are indeed σ^B dependent. In addition, a proteomics approach was used to analyze the protein expression profiles upon acid exposure. Although the GAD proteins were not recovered, nine proteins accumulated in the wild type but not in the $\Delta sigB$ strain. These proteins included Pfk, GalE, ClpP, and Lmo1580. Exposure to pH 4.5, in order to preload cells with active σ^B and consequently with σ^B -dependent general stress proteins, also provided considerable protection against high-hydrostatic-pressure treatment and freezing. The combined data argue that the expression of σ^B -dependent genes provides *L. monocytogenes* with nonspecific multiple-stress resistance that may be relevant for survival in the natural environment as well as during food processing.

Listeria monocytogenes is a gram-positive, nonsporulating, facultatively anaerobic microorganism. It is the causative agent of listeriosis, a serious illness for which the young, elderly, and immunocompromised are especially at risk (31). *L. monocytogenes* is of particular concern for the food industry due to the severity of the illness, as well as the wide distribution of the pathogen in the environment and consequently its presence on raw and minimally processed foods. Moreover, *L. monocytogenes* is known for its ability to survive and proliferate in adverse environmental conditions, including acidic conditions, refrigeration temperatures, and high osmolarity (up to 10%) (4, 36). These characteristics of *L. monocytogenes* make it a food-borne pathogen that is problematic for certain kind of foods, especially minimally processed, ready-to-eat foods.

Adaptation to (sudden) adverse conditions in the environment of a bacterium requires the ability to respond rapidly. Such a response of a bacterium to environmental changes involves activation of existing enzymes and enhanced rates of transcription of genes, resulting in enhanced levels of (defen-

sive) proteins. Initiation of transcription of mRNA from DNA is mediated by the holoenzyme RNA polymerase. The holoenzyme consists of a core enzyme and a sigma factor, and the sigma factor is primarily responsible for recognition and binding of the polymerase to the promoter sequence upstream of a gene. In the genome sequence of *L. monocytogenes* EGD-e there are genes encoding five sigma factors (15) (recovered by using the Ergo integrated genomics database). These genes include *rpoD* encoding σ^A , which regulates housekeeping genes; *sigH* encoding a sigma factor with an unknown specific function; *rpoN* encoding σ^{54} (SigL), which in *L. monocytogenes* has a role in resistance to the antibacterial peptide mesentericin Y105 (25); *sigV* (*lmo0423*) encoding an enzyme that regulates extracytoplasmic function genes; and the alternative sigma factor gene *sigB* encoding σ^B , which controls the transcription of genes involved in stress adaptation.

The role of σ^B in the stress responses of gram-positive bacteria, including *Bacillus subtilis*, *Staphylococcus aureus*, and *L. monocytogenes*, has received considerable attention in recent years. The best-studied system is that in *B. subtilis*. The activity of the σ^B protein is regulated by a posttranslational mechanism. The *B. subtilis* σ^B operon comprises *rsbR*, *rsbS*, *rsbT*, *rsbU*, *rsbV*, *rsbW*, *sigB*, and *rsbX*. The Rsb proteins are all involved in regulation of σ^B activity (32). RsbW binds under normal growth conditions with σ^B to form an inactive complex.

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However, the affinity of RsbW for its antagonists, σ^B and RsbV, can change via two independent processes that promote the binding of RsbW to RsbV, thus leaving free σ^B , which is then capable of forming holoenzyme complexes with core RNA polymerase (32). Carbon limitation and entry into the stationary phase correlate with a drop in the intracellular level of ATP, which may have a direct effect on the binding preference of RsbW, which shifts from σ^B to RsbV. Also, upon exposure to a number of environmental insults, such as ethanol treatment or salt or acid shock, RsbU dephosphorylates RsbV, which is then able to bind to RsbW, resulting in free σ^B (32). The other Rsb proteins have an indirect effect (positive or negative) on σ^B activity or gene expression. Activation of σ^B in *L. monocytogenes* might involve a similar process, as the structure and organization of the *rsbU*, *rsbV*, *rsbW*, and *rsbX* genes and the *sigB* gene exhibit a high level of similarity with the structure and organization of their *B. subtilis* counterparts; the encoded proteins exhibit predicted levels of identity of 53, 45, 47, 29, and 66%, respectively (2, 39).

In *B. subtilis*, the alternative sigma factor σ^B regulates expression of a large general stress operon that contributes to transcription of about 150 genes involved in heat, acid, ethanol, salt, and freezing stress resistance (23, 33, 34). These genes include the *katE* gene encoding a catalase (9); the *opuE* gene encoding a transporter dedicated to the transport of the osmoprotectant proline (35); the *clpC* gene, which encodes a protein similar to stress-induced ATPase subunits of ClpP-type proteases (18); and the *gtbB* gene encoding a UDP-glucose pyrophosphorylase believed to participate in trehalose biosynthesis (30).

In *L. monocytogenes*, a role for σ^B has been determined in response to several stresses; e.g., it plays a role in acid resistance of stationary-phase cells, in oxidative and osmotic stress resistance, in the response to carbon starvation, and in growth at low temperatures (3, 10, 39). *L. monocytogenes* displays an active acid tolerance response upon exposure to low, nonlethal pH values and subsequent exposure to a lethal pH. Recently, the contribution of σ^B to growth-phase-dependent acid resistance and to the adaptive acid tolerance response in *L. monocytogenes* 10403S was analyzed by Ferreira et al. (11). The survival of the $\Delta sigB$ strain upon exposure to brain heart infusion (BHI) at pH 2.5 (with and without prior acid adaptation) was consistently lower than the survival of the wild-type strain throughout all phases of growth. However, σ^B -mediated contributions to acquired acid tolerance appeared to be greatest in early logarithmic growth. The acid tolerance response has a great impact on food processing and the virulence of the bacterium, since exposure to acidic conditions not only enhances survival at a lethal pH but also provides protection against other challenges, such as heat, ethanol, oxidative, and osmotic stresses (6, 12, 19).

Here, we describe the role of σ^B in acid stress adaptation of exponentially growing *L. monocytogenes* EGD-e cells. Since the glutamate decarboxylase (GAD) acid resistance system is important in survival and adaptation of *L. monocytogenes* in acidic conditions (7), we tested whether this system plays a role in the acid adaptation of the wild-type strain and a strain with σ^B deleted. The putative σ^B dependence of the *gad* genes (*gadA*, *gadB*, *gadC*, *gadD* [*lmo2434*], and *gadE* [*lmo0449*]) was analyzed in silico, and subsequently reverse transcriptase PCR

was performed to analyze the transcription of the *gad* genes. In addition, a proteomics approach was used to analyze the protein expression profiles after exposure of the wild-type and $\Delta sigB$ strains to acid. Additionally, the role of σ^B in two industrially important processes, high-hydrostatic-pressure (HHP) treatment and freezing, was assessed in combination with pretreatment at a low pH in order to assess the protective effect of adaptation to these food-processing methods.

MATERIALS AND METHODS

Bacterial strains and generation of mutant. *L. monocytogenes* EGD-e and a $\Delta sigB$ mutant of this strain were used throughout this study. The $\Delta sigB$ mutant (U. Volk, S. S. Chatterjee, S. Otten, S. Wagner, S. Haas, B. Brors, T. Chakraborty, and T. Hain, submitted for publication) was constructed by using the temperature-sensitive suicide plasmid pAUL-A (5), which resulted in an in-frame chromosomal deletion.

Survival during acidic conditions and freeze-thaw cycles. Cells were grown in BHI at 30°C to an optical density at 620 nm (OD_{620}) of 0.4, centrifuged, and resuspended in fresh BHI at pH 2.5 (adjusted with HCl). Survival was measured during 3 h of incubation of this suspension at 30°C by determining the number of CFU. Survival in this acidic environment was also measured after adaptation for 1 h to BHI with a pH of 4.5 (adjusted with HCl). Survival of *L. monocytogenes* and survival of the $\Delta sigB$ mutant were also measured after freeze-thaw cycles. Exponential-phase cells, grown in BHI at 30°C, were centrifuged, resuspended in fresh BHI, and subsequently frozen for 24 h at -20°C. Then the preparation was thawed for 2 min at 30°C and the number of CFU was determined, and the preparation was subsequently frozen for another 24 h at -20°C. The numbers of CFU were determined after five freeze-thaw cycles. Freeze-thaw survival was also measured after exposure of the mid-exponential phase cells to BHI at pH 4.5 for 1 h and to BHI at 7°C for 4 h. The experiments were performed in duplicate, and standard errors are indicated below (see Fig. 1).

Analysis of protein extracts by 2D-E and matrix-assisted laser desorption ionization-time of flight analysis. Samples (10 ml) of a bacterial suspension (OD_{620} , 0.4) were removed, centrifuged, and suspended in water to an OD_{620} of 10. Total cellular proteins were extracted from the cells with a bead beater (B. Braun Biotech International, Melsungen, Germany) and zirconium beads (diameter, 0.1 mm; BioSpec Products, Bartlesville, Okla.) by using three 1-min treatments (with cooling on ice between treatments). After this the zirconium beads were allowed to sediment by gravity, and subsequently the supernatant, which contained the cellular proteins, was analyzed by two-dimensional gel electrophoresis (2D-E) by using a pI range of 4 to 7 and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12 to 14% polyacrylamide) for the second dimension. 2D-E was performed essentially as described previously (38). Protein spots were visualized by silver staining, and the gels were analyzed with PDQuest software (Bio-Rad, Richmond, Calif.). Proteins that were differentially expressed (more than a threefold difference) were identified as proteins that increased expression upon acid exposure or as proteins that decreased expression upon acid exposure. For identification of protein spots gels were loaded with 200 μ g of protein and stained with Coomassie blue. Differentially expressed proteins were cut out of the gel and analyzed at the Proteomics Center, Department of Human Biology, University of Maastricht, Maastricht, The Netherlands (J. Renes and F. Bouwman) by digestion with a MassPrep station (Micromass, Almere, The Netherlands) and subsequent analysis with a matrix-assisted laser desorption ionization-time of flight LR mass spectrometer (Micromass). The proteins were identified with PeptIdent.

Transcriptional analysis of the *gad* genes. *L. monocytogenes* EGD-e and the $\Delta sigB$ mutant were grown to the mid-exponential phase at 30°C. A portion (10 ml) of each culture was centrifuged and resuspended in 1 ml of BHI (with and without the pH adjusted to 4.5). After 1 h of incubation, the cells were centrifuged, and the RNA was extracted with an RNeasy RNA extraction kit (QIAGEN, Hilden, Germany). cDNA was synthesized by adding 2 μ l of total RNA to 1 μ l of a reverse primer mixture (10 pmol/ μ l), 1 μ l of 5 \times first strand buffer, and 1 μ l of water. This mixture was incubated for 2 min at 75°C, after which 3 μ l of first strand buffer, 2 μ l of dithiothreitol (0.1 M), 4 μ l of a preparation containing each deoxynucleoside triphosphate at a concentration of 2.5 mM, 0.4 μ l of Superscript II reverse transcriptase enzyme, and 5.6 μ l of water were added. The mixture was incubated for 1 h at 48°C, and then 3.55 μ l of water, 0.2 μ l of RNase H, and 1 μ l of first strand buffer were added for cleavage of the RNA in RNA-DNA hybrids during incubation for 10 min at room temperature.

The PCR was carried out by using the primers listed in Table 1, and 20, 25, and

TABLE 1. Primers used in this study

Gene	Primer	Sequence
<i>gadA</i> (GAD, <i>lmo0447</i>)	Forward	5'-CGG TGT TTG GCT CTT TT GA-3'
	Reverse	5'-CTC CGA TTC ATC CAC ATT CC-3'
<i>gadB</i> (GAD, <i>lmo2363</i>)	Forward	5'-GGC ATG CAC CTA AGG ACC AAA AAT-3'
	Reverse	5'-GAT ACC GAG GAT GCC GAC CAC AC-3'
<i>gadC</i> (antiporter, <i>lmo2362</i>)	Forward	5'-AAA TGG CGA CGG TGG ATG GT-3'
	Reverse	5'-TTT TGC GAT TTT AGC CGT GTT TT-3'
<i>gadD</i> (GAD, <i>lmo2434</i>)	Forward	5'-ACT TGG CAA AAA CTG TAG AAA A-3'
	Reverse	5'-TAG TGC GTA AAT CCG TAT GAA-3'
<i>gadE</i> (antiporter, <i>lmo0448</i>)	Forward	5'-ATT CGG CGG CGG TGG TA-3'
	Reverse	5'-AAA ACG GAA TTA AAA TAG TGA CGA-3'
16S RNA	Forward	5'-TTA GCT AGT TGG TAG GGT-3'
	Reverse	5'-AAT CCG GAC AAC GCT TGC-3'

30 cycles were performed to allow optimal quantification of PCR products. Template cDNA was used in the reaction mixtures at levels that gave similar band intensities for 16S RNA reactions. The experiment was performed in duplicate, and representative results are described below.

HHP treatment. Cells were harvested by centrifugation, resuspended in 50 mM *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES buffer; Sigma-Aldrich, Steinheim, Germany) (pH 7.0), and subsequently packed in plastic bags. The survival after high-pressure treatment (150, 200, 250, 300, 350, and 400 MPa for 20 min) was determined for exponentially growing cells, cells exposed to pH 4.5 for 1 h, and cells exposed to 10°C for 4 h. Depressurization of the HHP apparatus (Resato, Roden, The Netherlands) was achieved within seconds. The pressurization was performed at 20°C, while the temperatures during pressurization did not exceed 32°C. In addition, *L. monocytogenes* wild-type and $\Delta sigB$ mutant cells that were preexposed to pH 4.5 were exposed to 350 MPa for 3, 8, 14, 18, 23, and 28 min at 20°C. The experiments were performed in duplicate, and the standard errors are indicated below.

RESULTS

Survival of *L. monocytogenes* EGD-e at low pH is σ^B dependent. Survival of exponential-phase cells of *L. monocytogenes* EGD-e and the σ^B null mutant in BHI at pH 2.5 was determined in order to analyze the σ^B dependence of survival. About 5% of wild-type *L. monocytogenes* cells survived 1 h of

exposure to the acidic conditions (Fig. 1). For the σ^B mutant cells the survival rate was about 0.00005%; i.e., there was a 5-log difference. This clearly shows that the survival of *L. monocytogenes* at pH 2.5 is σ^B dependent. In addition, we determined the σ^B dependence of the acid tolerance response in *L. monocytogenes* EGD-e. Cells were preexposed to a non-lethal pH (pH 4.5) prior to exposure to pH 2.5. After this preexposure to pH 4.5, almost 100% of the EGD-e wild-type cells survived exposure to pH 2.5 for 1 h. However, only about 0.0005% of the preexposed cells with a σ^B null mutation survived, indicating the importance of induction and/or activation of σ^B during adaptation to a low pH for subsequent survival at a lethal pH (Fig. 1). Notably, the mutant still displayed a small acid tolerance response, indicating that σ^B is not solely responsible for the acid tolerance response.

σ^B -Dependent expression of *gad* genes. Adaptation of bacteria to acidic conditions involves maintenance of a relatively high intracellular pH (pH_i) (21). A specific mechanism for acid adaptation in *L. monocytogenes* aimed at maintenance of the pH_i is the GAD acid resistance system. This system involves an antiporter, which transports glutamate into the cell, and a glutamate decarboxylase (GAD), which converts glutamate into γ -aminobutyrate (GABA) upon consumption of a proton. The glutamate/GABA antiporter subsequently excludes GABA from the cell. Cotter et al. (7) identified the following three genes (in the pregenome era) involved in this system: *gadA* (*lmo447*), encoding a GAD; *gadB* (*lmo2363*), coding for another GAD; and *gadC* (*lmo2362*), encoding the associated glutamate/GABA antiporter. Using the genome data for *L. monocytogenes* EGD-e (<http://genolist.pasteur.fr/ListiList/>), Conte et al. (6) identified two other genes with high homology to the *gad* genes. *lmo2434* (designated *gadD* in this study) codes for another GAD, and *lmo448* (designated *gadE* in this study) encodes an additional antiporter. *gadA* and *gadE* are close to each other and are cotranscribed. However, a stem-loop-like structure within the intergenic spacer downstream of *gadA* stops readthrough transcription, resulting in single transcription of *gadA* (or in cotranscription with *lmo0446*) (6). Cotter et al. (7) described the cotranscription of *gadC* and *gadB*; this observation, together with the genome data, suggests that these two genes form an operon. *gadD* is oppositely oriented and is further downstream of the operon (Fig. 2A). Considering the low level of survival of a $\Delta sigB$ mutant at a low pH, the putative σ^B dependence of the *gad* genes was analyzed

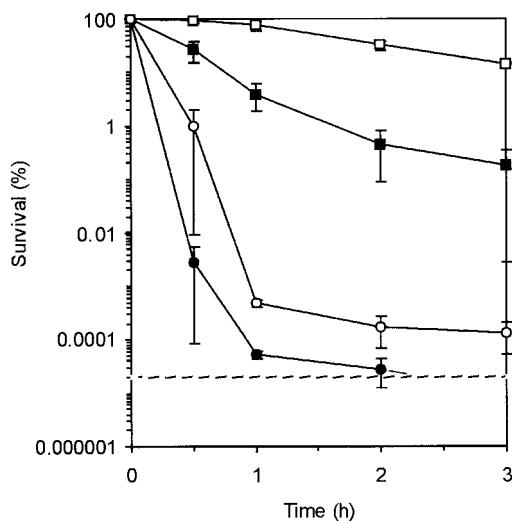


FIG. 1. Survival of *L. monocytogenes* EGD-e (squares) and the $\Delta sigB$ mutant (circles) during incubation at pH 2.5 with (□ and ○) and without (■ and ●) preexposure to pH 4.5 for 1 h. The error bars indicate standard errors.

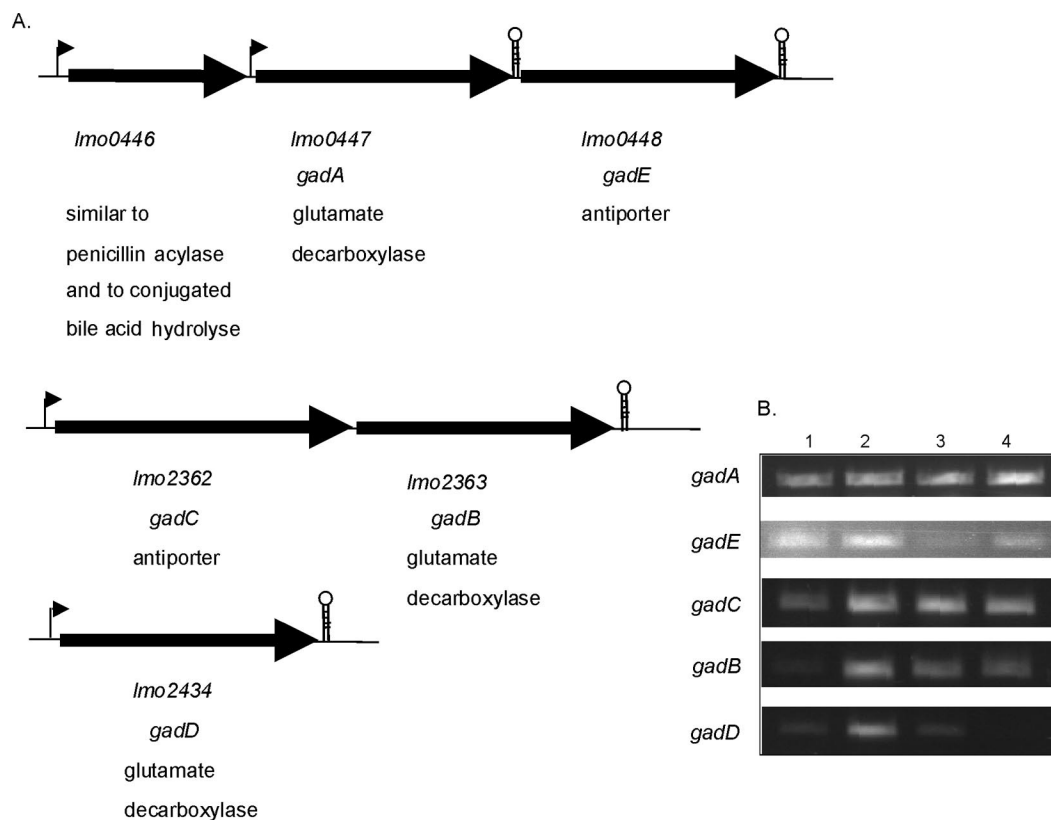


FIG. 2. (A) Organization of *gad* genes in *L. monocytogenes* EGD-e. The hairpin structures indicate putative terminators. The numbering of the open reading frames is according to the *L. monocytogenes* EGD-e genome sequence (15). (B) Transcriptional analysis of *gad* genes in *L. monocytogenes* EGD-e (lanes 1 and 2) and the $\Delta sigB$ mutant (lanes 3 and 4) in exponential-phase cells (lanes 1 and 3) and cells exposed to pH 4.5 for 1 h (lanes 2 and 4).

in silico. Comparison of the *L. monocytogenes* σ^B promoter sequence (GTTTTA-N₁₄-GGGTAA, as described by Becker et al. [2]) revealed only three and two mismatches upstream (within 200 bp) of the *gadCB* operon and *gadD*, respectively (Table 2). Using reverse transcriptase PCR, we analyzed the transcription of the *gad* genes (*gadA*, *gadB*, *gadC*, *gadD*, and *gadE*) upon exposure to pH 4.5 for 1 h for both the wild-type and $\Delta sigB$ strains. For wild-type strain EGD-e all *gad* genes except *gadA* were induced after exposure to low pH (Fig. 2B). This is an indication that GadA plays a minor role in acid adaptation, which is supported by the results of the acid sur-

vival experiments of Cotter et al. (7) in which *gad* deletion mutant strains were used. Of the four induced transcripts in the wild type, only one, *gadE*, was induced in the $\Delta sigB$ mutant strain. This indicates that transcription of the other three genes, *gadB*, *gadC*, and *gadD*, is at least partially σ^B regulated under the conditions tested. The apparent constitutive expression of the *gadCB* operon in the $\Delta sigB$ strain suggests that additional regulators are involved. Slight induction of *gadA* transcripts could be detected upon acid exposure of the $\Delta sigB$ strain. This might be compensation for the reduced transcription of the other genes coding for GADs (e.g., *gadD*).

2D-E and identification of proteins involved in acid adaptation. Previous research showed that the acid tolerance response of *L. monocytogenes* includes induction of several proteins (8, 22, 24). In this study the proteins present in the wild-type and $\Delta sigB$ strains after exposure to pH 4.5 were analyzed by 2D-E (Fig. 3) and were subsequently identified (Table 3). After exposure to pH 4.5 wild-type cells exhibited decreased accumulation of five proteins (spots 1 to 5), including Lmo1704; a protein similar to conserved hypothetical proteins, FbaA; a protein similar to fructose-1,6-biphosphate aldolase; and HPr, a phosphotransferase system (PTS) phosphocarrier protein (Table 3). However, the levels of 16 proteins were enhanced after exposure to low pH (spots a to p); these proteins included ValS, valyl-tRNA synthetase (Pfk), 6-phosphofructokinase (FlaA), flagellin protein (Lmo1709), a

TABLE 2. Alignment of putative σ^B -dependent promoters in *L. monocytogenes* (based on in silico analysis)

Gene	Promoter sequence			No. of mismatches
	-35 Region	Spacing	-10 Region	
<i>sigB</i>	GTTTTA	N ₁₄	GGGTAA	
<i>pfk</i>	GTTTTG	N ₁₁	GTTTTAA	3
<i>clpP</i>	GTTTGA	N ₁₆	GTGTAT	3
<i>lmo1580</i>	GGTTCT	N ₁₃	GGGTAA	3
<i>galE</i>	ATAGAT	N ₁₄	GGGTCT	4
<i>gadD</i>	TTTTTA	N ₁₂	CGGTAA	2
<i>gadC</i>	GTTTGT	N ₁₄	GGGTAT	3
<i>pykA</i>	GTTTTA	N ₁₂	TGGTAA	1
<i>lmo1339</i>	GTTTAA	N ₁₆	GGAGAA	3

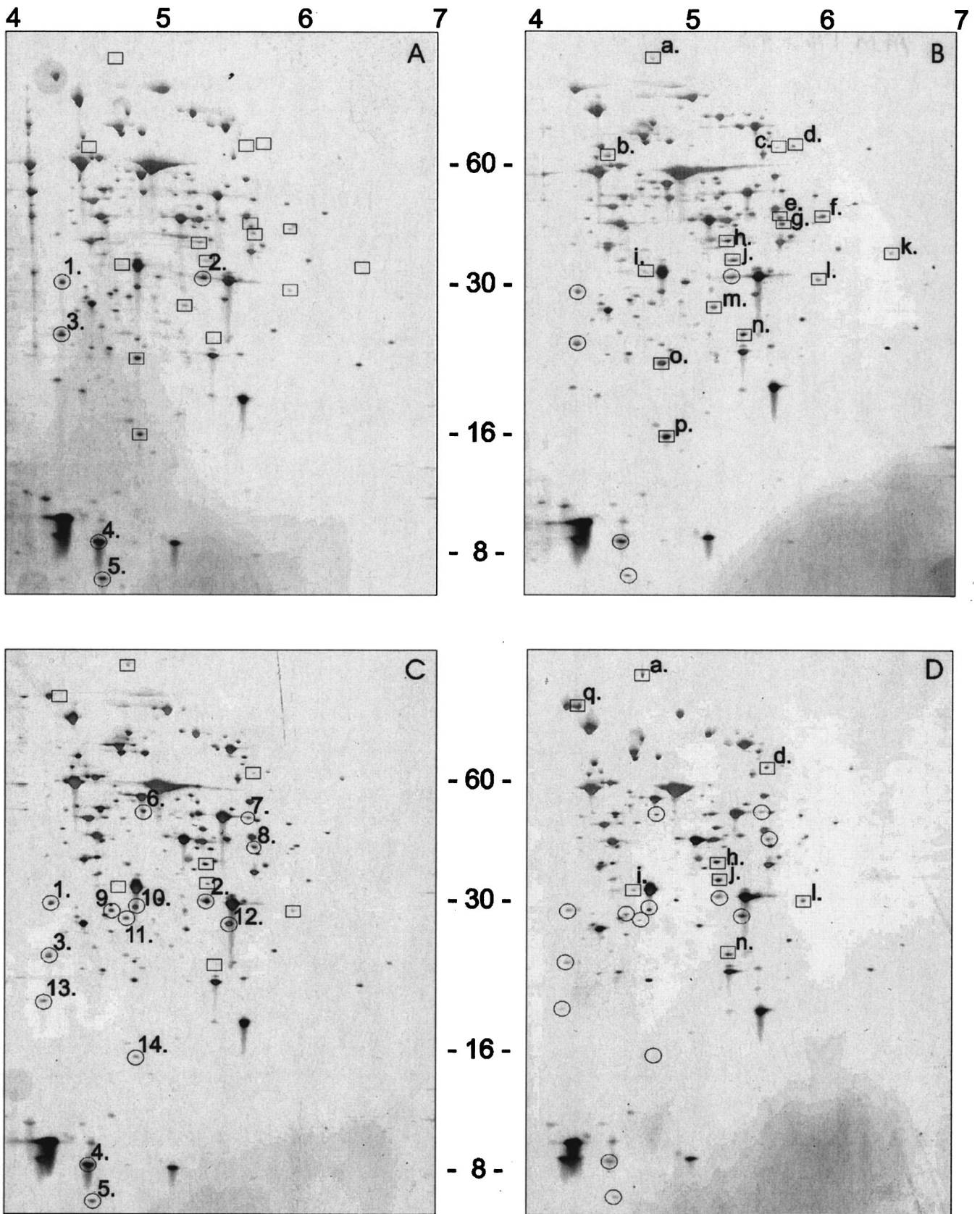


FIG. 3. Analysis of protein production in *L. monocytogenes* EGD-e exponential-phase cells (A), $\Delta sigB$ mutant exponential-phase cells (C), *L. monocytogenes* EGD-e cells exposed to pH 4.5 for 1 h (B), and $\Delta sigB$ mutant cells exposed to pH 4.5 for 1 h (D) by 2D-E. Differentially expressed proteins are indicated as follows: proteins whose levels were reduced are circled, and proteins that were induced upon exposure to low pH are enclosed in boxes.

TABLE 3. Identification of differentially expressed proteins in acid-shocked *L. monocytogenes* EGD-e and the $\Delta sigB$ mutant

Spot	Protein	Description	pI	Mol wt (10 ³)
Reduced protein production upon acid shock of EGD-e wild-type strain				
2	FbaA	Similar to fructose-1,6-biphosphate aldolase	5.1	30.0
4	Hpr	PTS phosphocarrier protein Hpr	4.5	9.4
5	Lmo1704	Similar to conserved hypothetical proteins	5.8	14.4
Increased protein production upon acid shock of EGD-e wild-type strain				
a	ValS ^a	Valyl-tRNA synthetase	4.7	102
f	Pfk	6-Phosphofructokinase	5.4	34.4
i	FlaA	Flagellin protein	4.7	30.4
l	Lmo1709	Similar to methionine aminopeptidases	5.4	27.9
m	GalE	UDP-glucose-4-epimerase	4.9	36.2
o	ClpP	ATP-dependent Clp protease proteolytic subunit	4.7	21.6
p	Lmo1580	Similar to unknown protein	4.7	16.9
Reduced protein production upon acid shock of the EGD-e $\Delta sigB$ mutant				
2	FbaA	Similar to fructose-1,6-biphosphate aldolase	5.1	30.0
4	Hpr	PTS phosphocarrier protein Hpr	4.5	9.4
5	Lmo1704	Similar to conserved hypothetical proteins	5.8	14.4
10	Lmo1011	Hypothetical protein, similar to tetrahydrodipicolinate succinylase	4.7	24.8
12	ThiD	Phosphomethyl pyrimidine kinase	5.0	28.8
14	Lmo1580	Similar to unknown protein	4.7	16.9
Increased protein production upon acid shock of the EGD-e $\Delta sigB$ mutant				
a	ValS ^a	Valyl-tRNA synthetase	4.7	102
i	FlaA	Flagellin protein	4.7	30.4
l	Lmo1709	Similar to methionine aminopeptidases	5.4	27.9
Putative σ^B -regulated proteins involved in acid adaptation				
f	Pfk	6-Phosphofructokinase	5.4	34.4
m	GalE	UDP-glucose-4-epimerase	4.9	36.2
o	ClpP	ATP-dependent Clp protease proteolytic subunit	4.7	21.6
p	Lmo1580	Similar to unknown protein	4.7	16.9

^a Only 6.2% of the sequence was covered.

protein similar to methionine aminopeptidases (GalE), UDP-glucose-4-epimerase (ClpP), the proteolytic subunit of the ATP-dependent Clp protease, and Lmo1580, which is similar to an unknown protein. In the $\Delta sigB$ strain the levels of 14 proteins were reduced after exposure to low pH, whereas the levels of eight proteins were higher. Of the 16 proteins that accumulated in the wild type, 7 were also present at enhanced levels in the $\Delta sigB$ mutant, indicating that these proteins are not strictly σ^B regulated. Of these 16 proteins, 9 were present at enhanced levels in the wild type (Fig. 3), whereas they were not induced in the $\Delta sigB$ mutant strain. These putatively σ^B -regulated proteins, which may participate in acid adaptation, include Pfk, GalE, ClpP, and Lmo1580. Using the *L. monocytogenes* σ^B promoter sequence for putative σ^B -regulated promoter site recognition (2), we matched the putative promoter sequences of the genes encoding these proteins in silico. Three mismatches were found in the promoter regions of *pfk*, *clpP*, and *lmo1580*, and four mismatches were found for *galE* (Table 2).

Role of σ^B in survival after HHP treatment and freeze-thaw cycles. To determine the role of σ^B in other important food-manufacturing processes, we assessed the survival of the parent strain and the mutant after HHP treatment and freeze-thaw cycles. The parent strain was about 100-fold less susceptible to 20 min at 300 MPa than the $\Delta sigB$ mutant strain (Fig. 4A).

Exposure to pH 4.5 for 1 h increased the survival of the parent strain after subsequent HHP exposure dramatically; i.e., treatment at 300 MPa did not significantly decrease the viable cell count. Notably, even treatment at 350 MPa only decreased the viable cell count to 10% of the initial count, whereas the cell counts for the $\Delta sigB$ mutant strain (preexposed to pH 4.5) were only just above the detection limit after treatment at 350 MPa; i.e., there was a >5-log difference. Additionally, we analyzed this difference in greater detail by varying the duration of exposure (Fig. 4C). There was a clear difference between the survival of EGD-e wild-type cells and the survival of the $\Delta sigB$ mutant; e.g., after 28 min of exposure to 350 MPa, there was a 4-log difference. These results indicate that acid-induced proteins encoded by σ^B -dependent genes provide considerable protection for cells exposed to HHP. Preexposure of the cells to a low temperature also resulted in increased survival after pressure treatment (more than 100-fold increase after treatment at 300 MPa) (Fig. 4B), as found previously for *L. monocytogenes* LO28 cells (38). Again, the $\Delta sigB$ mutant showed lower resistance to pressure treatment than the wild-type cells, indicating that the σ^B -dependent proteins formed during low-temperature adaptation are involved in tolerance to high pressure. However, the survival of both wild-type and $\Delta sigB$ mutant cells exposed to acid conditions was higher than the survival of refrigerated cells.

The survival of cells after exposure to freeze-thaw treatment

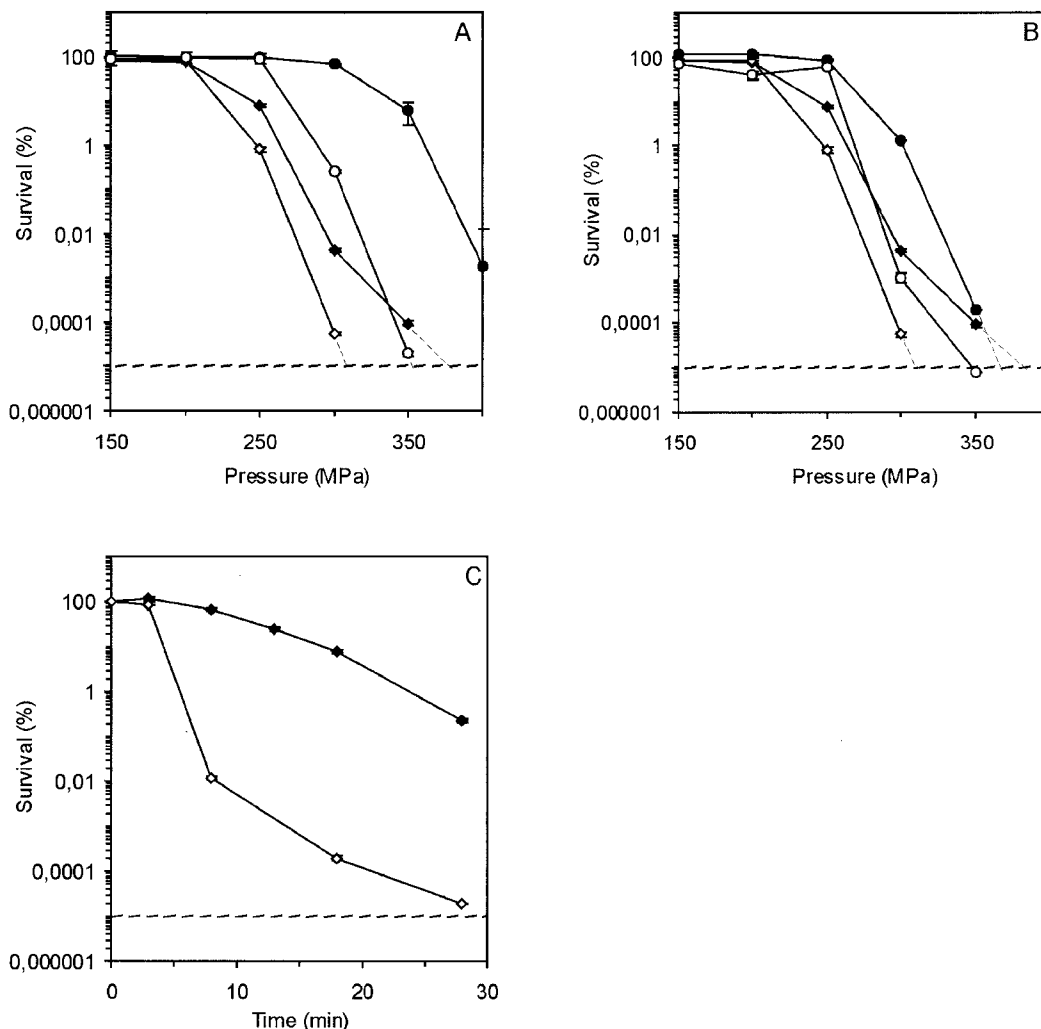


FIG. 4. Survival of *L. monocytogenes* EGD-e (solid symbols) and the $\Delta sigB$ mutant (open symbols) after HHP treatment. (A) Survival of exponential-phase cells (\blacklozenge and \diamond) and cells exposed to pH 4.5 for 1 h (\bullet and \circ). (B) Survival of exponential-phase cells (\blacklozenge and \diamond) and cells exposed to 10°C for 4 h (\bullet and \circ). (C) Survival of preexposed (1 h, pH 4.5) *L. monocytogenes* EGD-e (\blacklozenge) and the $\Delta sigB$ mutant (\diamond) after exposure to 350 MPa. The error bars indicate standard errors.

increased after preexposure to another insult, and exposure for 4 h at 7°C was as effective as exposure to low pH (Fig. 5). The protection provided by preexposure before freezing was partially σ^B dependent, as the level of survival of wild-type strain EGD-e after five freeze-thaw cycles was threefold higher than the level of survival of the $\Delta sigB$ mutant.

DISCUSSION

σ^B -Dependent expression of *gad* genes. Here we show that survival upon exposure to lethal acidic conditions and the acid tolerance response of *L. monocytogenes* EGD-e exponential-phase cells are highly σ^B dependent. In *L. monocytogenes*, maintenance of a relatively high pH_i is important for survival at a low pH. The GAD acid resistance system is a specific mechanism for acid adaptation in *L. monocytogenes* aimed at maintenance of the pH_i . This system involves an antiporter, which transports glutamate into the cell, and a GAD, which converts glutamate into GABA upon consumption of a proton. The glutamate/GABA antiporter subsequently excludes GABA

from the cell. As the $\Delta sigB$ mutant is significantly more acid sensitive than the wild type, the putative σ^B dependence of the *gad* genes was analyzed in silico. Putative σ^B -dependent promoter sites were found upstream of the *gadCB* operon and *gadD* and had three and two mismatches, respectively. Additionally, by performing transcription analysis with reverse transcriptase PCR, we confirmed the σ^B -dependent transcription of *gadCB* and *gadD* upon exposure to pH 4.5 for 1 h. This could partially explain the loss of acid tolerance by the $\Delta sigB$ mutant strain, as null mutations in the *gad* genes (*gadA*, *gadB*, and *gadC*) play an important role in acid resistance and adaptation (7). Ferreira et al. (11) did not find that σ^B contributes to the net proton movement across the cell membrane or to the GAD system. However, their experimental setup included incubation at pH 2.5 for 1 h of stationary phase cells that had not been preadapted to low pH; they showed that about 10% of the wild-type cells and only about 0.1% of the $\Delta sigB$ cells survived. Additionally, they analyzed the contribution of σ^B to growth-phase-dependent acid resistance and to the adaptive acid tol-

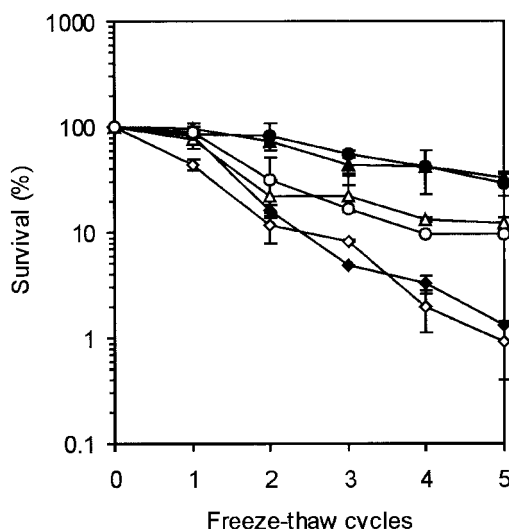


FIG. 5. Survival of *L. monocytogenes* EGD-e (solid symbols) and the $\Delta sigB$ mutant (open symbols) after freeze-thaw cycles. Freeze-thaw cycles were performed with exponential-phase cells (\blacklozenge and \diamond), cells preexposed to pH 4.5 for 1 h (\blacktriangle and \triangle), and cells preexposed to 10°C for 4 h (\bullet and \circ). The error bars indicate standard errors.

erance response in *L. monocytogenes* 10403S. Upon exposure to BHI at pH 2.5 (with and without prior acid adaptation) the survival of the $\Delta sigB$ strain was consistently lower than the survival of the wild-type strain throughout all phases of growth (11).

σ^B -Dependent proteins involved in acid adaptation. The acid tolerance response induces de novo protein synthesis, as demonstrated previously by 2D-E (8, 22, 24). Moreover, treatment with chloramphenicol during acid adaptation eliminates the protective effect (22). 2D-E of proteins from cells of the wild-type strain and the $\Delta sigB$ mutant exposed to acidic pH conditions revealed several proteins whose levels were increased (16 and 8 proteins, respectively) or reduced (5 and 14 proteins, respectively) at least threefold. The proteins whose levels were reduced in both the wild-type strain and the $\Delta sigB$ mutant included Lmo1704 (a protein similar to conserved hypothetical proteins), FbaA (a protein which is similar to fructose-1,6-biphosphate aldolase), the PTS phosphocarrier protein HPr, and two unidentified proteins. Although not previously described for *L. monocytogenes*, the level of HPr is also reduced in *Lactococcus lactis* upon exposure to acid. HPr not only has a role in the phosphoenolpyruvate-dependent sugar PTS but also has a regulatory role in cell metabolism (20, 40).

For the ValS, FlaA, and Lmo1709 proteins, increased accumulation during exposure to low pH was observed for both the wild-type and mutant strains. FlaA, the structural protein of the flagellum, might be induced for increased motility of the bacterium and subsequent movement away from growth-inhibiting conditions. The σ^B -dependent proteins that accumulated in the wild type after acid adaptation include Pfk, GalE, ClpP, and Lmo1580. Pfk (6-phosphofruktokinase) and GalE are enzymes that are involved in glycolysis and sugar metabolism, respectively. Pfk is one of the key enzymes involved in the control of glycolysis, together with hexokinase (glucose kinase)

and pyruvate kinase. Strikingly, comparison of the promoter sites of pyruvate kinase (*pykA*) and glucose kinase (*lmo1339*) with the sequence recognized by σ^B revealed one and three mismatches (Table 2), respectively, suggesting that these enzymes might also be σ^B regulated. These data together may suggest that the rate of glycolysis is partially σ^B regulated. However, additional experiments (e.g., reverse transcriptase PCR or primer extension analysis) should be performed to test this hypothesis. ClpP is the proteolytic subunit of the ATP-dependent Clp protease. The ATPases ClpC and ClpE and the proteolytic subunit ClpP are all required for stress survival, growth at a high temperature, and virulence (13, 21, 26). Clp ATPases regulate ATP-dependent proteolysis, preventing accumulation of misfolded proteins, and they also play a role as molecular chaperones involved in protein folding and assembly (37). The *clp* genes in *L. monocytogenes* form part of the CtsR (class three stress gene repressor) stress response regulon, in which CtsR negatively regulates the *clpP*, *clpC*, and *clpE* genes. In *B. subtilis*, two transcriptional start sites upstream of the *clpP* gene were identified, and they were preceded by sequences resembling the consensus sequences of promoters recognized by σ^A and σ^B transcriptional factors of the *B. subtilis* RNA polymerase (14). Transcription initiation occurred predominantly at the putative σ^A -dependent promoter. However, after exposure to stress, initiation of transcription also increased at the σ^B -dependent promoter (14). Alignment of the promoter regions of *clpP* of *L. monocytogenes* EGD-e revealed a putative promoter with three mismatches to the σ^B consensus promoter sequence. Along with our proteomics findings, this indicates that *clpP* in *L. monocytogenes* might also be, in addition to CtsR regulated, σ^B regulated.

Survival after HHP treatment and freeze-thaw cycles. Pre-exposure to a low pH not only provides protection against an otherwise lethal pH but also enhances survival during other challenges, such as heat, ethanol, oxidative, and osmotic stresses (12, 19). Here we determined the effect of preexposure of cells to acidic conditions on survival after HHP treatment and freezing. Cells exposed to a low pH prior to HHP treatment were significantly more piezotolerant. This effect was found to be conferred mainly by σ^B -dependent proteins, as $\Delta sigB$ mutant cells that were preexposed to a low pH were not as piezotolerant as wild-type cells. Piezotolerance was also observed for a mutant (AK01) that occurred naturally in an *L. monocytogenes* Scott A culture (16, 17). 2D-E showed that in this strain there were enhanced levels of ClpP that resulted from a single amino acid deletion in the highly conserved glycine-rich region of CtsR, a repressor of the *clp* genes. As we also found enhanced expression of ClpP in the wild type upon exposure to a low pH and not in the $\Delta sigB$ mutant, this protein may be an important factor in survival at high pressure. However, this could not be the only reason for increased pressure resistance, as the level of survival of AK01 was only about 2 logs higher than that of the wild type over a broad range of pressures (17), whereas the level of survival of acid-adapted wild-type EGD-e cells and the level of survival of $\Delta sigB$ mutant cells differed more than 5 logs at 350 MPa. Low-temperature adaptation of *L. monocytogenes* EGD-e cells also increased survival after HHP treatment. This is in agreement with previous results for HHP survival of low-temperature-adapted *L. monocytogenes* LO28 cells (38). Two cold shock proteins, Csp1

and Csp3, were found to be induced after a cold shock at 10°C. Strikingly, Csp1 was also induced after 10 min of exposure to 200 MPa (38). Analysis of the sequence of the putative promoter region of the *cspL* coding region revealed putative σ^B -regulated -10 and -35 promoter sequences. It can be speculated that the relative sensitivity of the $\Delta sigB$ mutant to high pressure after exposure to a low temperature may be due to the reduced expression of Csp1. Additionally, other (σ^B -regulated) proteins and other adaptation processes (e.g., membrane adaptations) might influence the survival during high-pressure treatment. In *L. monocytogenes*, the adaptation of membranes to low temperatures is accomplished by altering branching in the methyl end of the fatty acid from iso to anteiso and by shortening the fatty acid chains, resulting mainly in an increase in the level of anteiso-C15:0 fatty acids (1). In acid-adapted *L. monocytogenes* cells, the levels of the straight-chain C14:0 and C16:0 fatty acids were significantly increased, while the levels of C18:0 were decreased (29). In analogy, the fatty acids of barophilic microorganisms become more polyunsaturated with increases in the growth pressure (28). These observations together indicate that membrane adaptations that are induced in mildly acidic conditions may provide cross-protection against HHP. The influence of the membrane fluidity on piezotolerance may be exerted through the control of (ion) pumps in the membrane that are essential for maintaining pH homeostasis (27); e.g., the GAD system may have an impact on piezotolerance of acid-adapted cells, based on the importance of this system during adaptation to acidic conditions and its σ^B dependence.

The results of this study contribute to our understanding of survival under acid conditions and adaptation of *L. monocytogenes*. σ^B -Dependent protective mechanisms involved in survival and adaptation to low pH have been identified; these mechanisms include three genes of the GAD system and nine proteins induced at an acidic pH involved in stress protection and metabolism. Moreover, it is clear that acid adaptation provides, mainly in a σ^B -dependent manner, protection against HHP and freezing. The observed cross-adaptation phenomenon can have a significant impact on food processing (e.g., if acidification of food products is combined with a pressure treatment).

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