APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 2008, p. 594–604 Vol. 74, No. 3
0099-2240/08/$08.00+0 doi:10.1128/AEM.01921-07

Proteomic Analyses of a *Listeria monocytogenes* Mutant Lacking σ^B Identify New Components of the σ^B Regulon and Highlight a Role for σ^B in the Utilization of Glycerol[▽]

F. Abram,[1] Su Wan-Lin,[2] M. Wiedmann,[2] K. J. Boor,[2] P. Coote,[3] C. Botting,[3] K. A. G. Karatzas,[1] and C. P. O'Byrne[1]*

Bacterial Stress Response Group, Department of Microbiology, National University of Ireland—Galway, Galway, Ireland[1]; Department of Food Science, Cornell University, 412 Stocking Hall, Ithaca, New York 14853[2]; and Biomolecular Sciences Building, School of Biology, University of St. Andrews, North Haugh, St. Andrews, Fife KY16 9ST, United Kingdom[3]

Received 21 August 2007/Accepted 18 November 2007

In *Listeria monocytogenes* the alternative sigma factor σ^B plays important roles in both virulence and stress tolerance. In this study a proteomic approach was used to define components of the σ^B regulon in *L. monocytogenes* 10403S (serotype 1/2a). Using two-dimensional gel electrophoresis and the recently developed isobaric tags for relative and absolute quantitation technique, the protein expression profiles of the wild type and an isogenic Δ*sigB* deletion strain were compared. Overall, this study identified 38 proteins whose expression was σ^B dependent; 17 of these proteins were found to require the presence of σ^B for full expression, while 21 were expressed at a higher level in the Δ*sigB* mutant background. The data obtained with the two proteomic approaches showed limited overlap (four proteins were identified by both methods), a finding that highlights the complementarity of the two technologies. Overall, the proteomic data reaffirmed a role for σ^B in the general stress response and highlighted a probable role for σ^B in metabolism, especially in the utilization of alternative carbon sources. Proteomic and physiological data revealed the involvement of σ^B in glycerol metabolism. Five newly identified members of the σ^B regulon were shown to be under direct regulation of σ^B using reverse transcription-PCR (RT-PCR), while random amplification of cDNA ends-PCR was used to map four σ^B-dependent promoters upstream from lmo0796, lmo1830, lmo2391, and lmo2695. Using RT-PCR analysis of known and newly identified σ^B-dependent genes, as well as proteomic analyses, σ^B was shown to play a major role in the stationary phase of growth in complex media.

Listeria monocytogenes is a gram-positive facultative intracellular pathogen associated with a life-threatening disease in humans (43). Infections occur in immunocompromised individuals following ingestion of contaminated food. The success of *L. monocytogenes* as a human pathogen is determined in part by the presence of specialized virulence genes that allow it to multiply and spread within the host and in part by its ability to persist in harsh environmental conditions. It is now clear that there is a strong link between the virulence potential of *L. monocytogenes* and its ability to tolerate stress. Several studies have identified genes that play important roles in stress tolerance and virulence. For example, genes involved in osmotic stress tolerance (11, 37), acid tolerance (40), oxidative stress tolerance (17), and bile salt tolerance (36) have all been implicated in the virulence of *L. monocytogenes*. The link between stress tolerance and virulence is further underlined by the fact that several dedicated virulence genes are stress inducible (38, 39).

In all bacteria an effective response to stress requires rapid adaptation at the transcriptional level to changing environmental conditions. *L. monocytogenes* regulates the transcription of many genes with stress-related functions by employing an alternative sigma factor, σ^B. Mutants lacking σ^B display increased sensitivity to a range of stresses, such as osmotic pressure (4, 21, 42), acid (19, 42, 46), heat, ethanol, and oxidative stress (18), cold (4), and high hydrostatic pressure (44). Deletion of *sigB* also decreases the ability of *L. monocytogenes* to survive in the presence of bacteriocins and antibiotics (5).

More recently, roles for σ^B in regulating the expression of dedicated virulence genes have been described. A mutant lacking σ^B expresses the internalin gene, *inlA*, at a reduced level, and this correlates with a defect in its ability to invade human epithelial cells (25). Recently, σ^B was also shown to be solely responsible for transcription of the *inlC2* and *inlD* internalin genes, while it coregulates expression of the *inlA* and *inlB* internalin genes with PrfA, the main transcriptional regulator of key virulence genes (26). It is also clear that σ^B contributes directly to the transcription of *prfA*, which can be initiated from two distinct promoters (22), one of which (*prfA*p_2) is dependent on σ^B (28, 32, 35). The link between PrfA and σ^B is further highlighted by the finding that consensus σ^B promoter sequences are frequently found upstream from a subset of genes whose expression is reduced in a Δ*prfA* background (27). Examples of these genes include the *bilE* operon, *bsh*, and the *opuC* operon (21, 36, 37) encoding a bile exclusion system, a bile salt hydrolase, and a carnitine uptake system, respectively. All of these genes play an important role in host infection (6, 36, 37, 45), and their transcription is under both σ^B (21, 24)

* Corresponding author. Mailing address: Bacterial Stress Response Group, Department of Microbiology, National University of Ireland—Galway, Galway, Ireland. Phone: 353 91 493957. Fax: 353 91 494598. E-mail: conor.obyrne@nuigalway.ie.

▽ Published ahead of print on 7 December 2007.

and PrfA (16, 27, 36) control. Moreover, stress conditions such as high osmolarity and exposure to organic acids are known to enhance the ability of *L. monocytogenes* to invade human intestinal epithelial cells (23).

In view of the role played by σ^B in environmental stress tolerance and in virulence, a more comprehensive understanding of the σ^B regulon in *L. monocytogenes* is needed. In particular, elucidating the full extent of the σ^B regulon is likely to shed light on how *L. monocytogenes* is so adept at withstanding harsh environmental conditions and may also provide valuable insights into understanding the virulence of this pathogen. Limited gene set microarrays led to identification of 55 genes transcribed in a σ^B-dependent manner in *L. monocytogenes* (24). This study identified putative σ^B promoters by consensus searches of the published genome sequence and then tested the σ^B dependence of these genes using a 208-gene microarray. The σ^B consensus sequence used in this search was based on 29 known *Bacillus subtilis* σ^B promoters and four known *L. monocytogenes* σ^B promoters, a feature of the strategy that may have given rise to a bias in the genes identified. Proteomics have also been employed to help identify members of the σ^B regulon. Four proteins induced in exponential phase by low pH in a σ^B-dependent manner (ClpP, Lmo1580, Pfk, and GalE) were identified using a proteomics-based approach (44).

In the present study we employed a systematic proteomics-based approach to further define the σ^B regulon in *L. monocytogenes*. Analyses of σ^B-dependent changes in the pattern of protein expression identified 38 proteins that show altered expression in stationary phase. The identities of these proteins were established, and 31 of them were newly identified as proteins that are σ^B regulated. The functions of most of these proteins have not been defined, but their possible contributions to the biology of *L. monocytogenes* are discussed based on homologies to other proteins.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *L. monocytogenes* strain 10403S (serotype 1/2a) and an isogenic *sigB* mutant harboring an in-frame 297-bp deletion (46) were used throughout this study. Cells were grown in brain heart infusion (BHI) either in the presence or in the absence of 0.5 M NaCl with continuous shaking at 37°C. All cultures were inoculated to obtain a starting optical density at 600 nm (OD_{600}) of ~0.05, using 16-h overnight cultures as inocula. Two-dimensional gel electrophoresis (2-DGE), isobaric tags for relative and absolute quantitation (iTRAQ), and reverse transcription-PCR (RT-PCR) experiments were carried out with cells grown in BHI to exponential phase (OD_{600}, ~0.4) or 8 h into stationary phase (OD_{600}, ~2.0). In order to study glycerol utilization, cells were grown in a chemically defined medium (1) supplemented with 0.4% (wt/vol) glycerol instead of glucose, and growth was monitored in triplicate at OD_{595} using a 96-well plate reader (Genios; Tecan). Plates were shaken every 30 min for 30 s at 37°C, and measurements were automatically recorded for each well over a 48-h period.

Protein extraction. After growth to the required OD_{600}, 10 μg ml^{-1} chloramphenicol was added to the medium and cells were harvested by centrifugation (13,000 × *g*, 15 min, 4°C). Cell pellets were washed with sonication buffer (10 mM Tris-HCl, 0.1 mM EDTA, 5 mM $MgCl_2$) supplemented with 10 μg ml^{-1} chloramphenicol and subsequently resuspended in the same buffer containing 2 mg ml^{-1} lysozyme and 1% (vol/vol) protease inhibitor mixture (Amersham). Suspensions were incubated for 30 min at 37°C and then sonicated on ice using an MSE Soniprep 150 at an amplitude of 22 μm for 30 s. Ten pulses were used, with 30-s rest intervals between pulses. Soluble proteins were separated from the cell debris by centrifugation for 30 min at 13,000 × *g*. Benzonase (44 U ml^{-1}; Sigma) was added to each sample, and the samples were then incubated at 37°C for 30 min. The Benzonase treatment was repeated once, and protein samples were centrifuged at 13,000 × *g* for 30 min. Supernatants were stored at −80°C before analysis by 2-DGE. Protein concentrations were determined using a Bio-Rad RC DC kit with bovine serum albumin as a standard.

2-DGE. Proteins were separated by 2-DGE using a modified version of the O'Farrell method (29). The first dimension consisted of isoelectric focusing (IEF) using 11-cm IPG strips with linear pH gradients (pH 4 to 7; Amersham Biosciences). Prior to separation by IEF, proteins were precipitated and resuspended in IEF rehydration solution (Amersham Biosciences) to which IPG buffer (0.4%, vol/vol; Amersham Biosciences) and dithiothreitol (DTT) (2.5 mg ml^{-1}) had been added. Precipitation was performed using either acetone alone or acetone with 10% (vol/vol) trichloroacetic acid (TCA) and 20 mM DTT. Acetone-TCA-DTT precipitation was used for protein samples extracted from cultures grown in the presence of NaCl as an alternative to acetone precipitation, which did not seem to remove salt efficiently, a problem that led to poor-quality gels. Acetone precipitation was used for all other protein extracts. In both cases, appropriate volumes of protein samples (containing either 400 μg of protein for acetone precipitation or 1,600 μg of protein for acetone-TCA-DTT precipitation) were combined with 5 volumes of the precipitation reagent and incubated at −20°C for 1 h. The samples were then centrifuged at 18,000 × *g* for 10 min. The resulting protein pellets were dried at 37°C for 15 min and then resuspended in 230 μl IEF rehydration buffer. Each resulting suspension was centrifuged at 18,000 × *g* for 10 min to ensure that the insoluble material was removed. The IPG strips were then rehydrated overnight with 220 μl of rehydration solution containing the protein samples to be analyzed. Gels were run with an Investigator 5000 electrophoresis unit (Genomic Solutions) at 500 V overnight, followed by 1 h at 1,000 V and 3 h at 5,000 V, as recommended by the manufacturer (Amersham). IPG strips were stored at −20°C until they were needed (maximum, 5 days). Before sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis with 12% acrylamide in the second dimension was performed, IPG strips were equilibrated for 20 min in equilibration buffer A (6 M urea, 30% [vol/vol] glycerol, 2% SDS [wt/vol], 20 mg ml^{-1} DTT, 0.05% [wt/vol] bromophenol blue, 45 mM Tris base; pH 7) and then for 20 min in equilibration buffer B (like buffer A but containing 25 mg ml^{-1} iodoacetamide instead of DTT). The second-dimension gels were run in pairs at 70 mA for 5 h using a Protean II xi cell electrophoresis unit (Bio-Rad). Molecular mass markers with molecular masses ranging from 10 to 225 kDa (Broad Range protein molecular markers; Promega) were run in the second dimension as size standards. Prior to staining, gels were fixed for 15 min in a solution containing 50% (vol/vol) methanol and 7% (vol/vol) glacial acetic acid, stained overnight in GelCode Blue staining reagent (Pierce), and then destained in deionized, distilled water for several hours. Gel images were captured by scanning the gels with a Hewlett-Packard Scanjet 5300C scanner at a resolution of 600 dpi. For each growth condition and strain investigated six gels were run (i.e., 12 gels per comparison [6 wild-type strain gels and 6 Δ*sigB* mutant gels]), representing samples extracted from two independent cultures and three technical replicates. Gels were analyzed with PDQuest-Advanced software, version 8.0 (Bio-Rad). Data were normalized using the Local Regression Model, which is the most sophisticated normalization method available in PDQuest according to the software developer. This method was originally proposed by Cleveland (12) and was further developed by Cleveland and Devlin (13). It is less susceptible to outliers than a simple linear regression. Protein expression differences greater than twofold that were obtained for five pairs of replicates were considered significant. The proteins were identified using a combination of tryptic digestion and matrix-assisted laser desorption ionization–time of flight mass spectrometry, as previously described (8).

RNA extraction. Twenty milliliters of a culture grown to the required OD_{600} was mixed with 3.3 ml of a solution consisting of 5% (vol/vol) phenol and 95% (vol/vol) ethanol, and the suspension was incubated on ice for 15 min. Cells were harvested by centrifugation at 5,000 × *g* for 10 min and then at 12,000 × *g* for 2 min. RNA was extracted with a RiboPure yeast kit (Ambion) by following the manufacturer's instructions. The RNA was subsequently treated to remove DNA contamination with a Turbo DNA-free kit (Ambion) by following the manufacturer's recommended protocol. For modified random amplification of cDNA ends-PCR (RACE-PCR) experiments, cells were first treated using RNAprotect bacterial reagent (Qiagen), and RNA was extracted with an RNeasy bacterial midi kit (Qiagen) by following the manufacturer's protocol.

RT-PCR. First, RNA was proven to be free of DNA contamination by subjecting an RNA template to PCR using primers for the 16S rRNA gene (Table 1). Then cDNA was synthesized from 20 μl of RNA by using Expand reverse transcriptase with random hexanucleotide primers ($p[dN]_6$), both of which were supplied by Roche. cDNA concentrations were normalized by using primers for 16S rRNA. One-microliter aliquots of appropriate dilutions of cDNA were subjected to PCR for 18, 24, 30 and 36 cycles, and the resulting products were run on agarose (1%, wt/vol) gels. For each condition tested, the RNA and corre-

TABLE 1. Primers used in this study

Primer[a]	Sequence (5′ to 3′)
bsh FOR	CCGTGGATTCTTGGTCAATGC
bsh REV	CACAAAACGAGACATAGAAG
lmo2085 FOR	TTGCTTATTGGTTCACCCG
lmo2085 REV	GTCCCCTGGCAAAACATCTG
16S rRNA FOR	GGTGCATTAGCTAGTTGG
16S rRNA REV	AATCCGGACAACGCTTGC
lmo0796 GSP1	GTGACTGGTGACTTAACTATTCGC
lmo0796 GSP2	TTTCACGGCAACAAAAATCACAG
lmo0796 FOR	GACCCAGCACATAGTTC
lmo0796 REV	GCCAAGCTACCATGTTGCC
lmo0913 GSP1	TTTGACACGGTCAGCTAG
lmo0913 GSP2	TGCCCAATCAGGAAATGC
lmo0913 FOR	GAGGAGTGGTATTTTGAG
lmo0913 REV	GATTCTTTTAGCGGTTTACC
lmo1830 GSP1	ACTGGGCATTGACTCATTGA
lmo1830 GSP2	CCTGAGAAAAATGCGGTAAC
lmo1830 FOR	GGGGCTTCTGGTACAC
lmo1830 REV	CATTTCAATCGCAGCAG
lmo2391 GSP1	CCATCAAAGCAATCGAAACT
lmo2391 GSP2	CGCCTGCTTCCGAAACTGTT
lmo2391 FOR	GGCCGTCTTTTAGTCG
lmo2391 REV	CGTTTTAGGTTTTCCG
lmo2695 GSP1	CCAACTCCCGACCAAATTTA
lmo2695 GSP2	GAATGTTGTCTGCGGCTGTG
lmo2748 GSP1	AATACGTTCTTTGATTGAT
lmo2748 GSP2	TTCAAGAGACGCCAAACC
lmo2748 FOR	GGAAGGTGTTTGAGATG
lmo2748 REV	CCAATTAAATCGAGCGTG

[a] The FOR and REV primers were used for RT-PCR, while the GSP1 and GSP2 primers were used for RACE-PCR analysis.

sponding cDNA samples were prepared from two independent cultures, and each RT-PCR experiment was performed in duplicate for each cDNA preparation.

Modified RACE-PCR and TOPO cloning of cDNA fragments. Transcriptional start sites were mapped using the Invitrogen 5′ RACE-PCR system. The manufacturer's protocol was slightly modified by using a touchdown PCR protocol with AmpliTaq Gold DNA polymerase (Applied Biosystems). Specific primers were designed for lmo0796, lmo0913, lmo1830, lmo2391, lmo2695, and lmo2748 (Table 1). Aliquots of cDNA were subjected to PCR, and the resulting products were run on 3% agarose gels. PCR products present in the wild-type strain and absent in the Δ*sigB* mutant were gel purified using a QIAquick gel extraction kit (Qiagen) and were subsequently ligated into pCR2.1TOPO vectors (Invitrogen). *Escherichia coli* One Shot Top 10 competent cells (Invitrogen) were used in transformation reactions. Single colonies were inoculated into 5 ml of Luria-Bertani media and grown overnight at 37°C with continuous shaking. Plasmids were then harvested using the QIAprep spin miniprep kit protocol (Qiagen). The presence of an insert was confirmed using EcoRI restriction digests. Plasmid sequencing was carried out at the BioResource Center at Cornell University using an ABI 3700 automated sequencing unit (PerkinElmer Biosystems, Foster City, CA) with M13 forward and reverse primers. Sequences were proofread using DNAStar Seqman and were manually aligned using DNAStar Megalign.

Proteomic studies using iTRAQ. Proteins were separated and identified using the iTRAQ method described by Ross et al. (33). Protein extracts were prepared from duplicate stationary-phase cultures of *L. monocytogenes* 10403S (wt_1 and wt_2) and the Δ*sigB* mutant (Δ$sigB_1$ and Δ$sigB_2$) grown in BHI containing 0.5 M NaCl (BHIS) at 37°C. Cells were harvested by centrifugation at 10,000 × *g* for 15 min. Cell pellets were washed in 2 ml extraction buffer (EB), which consisted of 1 M triethyl ammonium bicarbonate (Sigma) buffer (pH 8.5) containing 0.1% (wt/vol) SDS and 10 μg ml^{-1} chloramphenicol. The cells were then pelleted and resuspended in 0.5 ml EB. Crude cell extracts were prepared from this cell suspension by bead beating. This procedure was performed using 0.75 ml of zirconia beads (Ambion) with 10 2-min bursts and 2-min rest intervals on ice. Protein concentrations were determined using a Calbiochem noninterfering protein assay kit with bovine serum albumin as the standard. The protein concentration of each preparation was normalized to 4.5 mg ml^{-1} by dilution in EB. Protein samples were then labeled with iTRAQ reagents by following the manufacturer's protocol (Applied Biosystems). Briefly, 20 μl (90 μg) of each sample was reduced, and the cysteine residues were blocked before digestion of each sample with trypsin. Following tryptic digestion overnight (16 h), each sample was labeled with one of four iTRAQ reagents designated 114, 115, 116, and 117 since they carry reporter groups with molecular masses of approximately 114, 115, 116, and 117 Da, respectively. In this experiment, iTRAQ reagents 114 and 115 were used to label the duplicate wild-type samples, while reagents 116 and 117 were used to label the duplicate Δ*sigB* samples. After labeling, the four samples were combined in one tube and then fractionated by cation-exchange chromatography in order to simplify the samples prior to analysis by liquid chromatography (LC)-tandem mass spectrometry (MS/MS). The combined sample was diluted with 7 ml of 10 mM KH_2PO_4–25% (vol/vol) acetonitrile (pH 3.0, adjusted with 1 M H_3PO_4) and then loaded onto an equilibrated cation-exchange column. The column was then washed with 2 ml of 10 mM KH_2PO_4–25% (vol/vol) acetonitrile buffer. Then the labeled peptides were eluted from the column by washing it with 12 600-μl aliquots of elution buffer (1 M KH_2PO_4, K_2HPO_4), which contained KCl at concentrations of 40, 50, 60, 70, 80, 90, 100, 120, 140, 165, 220, and 280 mM, respectively. The 12 eluted fractions were collected separately and stored at −20°C prior to analysis by LC-MS/MS.

LC-MS/MS analysis was performed with a Q-Star XL hybrid MS/MS instrument (Applied Biosystems, Foster City, CA), which was a hybrid quadrupole time-of-flight mass spectrometer. The peptides from each salt concentration were separated using an UltiMate nanoLC (LC Packings, Amsterdam, The Netherlands) equipped with a PepMap C_{18} trap and column, developing a gradient from 8 to 50% acetonitrile over 3.5 h, with an extended trap-washing period at the beginning of the run to remove the salt. The eluent was sprayed into the Q-Star Pulsar XL tandem mass spectrometer and analyzed in information-dependent acquisition mode. The high-performance liquid chromatography flow was controlled using Chromeleon software while the instrument was operated via the Analyst QS software (Applied Biosystems). The output data generated by Analyst QS (.wiff files) were then analyzed using ProQuant software (Applied Biosystems), which both identified the peptides and proteins present and quantified the peptides based on the iTRAQ reagent 114, 115, 116, and 117 peak intensities in the MS/MS spectra. The output data were viewed with the ProGroup Report viewer software (Applied Biosystems).

The data output from ProGroup Report included a list of all proteins identified (with a confidence of 99% or greater), as well as the ratio of the level of each identified protein in the wild-type samples (labeled 114 and 115) to the level of the protein in the Δ*sigB* samples (labeled 116 and 117). Each ratio was calculated from the ratios of the individual peptides identified as peptides derived from the protein. In ProGroup Report all ratios were returned with calculated *P* values, which indicated the likelihood that the ratios did not differ significantly from 1. For further analysis the data were exported from ProGroup Report to Microsoft Excel. For each protein a total of four expression ratios and their corresponding *P* values were considered in the analysis: 116/114 (Δ$sigB_1$/wt_1), 116/115 (Δ$sigB_1$/wt_2), 117/114 (Δ$sigB_2$/wt_1), and 117/115 (Δ$sigB_2$/wt_2). Using the method described by Bailey and Gribskov (3), *P* values were mathematically combined to obtain a combined *P* value (P_c) corresponding to the experimentally independent pair of ratios (Δ$sigB_1$/wt_1 and Δ$sigB_2$/wt_2). In this approach the combined *P* values were calculated using the relationship $P_c = k - k \times \ln(k)$, where $k = P_1 \times P_2$ (3). The *P* value cutoff used for data analysis was 0.05. The resulting list of proteins was filtered to remove all proteins showing a <1.5-fold difference in expression between strains (either increased or decreased in the mutant compared to the wild type). An analysis of the control ratios, 114/115 (wt_1/wt_2) and 116/117 (Δ$sigB_1$/Δ$sigB_2$), where no significant differences were expected, indicated that a 1.5-fold cutoff was likely to yield <3% false positives.

RESULTS

σ^B-dependent protein expression in stationary phase. We performed a proteomic comparison of *L. monocytogenes* wild-type strain 10403S (serotype 1/2a) and an isogenic mutant lacking a functional *sigB* gene (Δ*sigB*). In order to confirm that σ^B was active under the growth conditions used in the present investigation, the transcription of two genes known to be σ^B dependent was investigated using stationary-phase cultures, both with and without 0.5 M NaCl, of both the wild type and the Δ*sigB* mutant (Fig. 1A). Under these growth conditions the *bsh* transcript was found to be completely dependent on σ^B, while the lmo2085 transcript was strongly dependent on σ^B. For both genes the presence of 0.5 M NaCl in the growth

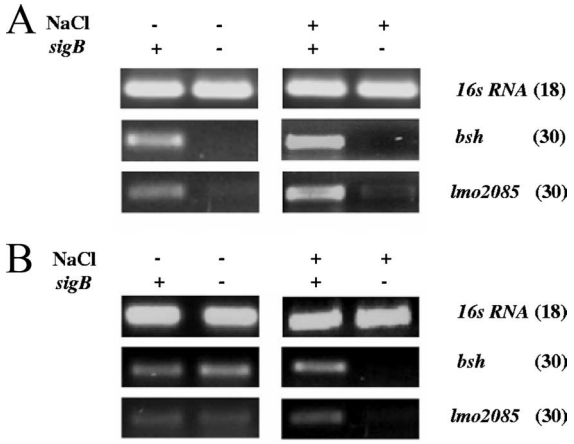

FIG. 1. Transcription of *bsh* and lmo2085 in BHI is σ^B dependent. mRNA was extracted from stationary-phase (A) and exponential-phase (OD_{600}, ~0.4) (B) *L. monocytogenes* 10403S wild-type (*sigB* +) and Δ*sigB* (*sigB* −) cells in the presence (NaCl +) or in the absence (NaCl −) of 0.5 M NaCl. The numbers in parentheses indicate the numbers of PCR cycles to which the cDNA templates were subjected. The results are representative results of at least three replicates.

medium appeared to stimulate transcription (Fig. 1), suggesting that σ^B may be more active under these conditions. In contrast, the effect of the Δ*sigB* deletion was less marked when transcripts from exponentially growing cells were analyzed; only cultures containing 0.5 M NaCl showed σ^B-dependent transcription (Fig. 1B).

Taken together, these data indicated that σ^B actively participates in transcription during stationary phase, making this phase of growth a good choice for identifying σ^B-dependent protein expression. 2-DGE analysis of extracts from stationary phase led to the identification of seven proteins that were found to have reproducibly ($n \geq 5$) different patterns of expression in the wild-type and Δ*sigB* mutant backgrounds (Fig. 2). These proteins were excised in duplicate and individually identified by tryptic digestion and matrix-assisted laser desorption ionization–time of flight mass spectrometry, and the results of these analyses are summarized in Table 2. These seven proteins were all found to be expressed at a lower level in the Δ*sigB* mutant background. No protein showing a higher level of expression in the Δ*sigB* mutant background could be reproducibly detected using the 2-DGE method.

In the absence of added NaCl, the following four proteins were found to be expressed at lower levels in a background

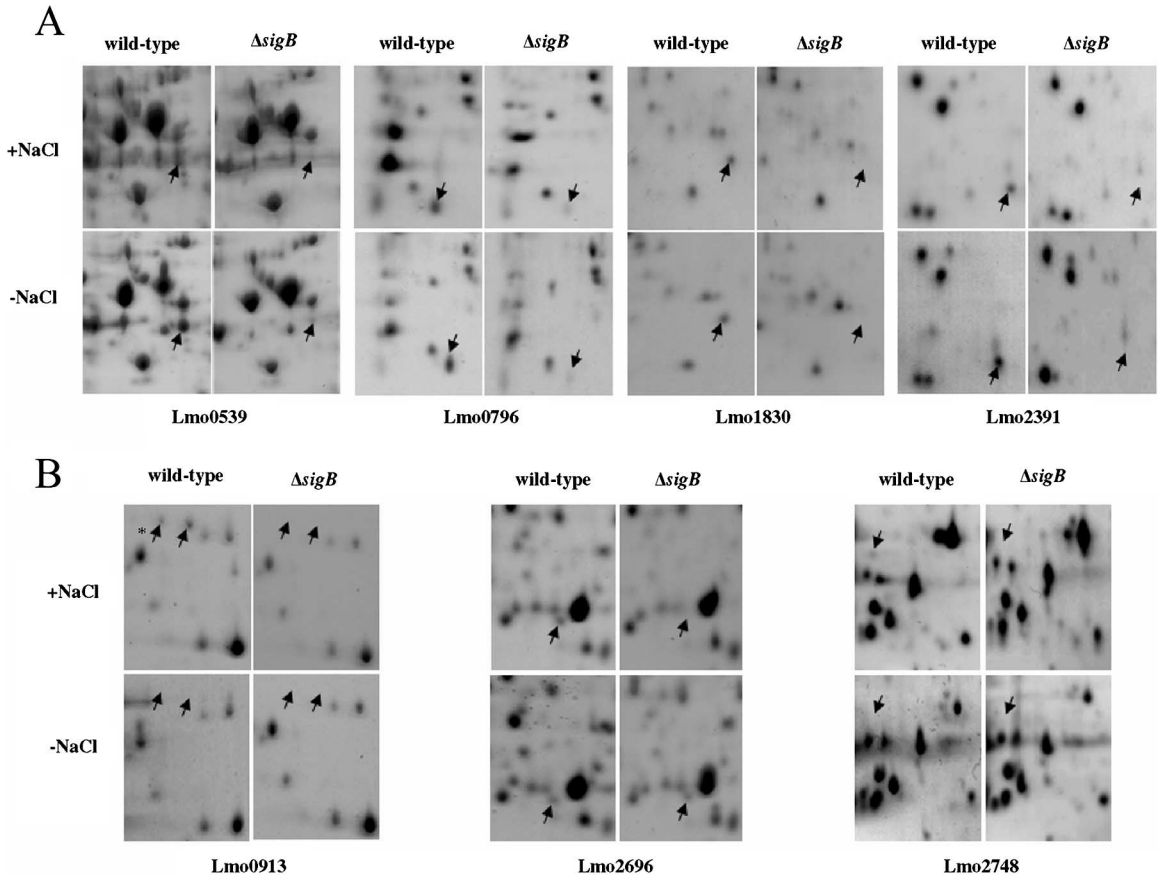

FIG. 2. Seven proteins (indicated by arrows) are expressed in a σ^B-dependent manner in stationary phase. The images are representative sections of 2-DGE profiles of proteins extracted from stationary-phase cells of *L. monocytogenes* wild-type strain 10403S and the Δ*sigB* mutant grown in BHI in the presence (+NaCl) or in the absence (−NaCl) of 0.5 M NaCl. Proteins showed σ^B-dependent expression in stationary phase in BHI either regardless of the presence of NaCl (A) or only in the presence of NaCl (B). The asterisk indicates the Lmo0913 protein referred to as Lmo0913a.

TABLE 2. Proteins identified in stationary-phase extracts from BHI and BHIS analyzed by 2-DGE

Protein	Homologous protein (% identity/% similarity)[a]	Microorganism with homologous protein	Mol wt (predicted/observed)	pI (predicted/observed)	% Coverage	% Matched peptides	Score[b]
Proteins expressed in a σ^B-dependent manner							
Lmo0539	Tagatose-1,6-diphosphate aldolase (44/61)	*Staphylococcus aureus*	37.8/39.0	4.9/4.9	45	54	824
Lmo0796	YceI (44/61)	*Escherichia coli*	19.3/19.4	4.6/4.4	60	69	102
Lmo1830	Short-chain dehydrogenase (47/67)	*Pseudomonas aeruginosa*	20.9/22.0	5.9/6.2	60	85	124
Lmo2391	YhfK (42/61)	*Bacillus subtilis*	22.7/25.0	6.0/6.5	65	62	110
Proteins expressed in a σ^B-dependent manner in the presence of salt							
Lmo0913	Succinate semialdehyde dehydrogenase (48/67)	*Bacillus subtilis*	53.2/55.0	5.8/6.4	23	26	78
Lmo0913a	Succinate semialdehyde dehydrogenase (48/67)	*Bacillus subtilis*	53.2/55.0	5.8/6.3	49	64	999
Lmo2696	Dihydroxyacetone kinase (50/68)	*Lactococcus lactis*	21.5/20.0	5.1/5.1	36	23	77
Lmo2748	YdaG (42/68)	*Bacillus subtilis*	15.7/10.0	4.6/4.0	54	78	81
Proteins expressed in a growth phase-dependent manner							
Fri	Nonheme iron-binding ferritin (100/100)	*Listeria monocytogenes*	16.0/15.5	4.7/5.0	69	15	77
Lmo1538	Glycerol kinase (74/88)	*Bacillus subtilis*	55.0/60.0	4.9/4.9	28	24	88
Lmo2101a	Pyridoxine biosynthesis protein (83/92)	*Bacillus subtilis*	31.8/32.0	5.3/4.9	34	24	78
Lmo2101b	Pyridoxine biosynthesis protein (83/92)	*Bacillus subtilis*	31.8/32.0	5.3/5.0	50	29	107
Lmo2101c	Pyridoxine biosynthesis protein (83/92)	*Bacillus subtilis*	31.8/32.0	5.3/5.2	29	27	104

[a] The identity and similarity values were obtained by performing a protein-protein BLAST search on the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/).
[b] The score was derived from ion scores that were equal to $-10 \times \log(P)$, where P is the probability that the observed match is a random event.

lacking σ^B: Lmo0539, Lmo0796, Lmo1830, and Lmo2391 (Fig. 2A and Table 2). Lmo0539 and Lmo0796 were present at lower levels in the Δ*sigB* mutant, while Lmo1830 and Lmo2391 were present at undetectable levels in extracts prepared from the Δ*sigB* mutant but were abundant in the wild-type strain (Fig. 2A). The presence of 0.5 M NaCl in the growth medium did not significantly influence the expression of these four proteins (Fig. 2A).

A further four protein spots were found to be differentially expressed in the Δ*sigB* mutant compared to the parent when cultures were grown in the presence of NaCl but not when NaCl was absent from the culture medium. The proteins were identified as Lmo2696, Lmo2748, and Lmo0913, which migrated as two spots that had very similar molecular weights and pIs that differed by approximately 0.1 U (Fig. 2B and Table 2). Lmo0913 and Lmo2748 were induced in the presence of NaCl in the wild type but were not induced in the Δ*sigB* strain (Fig. 2B). Lmo2696 was expressed at detectable levels in the wild type when 0.5 M NaCl was present in the growth medium, whereas it was undetectable in the Δ*sigB* mutant (Fig. 2B).

Growth phase-dependent changes in the proteome. The proteomes of the wild-type and Δ*sigB* strains grown in BHI to mid-exponential phase (OD_{600}, ~0.4) were also compared. Strikingly, no reproducible differences in protein expression patterns between the strains were detected under these conditions (data not shown). This series of experiments also allowed protein expression in exponential phase and protein expression in stationary phase to be compared. Several proteins were observed to be expressed in a growth phase-dependent manner. Interestingly, Lmo0539, Lmo0796, Lmo1830, and Lmo2391, the four proteins identified as being σ^B dependent in the absence of NaCl (Fig. 2A), were all found to be induced in the wild type during stationary phase (Fig. 3A). Lmo0913, Lmo2696, and Lmo2748, whose expression was found to be both σ^B dependent and NaCl dependent during stationary phase (Fig. 2B), were also found to be expressed in a growth phase-dependent manner. During the exponential phase in BHIS these proteins were not expressed at detectable levels, but they were present in protein extracts from stationary-phase cultures (Fig. 3B). Three other proteins were found to be expressed in a growth phase-dependent manner independent of σ^B (Fig. 3C), indicating that not all proteins showing growth phase-dependent expression were under the control of σ^B. These proteins were identified as Fri, a nonheme iron-binding ferritin (Table 2); Lmo1538, a protein with similarity to glycerol kinase (Table 2); and Lmo2101, a 32-kDa protein

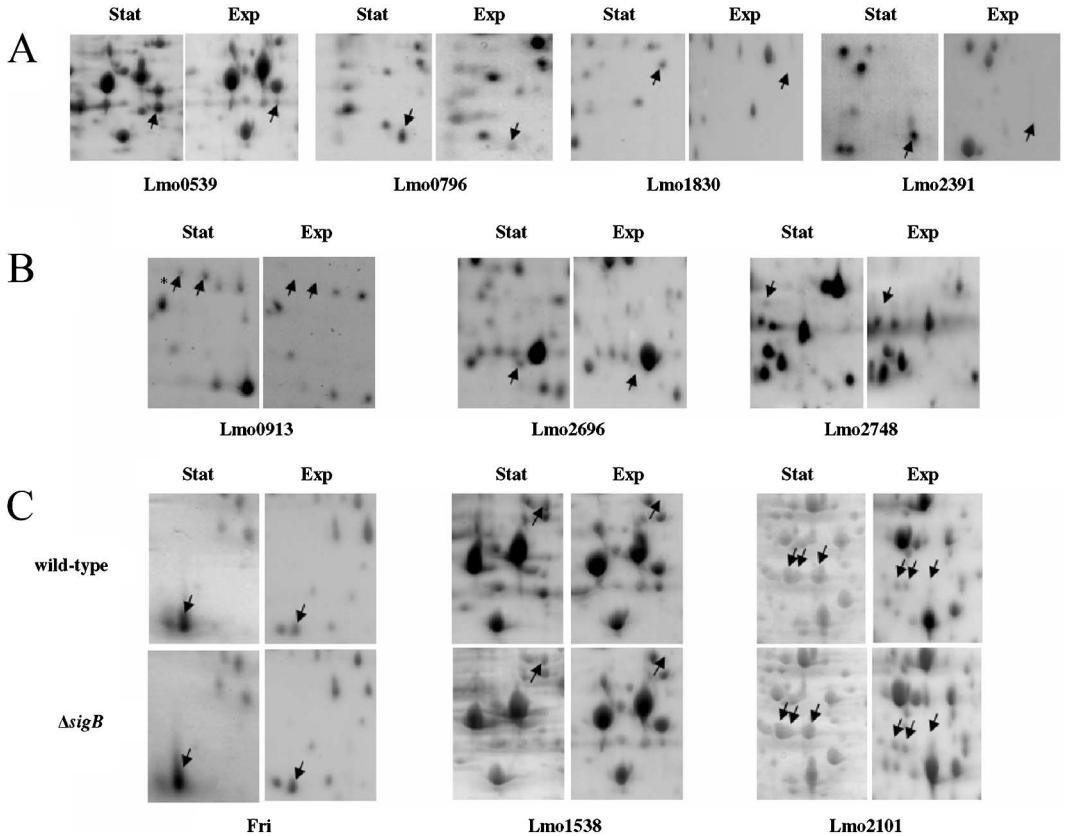

FIG. 3. Ten proteins (indicated by arrows) found to be expressed in a growth phase-dependent manner. The images are representative sections of 2-DGE profiles of proteins extracted from *L. monocytogenes* wild-type 10403S or Δ*sigB* cells grown to exponential phase (Exp) or stationary phase (Stat) in BHI in the presence or absence of 0.5 M NaCl. (A) σ^B-dependent proteins in the wild type showing growth phase-dependent expression in BHI. A similar pattern was observed for these proteins in BHIS (not shown). (B) σ^B-dependent proteins in the wild type showing growth phase-dependent expression in BHIS. (C) Proteins not affected by σ^B that were expressed in a growth phase-dependent manner in BHI. A similar pattern was observed for these proteins in BHIS (not shown). The asterisk indicates the Lmo0913 protein referred to as Lmo0913a. Lmo2101 migrated as three spots, referred to as Lmo2101a, Lmo2101b, and Lmo2101c from left to right.

with similarity to a pyridoxine biosynthesis protein (Table 2). The latter protein migrated on the two-dimensional polyacrylamide gels as three spots with different apparent molecular charges (Table 2). Thus, under these growth conditions growth phase-dependent protein expression was observed for 10 proteins, and the induction of 7 of these proteins was under the control of σ^B.

RT-PCR analysis of σ^B-dependent gene expression. In order to establish if σ^B had a direct role in regulating the transcription of the genes encoding these newly identified members of the σ^B regulon, RT-PCR was performed to assess the relative mRNA levels for each gene investigated in the wild-type and Δ*sigB* backgrounds. In exponential-phase extracts all five genes tested showed some degree of σ^B-dependent transcription (Fig. 4B); however, the Δ*sigB* mutation had a more dramatic impact on transcription in the stationary phase of growth (Fig. 4A). The transcription of lmo0796 was found to be influenced only slightly by σ^B in exponential phase, while in stationary phase in the presence of NaCl there was a clear dependence on σ^B for full transcription (Fig. 4A). The mRNA levels corresponding to lmo0913, lmo1830, lmo2391, and lmo2748 were all found to be strongly dependent on the presence of an intact *sigB* gene in stationary phase, regardless of whether NaCl was included in the growth medium (Fig. 4A). In exponential phase the most marked effect of the Δ*sigB* deletion on transcript levels was observed for lmo0913, lmo1830, and lmo2748 when NaCl was present in the growth medium (Fig. 4B). These results are consistent with the proteomic data (Fig. 2) and indicate that these genes are under the control of σ^B at the transcriptional level. They further suggest that σ^B may play a significant role during stationary phase or under osmotic stress conditions.

Confirmation of the presence of σ^B-dependent promoters. The DNA sequences upstream from the seven newly identified σ^B-dependent genes were inspected to search for possible σ^B promoter motifs. The consensus σ^B promoter sequence has been determined to be GTTT-$N_{13/17}$-GGGWAT in *L. monocytogenes* (24). The σ^B consensus sequence was identified upstream from all seven genes at positions −57, −35, −42, −35, −54, and −32 upstream from the start codons of lmo0539, lmo0796, lmo1830, lmo2391, lmo0913, and lmo2748, respectively. While no σ^B sequence was identified upstream from the gene encoding Lmo2696, a σ^B consensus sequence was identified at position −38 upstream from lmo2695, the gene immediately upstream from lmo2696. An inspection of the chromosomal region containing lmo2695 and lmo2696 suggested

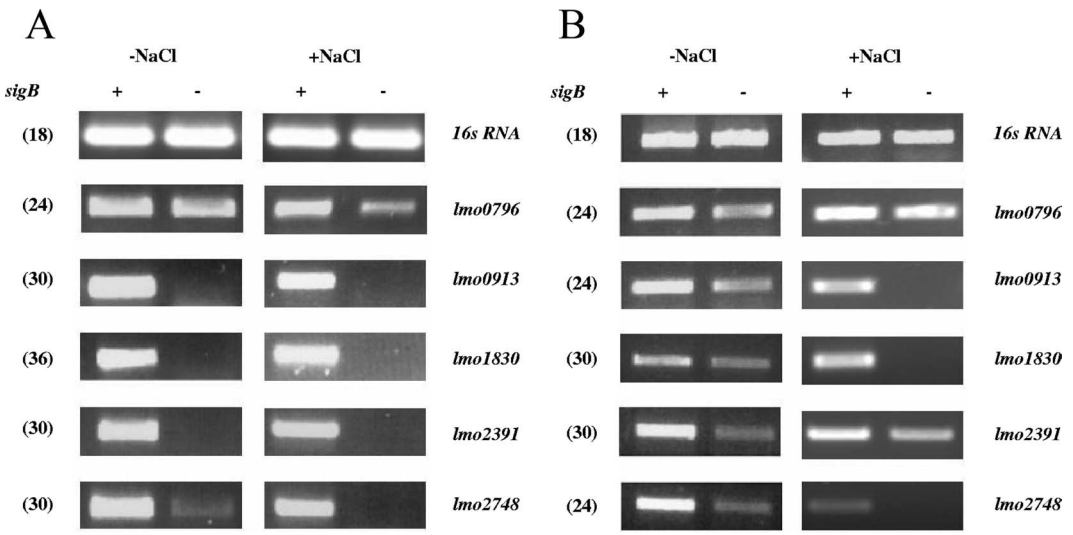

FIG. 4. lmo0796, lmo0913, lmo1830, lmo2391, and lmo2748 are transcribed in a σ^B-dependent manner. mRNA was extracted from *L. monocytogenes* wild-type strain 10403S (*sigB* +) and Δ*sigB* (*sigB* −) cells grown in BHI to stationary phase (A) and to exponential phase (OD_{600}, ~0.4) (B) in the presence and absence of 0.5 M NaCl. The numbers in parentheses indicate the numbers of cycles to which the cDNA templates were subjected. The results are representative results of at least three replicates.

that these genes were likely to be transcribed as part of the same operon, which includes a third gene downstream from lmo2696, lmo2697. It seemed possible, therefore, that the transcription of this operon was under the control of σ^B.

Promoter mapping experiments were performed using modified RACE-PCR to identify the transcriptional start sites upstream from six of the newly identified σ^B-dependent genes (lmo0796, lmo0913, lmo1830, lmo2391, lmo2695, and lmo2748). The primers for lmo0913 and lmo2748 (Table 1) failed to give reliable PCR products and were therefore eliminated from further analysis. The results of these analyses confirmed that the putative σ^B promoters identified upstream from lmo1830, lmo2391, and lmo2695 directed transcription (Table 3). The promoter sequence identified for lmo0796 appeared to be 12 bp further upstream than the sequence predicted by the in silico promoter search (Table 3) and had a somewhat unusual −10 sequence (GGCTAG) compared to the reported −10 consensus (GGGWAT) (24).

Proteomic analysis by the iTRAQ method. In order to complement the 2-DGE results, the proteomes of the 10403S and Δ*sigB* mutant strains were also compared using the newly developed iTRAQ proteomics method (33). In stationary phase in BHIS medium, the expression of 35 proteins was found to be influenced significantly by the *sigB* genotype (Table 4); 14 proteins were expressed at lower levels in the Δ*sigB* mutant, while 21 proteins were expressed at higher levels in the mutant strain. The following four proteins detected as being expressed in a σ^B-dependent manner using 2-DGE were also detected using this approach: Lmo0539, Lmo0796, Lmo2391, and Lmo2696. The substrate binding component of the OpuC carnitine transport system, which is known to depend on σ^B for expression (21), was also identified. Five proteins encoded by genes belonging to two putative operons (Lmo2695-Lmo2696-Lmo2697 and Lmo1601-Lmo1602) were among the σ^B-dependent proteins identified (Table 4). Together, these results show that the iTRAQ approach can reliably detect differentially expressed proteins in *L. monocytogenes* and that it is complementary to 2-DGE.

TABLE 3. Sequenced σ^B promoters

Gene	Promoter sequence[a]
lmo0796	.....TTTTTCTCAGGTTTAATTTCTTAAGATTT
	AGGCTAGATTATAA**GA**GA
lmo1830	.....TCGTGGAGCCGTTTTTTGTTTGTAATTTT
	AGGGTAGATGTGTAC**TGA**
lmo2391	.....GCGACTTGTGGTTTTATTTTTTACTCACC
	GGGAAAAGTTCTTT**GT**AG
lmo2695	.....AAGCAACCACGTTTTGACTTTCTAGTAA
	AGGGAAATTGAGGTA**A**GAG

[a] The −10 and −35 regions are underlined. The transcriptional start sites are indicated by bold type.

Δ*sigB* mutant utilizes glycerol inefficiently. The proteomic analyses identified Lmo2695, Lmo2696, and Lmo2697 as proteins that are under positive regulation of σ^B (Fig. 2B and Table 4). These three proteins are likely to be subunits of a dihydroxyacetone kinase, which is an enzyme involved in glycerol metabolism. Therefore, the ability of the wild-type and Δ*sigB* mutant strains to utilize glycerol was assessed (Fig. 5). When glycerol was added to a chemically defined growth medium as the sole carbon source, the Δ*sigB* mutant grew more slowly than the parent strain (Fig. 5). In addition, the Δ*sigB* mutant displayed a lag phase of approximately 5 h, which was not observed for the parent strain.

DISCUSSION

In this study we used a combination of 2-DGE and iTRAQ proteomics to elucidate the σ^B regulon in *L. monocytogenes*. A total of 38 proteins were identified as being expressed in a σ^B-dependent manner; 17 proteins were found to be positively regulated by σ^B, while 21 proteins were found to be negatively influenced by σ^B. For the 17 proteins that were under positive

TABLE 4. Proteins identified as being expressed in a σ^B-dependent manner in stationary-phase extracts from BHIS, analyzed by the iTRAQ method

Protein	Homology (% identity/% similarity)[a]	Microorganism with homologous protein[b]	Change (fold)[b]	*P* value
Lmo2391[c]	YhfK (42/61)	*Bacillus subtilis*	−14.3	1.59×10^{-7}
Lmo0265	Succinyldiaminopimelate desuccinylase, DapE (100/100)	*Listeria monocytogenes*	−14.3	3.36×10^{-4}
Lmo0539[c]	Tagatose-1,6-diphosphate aldolase (55/76)	*Staphylococcus aureus*	−6.3	1.59×10^{-7}
Lmo2695	Dihydroxyacetone kinase (60/78)	*Lactococcus lactis*	−4.0	1.59×10^{-7}
OpuCC	Glycine betaine/carnitine/choline ABC transporter (100/100)	*Listeria monocytogenes*	−3.8	9.80×10^{-4}
Lmo2205	Phosphoglycerate mutase (57/72)	*Bacillus anthracis*	−3.7	1.59×10^{-7}
Lmo0796[c]	YceI (44/61)	*Escherichia coli*	−3.3	1.59×10^{-7}
Lmo2696[c]	Dihydroxyacetone kinase (50/68)	*Lactococcus lactis*	−3.2	1.59×10^{-7}
Lmo2697	PTS-dependent dihydroxyacetone kinase phosphotransferase subunit (46/72)	*Lactococcus lactis*	−3.1	3.59×10^{-6}
Lmo1601	YtxH (49/62)	*Bacillus* sp. NRRL	−2.9	1.59×10^{-7}
Lmo1602	YtxG (40/66)	*Bacillus licheniformis*	−2.5	1.59×10^{-7}
Lmo0956	*N*-Acetylglucosamine-6-phosphate deacetylase (61/77)	*Lactobacillus salivarius*	−1.9	6.59×10^{-6}
Lmo0398	PTS fructose-specific IIABC component (37/63)	*Mycoplasma hyopneumoniae*	−1.6	2.19×10^{-5}
Lmo0292	Heat shock protein HtrA serine protease (100/100)	*Listeria monocytogenes*	−1.6	1.88×10^{-2}
Lmo2824	D-3-Phosphoglycerate dehydrogenase (56/76)	*Bacillus thuringiensis*	1.5	1.59×10^{-7}
PyrF	Orotidine 5′-phosphate decarboxylase (63/75)	*Bacillus subtilis*	1.5	1.38×10^{-3}
ProS	Prolyl-tRNA synthetase (100/100)	*Listeria monocytogenes*	1.5	7.42×10^{-6}
TcsA	$CD4^+$ T-cell-stimulating antigen, lipoprotein (100/100)	*Listeria monocytogenes*	1.6	1.59×10^{-7}
Lmo0344	Dehydrogenase/reductase (55/75)	*Pseudomonas syringae*	1.6	4.98×10^{-7}
Lmo0443	Transcription regulator LytR (35/56)	*Bacillus subtilis*	1.6	6.50×10^{-6}
DaaA	D-Amino acid aminotransferase (100/100)	*Listeria monocytogenes*	1.6	5.44×10^{-4}
Lmo0342	Transketolase (59/74)	*Bacillus thuringiensis*	1.6	4.08×10^{-3}
DeoD	Purine nucleoside phosphorylase (100/100)	*Listeria monocytogenes*	1.7	1.56×10^{-4}
Lmo1699	Methyl-accepting chemotaxis protein (41/59)	*Bacillus* sp. NRRL	1.7	2.84×10^{-3}
Lmo1681	Cobalamin-independent methionine synthase, MetE (53/71)	*Bacillus subtilis*	1.7	2.14×10^{-2}
DltD	DltD protein for D-alanine esterification of lipoteichoic acid and wall teichoic acid (100/100)	*Listeria monocytogenes*	1.7	4.50×10^{-4}
Lmo0560	NADP-specific glutamate dehydrogenase (70/83)	*Bacillus clausii*	1.8	1.59×10^{-7}
Lmo1057	L-Lactate dehydrogenase (32/54)	*Bacillus licheniformis*	1.8	1.23×10^{-5}
Lmo0521	6-Phospho-beta-glucosidase (67/80)	*Bacillus subtilis*	1.8	6.53×10^{-7}
Lmo2363	Glutamate decarboxylase, GadB (100/100)	*Listeria monocytogenes*	1.8	1.59×10^{-7}
Lmo1604	2-Cys peroxiredoxin (70/87)	*Bacillus halodurans*	1.8	2.21×10^{-2}
Lmo2258	Ribulose-phosphate 3-epimerase (90/95)	*Listeria welshimeri*	1.9	2.43×10^{-4}
Lmo0536	6-Phospho-beta-glucosidase (60/73)	*Bacillus subtilis*	1.9	1.20×10^{-2}
Lmo2362	Amino acid antiporter, GadC (100/100)	*Listeria monocytogenes*	2.3	3.40×10^{-2}
Lmo1867	Pyruvate phosphate dikinase (60/75)	*Clostridium difficile*	3.1	6.10×10^{-3}

[a] The identity and similarity values were obtained by performing a protein-protein BLAST search on the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/). It should be emphasized that most of the functions indicated are putative.

[b] The changes indicate the expression ratio for the Δ*sigB* mutant and the wild type (Δ*sigB*/wild type), expressed as a negative reciprocal for proteins that were present at reduced levels in the Δ*sigB* strain.

[c] The protein was also determined to be expressed in a σ^B-dependent manner using the 2-DGE technique.

σ^B control, six of the corresponding genes were previously shown to be regulated by σ^B: *opuCC* (21), lmo0956, lmo1602, lmo2205, and lmo2695 (24), and lmo2748 (34). Strikingly, several of the genes identified in this study (*opuCC*, lmo0539, lmo0796, lmo0913, lmo1601, lmo1602, lmo2391, lmo2695-7, and lmo2748) belong to a group of genes whose transcription is known to be influenced by the virulence regulator PrfA (27). These genes, designated the group III PrfA-dependent genes, frequently have potential σ^B promoter sequences upstream from their start codons (27). The present study confirmed that these genes are indeed expressed in a σ^B-dependent manner, a result that further highlights the overlap between the control of virulence and stress-related functions in *L. monocytogenes*.

Of the 38 differentially expressed proteins identified, 7 were identified using 2-DGE and 35 were identified using the iTRAQ method; 4 proteins were identified by both techniques. Thus, there was some overlap between the two approaches. A number of things might account for the comparatively small overlap between the results obtained with the iTRAQ and 2-DGE approaches. First, the iTRAQ method is more sensitive; a total of 511 proteins were detected and identified by this method, compared to the approximately 250 proteins detected by 2-DGE. Second, the intrinsic reproducibility of 2-DGE limits the extent to which differential expression can be said to be significant. Typically, changes in expression that are less than twofold cannot reliably be said to be significant. In contrast, the iTRAQ method is more robust, allowing greater confidence in the expression differences detected, and this enabled us to use a lower cutoff value, 1.5-fold. It is worth noting that both Lmo2748 and Lmo0913 were identified as proteins that were differentially expressed using the iTRAQ method but were eliminated from the final list because the statistical significance of the differences fell just below the threshold set for the analysis ($P_c > 0.05$). It is likely that if further iTRAQ experiments had been conducted, these proteins would also have appeared in the final list generated. Overall, this study

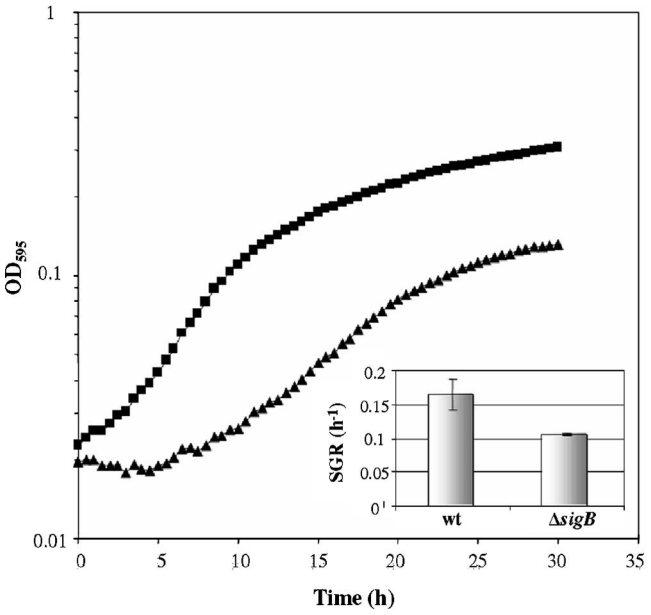

FIG. 5. Δ*sigB* mutant utilizes glycerol inefficiently. *L. monocytogenes* wild-type strain 10403S (squares) and Δ*sigB* (triangles) cells were grown in defined medium supplemented with 0.4% (wt/vol) glycerol at 37°C. The curves are representative growth curves for the conditions investigated; all growth curve experiments were performed in triplicate. The inset shows the specific growth rates (SGR) derived from the curves. The errors bars indicate the standard deviations from the means of triplicate measurements. wt, wild type.

highlighted the complementarity of the two proteomic methods since each method detected proteins that were not detected by the other method.

A number of observations suggest that σ^B plays a more significant role in regulating gene expression during the stationary phase of growth. First, no differentially expressed proteins were identified using 2-DGE when protein extracts from exponential-phase wild-type and Δ*sigB* mutant cultures were compared. Second, all seven σ^B-dependent proteins identified by 2-DGE were expressed in a growth phase-dependent manner, with higher levels of expression in stationary phase (Fig. 3A and 3B). Using RT-PCR, the transcript levels of known (Fig. 1) and newly identified (Fig. 4) σ^B-dependent genes were more strongly influenced by the *sigB* genotype in stationary phase than in exponential phase. This was particularly noticeable when no NaCl was added to the BHI growth medium. Taken together, these data highlight the important role played by σ^B during the stationary phase when *L. monocytogenes* is grown in this complex medium.

Previous studies have emphasized that σ^B has a central role in the stress responses of *L. monocytogenes* (4, 5, 18, 19, 21, 42, 44, 46). The results of the present study support this conclusion and also indicate that σ^B has an important role in regulating genes involved in metabolism. Most of the 38 differentially expressed proteins can be classified into two broad functional categories, metabolism (22 proteins) and stress-related functions (10 proteins). This classification is based purely on homology in many cases, since the functions of most of the identified proteins in *L. monocytogenes* have not yet been assigned genetically or biochemically. σ^B is also known to play a significant role in regulating the expression of virulence factors (25–28, 32, 35), but in the present study none of the well-characterized virulence-associated proteins of *L. monocytogenes* were found to be differentially expressed in the Δ*sigB* background. However, the expression of 12 genes identified here (*opuCC*, lmo1602, lmo1601, lmo2748, lmo0913, lmo2695, lmo2696, lmo2697, lmo0539, lmo2391, lmo0796, and lmo2205) is known to be modulated intracellularly within the host (10) and/or the genes are known to belong to the PrfA regulon (27). These results highlight overlaps in the cell's regulation of stress, metabolism, and virulence.

Consideration of the functions (or putative functions) of the differentially expressed proteins can provide new insight into the role played by σ^B in the biology of *L. monocytogenes*. Nine proteins with putative roles in metabolism were under positive σ^B control (Lmo0539, Lmo0956, Lmo0398, Lmo2695, Lmo2696, Lmo2697, Lmo0913, Lmo1830, and Lmo2205), while 13 proteins were negatively influenced by σ^B (Lmo2824, Lmo0344, Lmo0342, Lmo1057, Lmo2258, Lmo1867, Lmo0521, Lmo0536, PyrF, DeoD, Lmo0560, DaaA, and Lmo1681). Several of these differentially expressed proteins are likely to be involved in the metabolism or utilization of alternative carbon sources, such as glycerol (Lmo2695, Lmo2696, and Lmo2697), tagatose (Lmo0539), *N*-acetyl-D-glucosamine (Lmo0956), 6-phospho-β-D-glucosyl-1,4-D-glucose (Lmo0521 and Lmo0536), and fructose (Lmo0398). Two of the proteins under negative σ^B control are homologous to enzymes that participate in the pentose phosphate pathway (Lmo2258 and Lmo0342). Strikingly, two of the proteins identified are likely to use the glycolytic intermediate 3-phosphoglycerate as a substrate; Lmo2205 (putative phosphoglycerate mutase) is present at lower levels in the Δ*sigB* mutant background, while Lmo2824 (putative phosphoglycerate dehydrogenase) is expressed at higher levels in this background. This finding might suggest that the overexpression of Lmo2824 compensates for an increase in substrate availability that occurs when Lmo2205 is not expressed appropriately. Lmo2695, Lmo2696, and Lmo2697 are encoded by contiguous genes that are likely to form an operon preceded by a σ^B promoter (Table 3). These proteins share close homology to known members of the phosphotransferase system (PTS)-dependent dihydroxyacetone kinase family involved in the utilization of glycerol. The finding that these proteins are expressed at low levels in the Δ*sigB* mutant correlates well with the inability of this mutant to grow efficiently on glycerol as a sole carbon source (Fig. 5). This growth defect also represents a novel phenotype not previously described for strains lacking σ^B.

Ten proteins whose expression is under σ^B control are likely to have stress-related functions. Seven of these proteins (Lmo2748, Lmo1601, Lmo1602, Lmo2391, Lmo0796, Lmo0292, and OpuCC) were found to be expressed at a lower level in the Δ*sigB* mutant. Four of them (Lmo2748, Lmo1601, Lmo1602, and Lmo2391) show similarities with σ^B-dependent stress proteins with unknown functions in *B. subtilis* (31). Lmo0796 shows some similarity with YceI (61%) in *E. coli*, a protein with an unknown function that is induced in response to basic pH (41). Lmo0292 is a heat shock serine protease (HtrA) conferring multiple-stress resistance to *L. monocytogenes* by degrading misfolded proteins, preventing their accumulation on the bacterial cell surface (40). OpuCC is a subunit of an ABC transporter that is specifically involved in the uptake of osmoprotectants under high-osmolarity conditions (20, 21). The fol-

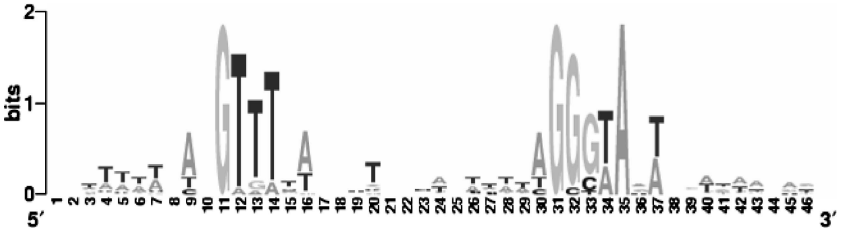

FIG. 6. Sequence logo for sequenced σ^B promoters resulting from alignments of the sequenced σ^B promoters presented in Table 3 with known *L. monocytogenes* σ^B promoters (upstream from lmo0596, *prfA*, *fri*, *gbuA*, lmo1421, lmo0699, lmo1433, lmo2230, *opuCA*, *inlA*, lmo2434, *bsh*, and *rsbV*) (4, 9, 24, 30, 32). The logo is composed of stacks of letters for the positions in the sequence. The overall height of each stack indicates the sequence conservation at that position (measured in bits), while the height of each letter reflects the relative frequency of the corresponding nucleic acid at that position.

lowing three stress-related proteins were regulated negatively by σ^B: Lmo2362 (GadC), Lmo2363 (GadB), and Lmo1604. The *gadCB* operon encodes a glutamate decarboxylase system that plays a central role in acid tolerance in *L. monocytogenes* (14). The finding reported here that σ^B exerts negative control on GadC and GadB expression is somewhat surprising since *gadCB* transcription has previously been reported to be under positive σ^B control in *L. monocytogenes* (44). Wemekamp-Kamphuis et al. (44) reported σ^B-dependent induction of *gadCB* in response to acid stress. A close inspection of the data presented in that study revealed that there was indeed a higher level of *gadCB* transcription in the Δ*sigB* mutant under the control conditions when no acid stress was applied, consistent with the findings of the present study. It is interesting to speculate about the reason for the increase in GadCB expression in the Δ*sigB* mutant. It is possible that the elevated expression compensates for the lack of Lmo0913 expression. Lmo0913 is homologous to succinate semialdehyde dehydrogenase, an enzyme that participates in the catabolism of the intermediate γ-aminobutyrate (GABA), which itself is produced as a by-product of glutamate decarboxylation. GABA is known to be toxic to *B. subtilis* (7) and other microorganisms (2). Cotter et al. (15) have suggested that Lmo0913 could potentially compensate for the absence of an antiporter such as GadC by ensuring the removal of GABA. Accordingly, it is possible that GadC is expressed to compensate for reduced levels of Lmo0913 in the Δ*sigB* mutant. In this scenario GadC would prevent GABA accumulation that might result from the lack of Lmo0913 by exchanging GABA for an extracellular glutamate.

Previous attempts to define the σ^B consensus promoter sequence have relied on promoter sequences of *B. subtilis*, as well as a limited number of promoter sequences of *L. monocytogenes* (24). It is possible, therefore, that the proposed σ^B sequence may have a slight bias toward promoters that are similar to the *B. subtilis* consensus sequence. In an effort to refine the σ^B consensus sequence for *L. monocytogenes*, the four newly identified promoter sequences were compiled with all other known *L. monocytogenes* σ^B promoters (4, 9, 24, 30, 32). The Weblogo alignment tool (http://weblogo.berkeley.edu) was used to generate a consensus sequence from a total of 17 known *L. monocytogenes* σ^B-dependent promoter sequences (Fig. 6). The resulting consensus sequence can be defined as GTTTNW-$N_{13/14}$-GGGWADW (where N is any base, W is A or T, and D is A, T, or G). This sequence is broadly similar to the previously defined sequence (24) but differs in one key respect; the T at position 6 of the −10 element is not highly conserved in *L. monocytogenes* σ^B promoter sequences, since it is present in only 3 of the 17 sequences examined. In addition, there is a detectable preference for adenine or thymine at position 6 of the −35 element and at position 7 of the −10 element (Fig. 6). This refined consensus sequence should aid in the identification of potential σ^B promoter sequences in the *L. monocytogenes* genome. An analysis of the promoter regions upstream from the genes identified as being under positive σ^B control in the iTRAQ experiments (Table 4) revealed that 12 of the 14 σ^B-dependent genes possessed putative σ^B promoter sequences (allowing two mismatches from the newly defined consensus sequence).

Using complementary proteomic approaches, this study identified 38 proteins whose expression was influenced by deletion of σ^B in *L. monocytogenes*. The data indicate that σ^B plays a very significant role in controlling gene expression during the stationary phase of growth in complex media. Proteomic and physiological evidence demonstrated a novel role for σ^B in glycerol metabolism. An analysis of the functional groups represented in the data highlighted a clear role for σ^B in modulating the expression of genes involved in carbon metabolism and provided further support for the idea that σ^B plays a central role in stress tolerance in this human pathogen.

ACKNOWLEDGMENTS

We are grateful to members of the Bacterial Stress Response Group at National University of Ireland—Galway for useful discussions and for providing critical comments on the manuscript. We also thank Cyril Carroll for allowing us unrestricted access to the 2-DGE apparatus.

This work was supported by National Institutes of Health award RO1-AI052151-01A1 to K.J.B., by a Science Foundation Ireland Research Frontiers Programme grant, and by a Framework 6 Marie Curie Transfer of Knowledge grant.

REFERENCES

1. **Amezaga, M., I. Davidson, D. McLaggan, A. Verheul, T. Abee, and I. R. Booth.** 1995. The role of peptide metabolism in the growth of *Listeria monocytogenes* ATCC 23074 at high osmolarity. Microbiology **141:**41–49.
2. **Arst, H. J.** 1976. Integrator gene in *Aspergillus nidulans*. Nature **262:**231–234.
3. **Bailey, T. L., and M. Gribskov.** 1998. Combining evidence using p-values: application to sequence homology searches. Bioinformatics **14:**48–54.
4. **Becker, L. A., M. S. Çetin, R. W. Hutkins, and A. K. Benson.** 1998. Identification of the gene encoding the alternative sigma factor σ^B from *Listeria monocytogenes* and its role in osmotolerance. J. Bacteriol. **180:**4547–4554.
5. **Begley, M., C. Hill, and R. P. Ross.** 2006. Tolerance of *Listeria monocytogenes* to cell envelope-acting antimicrobial agents is dependent on SigB. Appl. Environ. Microbiol. **72:**2231–2234.
6. **Begley, M., R. D. Sleator, C. G. Gahan, and C. Hill.** 2005. Contribution of three bile-associated loci, *bsh*, *pva*, and *btlB*, to gastrointestinal persistence and bile tolerance of *Listeria monocytogenes*. Infect. Immun. **73:**894–904.

7. **Belitsky, B. R., and A. L. Sonenshein.** 2002. GabR, a member of a novel protein family, regulates the utilization of gamma-aminobutyrate in *Bacillus subtilis*. Mol. Microbiol. **45:**569–583.
8. **Birch, R. M., C. P. O'Byrne, I. R. Booth, and P. Cash.** 2003. Enrichment of *Escherichia coli* proteins by column chromatography on reactive dye columns. Proteomics **3:**764–776.
9. **Cetin, M. S., C. Zhang, R. W. Hutkins, and A. K. Benson.** 2004. Regulation of transcription of compatible solute transporters by the general stress sigma factor, σ^B, in *Listeria monocytogenes*. J. Bacteriol. **186:**794–802.
10. **Chatterjee, S. S., H. Hossain, S. Otten, C. Kuenne, K. Kuchmina, S. Machata, E. Domann, T. Chakraborty, and T. Hain.** 2006. Intracellular gene expression profile of *Listeria monocytogenes*. Infect. Immun. **74:**1323–1338.
11. **Christiansen, J. K., M. H. Larsen, H. Ingmer, L. Sogaard-Andersen, and B. H. Kallipolitis.** 2004. The RNA-binding protein Hfq of *Listeria monocytogenes*: role in stress tolerance and virulence. J. Bacteriol. **186:**3355–3362.
12. **Cleveland, W. S.** 1979. Robust locally weighted regression and smoothing scatterplots. J. Am. Stat. Assoc. **74:**829–836.
13. **Cleveland, W. S., and S. J. Devlin.** 1988. Locally-weighted regression: an approach to regression analysis by local fitting. J. Am. Stat. Assoc. **83:**596–610.
14. **Cotter, P. D., C. G. M. Gahan, and C. Hill.** 2001. A glutamate decarboxylase system protects *Listeria monocytogenes* in gastric fluid. Mol. Microbiol. **40:** 465–475.
15. **Cotter, P. D., S. Ryan, C. G. Gahan, and C. Hill.** 2005. Presence of GadD1 glutamate decarboxylase in selected *Listeria monocytogenes* strains is associated with an ability to grow at low pH. Appl. Environ. Microbiol. **71:**2832–2839.
16. **Dussurget, O., D. Cabanes, P. Dehoux, M. Lecuit, C. Buchrieser, P. Glaser, and P. Cossart.** 2002. *Listeria monocytogenes* bile salt hydrolase is a PrfA-regulated virulence factor involved in the intestinal and hepatic phases of listeriosis. Mol. Microbiol. **45:**1095–1106.
17. **Dussurget, O., E. Dumas, C. Archambaud, I. Chafsey, C. Chambon, M. Hebraud, and P. Cossart.** 2005. *Listeria monocytogenes* ferritin protects against multiple stresses and is required for virulence. FEMS Microbiol. Lett. **250:**253–261.
18. **Ferreira, A., C. P. O'Byrne, and K. J. Boor.** 2001. Role of σ^B in heat, ethanol, acid, and oxidative stress resistance and during carbon starvation in *Listeria monocytogenes*. Appl. Environ. Microbiol. **67:**4454–4457.
19. **Ferreira, A., D. Sue, C. P. O'Byrne, and K. J. Boor.** 2003. Role of *Listeria monocytogenes* σ^B in survival of lethal acidic conditions and in the acquired acid tolerance response. Appl. Environ. Microbiol. **69:**2692–2698.
20. **Fraser, K. R., D. Harvie, P. J. Coote, and C. P. O'Byrne.** 2000. Identification and characterization of an ATP binding cassette L-carnitine transporter in *Listeria monocytogenes*. Appl. Environ. Microbiol. **66:**4696–4704.
21. **Fraser, K. R., D. Sue, M. Wiedmann, K. Boor, and C. P. O'Byrne.** 2003. Role of σ^B in regulating the compatible solute uptake systems of *Listeria monocytogenes*: osmotic induction of *opuC* is σ^B dependent. Appl. Environ. Microbiol. **69:**2015–2022.
22. **Freitag, N. E., and D. A. Portnoy.** 1994. Dual promoters of the *Listeria monocytogenes prfA* transcriptional activator appear essential in vitro but are redundant in vivo. Mol. Microbiol. **12:**845–853.
23. **Garner, M. R., K. E. James, M. C. Callahan, M. Wiedmann, and K. J. Boor.** 2006. Exposure to salt and organic acids increases the ability of *Listeria monocytogenes* to invade Caco-2 cells but decreases its ability to survive gastric stress. Appl. Environ. Microbiol. **72:**5384–5395.
24. **Kazmierczak, M. J., S. C. Mithoe, K. J. Boor, and M. Wiedmann.** 2003. *Listeria monocytogenes* σ^B regulates stress response and virulence functions. J. Bacteriol. **185:**5722–5734.
25. **Kim, H., K. J. Boor, and H. Marquis.** 2004. *Listeria monocytogenes* σ^B contributes to invasion of human intestinal epithelial cells. Infect. Immun. **72:**7374–7378.
26. **McGann, P., M Wiedmann, and K. J. Boor.** 2007. The alternative sigma factor σ^B and the virulence gene regulator PrfA both regulate transcription of *Listeria monocytogenes* internalins. Appl. Environ. Microbiol. **73:**2919–2930.
27. **Milohanic, E., P. Glaser, J. Y. Coppee, L. Frangeul, Y. Vega, J. A. Vazquez-Boland, F. Kunst, P. Cossart, and C. Buchrieser.** 2003. Transcriptome analysis of *Listeria monocytogenes* identifies three groups of genes differently regulated by PrfA. Mol. Microbiol. **47:**1613–1625.
28. **Nadon, C. A., B. M. Bowen, M. Wiedmann, and K. J. Boor.** 2002. Sigma B contributes to PrfA-mediated virulence in *Listeria monocytogenes*. Infect. Immun. **70:**3948–3952.
29. **O'Farrell, P. H.** 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250:**4007–4021.
30. **Olsen, K. N., M. H. Larsen, C. G. M. Gahan, B. Kallipolitis, X. A. Wolf, R. Rea, C. Hill, and H. Ingmer.** 2005. The Dps-like protein Fri of *Listeria monocytogenes* promotes stress tolerance and intracellular multiplication in macrophage-like cells. Microbiology **151:**925–933.
31. **Petersohn, A., M. Brigulla, S. Haas, J. D. Hoheisel, U. Volker, and M. Hecker.** 2001. Global analysis of the general stress response of *Bacillus subtilis*. J. Bacteriol. **183:**5617–5631.
32. **Rauch, M., Q. Luo, S. Muller-Altrock, and W. Goebel.** 2005. SigB-dependent in vitro transcription of *prfA* and some newly identified genes of *Listeria monocytogenes* whose expression is affected by PrfA in vivo. J. Bacteriol. **187:**800–804.
33. **Ross, P. L., Y. N. Huang, J. N. Marchese, B. Williamson, K. Parker, S. Hattan, N. Khainovski, S. Pillai, S. Dey, S. Daniels, S. Purkayastha, P. Juhasz, S. Martin, M. Bartlet-Jones, F. He, A. Jacobson, and D. J. Pappin.** 2004. Multiplexed protein quantitation in *Saccharomyces cerevisiae* using amine-reactive isobaric tagging reagents. Mol. Cell. Proteomics **3:**1154–1169.
34. **Satorhelyi, P.** 2005. Microarray-analyse der pH-stressantwort von *Listeria monocytogenes* und *Corynebacterium glutamicum*. Technische Universitat Munchen, Munich, Germany.
35. **Schwab, U., B. Bowen, C. Nadon, M. Wiedmann, K. J. Boor.** 2005. The *Listeria monocytogenes prfA*P2 promoter is regulated by sigma B in a growth phase dependent manner. FEMS Microbiol. Lett. **245:**329–336.
36. **Sleator, R. D., H. H. Wemekamp-Kamphuis, C. G. M. Gahan, T. Abee, and C. Hill.** 2005. A PrfA-regulated bile exclusion system (BilE) is a novel virulence factor in *Listeria monocytogenes*. Mol. Microbiol. **55:**1183–1195.
37. **Sleator, R. D., J. Wouters, C. G. M. Gahan, T. Abee, and C. Hill.** 2001. Analysis of the role of OpuC, an osmolyte transport system, in salt tolerance and virulence potential of *Listeria monocytogenes*. Appl. Environ. Microbiol. **67:**2692–2698.
38. **Sokolovic, Z., and W. Goebel.** 1989. Synthesis of listeriolysin in *Listeria monocytogenes* under heat shock conditions. Infect. Immun. **57:**295–298.
39. **Sokolovic, Z., J. Riedel, M. Wuenscher, and W. Goebel.** 1993. Surface-associated, PrfA-regulated proteins of *Listeria monocytogenes* synthesized under stress conditions. Mol. Microbiol. **8:**219–227.
40. **Stack, H. M., R. D. Sleator, M. Bowers, C Hill, and C. G. M. Gahan.** 2005. Role for HtrA in stress induction and virulence potential in *Listeria monocytogenes*. Appl. Environ. Microbiol. **71:**4241–4247.
41. **Stancik, L. M., D. M. Stancik, B. Schmidt, D. M. Barnhart, Y. N. Yoncheva, and J. L. Slonczewski.** 2002. pH-dependent expression of periplasmic proteins and amino acid catabolism in *Escherichia coli*. J. Bacteriol. **184:**4246–4258.
42. **Sue, D., D. Fink, M. Wiedmann, and K. J. Boor.** 2004. σ^B-dependent gene induction and expression in *Listeria monocytogenes* during osmotic and acid stress conditions simulating the intestinal environment. Microbiology **150:** 3843–3855.
43. **Vazquez-Boland, J. A., M. Kuhn, P. Berche, T Chakraborty, G. Dominguez-Bernal, W. Goebel, B. Gonzalez-Zorn, J. Wehland, and J. Kreft.** 2001. *Listeria* pathogenesis and molecular virulence determinants. Clin. Microbiol. Rev. **14:**584–640.
44. **Wemekamp-Kamphuis, H. H., J. A. Wouters, P. P. de Leeuw, T. Hain, T. Chakraborty, and T. Abee.** 2004. 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. Appl. Environ. Microbiol. **70:** 3457–3466.
45. **Wemekamp-Kamphuis, H. H., J. A. Wouters, R. D. Sleator, C. G. M. Gahan, C. Hill, and T. Abee.** 2002. Multiple deletions of the osmolyte transporters BetL, Gbu, and OpuC of *Listeria monocytogenes* affect virulence and growth at high osmolarity. Appl. Environ. Microbiol. **68:**4710–4716.
46. **Wiedmann, M., T. J. Arvik, R. J. Hurley, and K. J. Boor.** 1998. General stress transcription factor σ^B and its role in acid tolerance and virulence of *Listeria monocytogenes*. J. Bacteriol. **180:**3650–3656.