THE ALTERNATIVE SIGMA FACTORS OF *LISTERIA MONOCYTOGENES*: STRESS RESPONSE REGULATION AT THE LEVEL OF NON-CODING RNAs AND PROTEINS

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THE ALTERNATIVE SIGMA FACTORS OF *LISTERIA MONOCYTOGENES*: STRESS RESPONSE REGULATION AT THE LEVEL OF NON-CODING RNAs AND PROTEINS

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The foodborne pathogen *Listeria monocytogenes* is ubiquitous in the environment and can cause a life-threatening invasive infection in humans. *L. monocytogenes* has the ability to survive a wide range of environmental and physiological stress conditions through complex stress response and virulence mechanisms. Among the key mechanisms *L. monocytogenes* employs to respond to changing conditions, including those encountered during growth on food and in the infection process, is the regulation of gene, protein, and non-coding RNA (ncRNA) expression through the regulatory network of the alternative sigma factors, σ^B, σ^C, σ^H, and σ^L. In these studies we (i) explored σ^B-dependent regulation of the ncRNA SbrE, and (ii) determined σ^B, σ^C, σ^H, and σ^L regulons at the protein level using a quantitative proteomics approach.

We demonstrated σ^B-dependent transcription of SbrE and identified putative targets of SbrE at the transcriptomic and proteomic level. We also found increased transcript levels of SbrE in stationary phase and after exposure of *L. monocytogenes* to oxidative stress. Our proteomic analysis of *L. monocytogenes* parent strain 10403S and a Δ*sigB* mutant combined with a meta-analysis of published transcriptomic studies identified 149 genes and proteins as positively regulated by σ^B at either or both the

transcript and protein level, and suggested contributions of σ^B to gene expression through direct regulation of gene transcription and through indirect mechanisms, including regulation of ncRNA.

Finally, our comparison of the protein expression profiles of parent strain 10403S and a quadruple mutant Δ*sigBCHL*, and comparisons of Δ*sigBCHL* with triple mutants, Δ*sigBCH*, Δ*sigBCL*, and Δ*sigBHL*, characterized the independent regulons of σ^L, σ^H, and σ^C at the protein level, as well as co-regulation and protein expression in the absence of all four alternative σ factors. Co-regulated proteins identified included MptA, which has a potential role in regulation of PrfA, a transcriptional activator of *L. monocytogenes* virulence genes. These studies identify and characterize components of the complex regulatory network of *L. monocytogenes* alternative σ factors and illustrate co-regulation of gene expression by multiple alternative σ factors, which contributes to our understanding of the alternative σ factor dependent stress response and virulence abilities of *L. monocytogenes*.

BIOGRAPHICAL SKETCH

Sana Mujahid earned a Bachelor of Science degree in Biological Sciences with a minor in Psychology from Mississippi State University. She went on to complete a Master of Science degree in Veterinary Medical Science with a research concentration in Molecular Microbiology and a minor in Biochemistry and Molecular Biology, at Mississippi State University. During her time as a master's student, Sana also interned with the United States Agency for International Development on an international public health project. Sana's interest in public health and microbiology brought her to the Food Science Department at Cornell University in 2008 to start her doctoral degree with a concentration in Food Microbiology and minors in Nutrition and International Development.

For Nani jaan, Amma, Baba, and the little Khanums

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CHAPTER 1

INTRODUCTION

Listeria monocytogenes, the causative agent of listeriosis, is a foodborne pathogen that is responsible for approximately 1,500 illnesses and 250 deaths per year in the United States [1]. Listeriosis develops as non-invasive febrile gastroenteritis in immunocompetent individuals or manifests in its invasive form, as septicemia or meningoencephalitis, in immunocompromised adults. Abortion, stillbirth, and invasive neonatal infection can also occur due to perinatal listeriosis [2]. It is estimated that 99% of all human listeriosis cases are due to the consumption of contaminated food products [1]. Along with its ability to invade and survive within mammalian host cells, *L. monocytogenes* is ubiquitous in the environment and has been isolated from various environmental locations, including soil, ground water, and silage [3]. *L. monocytogenes* strains can be grouped into four genetic lineages, i.e., lineage I, II, III, and IV [4]. Strains belonging to lineage II are most commonly found among food products as well as food-related and natural environments, and are also associated with sporadic human clinical listeriosis cases and animal clinical cases. Lineage I strains are overrepresented among human clinical cases, while lineage III and IV strains are commonly isolated from animals [4].

L. monocytogenes has the ability to survive and multiply under a diverse range of environmental stresses, making it easy for the bacterium to overcome stresses of common food storage and preservation conditions such as refrigeration, low pH, and salt. *L. monocytogenes* also overcomes stresses inside the host to cause infection. In

particular, orally ingested *L. monocytogenes* has the ability to survive the gastric passage and intestinal tract and, in a series of steps, invade and colonize host cells [5]. The development of effective strategies for the prevention and treatment of listeriosis thus requires an in-depth understanding of the regulatory mechanisms that allow *L. monocytogenes* to persist in food processing environments, be transmitted through the food chain, and cause human infections [5].

Among the central mechanisms *L. monocytogenes* employs to respond to changing environmental conditions is the regulation of gene expression through the alternative sigma factors, σ^B, σ^C, σ^H, and σ^L. The association of a particular alternative sigma factor with the core RNA polymerase reprograms the RNA polymerase holoenzyme to recognize specific promoter regions and allows for direct rapid induction of transcription of groups of genes, under specific environmental conditions [6]. As bacteria are exposed to changing environmental conditions, alternative sigma factors can thus directly or indirectly, through regulatory networks, modulate the expression of certain genes, proteins, and small non-coding RNAs (ncRNAs) that enable the bacteria to rapidly adapt to the specific conditions. The four alternative sigma factors of *L. monocytogenes*, σ^B, σ^C, σ^H, and σ^L, accordingly contribute to the response of *L. monocytogenes* to various environmental stress conditions. A number of studies exploring σ^B mediated stress response in *L. monocytogenes* have shown that this alternative sigma factor contributes to the survival of the bacterium under several stress conditions, including acid, osmotic, oxidative, and energy stress [6-9]. The contributions of σ^H, σ^L, and σ^C to *L. monocytogenes* stress response have not been as extensively explored as the contributions of σ^B. However, studies suggest that σ^H,

σ^L, and σ^C play a role in the response to specific stresses encountered by *L. monocytogenes* [9-15]. σ^C has only been described in *L. monocytogenes* strains that group into lineage II [6].

Once consumed, *L. monocytogenes* has the ability to cross the intestinal, blood-brain, and placental barriers in humans [16]. Among the *L. monocytogenes* alternative sigma factors, σ^B has been found to contribute to *L. monocytogenes* virulence, in a guinea pig listeriosis infection model, across strains representing the four lineages of *L. monocytogenes* [8]. σ^B directly regulates the transcription of several *L. monocytogenes* virulence genes, including the gene encoding PrfA, the major regulator of virulence genes in *L. monocytogenes* [6, 17]. σ^B has also been shown to contribute to the regulation of ncRNA expression during *L. monocytogenes* infection of host cells [18]. Limited contributions of σ^C, σ^H, and σ^L to *L. monocytogenes* virulence have been identified to date [9]. Along with the independent regulons of each of the alternative sigma factors, overlapping regulons exist among σ^B, σ^C, σ^H, and σ^L and among these alternative sigma factors and other transcriptional regulators [9]. Many *L. monocytogenes* stress response and virulence genes may be co-regulated by multiple alternative sigma factors, allowing *L. monocytogenes* to rapidly regulate gene expression via a complex regulatory network.

The alternative sigma factors, σ^B, σ^C, σ^H, and σ^L, thus work together in a network that is critical for *L. monocytogenes* stress response and virulence and that includes regulatory roles of genes, ncRNAs, and proteins. To date, there are no published studies on the response of *L. monocytogenes* to the loss of all four alternative sigma factors together and limited information on regulation by the

alternative sigma factors at the protein level. There is also limited information available on alternative sigma factor dependent ncRNAs in *L. monocytogenes*. In this work, we i) explored the role of the σ^B-dependent ncRNA SbrE in *L. monocytogenes* stress response, ii) developed a comprehensive definition of the *L. monocytogenes* σ^B regulon at the transcriptomic and protein level using quantitative proteomics analysis and a meta-analysis of published transcriptomic studies, and iii) characterized protein regulation by *L. monocytogenes* in the absence of all four alternative sigma factors and determined the regulons of σ^C, σ^H, and σ^L at the protein level, using quantitative proteomics.

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CHAPTER 2

EXPLORATION OF THE ROLE OF THE NON-CODING RNA SBRE IN *L. MONOCYTOGENES* STRESS RESPONSE[1]

ABSTRACT

SbrE is a ncRNA in *Listeria monocytogenes*, reported to be up-regulated by the alternative sigma factor σ^B. Initial quantitative RT-PCR (qRT-PCR) experiments on parent strains and isogenic Δ*sigB* strains demonstrated σ^B-dependent expression of SbrE across the four *L. monocytogenes* lineages and in *L. innocua*. Microarray and proteomics (MDLC/MS/MS with iTRAQ labeling) experiments with the *L. monocytogenes* parent strain and an isogenic Δ*sbrE* strain identified a single gene (*lmo0636*) and two proteins (Lmo0637 and Lmo2094) that showed lower expression levels in the Δ*sbrE* strain. qRT-PCR demonstrated an increase in SbrE transcript levels in stationary phase *L. monocytogenes* and in bacteria exposed to oxidative stress (mean log2 transcript levels 7.68 $\pm$ 0.57 and 1.70 $\pm$ 0.71 greater than in mid-log phase cells, respectively). However, no significant differences in growth or survival between the parent strain and Δ*sbrE* strain were confirmed under a variety of environmental stress conditions tested. Our data suggest that σ^B-dependent transcription of SbrE represents a conserved mechanism that contributes, across *Listeria* species, to fine-tuning of gene expression under specific environmental conditions that remain to be defined.

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INTRODUCTION

Listeria monocytogenes is a Gram-positive foodborne pathogen that causes listeriosis, a life threatening invasive illness in humans and animals [1]. *L. monocytogenes* is ubiquitous in the environment and has the ability to adapt to harsh and stressful conditions. For instance, the bacterium is able to grow at refrigeration temperatures and can survive high salt concentrations as well as acidic conditions [2-5]. This ability to adapt to harsh and stressful conditions facilitates *L. monocytogenes* survival under environmental, food, and host associated stress conditions. A complex transcriptional response network consisting of various signaling pathways and transcriptional regulators, including alternative sigma factors and non-coding RNAs (ncRNAs), supports the ability of *L. monocytogenes* to respond to and survive under a wide range of stress conditions [6-14].

ncRNAs have been shown to be involved in a variety of regulatory functions in bacteria, including regulation of bacterial response to stress and virulence related functions, through transcriptional, translational, and post-transcriptional regulation of gene expression [15-27]. In *L. monocytogenes*, more than 100 ncRNAs have been identified to date, including ncRNAs involved in virulence and stress response [7,11,14,28-34]. The alternative sigma factor Sigma B (σ^B) is estimated to regulate transcription of 100 to 200 *L. monocytogenes* genes and contributes critically to the ability of this pathogen to survive stressful conditions encountered inside and outside the host [7,8,11-13]. σ^B is involved in the transcriptional response of *L. monocytogenes* to a variety of stresses, including osmotic and acid stress, as well as the regulation of metabolism and virulence [6,8,13]. *In vitro* and/or *in vivo* studies

indicate that σ^B also directly regulates at least four ncRNAs in *L. monocytogenes* [7,11,30,31], in addition to possibly regulating ncRNAs indirectly by affecting transcription of *hfq*, which encodes a protein (Hfq) that binds to and regulates ncRNAs [7,11,27,35]. One σ^B-dependent *L. monocytogenes* ncRNA is SbrE (also referred to as *rli47*), which was found to be highly transcribed in stationary phase cells using RNA-Sequencing (RNA-Seq) [7]. A study using tiling arrays also found SbrE to be expressed at higher levels in stationary phase cells and in the intestinal lumen compared to exponential phase cells [11]. In addition, SbrE appears to be transcribed at higher levels in macrophages compared to exponential phase cells [36]. The 514 nucleotide sequence for SbrE is 96.6% conserved among 18 *L. monocytogenes* genomes, including EGD-e and F2365, and was found to be present in one *L. innocua* and one *L. welshimeri* genome [7]. In addition to identification of a putative σ^B-dependent promoter upstream of SbrE, SbrE has been reported to show σ^B-dependent transcript levels in *L. monocytogenes* strain 10403S [7] and EGD-e [11]. SbrE was also found to show σ^B-dependent transcript levels in exponential phase cells and in *L. monocytogenes* present in the intestinal lumen, but not in *L. monocytogenes* inoculated into human blood [11]. As the role of SbrE has not yet been defined, we employed transcriptomic, proteomic, and phenotypic approaches to characterize the role of SbrE in σ^B-dependent stress responses.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

Strains used in this study are listed in Table 2.1. Stock cultures of all strains were stored at -80°C in Brain Heart Infusion (BHI) medium containing 15% glycerol. Cultures were streaked onto BHI agar and incubated at 37°C for 24 h to obtain isolated colonies for inoculation of overnight cultures. Specific growth conditions for each experiment are described below.

Table 2.1: Strains used in this study

Strain	Lineage	Serotype	Origin
10403S	II	1/2a	Laboratory type strain
FSL A1-254, *ΔsigB*	II	1/2a	10403S (Wiedmann *et al*. [42])
FSL B2-236, *ΔsbrE*	II	1/2a	10403S
FSL J1-194	I	1/2b	Human clinical case
FSL C6-001, *ΔsigB*	I	1/2b	FSL J1-194 (Oliver *et al*. [8])
FSL J2-071	IIIA	4c	Bovine clinical case
FSL O1-006, *ΔsigB*	IIIA	4c	FSL J2-071 (Oliver *et al*. [8])
FSL J1-208	IV	4a	Caprine clinical case
FSL O1-005, *ΔsigB*	IV	4a	FSL J1-208 (Oliver *et al*. [8])
FSL C2-008			*L. innocua* DD 680
FSL R4-009, *ΔsigB*			*L. innocua* DD 680 (Raengpradub *et al*. [10])
DP-L3903, Erm^r			10403S (Auerbuch *et al*. [43])

Construction of *L. monocytogenes* mutants.

A nonpolar internal deletion mutant allele of *sbrE* was created by splicing by overlap extension (SOE) PCR and allelic mutagenesis, using previously described procedures [44]. Allelic exchange mutagenesis of the wildtype *sbrE* allele with the mutant allele was confirmed by PCR amplification and direct sequencing of the PCR product (see Appendix 2.7 for primers).

TaqMan quantitative RT-PCR (qRT-PCR) to measure *sbrE* and *lmo0636* transcript levels.

qRT-PCR was used to quantify (i) *sbrE* transcript levels in parent and Δ*sigB* mutant strains representing the different lineages of *L. monocytogenes* as well as one *L. innocua* strain, and (ii) *lmo0636* transcript levels in *L. monocytogenes* parent strain10403S and its isogenic Δ*sbrE* null mutant. Briefly, cells were grown to stationary phase at 37°C as previously described [10], with shaking at 230 rpm. After cells reached stationary phase, RNAProtect bacterial reagent (Qiagen) was used to stabilize the mRNA according to manufacturer's instructions. Bacterial cells were collected by centrifugation and stored at -80˚C prior to RNA isolation. RNA extraction was performed using TRI reagent as described previously [45]. Total RNA was incubated with RNasin (Promega, Madison, WI) and RQ1 DNase (Promega) to inhibit RNases and remove DNA contamination, respectively. Further RNA cleanup and concentration was performed using the RNeasy MinElute Cleanup Kit (Qiagen). A NanoDrop ND-1000 spectrophotometer (NanoDrop, Rockland, DE) was used to quantify and assess purity of the RNA. RNA quality and integrity was assessed by the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA).

One microgram of RNA from each sample was reverse transcribed to cDNA using random hexamers and reverse transcriptase (TaqMan Reverse Transcription Reagents, Applied Biosystems, Carlsbad, CA) prior to qRT-PCR. To evaluate residual genomic DNA contamination, control reactions without reverse transcriptase were included for each template. qRT-PCR was performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems), using the TaqMan Universal PCR Master Mix Reagent (Applied Biosystems). Duplicate qRT-PCR reactions were loaded into MicroAmp optical 96-well reaction plates and run using the following program: 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Standard curves for each target template were included to determine the amplification efficiency. All qRT-PCR analyses were performed in triplicate using RNA isolated from three independent biological replicates of cells (see Appendix 2.7 for primers and probes). Relative gene transcription levels, i.e. fold changes, were calculated using the efficiency calibrated mathematical model described by Pfaffl [46]. Target transcript levels were normalized to transcript levels of the housekeeping gene *rpoB*, which displays relatively stable transcript levels under varying experimental conditions [8].

qRT-PCR to determine growth phase and environmental stress dependent *sbrE* transcript levels.

qRT-PCR was used to measure *sbrE* transcript levels in mid-log phase (OD_{600} 0.4), late log phase (OD_{600} 1.0), early stationary phase (OD_{600} 1.0 + 3h), and after exposure of mid-log phase cells to either (i) 13 mM cumene hydroperoxide (CHP) (Sigma-Aldrich), 15 min (as described by Oliver *et al.*, [8]), or (ii) 10% NaCl, 15 min.

L. monocytogenes 10403S cells were grown as described above. To apply salt stress, an equal volume inoculum of mid-log phase cells was transferred to 5 ml 20% NaCl, and cultures were then incubated at 37°C with shaking for 15 min. RNA extraction, cDNA synthesis, and qRT-PCR were performed as described above, with the exception that RNA was purified using two phenol-chloroform extractions and one chloroform extraction, followed by RNA precipitation and resuspension in RNase-free water, instead of the RNeasy MinElute Cleanup Kit (Qiagen) procedure described above. Relative gene transcription levels were calculated using the Pfaffl model as described above [46] with target transcript levels normalized to transcript levels of *rpoB* within samples. Results from all samples were normalized to SbrE transcript levels from a single replicate at mid-log phase (OD 0.4) [47].

Microarray.

L. monocytogenes 10403S parent strain, Δ*sbrE*, and Δ*sigB* cells were grown to stationary phase and total RNA was extracted as described above for qRT-PCR analysis. DNA Microarray design and construction were described in a previous study [10]. cDNA synthesis, labeling with dyes, and hybridization were performed as described by Ollinger *et al*. [9], with the exception that samples were labeled with Cy3 and Cy5 dyes (Amersham Biosciences, Piscataway, NJ). Three replicates using three independent RNA isolations were performed for each microarray comparison (Δ*sbrE* versus parent strain; Δ*sbrE* versus Δ*sigB*). Microarray statistical analysis was performed as described previously [10]. A *n*-fold change of $\geq$ 1.5 was used as the cutoff for the identification of differentially expressed genes.

Protein isolation, digestion, and iTRAQ labeling.

L. monocytogenes 10403S and Δ*sbrE* were grown to stationary phase as described above. After growth to stationary phase, bacterial cells from 25 ml of culture were collected by centrifugation. Cell pellets were quick-frozen with liquid nitrogen and stored at -80˚C prior to protein isolation. Proteins were isolated as previously described [48] with slight modifications. Briefly, cell pellets were washed in 1 M triethyl ammonium bicarbonate buffer (pH 8.5) (Sigma) with 0.1% (wt/vol) SDS and 10 µg/ml chloramphenicol (extraction buffer [EB]). Cells were then lysed using a bead beater (BioSpec Mini-Beadbeater-1) in a mixture of 0.5 mm zirconia/silica beads and 1 ml EB. The protein sample was separated from beads by centrifugation and protein concentrations were determined using a noninterfering protein assay kit with bovine serum albumin as the standard (Calbiochem, USA) according to the manufacturer's instructions. 1D SDS-PAGE was used to verify sample protein concentration and quality.

Protein samples were analyzed at the Cornell University Proteomics and Mass Spectrometry Core Facility using shotgun-based quantitative proteomics. A total of 100 µg protein of each sample was denatured, reduced with 5 mM tris-(2-carboxylethyl) phosphine at 37ºC for 1 h and the cysteine residues were blocked with 8 mM methyl methanethiosulfonate for 10 min at room temperature. Protein samples were digested with 10 µg of sequence-grade-modified trypsin at 37ºC for 16 h. Efficiency of protein digestion was assessed by SDS-PAGE. Tryptic peptides from *L. monocytogenes* parent strain 10403S and Δ*sbrE* were each labeled with iTRAQ reagents, according to the manufacturer's protocols (document #4351918A and

4350831C downloaded from http://docs.appliedbiosystems.com/search.taf; Applied Biosystems). The labeled samples were then combined and fractionated via Isoelectric focusing (IEF) OffGel electrophoresis (OGE) as described below.

OGE fractionation and Nano-scale reverse phase chromatography and tandem mass spectrometry (nanoLC-MS/MS).

The pooled iTRAQ labeled peptides were separated using an Agilent 3100 OFFGEL Fractionator (Agilent, G3100AA) as described by Yang *et al*. [49]. Subsequent nanoLC-MS/MS was carried out using a LTQ-Orbitrap Velos (Thermo-Fisher Scientific) mass spectrometer equipped with a nano-ion source as reported previously [49], with the Orbitrap Velos operated in positive ion mode with nano spray voltage set at 1.5 kV and source temperature at 175 °C.

Data processing, protein identification, and data analysis.

All MS and MS/MS raw spectra from iTRAQ experiments were processed using Proteome Discoverer 1.1 (PD1.1, Thermo) for subsequent database search using in-house licensed Mascot Daemon (version 2.2.04, Matrix Science); quantitative processing, protein identification, and data analysis were conducted as described by Yang *et al*. [49], with some modifications. Briefly, the *L. monocytogenes* protein sequence database containing 4,177 sequence entries downloaded from the Broad Institute (http://www.broadinstitute.org/annotation/genome/listeria_group/GenomesIndex.html) on May 22nd, 2009 was used for database search. The default Mascot search settings included (i) one missed cleavage for full trypsin with fixed MMTS modification of cysteine, (ii) fixed 4-plex iTRAQ modifications on lysine and N-terminal amines, and

(iii) variable modifications of methionine oxidation and 4-plex iTRAQ on tyrosine. The peptide mass tolerance and fragment mass tolerance values were 10 ppm and 30 mDa, respectively. To estimate the false discovery rate (FDR), an automatic decoy database search was performed in Mascot. The relative quantitation ratios were normalized (bias-corrected) using the "median ratio" procedure for the iTRAQ 4-plex in each set of experiments. Two biological replicates were analyzed independently.

The Wilcoxon signed rank test was applied to peptide ratios for each identified protein to determine significant changes between strains. The Fisher's Combined Probability Test was used to combine FDR adjusted Wilcoxon *p*-values from each replicate into one test statistic for every protein to obtain a combined *p*-value. Proteins with peptide ratios exhibiting a Fisher's Combined Probability Test *p*-value < 0.05 and an iTRAQ protein ratio ≥ 1.2 in both replicates were considered significantly differentially expressed. Statistical analyses were conducted using R statistical software.

Determination of acid and oxidative stress resistance as well as phage resistance.

Acid and oxidative stress survival of Δ*sbrE* was compared to Δ*sigB* and the 10403S parent strain. Cells were grown to stationary phase (OD_{600} of 1.0 + 3 h) as described above. For acid stress experiments, 12N HCl was added to 5 ml aliquot of stationary phase cells to reduce the culture pH to 2.5 as described previously [8]. Bacterial cells were quantified at 10, 30 and 60 min after addition of HCl by plating on BHI agar using a spiral plater (Autoplate 4000; Spiral Biotech, Inc., Norwood, MA). Three independent replicates were performed.

For oxidative stress experiments, 900 µl of stationary phase cells were exposed to 13 mM CHP for 15 min at 37°C as described previously [8]. Bacterial numbers were quantified by plating as described above. Three independent replicates were performed. In separate experiments, stationary phase cells were also exposed to CHP as described above over a 60 min period, and bacterial numbers were quantified by plating as described above at 15 min, 30 min, and 60 min. At least three independent replicates were performed.

Survival of Δ*sbrE* and Δ*sigB* mutants after CHP stress was also examined using competitive-index experiments. Strains used for these experiments included the erythromycin sensitive parent strain 10403S (Erm^S 10403S) and an erythromycin-resistant 10403S derivative (DP-L3903; Erm^R 10403S). Oxidative stress exposure (13 mM CHP) was applied as described above to strains mixed in a 1:1 ratio including (i) Erm^S Δ*sbrE* and Erm^R 10403S and (ii) Erm^S Δ*sigB* and the Erm^R 1043S (to evaluate the relative survival of the Δ*sbrE* and Δ*sigB* compared to 10403S). A control competition experiment was conducted with a 1:1 mixture of Erm^S 10403S and Erm^R 10403S. Competition experiments conducted with and without 13mM CHP were plated on BHI and incubated at 37°C for 24 h. A hundred colonies from BHI plates were patched onto BHI agar containing 1 µg erythromycin/ml (BHI-erm). For differential enumeration, total colonies on BHI-erm were subtracted from total colonies on BHI (100). The competitive index was then calculated as the ratio of Erm^R to Erm^S colonies [43,50].

L. monocytogenes 10403S as well as Δ*sbrE* and Δ*sigB* strains were also tested for resistance against 22 diverse listeriaphages, using the procedures described by Vongkamjan *et al.* [51].

Salt, cold, and energy stress growth experiments.

Growth of 10403S Δ*sbrE*, and Δ*sigB* strains under salt, cold, and energy stress conditions was compared. For salt growth experiments, cells were grown to mid-log phase (OD_{600} of 0.4) as described above. A 0.01% inoculum (vol/vol) was transferred to 50 ml pre-warmed BHI broth supplemented with 1.75 M NaCl in a 300 ml nephelo flask (5 ul into 50 ml). Cells were then incubated for 48 h at 37°C with shaking (230 rpm). Cell numbers were determined, by plating on BHI agar, at specific time points over 48 h.

For cold growth experiments cells were grown to mid-log phase (OD_{600} of 0.4) as described above. A 0.01% inoculum (vol/vol) was transferred to pre-chilled 50 ml BHI broth in a 300 ml nephelo flask. Cells were then incubated at 7°C for 12 days without shaking, and cell numbers were determined by plating on BHI agar using a spiral plater. For energy stress experiments, carbon starvation was induced by growing cells in defined medium (DM) containing a growth-limiting concentration of glucose (0.04%, wt/vol) [52]. Cells were initially grown in 5 ml of BHI broth at 37°C overnight with shaking (230 rpm). A 0.1 ml aliquot of the overnight culture was inoculated into 10 ml DM supplemented with 0.4% (wt/vol) glucose and incubated for 12 h with shaking (230 rpm), followed by inoculation of a 0.01 ml aliquot into 10 ml pre-warmed DM containing 0.04% glucose and subsequent incubation for 30 h at 37°C with aeration. Cell numbers were determined by plating on BHI agar using a

spiral plater at specific time points over 30 h. Three biological replicates were performed for each growth experiment.

Statistical analyses of stress experiments.

The Baranyi model [53] was used to estimate maximum growth rates (μ_{max}) for cold stress experiments, using the NLStools package (v 0.0-5) in R v2.6.2. All other statistical analyses were performed with the Statistical Analysis Software (SAS) 9.0 (SAS Institute, Inc., Cary, NC). Regression analysis was used to calculate the death rate of cells exposed to pH 2.5, which was expressed as average log CFU death per hour for each strain. Analysis of variance (ANOVA) was used to test if there was significant difference in the death rates between the parent strain (10403S), Δ*sbrE*, and Δ*sigB*. ANOVA was also used to test for (i) differences in cell death due to oxidative stress; (ii) differences in growth rate (μmax) of cells exposed to cold stress, which was expressed as increase in cell density in log10 CFU/ml per day, (iii) differences in bacterial numbers after exposure to salt stress and growth under energy stress. Significance was set at $p < 0.05$ for all statistical analyses.

RESULTS AND DISCUSSION

In this study, we demonstrate that (i) SbrE is σ^B-dependent across *L. monocytogenes* lineages and in the non-pathogenic species *L. innocua*, and SbrE transcript levels are induced in stationary phase and under oxidative stress; (ii) SbrE contributes to the expression of an operon composed of *lmo0636* and *lmo0637*; (iii) contributions of SbrE to *L. monocytogenes* survival and growth under different stress conditions could not be identified, suggesting that SbrE may play a role in "fine-

tuning of gene expression" in *L. monocytogenes*, which may only have phenotypic consequences under very specific growth conditions, as previously suggested for SbrA, another σ^B-dependent ncRNA in *L. monocytogenes* [30].

SbrE is σ^B-dependent across *L. monocytogenes* lineages and induced in stationary phase and under oxidative stress.

qRT-PCR showed that, in stationary phase bacteria, SbrE transcript levels were significantly higher in parent strains relative to their Δ*sigB* mutants in (i) four strains representing all four *L. monocytogenes* lineages (4.8 ± 1.76 to 8.6 ± 0.67 higher log2 SbrE transcript in the parent strain) and (ii) an *L. innocua* strain (Figure 2.1), supporting σ^B-dependent transcription of SbrE across *L. monocytogenes* lineages and in *L. innocua*. While these findings were not necessarily unexpected, they are still valuable as other studies have shown some diversification of the σ^B regulon and variation in σ^B-dependent regulation of conserved genes, among *L. monocytogenes* lineages and *Listeria* species [10,37].

qRT-PCR of SbrE transcripts in the *L. monocytogenes* strain 10403S showed significantly higher transcript levels in early stationary phase cells (OD 1.0 + 3h) as compared to mid-log phase (OD 0.4) or late log phase (OD 1.0) cells, consistent with σ^B-dependent transcription of SbrE (as σ^B is induced in stationary phase cells [7,11]. While SbrE transcript levels were not induced after exposure of mid-log phase cells to salt stress (Appendix 2.1), they were induced after exposure to oxidative stress (13 mM cumene hydroperoxide [CHP]). SbrE transcript levels were 1.70 $\pm$ 0.71 log2 (approximately 2.0 to 5.5 absolute, non log-transformed fold changes) higher in CHP treated cells, relative to mid-log phase cells.

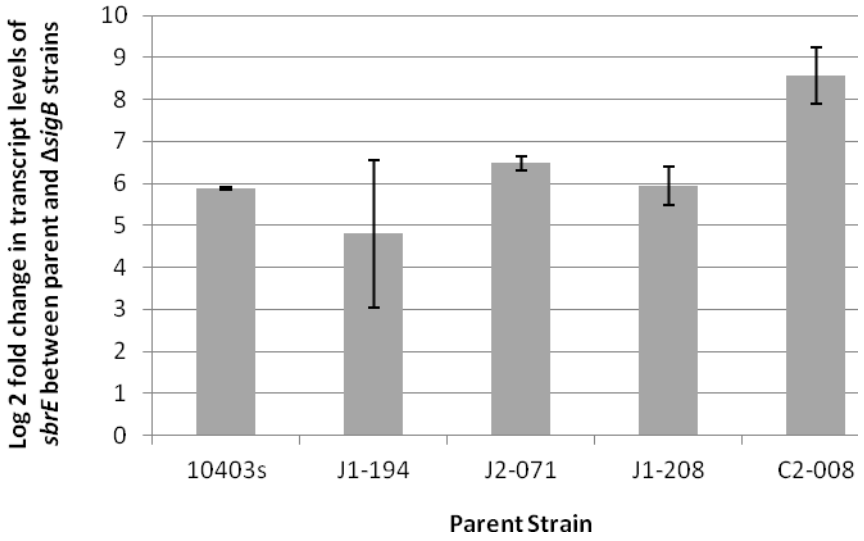

Figure 2.1. *sbrE* transcript levels detected using TaqMan qRT-PCR in parent strains relative to their Δ*sigB* null mutants. The y-axis shows the log2 fold change in *sbrE* transcript levels between parent strains and Δ*sigB* null mutant strains, calculated using the Pfaffl method. Data shown are mean values obtained from three independent experiments. The average log2 fold changes ranged from 4.8 to 8.6, which equals absolute (non-log transformed) fold changes of approximately 30 to 400.

SbrE contributes to the expression of an operon composed of *lmo0636* and *lmo0637*.

Microarray experiments comparing transcript levels in *L. monocytogenes* 10403S parent and Δ*sbrE* strains identified a single gene that showed differential transcript levels (FC $\geq$ 1.5 and $p < 0.05$). Specifically, *lmo0636* showed 2 fold lower transcript levels in Δ*sbrE* as compared to the parent strain. *lmo0636* transcript levels were also found to be downregulated in Δ*sigB*, as compared to the parent strain (FC = -2.17; $p < 0.05$). *lmo0636* transcript levels were not found to be significantly different in the microarray comparison between Δ*sigB* and Δ*sbrE*. qRT-PCR confirmed lower

lmo0636 transcript levels in Δ*sbrE* as compared to the parent strain (1.38 $\pm$ 0.16 log2 lower in Δ*sbrE*; $p < 0.05$ one sample t-test). These data indicate that *lmo0636* is positively regulated by SbrE.

Proteomics experiments identified two proteins that were differentially expressed (FC $\geq$ 1.2 and $p < 0.05$) between the *L. monocytogenes* parent strain and Δ*sbrE*. Lmo0637 and Lmo2094 both showed lower protein levels in the Δ*sbrE* strain (1.45 and 1.2 fold, respectively). Lmo2094 has been annotated as a metal ion binding, class II aldolase/adducin domain protein (Uniprot, www.uniprot.org). Lmo0637, annotated as an UbiE/COQ5 family methyltransferase, is encoded by a gene that forms a 2 gene operon with *lmo0636* [11]. Hence, the combination of transcriptional and proteomics results indicates that SbrE regulates the expression of the *lmo0636-lmo0637* operon. *lmo0636* encodes a protein that was annotated as a hypothetical 2Fe-2S cluster/DNA binding protein of the Rrf2 family of regulators, which belongs to the winged helix-turn-helix superfamily of transcriptional regulators [38]. The N-terminal and C-terminal regions of Rrf2 family proteins are generally involved in DNA binding and signaling, respectively, and may function as redox sensors [39]. Interestingly, previous studies were not able to identify *lmo0636*/*lmo0637* transcription patterns that would point towards a specific mechanism for regulation of this operon. While Raengpradub *et al*. [10] did not find *lmo0636* and *lmo0637* to be significantly differentially expressed in comparisons of *L. monocytogenes* 10403S and Δ*sigB*, in an *L. monocytogenes prfA** genetic background (which expressed a constitutively active PrfA), both genes were found to have significantly higher transcript levels in Δ*sigB* strains, suggesting that they are negatively regulated by σ^B in the presence of an active

PrfA [9]. On the other hand, *lmo0637* was found to be up-regulated in the host during mouse infection with *L. monocytogenes* EGD-e, as compared to stationary phase and exponential phase cells grown in BHI, while *lmo0636* was reported to be downregulated in the host as compared to stationary phase cells grown in BHI [40]. These data suggest that transcriptional regulation of *lmo0636*/*lmo0637* is highly dependent on environmental conditions and may be fine-tuned by SbrE and σ^B-dependent transcription of *sbrE*.

As *trans*-encoded ncRNAs largely act through base pairing with target RNAs, typically the 5'UTR, consequently affecting their translation and/or stability [27], we modeled the putative interaction between SbrE and lmo0636 *in silico*, using IntaRNA version 1.2.2 [41] (Figure 2.2). We only found an interaction with a ΔEnergy of -11.75 kcal/mol, indicating limited complementarily between SbrE and *lmo0636* (including its 5' UTR). A preliminary target capture experiment that used biotin-labeled SbrE bound to BioMag Streptavidin beads (Qiagen, Valencia, CA) to capture *lmo0636* RNA (with subsequent detection by qRT-PCR) also found no evidence for a specific interaction between SbrE and lmo0636. Specifically, levels of *lmo0636* RNA recovered were not different from levels of RNA recovered for another *L. monocytogenes* gene with no evidence for SbrE dependent expression (i.e., *lmo0514*). Future experiments are thus needed to identify the direct or indirect mechanism by which SbrE may influence the expression of the *lmo0636-lmo0637* operon or to identify other SbrE targets.

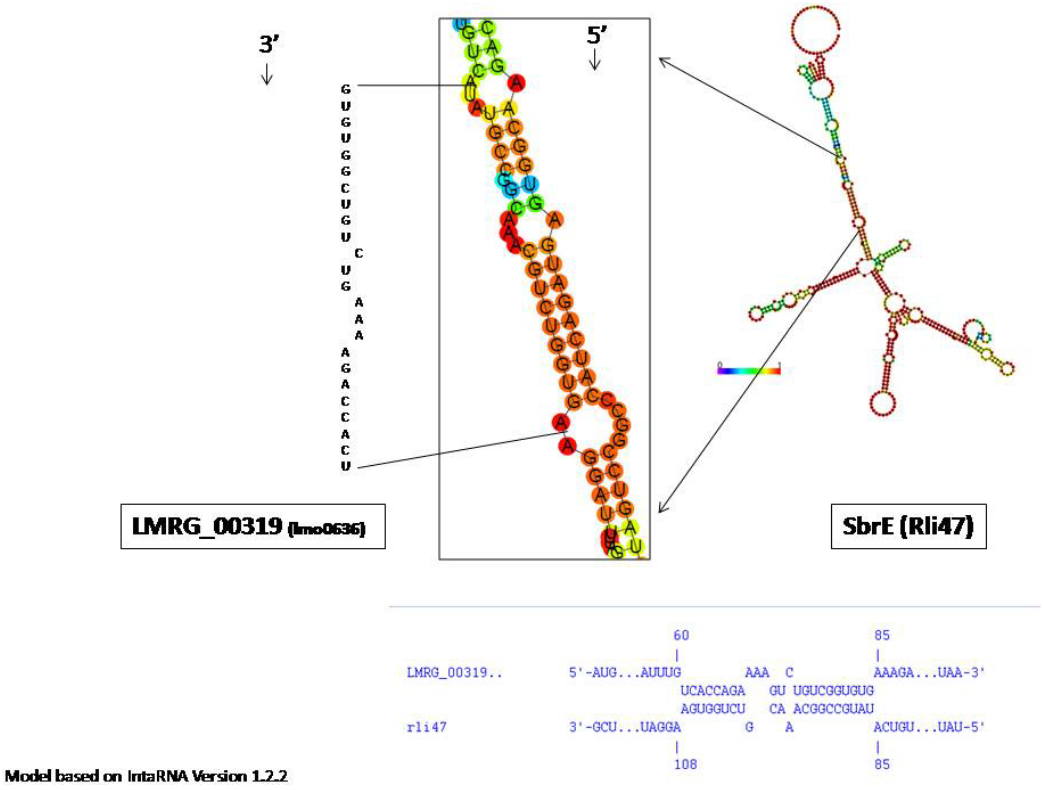

Figure 2.2. SbrE interaction with *LMRG_00319* (*lmo0636*) predicted using IntaRNA software. The ΔEnergy [kcal/mol] of the interaction is -11.75.

Contributions of SbrE to *L. monocytogenes* survival and growth under different stress conditions could not be identified.

As Lmo0636 is annotated as a protein that may play a role in oxidative stress response, we initially focused on characterizing the oxidative stress survival phenotype of the Δ*sbrE* mutant constructed here. Initial assays showed relative killing of 1.95 log CFU/ml for the Δ*sbrE* mutant as compared to 1.11 log CFU/ml for the parent strain after oxidative stress (13mM CHP) exposure for 15 min, indicating a

potentially small but significantly (p = 0.0084) reduced ability to survive oxidative stress for the Δ*sbrE* mutant (Appendix 2.2).

As the difference in survival between the parent and the Δ*sbrE* strain was <1 log (i.e., 0.84 ± 0.29 log CFU/ml), follow up experiments were conducted to monitor oxidative stress (13mM CHP exposure) survival over 60 min. In these experiments, we found no significant differences in log reduction for the parent and the Δ*sbrE* strain after CHP exposure for 15, 30, and 60 min (p >0.05), even though the Δ*sbrE* strain showed numerically higher log CFU reductions, as compared to the parent strain at each time point, with the difference being <1 log at each time point (Table 2.2 and Appendix 2.3). We thus used a competitive index experiment, which provides a more sensitive approach to identify phenotypic differences between two strains, to compare the oxidative stress resistance between the parent and the Δ*sbrE* strain. After 13mM CHP exposure for 15 min the competitive index comparing the Erm^r parent strain and the Erm^s Δ*sbrE* mutant was 1.63, virtually the same as for the control comparing the Erm^r parent strain to an Erm^s parent (1.50), suggesting no difference in oxidative stress survival between the parent and Δ*sbrE* strains in this experiment. In all three experiments detailed above we did find evidence for significantly reduced oxidative stress resistance of the Δ*sigB* strain, including a competitive index of 13.29 for the comparison between the parent strain and the Δ*sigB* strain. These findings are consistent with previous reports, which showed that σ^B contributes to oxidative stress resistance in *L. monocytogenes* [8].

Further phenotypic evaluation of the Δ*sbrE* strain showed no significant effect of the *sbrE* deletion on (i) ability to survive acid stress (pH 2.5, 1 h; see Table 2.2 and

Figure 2.3), (ii) ability to survive under salt stress (1.75 M NaCl, 12 h; see Table 2.2 and Appendix 2.4); (iii) growth under glucose-limiting conditions (0.04% wt/vol glucose, 30 h; see Table 2.2 and Appendix 2.5), and (iii) growth at 7°C for 12 days (Table 2.2; Appendix 2.6). On the other hand, the Δ*sigB* strain, which was included as a control, showed (i) significantly higher death rate under acid stress as compared to the parent strain ($p = 0.0054$) and Δ*sbrE* ($p = 0.0022$) (Table 2.2 and Figure 2.3); (ii) significantly reduced ability to survive salt stress as compared to the parent strain ($p = 0.0039$) and Δ*sbrE* ($p = 0.0039$) (Table 2.2; Appendix 2.4), and (iii) significantly greater increase in cell density under glucose limiting conditions as compared to the parent strain ($p = 0.0008$) and Δ*sbrE* ($p = 0.0014$) (Table 2.2; Appendix 2.5). The Δ*sigB* strain showed a small but significant ($p = 0.0371$) reduction in growth rate under cold stress compared to wildtype, with a difference of 0.06 ± 0.03 log CFU/ml/day (Table 2.2; Appendix 2.6). Susceptibility to infection from the 22 Listeriaphages tested did not differ between wildtype, Δ*sbrE*, and Δ*sigB* strains (Appendix 2.8)

Table 2.2: Environmental stress survival and growth of *L. monocytogenes* 10403S parent strain, Δ*sbrE*, and Δ*sigB*

Strain	Reduction in cell numbers (log CFU/ml) after oxidative stress (13 mM CHP)[a]			Death rate (log CFU/h) after acid stress (pH 2.5 for 1h)[a]	Average μmax (log CFU/ml/day) at 7°C[a]	Increase in cell density (log CFU/ml) over 27 h growth in DM/0.04% glucose (Energy Stress)[a,b]	Cell numbers (log CFU/ml) after 12h of growth in BHI with 1.75 M NaCl (Salt Stress)[a]
	15 min	30 min	60 min				
Parent strain	2.46 ± 0.36	2.74 ± 0.08	2.69 ± 0.24	1.70 ± 0.08	0.73 ± 0.02	0.53 ± 0.04	4.86 ± 0.07
Δ*sbrE*	2.75 ± 0.62	3.02 ± 0.05	3.39 ± 0.13	1.01 ± 0.32	0.68 ± 0.01	0.64 ± 0.17	4.61 ± 0.11
Δ*sigB*	3.99 ± 0.88	3.62 ± 0.15[#^]	4.76 ± 0.20[#^]	4.45 ± 1.02[#^]	0.67 ± 0.01[#]	1.45 ± 0.06[#^]	3.82 ± 0.12[#^]

[a]Data shown are means of at least three biological replicates ± standard deviation

[#] Indicates significant difference between the parent strain and Δ*sigB*

[^] Indicates significant difference between Δ*sigB* and Δ*sbrE*

[b]Increase in cell density was calculated as CFU/ml after 30 h minus CFU/ml after 3 h in DM

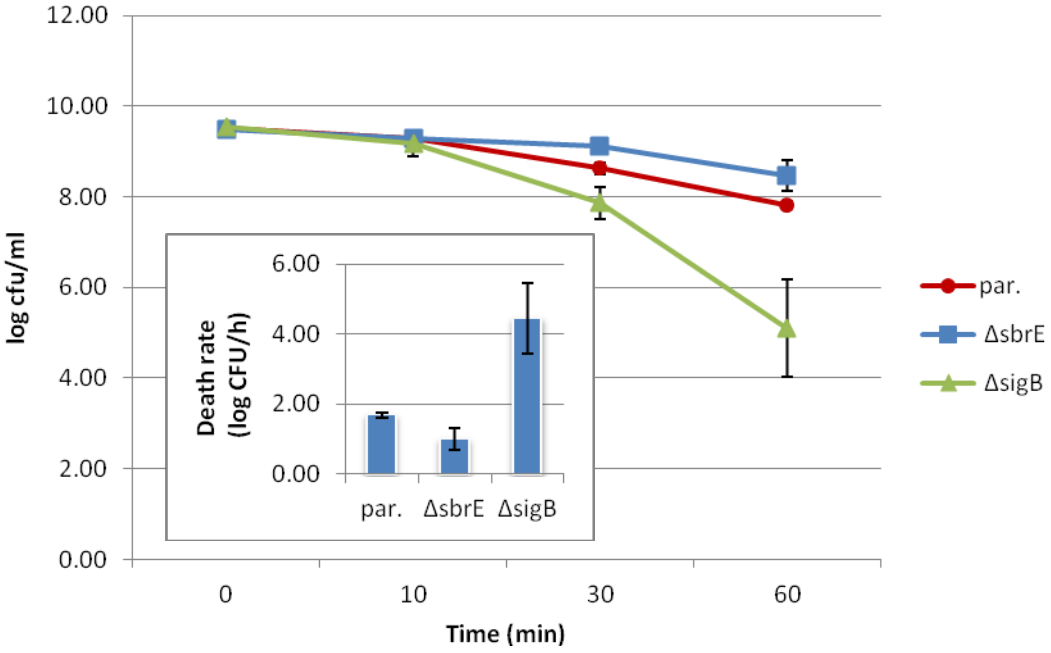

Figure 2.3. Acid stress survival of parent strain (par., circle), Δ*sbrE* (square), and Δ*sigB* (triangle) strains. Bacterial numbers in log CFU/ml after exposure to pH 2.5 for 1 h are plotted. The inset shows the average death rate of each strain in log CFU/h. Values are means from three independent experiments.

Our data suggest that SbrE does not contribute to *L. monocytogenes* survival and growth under a number of stress conditions that are well established to require σ^B for optimal growth and survival. Overall, we found that a SbrE deletion does not affect *L. monocytogenes* growth under cold stress or energy stress or *L. monocytogenes* acid stress survival or phage resistance. SbrE did however show a small but significant contribution to the survival of *L. monocytogenes* 10403S under oxidative stress in our initial experiments; however, this phenotype was not confirmed by subsequent experiments. These data indicate potential contributions of *L. monocytogenes* SbrE to growth and survival under very specific and defined environmental stress conditions.

Interestingly, the characterization of the σ^B-dependent ncRNA SbrA also found no phenotypes for a Δ*sbrA* mutant strain under the conditions tested [30].

CONCLUSIONS

Our work showed that SbrE is a conserved part of the σ^B regulon, being σ^B-dependent across *L. monocytogenes* lineages and in *L. innocua*. A combination of proteomics and microarray approaches indicates that SbrE contributes to regulating the expression of an operon composed of *lmo0636* and *lmo0637*, which encodes two proteins annotated as a hypothetical 2Fe-2S cluster/DNA binding protein and methyltransferase, respectively. SbrE dependent regulation of this operon likely occurs directly or indirectly at the mRNA level through the regulation of transcription or mRNA stability, as both microarray and qRT-PCR showed lower transcript levels for *lmo0636* in the Δ*sbrE* strain. While transcription of *sbrE* was found to be induced under oxidative stress conditions, phenotypic data could not find consistent evidence for contributions of SbrE to oxidative stress resistance, even though a trend towards reduced oxidative stress resistance was found in some experiments. As no phenotypic consequences of an *sbrE* deletion were found for environmental stress conditions under which σ^B had previously been demonstrated to be important for survival or growth, we hypothesize that SbrE may play a role in "fine-tuning of gene expression" in *L. monocytogenes* or that it may play a role for *L. monocytogenes* fitness under very specific growth conditions that were not tested here as previously proposed by Nielsen *et al*. [30] for SbrA, another σ^B-dependent ncRNA. Our data thus support that in addition to playing a role as a major regulator of certain stress response pathways

(e.g., acid stress), σ^B is also likely to contribute more subtly to *L. monocytogenes* adaptation to other environmental stress conditions, including through complex regulatory networks. Additional experiments that utilize overexpression of SbrE will be needed, however, to gain further insight into the role of SbrE in *L. monocytogenes*.

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CHAPTER 3

REFINEMENT OF THE *LISTERIA MONOCYTOGENES* σ^B REGULON THROUGH QUANTITATIVE PROTEOMIC ANALYSIS

ABSTRACT

σ^B is an alternative σ factor that regulates stress response and virulence genes in the foodborne pathogen *Listeria monocytogenes*. To gain further insight into σ^B-dependent regulatory mechanisms in *L. monocytogenes*, we (i) performed quantitative proteomic comparisons between the *L. monocytogenes* parent strain 10403S and an isogenic Δ*sigB* mutant and (ii) conducted a meta-analysis of published microarray studies on the 10403S σ^B regulon. A total of 134 genes were found to be significantly positively regulated by σ^B at the transcriptomic level with > 75% of these genes preceded by putative σ^B-dependent promoters; 21 of these 134 genes were also positively regulated by σ^B at the protein level. In addition, 15 proteins were only found to be positively regulated by σ^B at the protein level including Lmo1349, a putative glycine cleavage system protein. The lmo1349 gene is preceded by a 5' UTR that functions as a glycine riboswitch, which suggests regulation of glycine metabolism by σ^B in *L. monocytogenes*, including a model where σ^B upregulates pathways that facilitate biosynthesis and uptake of glycine, which may then activate this riboswitch. Our data also (i) identified a number of σ^B-dependent proteins that appear to be encoded by genes that are co-regulated by multiple transcriptional regulators, in particular PrfA, and (ii) found σ^B-dependent genes and proteins to be overrepresented

in the "energy metabolism" role category, highlighting contributions of the σ^B regulon to *L. monocytogenes* energy metabolism as well as a role of PrfA and σ^B interaction in regulating aspects of energy metabolism in *L. monocytogenes*.

INTRODUCTION

The general stress response alternative σ factor σ^B is an important transcriptional regulator in the facultative intracellular pathogen *Listeria monocytogenes* [25]. *L. monocytogenes* is not only the causative agent of listeriosis, a life-threatening foodborne infection in humans and animals, but is also commonly found in many non-host environments and has the ability to survive under a variety of environmental stress conditions that are lethal to other bacteria. σ^B contributes to the resistance of *L. monocytogenes* to different stress conditions, including acid, osmotic, and energy stress, and also plays an important role in survival during stationary phase [25]. σ^B has also been shown to directly regulate transcription of some virulence genes as well as other genes that contribute to *L. monocytogenes* growth and survival during gastrointestinal passage as well as intracellular survival and replication [7, 10, 15, 38].

As a transcriptional activator, σ^B directly up-regulates and indirectly down-regulates *L. monocytogenes* genes involved in stress response and virulence. Several transcriptomic studies have probed the σ^B regulon of *L. monocytogenes*, including four studies with *L. monocytogenes* strain 10403S. A microarray study by Raengpradub *et al*. (2008) [33], comparing transcript levels in *L. monocytogenes* 10403S and an isogenic Δ*sigB* null mutant grown to stationary phase or exposed to salt stress, identified 168 genes as positively regulated by σ^B ($p < 0.05$; fold change ≥ 2.0). A

microarray study by Ollinger *et al*. (2009) [28] characterized transcript levels in stationary phase cells of *L. monocytogenes* 10403S and ΔsigB mutant strains with a constitutively active PrfA protein, which forms a regulatory network with σ^B that contributes to virulence gene expression and host infection by *L. monocytogenes.* In this study > 200 genes were identified as having significant σ^B effects with fold changes $\geq$ 1.5 between the parent and mutant strain. In addition, Oliver *et al*. (2010) [26] used microarray analyses to compare transcript levels in parent and Δ*sigB* mutant strain pairs representing *L. monocytogenes* lineages I, II, IIIA, and IIIB; lineages IIIA and IIB have recently been reclassified as lineages III and IV [39]. In *L. monocytogenes* 10403S, a lineage II strain, 252 genes were found to be significantly positively regulated by σ^B in stationary phase cells, with a fold change $\geq$ 1.5. Furthermore, in an RNA sequencing (RNA-Seq) study of *L. monocytogenes* 10403S and an isogenic Δ*sigB* strain, Oliver *et al*. (2009) [27] identified 96 genes as positively regulated by σ^B in stationary phase cells. Along with transcriptional analyses, proteomic studies have also been used to define the σ^B regulon in *L. monocytogenes* 10403S. Using non-gel based quantitative proteomics and two-dimensional gel electrophoresis (2-DE), Abram *et al*. (2008a) [1] identified 17 proteins that showed higher expression levels in the parent strain than the Δ*sigB* strain, using bacteria grown to stationary phase in Brain Heart Infusion (BHI) medium with or without osmotic stress (0.5 M NaCl). Additionally, a 2-DE study of bacteria grown to exponential or stationary phase in chemically defined media (DM) with or without 0.5 M NaCl (Abram *et al*., 2008b) [2] identified 10 proteins that showed higher levels in the parent strain as compared to the Δ*sigB* mutant.

Overall, the studies detailed above show that σ^B in *L. monocytogenes* is responsible for positive regulation of a large regulon with > 100 genes, with a considerable number of these genes directly regulated by σ^B, as supported by identification of σ^B consensus promoters upstream of many of these genes [1, 26, 27, 33]; however, a comprehensive analysis that formally integrates data on the σ^B regulon from multiple studies has been missing so far. Increasing evidence further suggests that σ^B also makes important contributions to gene regulation in *L. monocytogenes* via mechanisms other than direct regulation of a gene or operon through σ^B-dependent transcription from an upstream promoter, including, but not limited to, regulation of non-coding RNAs (ncRNAs), which can regulate gene expression at both the transcriptional and post-transcriptional stage [22, 24, 27, 37]. We thus performed a non-gel based quantitative proteomic analysis of stationary phase *L. monocytogenes* 10403S and Δ*sigB mutant* cells to further refine the definition of the σ^B regulon for this *L. monocytogenes* strain and to explore additional σ^B-dependent mechanisms of gene regulation, including through a meta-analysis of previous microarray studies on the σ^B regulon and a comparison with RNA-Seq data, all generated for the strain 10403S genetic background. Our hypothesis was that this approach would allow for identification of additional transcriptional and post-transcriptional regulatory pathways governed by *L. monocytogenes* σ^B.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

The *L. monocytogenes* parent strain 10403S and its isogenic Δ*sigB* mutant [40] were stored at -80°C in BHI medium containing 15% glycerol. Cells were grown to stationary phase with aeration at 37°C, as previously described [33].

Protein isolation, iTRAQ labeling, and Nano-scale reverse phase chromatography and tandem mass spectrometry (nanoLC-MS/MS).

Proteins were isolated from stationary phase *L. monocytogenes* cells from 25 ml of culture using the method of Abram *et al.* (2008a) [1] with slight modifications as previously described [23]. Protein concentrations were determined using a noninterfering protein assay kit (Calbiochem). Protein concentration and quality were verified using1D SDS-PAGE.

Protein samples were analyzed at the Cornell University Proteomics and Mass Spectrometry Core Facility using shotgun-based quantitative proteomics as previously described [23]. Briefly, a total of 100 µg protein of each sample was denatured, reduced, and the cysteine residues were blocked, after which protein samples were digested with sequence-grade-modified trypsin at 37ºC for 16 h. SDS-PAGE was used to assess the efficiency of protein digestion. The tryptic peptides from *L. monocytogenes* parent strain 10403S and Δ*sigB* were each labeled with iTRAQ reagents, according to the manufacturer's protocols. The labeled samples were combined and fractionated via Isoelectric focusing (IEF) OffGel electrophoresis (OGE) using an Agilent 3100 OFFGEL Fractionator (Agilent, G3100AA). nanoLC-

MS/MS was carried out using a LTQ-Orbitrap Velos (Thermo-Fisher Scientific) mass spectrometer.

Protein identification and data analysis.

Data processing, protein identification, and subsequent data analysis were carried out as described previously [23]. Briefly, all MS and MS/MS raw spectra from iTRAQ experiments were processed using Proteome Discoverer 1.1 for subsequent database search using in-house licensed Mascot Daemon. The *L. monocytogenes* protein sequence database downloaded from the Broad Institute was used for the database search. The false discovery rate (FDR) was estimated using an automatic decoy database search in Mascot, and the relative quantitation ratios were normalized using the "median ratio" procedure in each set of experiments. Two biological replicates of the parent strain 10403S and Δ*sigB* comparison were analyzed independently.

The Wilcoxon signed rank test was applied to peptide ratios for each identified protein to determine significant changes between parent strain 10403S and Δ*sigB*. The Fisher's Combined Probability Test was used to combine FDR adjusted Wilcoxon *p*-values from each replicate into one test statistic for every protein to obtain a combined *p*-value (*p*-value^{c}). Proteins with peptide ratios exhibiting a Fisher's Combined Probability Test *p*-value^{c} < 0.05 and an average iTRAQ protein ratio ≥ 1.5 were considered significantly differentially expressed. Statistical analyses were conducted using R statistical software.

Meta-analysis of published studies.

A meta-analysis of three published microarray studies was performed to identify genes that showed differential transcript levels between the *L. monocytogenes* parent strain 10403S and an isogenic Δ*sigB* strain. Two of these studies used 10403S and an isogenic Δ*sigB* strain [26, 33], while one study [28] used an *L. monocytogenes* 10403S *prfA** strain, which constitutively expresses an active form of the virulence gene transcriptional activator PrfA, and an isogenic *prfA**Δ*sigB* strain. Two of these studies [28, 33] used the same set of microarray probes, which were designed based on the genome sequence of strain EGD-e, while the other study [26] used a set of microarray probes designed based on four different genomes. The meta-analysis used the Fisher's Combined Probability Test to combine *p*-values reported in each microarray study into one test statistic (combined microarray *p*-value, $p\text{-value}^c$) for every gene. Genes with a $p\text{-value}^c < 0.05$ and fold change (FC) ≥ 1.5 in each of the three array studies were considered significantly differentially expressed. To further refine the σ^B regulon in *L. monocytogenes* 10403S, microarray meta-analysis data were also compared to RNA-Seq data collected to compare transcript levels between the *L. monocytogenes* parent strain 10403S and an isogenic Δ*sigB* strain [27]. As previously described, genes were considered positively regulated by σ^B in the RNA-Seq study if they had a Q-value < 0.05 and FC ≥ 2.0 in all four comparisons of parent and Δ*sigB* strain transcript levels [27].

Promoter mapping and analysis of 5' untranslated regions (UTRs).

Data for σ^A and σ^B promoters as well as 5' UTRs were obtained from an analysis of a manually annotated genome sequence for *L. monocytogenes* 10403S (Broad Institute; see http://www.broadinstitute.org/annotation/genome/listeria_group/MultiHome.html) and RNA-Seq transcriptome data ([27]; Orsi *et al*., in preparation). 5' UTR nucleotide sequences for selected σ^B-dependent genes were examined for self-binding ability through the RNAFold Server (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi). The lengths of 5' UTRs among different groups of genes was compared using a two-sided and one-sided Wilcoxon rank sum test, as the Shapiro-Wilkes test for normality showed that 5' UTR lengths were not normally distributed.

Association of σ^B-dependent genes and proteins with JCVI role categories.

A Monte Carlo simulation of Fisher's exact test was used to determine whether the distribution of role categories among the 149 genes identified as positively regulated by σ^B by RNA-based or proteomic approaches was different from the role category distribution that would be expected by chance (based on the role category primary annotation for all *L. monocytogenes* EGD-e genes, available at http://cmr.jcvi.org). Individual Fisher's exact tests were subsequently used to determine whether individual role categories were over- or under-represented among the σ^B-dependent genes. While 21 comparisons were performed (based on 21 role categories), we did not adjust *p*-values for multiple comparisons, rather we reported actual *p*-values to allow readers to apply corrections if deemed appropriate. Analyses

were performed using all role categories assigned to a given gene in the JCVI-CMR *L. monocytogenes* EGD-e database.

RESULTS AND DISCUSSION

Proteomics identified 36 proteins that show higher expression levels in the presence of σ^B, suggesting positive regulation by σ^B.

Our quantitative proteomic comparison identified 36 proteins that show higher expression levels in the parent as compared to the Δ*sigB* strain, suggesting σ^B-dependent regulation, including (i) 21 proteins for which the corresponding genes were found to be positively regulated by σ^B through microarray meta-analysis and/or RNA-Seq, and (ii) 15 proteins found to be σ^B-dependent through proteomics only (Group D; see Fig. 3.1 and 3.2; Appendix 3.3); these 15 proteins are discussed in further detail below. The 21 proteins for which the corresponding genes were found to be positively regulated by σ^B through microarray meta-analysis and/or RNA-Seq include (i) 15 proteins encoded by genes that were found to be under positive regulation by σ^B through RNA-Seq and microarray meta-analysis (Group A; Appendix 3.3); (ii) three proteins encoded by genes that were found to be positively regulated by σ^B through microarray meta-analysis but not RNA-Seq (Group E; Appendix 3.3); and (iii) three proteins (Lmo1426 (OpuCC), Lmo0819, and Lmo0265) encoded by genes that were found to be positively regulated by σ^B through RNA-Seq but not microarray meta-analysis (Group F; Appendix 3.3). The genes encoding the three proteins in Group F likely represent false negatives in the microarray analysis as both lmo0819 and *opuCC* had a significant combined microarray *p*-value (*p*-value^c) and positive fold

changes in all three microarray studies, but missed the fold change cutoff of ≥ 1.5 in one and two microarray studies, respectively. *opuCC* is also (i) preceded by a putative σ^B-dependent promoter, and (ii) belongs to the four-gene *opuC* operon that has been identified in multiple studies as σ^B-dependent [9, 36]. Lmo0265, which had a significant *p*-value[c] and a fold change of 8.0 reported in the Oliver *et al*. (2010) [26] microarray study, did not show evidence for differential expression in the two other array studies [28, 33], which used an EGD-e array that had a lmo0265 probe with low (71%) homology to the 10403S lmo0265 gene, likely leading to a false negative result in these two studies.

Two previous proteomic studies aimed to identify σ^B-dependent genes in *L. monocytogenes* 10403S. One study only used 2-DE [2], and while the other study used iTRAQ as well as 2-DE, it used *L. monocytogenes* cells grown to stationary phase in 0.5M salt for iTRAQ analysis, which is different from the growth conditions used to generate the microarray data that were used for the meta-analysis detailed below. The study that used both iTRAQ as well as 2-DE [1] identified 17 proteins as positively regulated by σ^B, compared to 36 proteins identified here; 14 of these 36 proteins had also been reported as positively regulated by σ^B in this previous study. The 2-DE study by Abram *et al*. (2008b) [2], on the other hand, found 10 proteins to be positively regulated by σ^B; 7 of these proteins were also found among the 36 proteins we identified here as positively regulated by σ^B. Our study thus identified substantially more proteins as positively regulated by σ^B as compared to previous studies. Importantly, the σ^B-dependent proteins newly identified here provide novel insight into the direct and indirect regulation of cellular pathways by σ^B, as discussed below.

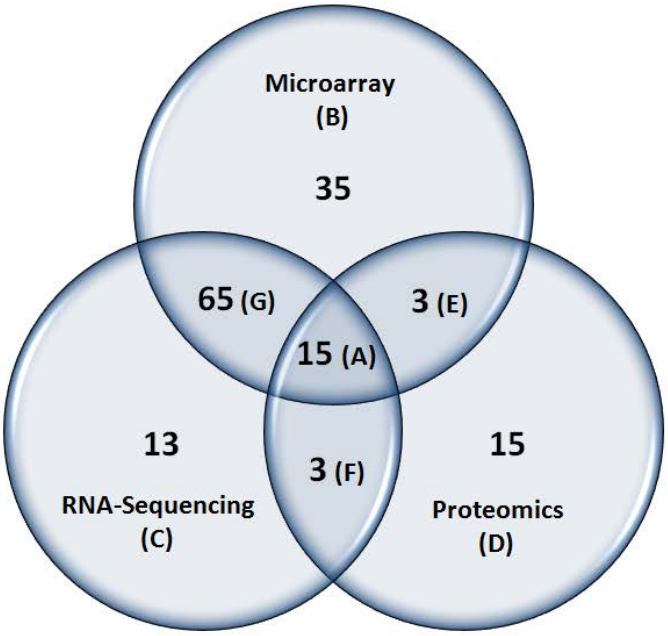

Figure 3.1. Genes or proteins identified as positively regulated by σ^B through microarray meta-analysis, RNA-Sequencing, or proteomics. Venn diagram of genes and proteins identified as positively regulated by σ^B, in *L. monocytogenes* 10403S stationary phase cells, through (i) proteomic data reported here ($p < 0.05$; average fold change [FC] ≥ 1.5), (ii) a meta-analysis of previous microarray studies (Oliver *et al*., 2010 [26], Ollinger *et al*., 2009 [28], Raengpradub *et al*., 2008 [33]) ($p^C < 0.05$; FC ≥ 1.5 in each of the three microarray studies), and (ii) previously reported RNA-Sequencing data (Oliver *et al*., 2009 [27]) (Q < 0.05; FC ≥ 2.0 in each of the four comparisons). Group names (A-G) correspond to the groups in Fig. 3.2 and Appendix 3.3.

Figure 3.2. Heat map comparing fold changes of genes or proteins identified as positively regulated by σ^B through microarray, RNA-Sequencing, or proteomics. Heat map comparing fold changes (FC) of genes and proteins identified, in *L. monocytogenes* 10403S stationary phase cells, as positively regulated by σ^B through (i) proteomics data reported here (reported as both replicate 1 and 2; shown as Rep. 1 and Rep. 2), (ii) a meta-analysis of previous microarray studies (Raengpradub *et al*., 2008 [33], Ollinger *et al*., 2009 [28], and Oliver *et al*., 2010 [26]), and (iii) previously reported RNA-Sequencing data (all four comparisons reported by Oliver *et al*. (2009) [27] are shown; indicated as Comp. 1 through 4). Blank cells indicate that a given protein was not identified in the proteomic experiments. The color scale applies to microarray data columns, proteomics data columns, and RNA-Sequencing data columns separately. Detailed descriptions of genes and encoded proteins can be found in Appendix 3.3.

Gene	Gene name or protein encoded	Microarray FC reported by			RNA-Seq fold changes (Oliver, 2009)				Proteomics FC (this study)	
		Ollinger, 2009	Raengpra-dub, 2008	Oliver, 2010	Comp. 1	Comp. 2	Comp. 3	Comp. 4	Rep. 1	Rep. 2
Group A. Genes or proteins identified as positively regulated by SigB through microarray, proteomics, and RNA-Sequencing (15)										
lmo0134	hypothetical protein	8.70	6.20	5.64	72.00	72.00	52.00	52.00	12.82	2.96
lmo0539	*lacD*	15.40	13.70	7.50	18.00	13.79	14.93	11.44	4.35	3.29
lmo0554	similar to NADH-dependent butanol dehydrogenase	3.80	4.40	6.03	106.00	53.00	214.00	107.00	2.16	2.26
lmo0654	hypothetical protein	4.60	2.10	3.77	5.32	6.73	7.21	9.13	7.87	3.73
lmo0722	pyruvate oxidase	3.10	2.30	5.39	21.25	9.44	33.63	14.94	12.20	2.13
lmo0783	mannose-specific PTS system IIB component	12.60	10.80	11.99	57.00	114.00	64.00	128.00	3.33	2.84
lmo0794	hypothetical protein	15.80	5.50	12.83	32.71	114.50	26.86	94.00	5.13	2.81
lmo0796	YceI like family protein	4.60	3.70	4.03	4.31	3.30	5.22	4.00	4.33	2.44
lmo0913	succinate-semialdehyde dehydrogenase	11.40	5.80	13.43	365.00	182.50	516.00	258.00	1.98	1.74
lmo1602	hypothetical protein	18.60	5.10	4.56	4.71	3.07	8.55	5.56	2.41	1.77
lmo1830	short chain dehydrogenase	3.20	2.40	7.15	23.00	46.00	19.25	38.50	4.29	4.65
lmo2158	stress response protein	8.80	15.60	7.60	737.00	8.77	1158.00	13.79	3.76	2.49
lmo2213	similar to antibiotic biosynthesis monoxygenase subfamily	12.40	11.60	2.30	91.00	91.00	98.00	98.00	2.70	3.00
lmo2398	*ltrC*	4.60	2.30	2.38	2.03	3.22	2.29	3.64	2.43	1.63
lmo2748	general stress protein 26	9.50	8.90	10.02	281.00	281.00	134.00	134.00	8.70	2.84
Group B. Genes identified as positively regulated by SigB through microarray only (35)										
lmo0210	*ldh1*	5.20	3.10	2.63	-1.67	4.58	-2.67	2.86	1.15	1.11
lmo0342	transketolase	1.60	3.10	2.14	13.73	2.29	7.09	1.18	1.24	-1.01
lmo0343	*tal2*	1.50	3.80	2.01	9.60	1.71	4.80	-1.17	1.26	-1.01
lmo0345	ribose 5-phosphate isomerase B	2.20	3.30	1.53	14.00	3.50	3.50	-1.14	1.41	1.01
lmo0346	*tpiA2*	1.90	2.40	1.89	34.00	2.43	16.00	1.14	2.30	1.03
lmo0347	dihydroxyacetone kinase L subunit	1.80	2.90	2.11	8.75	3.18	3.50	1.27	-1.53	-1.03
lmo0348	dihydroxyacetone kinase	2.00	2.80	1.76	66.00	2.54	33.00	1.27	1.57	-1.08
lmo0406	lactoylglutathione lyase	1.50	1.60	1.63	1.83	5.50	4.50	13.50		
lmo0408	hypothetical protein	2.80	1.50	1.55	1.04	2.00	2.08	4.00		
lmo0524	similar to putative sulfate transporter	3.00	1.90	2.33	1.69	1.26	4.39	3.29	1.45	1.00
lmo0579	hypothetical protein	3.00	1.50	1.51	1.19	2.27	2.10	4.00	1.50	
lmo0580	weakly similar to carboxylesterase	3.30	1.60	1.61	1.24	1.64	1.88	2.49		
lmo0590	hypothetical protein	1.50	1.90	1.66	-1.61	-1.01	1.71	2.71	-1.37	
lmo0648	*corA*	1.90	1.60	1.65	1.91	3.50	5.91	10.83		
lmo0896	*rsbX*	2.30	2.20	2.34	2.89	1.82	5.27	3.33		1.04
lmo0956	N-acetylglucosamine-6-phosphate deacetylase	3.90	2.30	2.06	1.22	1.93	1.44	2.28	1.21	1.18
lmo0957	*nagB*	3.20	1.70	1.56	1.15	1.86	1.37	2.21	-1.06	1.06
lmo0995	YkrP protein	3.90	2.80	3.45	2.20	-1.09	15.20	6.33		
lmo1261	hypothetical protein	2.80	1.80	3.02	2.62	1.58	6.50	3.93	5.46	2.09
lmo1376	6-phosphogluconate dehydrogenase, decarboxylating	2.30	1.50	1.68	-1.13	1.07	1.68	2.05	1.00	1.00
lmo1388	*tcsA*	2.00	1.60	1.68	3.18	1.14	3.73	1.34	-1.21	-1.26
lmo1432	hypothetical protein	4.50	2.10	1.86	1.34	1.30	2.46	2.39	1.41	1.20
lmo1580	universal stress protein	2.30	2.60	1.96	2.77	2.06	2.32	1.72	1.23	1.30
lmo1605	*murC*	2.00	1.70	2.22	2.04	1.83	3.55	3.18	1.04	1.00
lmo1636	ABC-2 type transport system ATP-binding protein	1.50	1.50	1.89	-1.21	-2.04	5.28	3.12	-1.13	1.04
lmo1666	*lapB*	2.20	1.50	1.65	3.20	1.66	7.06	3.67		1.26
lmo1929	*ndk*	2.10	1.50	1.84	3.31	1.95	6.31	3.73	1.37	1.21
lmo1930	heptaprenyl diphosphate synthase component II	1.70	1.50	1.59	1.00	-1.04	3.92	3.76		1.00
lmo1933	*folE*	2.10	2.10	1.95	1.50	3.43	2.81	6.43	1.03	1.06
lmo2041	*mraW*	2.40	1.60	1.79	1.29	-1.15	3.57	2.41	-1.03	1.07
lmo2169	hypothetical protein	1.50	2.00	1.73	1.20	1.11	1.82	1.68		
lmo2191	*spxA*	3.20	2.70	3.03	1.79	3.23	1.03	1.86	1.26	1.21
lmo2386	hypothetical protein	2.40	1.60	1.91	-1.05	2.17	1.28	2.90	1.44	1.40
lmo2389	similar to NADH dehydrogenase	1.70	1.50	1.77	-1.40	2.41	-1.52	2.22	1.07	1.03
lmo2539	*glyA*	1.90	1.50	1.51	1.68	-1.06	2.69	1.51	1.12	1.00
Group C. Genes identified as positively regulated by SigB through RNA-Sequencing only (13)										
lmo0122	similar to phage proteins	-1.25	1.10	1.08	4.60	4.60	3.20	3.20		
lmo0133	hypothetical protein	3.20	1.20	5.05	71.00	71.00	34.00	34.00		1.90
lmo0274	hypothetical protein	1.20	1.20	1.98	3.33	3.33	3.00	3.00		
lmo0372	beta-glucosidase	1.00	1.10	1.15	5.50	2.54	6.17	2.85		
lmo0405	PiT family inorganic phosphate transporter	2.10	2.10	1.38	4.42	10.60	3.25	7.80		
lmo0433	*inlA*	-2.50	1.30	3.08	8.46	7.52	3.96	3.52		
lmo0434	*inlB*	-3.33	1.40	1.46	8.00	8.00	4.00	4.00		
lmo0439	weakly similar to a module of peptide synthetase	2.40	1.40	5.63	35.00	35.00	55.00	55.00		1.21
lmo1421	*bilEA*	1.40	1.50	2.19	34.00	2.83	71.00	5.92	3.34	1.65
lmo1866	phosphotransferase	2.70	-1.11	-1.07	2.80	2.80	2.47	2.47		
lmo2003	similar to transcription regulator GntR family	-1.11	1.00	-1.11	33.00	11.00	11.00	3.67	-1.55	-1.28
lmo2572	similar to chain A, dihydrofolate reductase	7.00	3.50	1.18	7.40	8.22	7.85	8.72		2.69
lmo2733	similar to PTS system, fructose-specific IIABC component	1.20	1.10	1.57	14.00	2.80	12.50	2.50		
Group D. Proteins identified as positively regulated by SigB through proteomics only (15)										
lmo0398	fructose-specific PTS system IIA component	-2.00	11.60	13.48	1452.00	19.11	29.00	-2.62	2.04	1.65
lmo0399	fructose-specific PTS system IIB component	-2.00	13.50	10.52	374.20	22.01	8.00	-2.13	1.92	2.41
lmo0643	transaldolase	-3.33	1.30	1.06	4.17	-5.08	9.50	-2.23	2.05	1.53
lmo1046	*moaC*	1.10	1.20	1.36	7.88	-1.46	18.00	1.57	1.53	1.49
lmo1349	*gcvPA*	-1.11	1.10	-1.14	7.87	-2.39	18.32	-1.02	1.70	1.69
lmo1422	similar to glycine betaine/carnitine/choline ABC transporter	1.50	1.30	1.40	5.00	1.25	52.00	13.00	2.40	2.04
lmo2047	*rpmF2*	-1.11	-1.43	-1.03	-1.56	-1.39	1.10	1.23	2.04	1.27
lmo1539	glycerol uptake facilitator protein	1.30	-5.00	-1.95	8.76	2.91	3.19	1.06	1.53	1.67
lmo2743	*tal1*	1.00	1.60	1.35	4.01	1.53	2.05	-1.28	1.67	1.41
lmo2697	dihydroxyacetone kinase	1.10	1.90	1.46	20.35	-1.23	20.10	-1.25	3.00	2.40
lmo2696	dihydroxyacetone kinase L subunit	1.30	1.50	1.15	18.30	-1.07	18.30	-1.07	4.29	2.57
lmo2695	dihydroxyacetone kinase DhaK subunit	1.30	1.30	1.27	16.20	-1.19	16.61	-1.16	3.55	1.93
lmo2666	galactitol-specific PTS system IIB component	-10.00	1.40	-1.50	9.55	-7.18	9.09	-7.54	2.18	1.81
lmo0110	esterase/lipase	-1.43	1.10	1.31	4.66	-1.33	8.89	1.43	4.08	2.83
lmo1730	similar to sugar ABC transporter binding protein	-1.67	-1.43	-1.80	5.85	-3.80	8.54	-2.60	1.77	1.58

Figure 3.2 (continued)

Group E. Genes or proteins identified as positively regulated by SigB through microarray and proteomics and not RNA-Sequencing (3)										
lmo1601	similar to general stress protein	12.00	4.20	4.01	3.36	1.86	6.68	3.70	2.91	1.86
lmo2205	*gpmA*	2.70	2.20	2.05	2.40	2.94	1.93	2.37	3.95	2.20
lmo1428	*opuCA*	2.30	2.90	2.87	1.85	2.49	4.26	5.73	4.93	2.33
Group F. Genes or proteins identified as positively regulated by SigB through RNA-Sequencing and proteomics and not microarray (3)										
lmo1426	*opuCC*	1.20	1.00	3.12	3.05	2.08	5.90	4.03	6.54	1.81
lmo0819	hypothetical protein	1.80	1.40	1.65	2.79	2.39	3.69	3.16	1.47	1.63
lmo0265	*dapE*	-1.11	1.00	8.25	226.00	226.00	183.00	183.00	4.93	2.67
Group G. Genes identified as positively regulated by SigB through RNA-Sequencing and microarray and not proteomics (65)										
lmo0169	sugar uptake protein, similar to a glucose uptake protein	2.40	2.10	3.37	13.67	20.50	14.83	22.25		
lmo0170	hypothetical protein	5.40	1.90	3.05	2.14	3.90	3.38	6.16	1.02	1.11
LMRG_02646	*inlC2* (lmo0263)	7.10	6.90	3.73	278.00	92.67	144.00	48.00		
LMRG_02851	*inlD* (lmo0263)	7.10	6.90	5.29	16.00	8.00	25.00	12.50		
lmo0321	hypothetical protein	5.80	4.30	5.31	4.00	20.00	3.60	18.00		
lmo0445	putative M protein trans-acting positive regulator	3.30	3.00	5.03	22.25	89.00	19.00	76.00		
lmo0515	universal stress protein	4.30	2.10	3.41	19.00	31.67	7.60	12.67	1.31	
lmo0555	*dtpT*	9.10	4.10	4.11	7.50	6.08	11.00	8.92	1.62	
lmo0593	formate/nitrite transporter	3.80	4.30	5.69	3.95	9.22	2.95	6.89		
lmo0596	membrane protein	5.70	12.30	22.75	137.00	137.00	204.00	204.00		
lmo0602	weakly similar to transcription regulator	10.20	3.00	3.66	136.00	136.00	95.00	95.00	1.94	4.31
lmo0610	internalin, putative peptidoglycan bound protein	2.00	2.30	3.66	3.87	11.60	4.00	12.00		
lmo0628	hypothetical protein	3.40	3.80	3.31	30.00	30.00	18.00	18.00		
lmo0629	hypothetical protein	3.20	2.30	1.61	3.77	4.08	2.38	2.58		1.47
lmo0655	serine/threonine protein phosphatase 1	2.60	2.00	2.90	3.83	3.83	4.92	4.92		
lmo0669	uncharacterized oxidoreductase	19.70	34.40	15.79	25.80	43.00	88.10	146.83	2.53	-1.22
lmo0670	hypothetical protein	13.50	8.30	11.06	56.00	56.00	155.00	155.00		1.14
lmo0781	manse-specific PTS system IID component	5.60	3.30	15.62	33.88	90.33	31.13	83.00	2.50	2.29
lmo0782	manse-specific PTS system IIC component	13.20	12.60	13.47	19.00	13.93	24.82	18.20	4.98	2.84
lmo0784	manse-specific PTS system IIA component	7.90	10.20	5.73	112.00	56.00	124.00	62.00		
lmo0880	similar to wall associated protein precursor	9.90	13.00	6.72	155.00	155.00	383.00	383.00		
lmo0911	hypothetical protein	2.80	2.70	2.15	3.59	4.76	3.37	4.46	-1.70	-1.33
lmo0937	predicted protein	3.90	3.80	10.41	26.00	13.00	119.00	59.50		
lmo0953	hypothetical protein	17.10	3.00	6.46	211.00	211.00	123.00	123.00		
lmo0994	hypothetical protein	14.00	7.30	14.06	82.00	82.00	81.00	81.00		
lmo1140	hypothetical protein	13.00	5.90	3.53	11.18	18.92	6.55	11.08		
lmo1241	hypothetical protein	7.00	2.60	2.22	10.50	28.00	12.88	34.33		
lmo1295	*hfq*	7.50	3.90	3.44	3.84	2.21	8.42	4.85		
lmo1375	similar to aminotripeptidase	3.30	3.40	3.02	8.89	11.43	16.67	21.43		
lmo1425	*opuCD*	1.50	1.80	3.43	3.16	2.34	5.03	3.72	3.32	2.68
lmo1433	glutathione reductase	5.80	2.60	4.25	11.00	22.00	25.00	50.00	2.17	1.21
lmo1526	hypothetical protein	3.30	1.90	3.99	9.75	4.88	16.75	8.38	3.85	1.89
lmo1606	similar to DNA translocase	4.20	2.80	5.57	7.18	4.40	12.38	7.58	1.02	1.15
lmo1694	similar to CDP-abequose synthase	9.60	2.60	11.26	55.00	220.00	69.25	277.00	2.30	
lmo1698	ribosomal-protein-alanine N-acetyltransferase	2.10	2.00	3.52	2.10	3.06	4.57	6.67	3.76	2.07
lmo1883	chitinase	11.00	12.00	3.17	139.00	139.00	172.00	172.00		
lmo2067	choloylglycine hydrolase with bile hydrolase activity	4.80	4.40	3.52	22.67	136.00	15.00	90.00		1.79
lmo2085	peptidoglycan binding protein	6.50	11.10	12.19	106.50	106.50	102.25	102.25		1.02
lmo2130	hypothetical protein	2.80	2.50	2.55	4.25	2.04	7.83	3.76		
lmo2132	hypothetical protein	4.60	3.80	5.06	8.00	16.00	7.75	15.50		
lmo2157	*sepA*	10.20	11.60	14.96	24.57	49.14	41.36	82.71		1.04
lmo2230	arsenate reductase	44.20	21.80	18.66	355.00	355.00	467.00	467.00		2.16
lmo2231	cation efflux family protein	2.70	2.30	3.06	18.00	18.00	25.00	25.00		
lmo2269	hypothetical protein	1.90	5.70	5.70	13.00	26.00	14.00	28.00		
lmo2387	hypothetical protein	3.50	2.10	4.02	51.00	17.00	37.00	12.33		
lmo2391	hypothetical protein	19.40	7.50	9.08	7.28	22.36	4.28	13.14	1.16	1.32
lmo2434	glutamate decarboxylase	1.90	3.40	2.75	204.00	102.00	132.00	66.00		-1.18
lmo2454	hypothetical protein	4.10	4.40	4.62	110.00	110.00	59.00	59.00		
lmo2463	similar to transport protein	2.40	2.30	3.90	7.60	8.44	10.60	11.78	4.31	1.08
lmo2484	membrane protein	5.60	5.20	5.09	4.40	3.14	5.67	4.05		
lmo2485	PspC domain-containing protein	5.40	3.70	4.42	2.88	2.56	5.44	4.83		
lmo2494	*phoU*	3.00	2.70	2.34	5.75	7.67	8.75	11.67		1.43
lmo2570	hypothetical protein	3.00	1.90	4.51	12.80	5.82	18.60	8.45		
lmo2571	nicotinamidase	8.50	4.00	5.84	5.84	12.17	6.92	14.42		
lmo2573	similar to zinc-binding dehydrogenase	8.90	4.00	4.58	5.39	10.58	4.76	9.35		
lmo2602	Mg2+ transporter-C family protein	4.00	2.20	7.00	27.50	55.00	16.00	32.00		
lmo2603	amidase	3.40	3.80	6.75	47.50	47.50	41.00	41.00		
lmo2670	hypothetical protein	2.10	1.90	2.32	3.00	3.64	2.41	2.93		
lmo2671	hypothetical protein	2.40	2.80	2.20	2.83	2.43	3.90	3.34		
lmo2672	weakly similar to transcription regulator	2.40	1.70	2.67	4.13	3.35	3.63	2.95		
lmo2673	universal stress protein	18.70	13.20	9.70	211.00	211.00	90.00	90.00		2.15
lmo2674	similar to ribose 5-phosphate epimerase	7.40	2.70	3.76	6.48	8.64	2.81	3.74	-1.10	-1.04
lmo2724	DNA binding 3-demethylubiquine-9 3-methyltransferase domain-containing protein	3.40	1.80	2.86	4.42	11.78	3.17	8.44	3.28	2.26
lmo0019	transmembrane protein	2.30	3.50	2.92	31.00	15.50	48.00	24.00		
lmo0043	*arcA*	3.90	3.50	3.68	119.00	59.50	81.00	40.50		2.72

Consistent with previous transcriptional studies, proteomics identified few genes that show evidence for negative regulation by σ^B.

Our quantitative proteomic comparison conducted in this study found only 6 proteins that displayed significantly lower levels in the parent strain as compared to the Δ*sigB* mutant; three of these proteins are encoded by a single operon (Lmo1997, a mannose-specific PTS system IIA component; Lmo1998, a sugar isomerase domain-containing protein; Lmo2002, a mannose-specific PTS system IIB component; these showed fold changes of -1.56, -1.60, and -1.59, respectively). The other proteins with lower expression levels were Lmo0427 (fructose-specific PTS system IIB component), Lmo0484 (heme-degrading monooxygenase, IsdG), and Lmo2648 (similar to phosphotriesterase), which showed fold changes of -1.56, -1.53, and -1.60, respectively. The genes encoding these six proteins also had significant *p*-values[c] in the microarray meta-analysis, and displayed negative fold changes ranging from -1.6 to -1.1 in three (lmo2648; lmo1998; lmo2002) or two (lmo0484; lmo0427; lmo1997) microarray studies in Δ*sigB* stationary phase cells [26, 28, 33]. No genes were identified as negatively regulated by σ^B in the RNA-Seq study [27]. These findings are similar to previous analyses that show that few genes are consistently identified as being negatively regulated by σ^B [29], which is likely due to the fact that negative regulation would be indirect. The only example of negative regulation by σ^B for which the mechanism has been characterized was described for genes required for the synthesis of flagellum, which are negatively regulated by σ^B though an antisense RNA mechanism [37].

Meta-analysis of microarray data and comparison with RNA-Seq data identified 134 genes that show evidence for σ^B-dependent transcript levels.

A meta-analysis of three microarray studies [26, 28, 33] and comparison with RNA-Seq data [27] that also probed the stationary phase σ^B regulon in *L. monocytogenes* 10403S identified 134 genes that show strong evidence for being positively regulated by σ^B. These 134 genes include (i) 118 genes that were identified by our meta-analysis as being under positive control by σ^B and (ii) 16 genes that were identified as showing σ^B-dependent transcript levels by RNA-Seq but not by the microarray meta-analysis (Fig. 3.1; Fig. 3.2). The 118 genes identified by our meta-analysis as being under positive control by σ^B include (i) 65 genes identified by both microarray meta-analysis and RNA-Seq, but not by proteomics (Group G; Appendix 3.3); (ii) 35 genes identified by microarray meta-analysis, but not by RNA-Seq or proteomics (Group B; Appendix 3.3); (iii) 15 genes identified by microarray meta-analysis, RNA-Seq, and the proteomics comparison in this study (Group A; Appendix 3.3); and (iv) 3 genes identified by microarray meta-analysis and proteomics, but not by RNA-Seq (Group E; Appendix 3.3). Among these 118 genes, 101 were preceded by putative σ^B-dependent promoters, including 63/65 genes in Group G, 20/35 in Group B, 15/15 in Group A and 3/3 in Group E. The 16 genes that were identified as showing σ^B-dependent transcript levels by RNA-Seq but not by the microarray meta-analysis included (i) 13 genes identified by RNA-Seq but not by microarray meta-analysis and proteomics (Group C; Appendix 3.3) and (ii) 3 genes identified by RNA-Seq and proteomics but not by microarray meta-analysis (Group F; Appendix 3.3).

Overall, the 134 genes identified here as showing σ^B-dependent transcript levels include two groups that are only supported by a single experimental approach, i.e., 35 genes that were identified only by microarray meta-analysis (Group B; Appendix 3.3) and 13 genes only identified by RNA-Seq (Group C; Appendix 3.3). A number of pieces of evidence support that the genes in Group B are indeed σ^B-dependent, including (i) 20/35 genes in Group B are preceded by putative σ^B consensus promoters and (ii) 4/35 genes in Group B are in operons that include genes that were identified as σ^B-dependent by other experimental approaches (i.e., RNA-Seq or proteomics). In addition, we found that the 38 genes identified as σ^B-dependent by microarray, but not by RNA-Seq (representing the 35 genes in Group B as well three genes in Group E) show significantly higher RNA-Seq fold change values ($p < 0.05$; one-sided Wilcoxon ranked sum test) as compared to genes not identified as σ^B-dependent by microarray or RNA-Seq, suggesting that many of these genes represent false negatives for σ^B dependence in RNA-Seq. A number of pieces of evidence also support that the genes in Group C (identified as positively regulated by σ^B through RNA-Seq but not by microarray meta-analysis or proteomics) are indeed σ^B-dependent, including (i) 8/13 genes in Group C are preceded by σ^B consensus promoters, (ii) 3/13 genes in Group C are in operons that include genes that were identified as σ^B-dependent by other experimental approaches (i.e., microarray meta-analysis or proteomics), and (iii) 7/13 genes had a significant *p*-value[c] and fold change above 1.0 in all three microarray studies, but missed the fold change cutoff of ≥ 1.5 in one or more microarray study. Two of the genes in Group C (*inlA* and *inlB*) are located in the same operon and are co-regulated by PrfA and σ^B [16, 27, 28, 33] and

were thus not identified as positively regulated by σ^B in the one microarray study that used a *prfA** genetic background, which expresses a constitutively active PrfA [28].

The 134 genes identified as positively regulated by σ^B by the meta-analysis reported here included 21 genes where the corresponding proteins were also identified here as showing higher expression levels in the parent strain, as compared to the Δ*sigB* strain; an additional 15 proteins were identified as σ^B-dependent by proteomics only, for a total of 149 σ^B-dependent genes and proteins (Appendix 3.1). Statistical analyses showed that role categories (n=21) were not randomly distributed among these 149 genes ($p = 0.0210$; Monte-Carlo simulation of Fisher's exact test). Individual Fisher's exact tests for each role category showed that the role category "energy metabolism" was significantly overrepresented among the 149 σ^B-dependent genes and proteins (Appendix 3.4), similar to data reported by Oliver *et al*., 2010 [26], who found σ^B-dependent genes to be overrepresented in the "energy metabolism" and "cellular processes" role categories. The contributions of the σ^B regulon to energy metabolism are also supported by phenotypic data. For example, under energy stress in glucose-limiting media, a *L. monocytogenes* Δ*sigB* mutant was shown to grow more rapidly than the parent strain, while the parent strain displayed greater survival after the carbon source was depleted [6]. In addition to 30 genes that grouped into the "energy metabolism" category, the 149 σ^B-dependent genes and proteins identified here also represented 18 other role categories, including transport and binding proteins (22 genes) and cellular processes (14 genes) (Appendix 3.4), illustrating the extensive network of cellular functions regulated by σ^B.

Genes identified as positively regulated by σ^B by proteomics but not by transcriptional analyses largely represent false negatives in transcriptional approaches, but also include at least some genes that may show indirect regulation by σ^B.

Considerable evidence supports that the 15 proteins that were identified as positively regulated by σ^B through proteomics only (Group D; Appendix 3.3) represents a number of genes that were likely false negatives for σ^B-dependent transcript levels in the microarray meta-analysis and/or the RNA-Seq data analysis. Evidence supporting this notion includes (i) 4/15 genes encoding these proteins are preceded by a putative σ^B-dependent promoter, (ii) 8/15 genes had a significant *p*-value[c] and fold change above 1.0 in at least two microarray studies, but missed the fold change cutoff of $\geq$ 1.5 in one or more microarray study, and (iv) 14/15 genes only failed one or two FC or Q-value cut-offs in the RNA-Seq study by Oliver *et al*., 2009 [27], which considered genes as σ^B-dependent if they met both Q-value and FC cut-offs in all four comparisons carried out in that study. When including studies that used *L. monocytogenes* strains other than 10403S (e.g., EGD-e [11]; FSL J1-194, FSL J1-208, FSL J2-071 [26]), we also found that eight genes in Group D had previously been reported as positively regulated by σ^B in at least one study; the only 7 genes not previously reported, in any study reviewed here, as positively regulated by σ^B are lmo0643, lmo1046, lmo1349, lmo2047, lmo2743, lmo0110, and lmo1730 (Appendix 3.3); lmo1046, lmo2743, and lmo0110 are part of the 8 genes in category (ii) listed above.

Interestingly, a number of proteins that were identified as showing σ^B-dependent expression levels and were classified into Group D are annotated as having functions that relate to energy metabolism and transport and binding functions. For example, Group D proteins Lmo2695, Lmo2696, Lmo2697 are encoded as part of the *dhaK* operon, which includes a σ^B consensus promoter upstream of lmo2695. Among the genes encoding these three proteins, lmo2696, and lmo2697 had a significant *p*-value[c] (< 0.05) in the microarray meta-analysis and this operon was previously reported to be σ^B-dependent in strain EGD-e [11]. σ^B thus appears to regulate transcription of these genes, which encode subunits of dihydroxyacetone kinase, an enzyme that plays a role in energy metabolism. The Group D protein Lmo1422 is encoded in an operon that includes lmo1421 (which was also identified as σ^B-dependent; Group C, Appendix 3.3). This operon is preceded by a σ^B consensus promoter upstream of lmo1421; and lmo1421 has been reported to be transcribed in a σ^B-dependent manner [9, 36]. While lmo1421 and lmo1422 were initially annotated as glycine betaine/carnitine/choline ABC transporters, they have been reported to be part of a bile exclusion system coordinately regulated by σ^B and PrfA [9, 35, 36]. The gene encoding the Group D protein Lmo2666, annotated as a galactitol-specific PTS system IIB component, was previously found to be positively regulated by σ^B in *L. monocytogenes* lineage I and lineage IIIB strains, but not in 10403S [26]. lmo2666 has also been found to be differentially regulated in comparisons between *L. monocytogenes* EGD-e and mutants that did not express the regulators CodY and DegU [3, 41], suggesting regulatory interactions that govern transcription of this gene.

The Group D proteins Lmo0398 and Lmo0399 (annotated as fructose-specific PTS system IIA and IIB components) are also encoded by an operon. lmo0398 and lmo0399 showed a significant *p*-value[c] in our microarray meta-analysis and showed FC values > 10 in comparisons of wildtype and Δ*sigB* strains reported by Raengpradub *et al*. (2008) [33] and Oliver *et al*. (2010) [26] (*p*-value < 0.01 in both studies), but were not found to have significantly different transcript levels in the study by Ollinger *et al*. (2009) [28], which used a *L. monocytogenes prfA** strain. As described above for the *inlAB* operon (Group C), these genes thus are likely co-regulated by PrfA and σ^B and hence were not identified as positively regulated by σ^B in the microarray study with the *prfA** strain. lmo0398 and lmo0399 were previously proposed to play a role in σ^B-dependent regulation of carbohydrate metabolism during low nutrient energy stress conditions encountered during stationary phase [7]. Interestingly, the gene encoding Lmo1539, a Group D protein involved in the uptake of glycerol, which *L. monocytogenes* can utilize as a carbon source for growth [14, 32], was found to be negatively regulated by σ^B in the 10403S microarray analyses conducted by Raengpradub *et al.* (2008) [33] and Oliver *et al.* (2010) [26] (FC= 0.2 and 0.5, respectively; $p < 0.01$ in both studies), but was not found to be significantly differentially expressed in the microarray comparison of a *prfA** and *prfA**Δ*sigB* strain [28]. However, lmo1539 was found to be up-regulated during intracellular replication [5] and in a σ^B-dependent manner in the intestine [37] using *L. monocytogenes* EGD-e, during stationary phase stress using *L. monocytogenes* FSL J1-194, FSL J2-071, and FSL J1-208 [26], and showed evidence for positive regulation by σ^B using *L. monocytogenes* 10403S when FPSS (fluoro-phenyl-styrene-

sulfonamide) was used to inhibit σ^B activation [29]. The gene encoding the Group D protein Lmo0643, annotated as a transaldolase, was found to be significantly downregulated in the *prfA** parent stain as compared to the *prfA**Δ*sigB* strain (FC = 0.3, *p*-value < 0.05) [28], whereas it was not found to be significantly differentially expressed in the parent and Δ*sigB* strain comparisons by Raengpradub *et al*. (2008) [33] and Oliver *et al*. (2010) [26]. Overall, a number of the proteins classified in Group D thus appear to be encoded by genes that are co-regulated by multiple transcriptional regulators, in particular PrfA and σ^B, supporting previously proposed models that hypothesized that σ^B plays a role in a number of regulatory networks (Appendix 3.2) and highlighting a role of PrfA and σ^B interaction in regulating some aspects of energy metabolism in stationary phase *L. monocytogenes*.

Identification of a 5' UTR, upstream of lmo1349, that functions as a metabolite-binding riboswitch, which is linked to a σ^B-dependent glycine cleavage pathway.

While some of the 15 proteins that were identified here as σ^B-dependent by proteomics only (Group D, Appendix 3.3) appear to actually be encoded by genes that show σ^B-dependent transcription (representing false negatives in the microarray meta-analysis and /or RNA-Seq analysis), we hypothesized that some of the proteins classified in Group D are regulated by σ^B through post-transcriptional mechanisms. Genes that are regulated post-transcriptionally would be expected to show discrepancies between mRNA expression levels and the expression levels of their protein products [12, 31, 42]. In our case, we thus initially analyzed genes that showed σ^B-dependent protein levels, but not σ^B-dependent transcript levels (i.e., proteins in Group D). As transcripts of several *L. monocytogenes* genes are known to

be regulated post-transcriptionally, particularly through mechanisms affecting the 5' UTR of the transcripts [19], we initially compared 5' UTR lengths among three categories of genes including genes identified as positively regulated by σ^B through (i) RNA-based methods, but not by our proteomics data (Groups B, C, G; Appendix 3.3); (ii) proteomics data only (Group D, Appendix 3.3); and (iii) all three methods (RNA-Seq, microarray meta-analysis, and proteomics) (Group A, Appendix 3.3). A two-sided and one-sided Wilcoxon ranked sum test found no significant differences in 5' UTR lengths between all three groups ($p > 0.05$).

As one mechanism for post-transcriptional regulation is through riboswitches, we further evaluated the 5' UTR regions of the genes encoding the nine Group D proteins that (i) are identified as positively regulated by σ^B in our proteomics analysis; (ii) are encoded by genes that were not identified by RNA-based methods as showing σ^B-dependent transcript levels; and (iii) are not preceded by a σ^B consensus promoter. One of these proteins, *Lmo1349,* was found to be encoded by the second gene in the three gene *gcvT* operon. In addition to lmo1349 (*gcvPA*), which encodes the glycine dehydrogenase subunit 1, this operon includes lmo1348 (*gcvT*), which encodes the glycine cleavage system T protein, and lmo1350 (*gcvPB*), which encodes the glycine dehydrogenase subunit 2 (glycine cleavage system P2-protein). Interestingly, the first gene in this operon has a 5' UTR that has previously been annotated as a glycine riboswitch [20] (Fig. 3.3). This glycine riboswitch is conserved across many bacterial species and occurs upstream of genes encoding proteins of the glycine cleavage system, which facilitates use of glycine as an energy source [4, 8, 13, 18, 20, 34]. In *B. subtilis*, it has been shown that this riboswitch regulates transcription of the

downstream *gcvT* operon [20, 30]. In the absence of glycine, this riboswitch allows for formation of a terminator upstream of *gcvT*; consequently, approximately 70% of the transcripts from the upstream promoter are represented by a short, ~ 200 nt, transcript, while the other 30% of transcripts represent the full length 4 kb *gcvT* operon. In the presence of glycine, which binds to the riboswitch, the terminator appears to be destabilized and consequently ~70% of the transcript is full length [20]. Glycine dependent activation of this riboswitch has also been reported in other species [34]. We hypothesize that σ^B-dependent formation of glycine is responsible for activating transcription of the *gcvT* operon via this riboswitch, as we found lmo2539 (*glyA;* Group B, Appendix 3.3), which encodes a serine hydroxymethyltransferase that catalyzes the interconversion of L-serine to glycine [21], to be positively regulated by σ^B. In addition to the σ^B-dependent production of glycine, *L. monocytogenes* also includes glycine betaine/carnitine/choline ABC transporters that are encoded by σ^B-dependent genes, suggesting that the multiple σ^B-dependent glycine synthesis and uptake systems may facilitate indirect σ^B-dependent activation of the glycine riboswitch upstream of the *gcvT* operon in *L. monocytogenes.*

As the mechanism proposed here for σ^B-dependent expression of the proteins in the *gcvT* operon involves transcriptional regulation through the glycine riboswitch, one might expect that the three genes in the *gcvT* operon should actually show differential expression in transcriptome analyses of *L. monocytogenes* Δ*sigB* strains; neither our microarray meta-analysis nor previous RNA-Seq data found evidence for σ^B-dependent transcription of genes in the *gcvT* operon, even though lmo1348 (*gcvT*) was found to show significantly higher transcript levels in the parent strain as

compared to a Δ*sigB* strain (FC = 2.1; $p = 0.002$) in one microarray study [33]. We propose that the relatively minor differences in transcript levels for these three genes (as supported by *B. subtilis* data that show that even in the absence of glycine a considerable proportion of the *gcvT* operon transcripts were still of full length) were not detectable by microarray, but were detectable by the proteomics tools. Additional indirect σ^B-dependent post-transcriptional regulatory mechanisms cannot be excluded though, and future detailed dissection of the σ^B-dependent regulation of glycine cleavage systems will be necessary, particularly since one previous proteomics study [2] also found evidence for σ^B-dependent protein levels, in exponential phase *L. monocytogenes* grown in DM with salt, for GcvH, which encodes another component of the glycine cleavage system outside the *gcvT* operon.

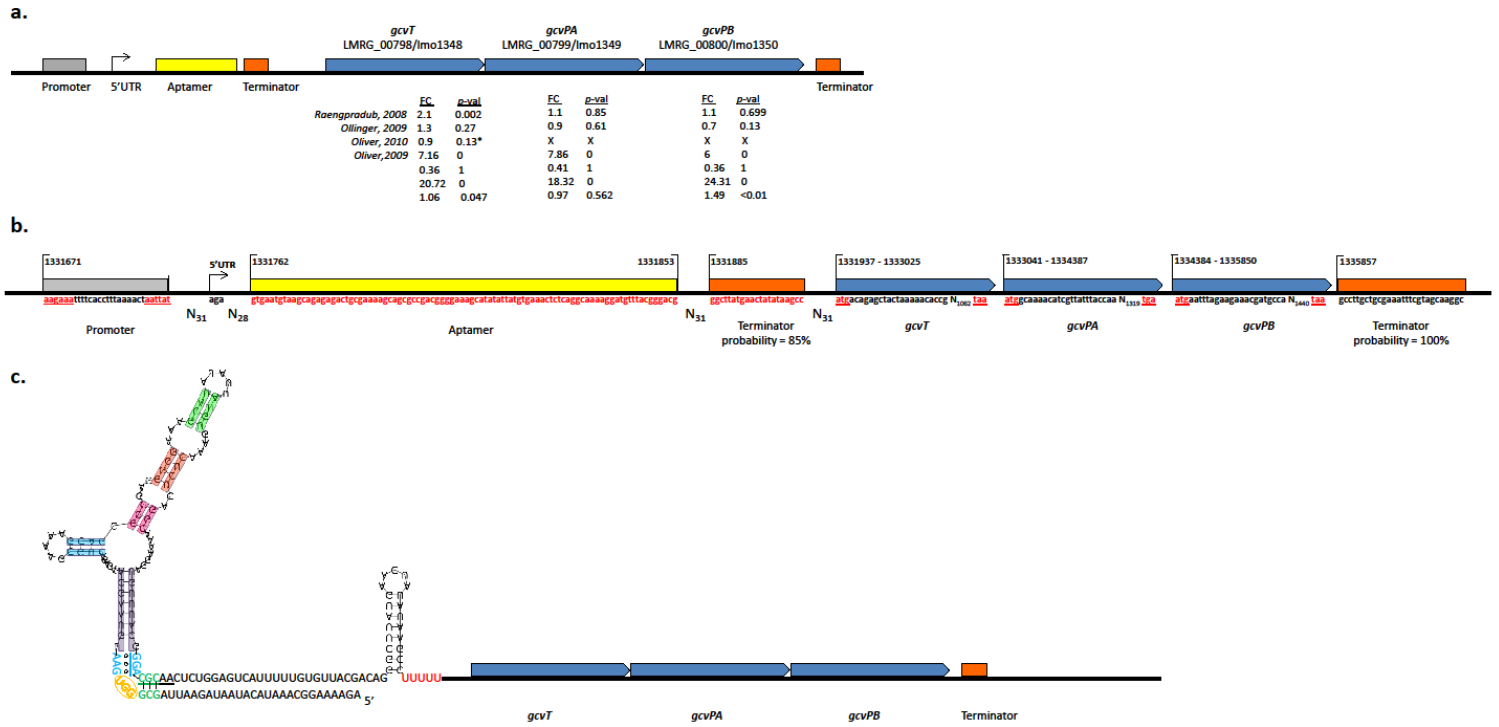

Figure 3.3. Glycine cleavage system in *L. monocytogenes* 10403S. (a) Operon map for glycine cleavage system genes, *gcvT*, *gcvPA*, and *gcvPB*, including the putative promoter, and the aptamer and putative terminator of the glycine riboswitch upstream of the genes. The fold changes and *p*-values for the genes from Raengpradub *et al*. (2008) [33], Ollinger *et al*. (2009) [28], Oliver *et al*. (2009) [27] and Oliver *et al*. (2010) [26] are included. *In the Oliver *et al*. (2010) study [26], the fold changes (FC) and *p*-values for *gcvT* in lineage I, IIIA, and IIIB strains were FC= 1.7, *p*= 0.044; FC= 0.5, *p*= 0.006; and FC= 1.4, *p*= 0.625, respectively. (b) Extended operon map showing the sequences and positions for the glycine riboswitch (aptamer and putative terminator) based on RNA-Seq data (unpublished). (c) Structure of the glycine riboswitch aptamer based on the putative base pairing for lmo1348 provided in Mandal *et al*. (2004) [20]; the leader linker interaction is based on the data for lmo1348 provided in Sherman *et al*. (2012) [34]. The terminator was identified using TransTermHP [17].

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CHAPTER 4

PROTEIN LEVEL IDENTIFICATION OF THE *LISTERIA MONOCYTOGENES* SIGMA H, SIGMA L, AND SIGMA C REGULONS

ABSTRACT

Transcriptional regulation by alternative σ factors represents an important mechanism that allows bacteria to rapidly modify expression of large groups of genes in response to changing environmental conditions. While the role of the alternative σ factor σ^B has been comparatively well characterized in *L. monocytogenes*, our understanding of the roles of the three other *L. monocytogenes* alternative σ factors is still limited. In this study, we employed a quantitative proteomics approach using Isobaric Tags for Relative and Absolute Quantitation (iTRAQ) to characterize the *L. monocytogenes* σ^L, σ^H, and σ^C protein regulons. Proteomic comparisons used a quadruple alternative σ factor mutant strain (Δ*BCHL*) and strains expressing a single alternative σ factor (i.e., σ^L, σ^H, and σ^C; strains Δ*BCH,* Δ*BCL*, and Δ*BHL*) to eliminate potential redundancies between σ factors. Among the three alternative σ factors studied here, σ^H provides positive regulation for the largest number of proteins, consistent with previous transcriptomic studies, while σ^L appears to contribute to negative regulation of a number of proteins. σ^C was found to regulate a small number of proteins in *L. monocytogenes* grown to stationary phase at 37°C. Proteins identified as being regulated by multiple alternative σ factors include MptA, which is a component of a PTS system with a potential role in regulation of PrfA activity. This study provides initial insights into global regulation of protein expression by the *L. monocytogenes*

alternative σ factors σ^L, σ^H, and σ^C. While, among these σ factors, σ^H appears to positively regulate the largest number of proteins, we also identified PTS systems that appear to be co-regulated by multiple alternative σ factors. Future studies should not only explore potential roles of alternative σ factors in activating a "cascade" of PTS systems that potentially regulate PrfA, but also may want to explore the σ^L and σ^C regulons under different environmental conditions to identify conditions where these σ factors may regulate larger numbers of genes.

INTRODUCTION

The foodborne pathogen *Listeria monocytogenes* uses complex regulatory mechanisms to adapt to a variety of environmental conditions and to cause listeriosis, a life-threatening infection, in humans and animals. A key mechanism used by *L. monocytogenes* to regulate gene expression in order to adapt to changing environmental conditions is through alternative sigma (σ) factors. Alternative σ factors reprogram the RNA polymerase holoenzyme to recognize specific promoters and hence allow for rapid induction of transcription of potentially large groups of genes under specific environmental conditions [1]. In *L. monocytogenes*, four alternative σ factors, σ^B, σ^C, σ^H, and σ^L, have been identified. However, σ^C has only been described in *L. monocytogenes* strains that group into lineage II, a well defined phylogenetic group that includes serotypes 1/2a and 1/2c [2-4]. A number of studies that have explored σ^B-mediated stress response as well as σ^B-mediated gene and protein expression in *L. monocytogenes* [1, 5-16] have shown that this alternative σ factor controls a large regulon and contributes to both stress response and virulence.

σ^H, σ^L, and σ^C have not been as extensively characterized as σ^B in *L. monocytogenes*, at least partially because studies to date have only identified limited phenotypic consequences of null mutations in these σ factors in *L. monocytogenes*. Among these three alternative σ factors, σ^H appears to control the largest regulon; Chaturongakul *et al*. (2011) identified 97 and 72 genes as positively and negatively regulated by σ^H, respectively, in *L. monocytogenes* strain 10403S [7]. While a *L. monocytogenes* EGD-e *sigH* mutant was reported to have significantly impaired growth in minimal medium and under alkaline stress conditions as well as slightly reduced virulence potential in a mouse model [17], phenotypic studies in a *L. monocytogenes* 10403S Δ*sigH* strain did not find evidence for an effect of this mutation on virulence in a guinea pig model, cell invasion and intracellular growth, or resistance to heat stress [7]. With regard to σ^L, 31 and 20 genes were identified as positively and negatively regulated, respectively, by this σ factor, in *L. monocytogenes* 10403S [7]. A more recent study in *L. monocytogenes* EGD-e identified 237 and 203 genes as positively regulated by σ^L when the parent and Δ*sigL* mutant strains were grown at 3°C and 37°C, respectively; most of the 47 genes that showed positive regulation by σ^L under both temperatures were located within prophage A118 [18]. Phenotypic and gene expression studies also support a potential contribution of σ^L to *L. monocytogenes* growth under different stress conditions, most notably osmotic and low temperature stress [19, 20]. *L. monocytogenes* σ^L has also been reported to be involved in resistance to the antimicrobial peptide mesentericin Y105 [21]. Finally, studies conducted to date on the *L. monocytogenes* σ^C regulon typically identified few genes as σ^C-dependent. Chaturongakul *et al*. (2011) were only able to identify and

confirm, by qRT-PCR, a single gene (lmo0422) as σ^C-dependent; lmo0422, which encodes LstR, a lineage II specific thermal regulator, is in the same operon as *sigC* and this finding is consistent with previous data suggesting that the *sigC* operon is auto-regulated [3, 7]. Zhang *et al*. (2005) also found some evidence that σ^C may contribute to thermal resistance in the *L. monocytogenes* lineage II strain 10403S, when grown to log phase [3]; by contrast, Chaturongakul *et al*. (2011) did not find any evidence for reduced heat resistance when an independent *L. monocytogenes* 10403S Δ*sigC* strain was grown to stationary phase prior to heat exposure [7].

Previous studies have suggested considerable overlap between different *L. monocytogenes* alternative σ factor regulons (e.g., between the σ^B and the σ^H regulon), suggesting the potential for redundancies as well as compensation for deletion of a single alternative σ factor by other σ factors. We thus hypothesized that an experimental approach that eliminates these potential redundancies is needed to gain a better understanding of the roles of σ^C, σ^H, and σ^L in regulating protein expression in *L. monocytogenes*. We particularly focused on exploring the contributions of these alternative σ factors to protein expression as, despite availability of a number of proteomics data sets on the σ^B regulon [15, 16], only a single proteomics study on the σ^L regulon is available [22]. As an experimental approach, we selected to create an *L. monocytogenes* 10403S quadruple mutant with a non-polar deletion of all four genes that encode alternative σ factors (i.e., strain Δ*BCHL*) as well as corresponding mutants with deletions of three alternative σ factors (Δ*BCH*, Δ*BCL*, and Δ*BHL*), which thus expressed only σ^L, σ^H, and σ^C, respectively. These strains where then used for

proteomic comparisons between the quadruple mutant strain and the three different strains expressing only a single alternative σ factor.

MATERIALS AND METHODS

Bacterial strains, mutant construction, and growth conditions.

Splicing by overlap extension (SOE) PCR and allelic exchange mutagenesis was used to construct Δ*BCL*, Δ*BHL*, Δ*BCH*, and Δ*BCHL* mutant strains in a *L. monocytogenes* 10403S background as described previously [13] (Appendix 4.1). All mutations were confirmed by PCR amplification and sequencing of the PCR product. Strains were grown to stationary phase in BHI at 37°C as described previously [31].

Protein isolation, iTRAQ labeling, and Nano-scale reverse phase chromatography and tandem mass spectrometry (nanoLC-MS/MS).

Protein isolation, digestion, and iTRAQ labeling were performed as previously described [31]**.** Briefly, proteins were isolated from a 25 ml culture of *L. monocytogenes* stationary phase cells. A noninterfering protein assay kit (Calbiochem) and 1D SDS-PAGE were used to verify protein concentration and quality. A total of 100 µg protein of each sample was denatured, reduced, and the cysteine residues were blocked. Protein samples were then digested with sequence-grade-modified trypsin at 37ºC for 16 h, and protein digestion efficiency was assessed by SDS-PAGE. Tryptic peptides from *L. monocytogenes* parent strain 10403S and Δ*BCL*, Δ*BHL*, Δ*BCH*, and Δ*BCHL* mutant strains were each labeled with iTRAQ reagents, according to the manufacturer's protocols.

Four labeled protein samples were combined for a single run and fractionated via Isoelectric focusing OffGel electrophoresis (OGE) using an Agilent 3100 OFFGEL Fractionator (Agilent, G3100AA), and subsequent nanoLC-MS/MS was carried out using a LTQ-Orbitrap Velos (Thermo-Fisher Scientific) mass spectrometer as previously described [31]. Two separate biological replicates of the entire proteomics experiment were run for each strain.

Protein identification and data analysis.

All MS and MS/MS raw spectra from iTRAQ experiments were processed using Proteome Discoverer 1.1 for subsequent database search using in-house licensed Mascot Daemon; and quantitative processing, protein identification, and data analysis were conducted as previously described [31].

The biological replicates of each experiment were analyzed independently. As described in [31], the Wilcoxon signed rank test was applied to peptide ratios for each identified protein to determine significant changes between strains. The Fisher's Combined Probability Test was then used to combine FDR adjusted Wilcoxon *p*-values from each replicate into one test statistic for every protein to obtain a combined *p*-value (p-value^c). Proteins with peptide ratios exhibiting a Fisher's Combined Probability Test p-value$^c < 0.05$ and an iTRAQ protein ratio ≥ 1.5 in both replicates were considered significantly differentially expressed. Statistical analyses were conducted using R statistical software.

A Monte Carlo simulation of Fisher's exact test was used to determine whether the distribution of role categories among proteins identified as differentially regulated by a given σ factor was different from the role category distribution that would be

expected by chance (based on the role category primary annotation for all *L. monocytogenes* EGD-e genes [32]). Individual Fishers exact tests were then used to determine whether individual role categories were over- or underrepresented; uncorrected *p*-values were reported, allowing readers to apply corrections if deemed appropriate. Analyses were performed using all role categories assigned to a given gene in the JCVI-CMR *L. monocytogenes* EGD-e database. Analyses were only performed for regulons that contained 10 or more proteins (i.e., proteins positively regulated by σ^H; proteins negatively regulated by σ^L; proteins with higher or lower expression level in the parent strain).

RESULTS AND DISCUSSION

Proteomic comparisons between *L. monocytogenes* mutants expressing only σ^L, σ^H, and σ^C and a quadruple mutant that does not express any alternative σ factors, all grown to stationary phase at 37°C, showed that (i) σ^H provides, among these three alternative σ factors, positive regulation for the largest number of proteins, consistent with previous transcriptomic studies; (ii) σ^L appears to contribute to negative regulation of a number of proteins; (iii) σ^C regulates a small number of proteins in *L. monocytogenes* grown to stationary phase at 37°C; and (iv) proteins regulated by multiple alternative σ factors include MptA, which has a potential role in regulation of PrfA.

σ^H positively regulates a large number of proteins.

Our proteomic comparison identified 15 proteins as positively regulated by σ^H, as supported by higher protein expression levels (Fold change (FC) $\geq$ 1.5; *p*-value[c] (p^c) < 0.05) in *L. monocytogenes* Δ*BCL* as compared to the Δ*BCHL* strain (Table 4.1); 4 of these 15 proteins also showed higher expression levels in the parent strain (which expresses all four alternative σ factors) as compared to the quadruple mutant. Overall, positive fold changes for these proteins (in Δ*BCL* versus Δ*BCHL*) ranged from 1.55 to 3.39. These 15 proteins represented nine role categories (e.g., "energy metabolism"; "amino acid biosynthesis"; "transport and binding proteins", see Fig. 4.1); a Monte Carlo simulation of Fisher's exact test did not find a significant association between positively regulated genes and role categories ($p = 0.06$); however, individual Fisher's exact tests did show overrepresentation of proteins in the role category "amino acid biosynthesis" among the 15 proteins that were found to be positively regulated by σ^H (with a significant *p*-value; $p < 0.01$; Odds Ratio = 6.26). Some of the 15 proteins positively regulated by σ^H have likely roles in stress adaptation and virulence, including Lmo1439 (superoxide dismutase, SodA) [23] and Lmo0096 (mannose-specific PTS system IIAB component, MptA), which has been linked to regulation of the virulence gene regulator PrfA [24]. Previously reported transcriptomic studies [7] only identified the coding gene for one of these 15 proteins (i.e., Lmo1454) as σ^H-dependent; lmo1454 was also identified as preceded by a σ^H consensus promoter, suggesting direct transcriptional regulation by σ^H. In addition, the coding gene for Lmo2487, one of these 15 proteins, is in an operon with lmo2485, which was

previously reported to be positively regulated by σ^H, even though no upstream σ^H consensus promoter was identified, suggesting indirect regulation [7].

Our proteomic comparison also identified four proteins that showed lower expression levels in the strain expressing σ^H, suggesting (indirect) negative regulation by σ^H; 3 of these 4 proteins also showed lower expression levels in the parent strain (which expresses all 4 alternative σ factors) as compared to the quadruple mutant. None of the genes encoding these proteins showed significantly higher transcript levels in a Δ*sigH* strain in a transcriptomic study [7]. However, the coding gene for Lmo1877, one of these 4 proteins, is in an operon with lmo1876, which was previously reported to be negatively regulated by σ^H [7]. Overall, global indirect downregulation of proteins by σ^H does not seem to play an important role in stationary phase *L. monocytogenes* 10403S.

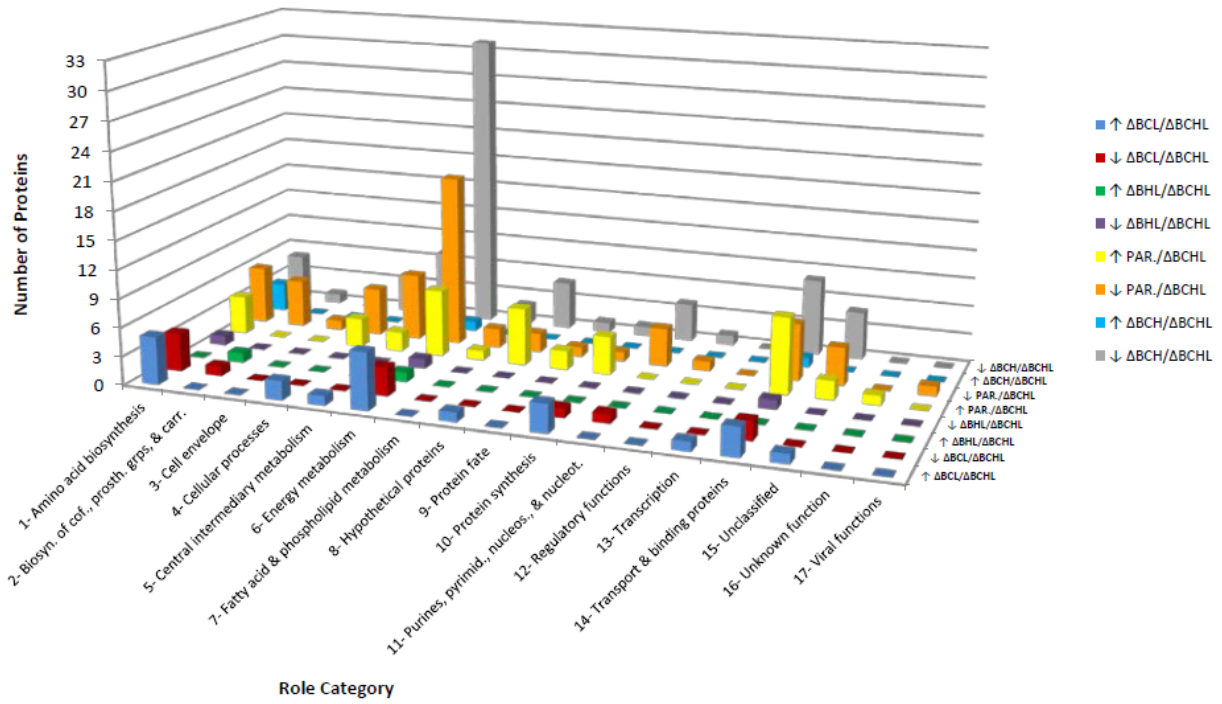

Figure 4.1. Functional role category classification of alternative σ factor dependent proteins. Functional role category classification of σ^H positively-regulated (blue), σ^H negatively-regulated (red), σ^C positively-regulated (green), σ^C negatively-regulated (purple), σ^L positively-regulated (turquoise), and σ^L negatively-regulated (gray) proteins; and proteins with higher expression in *L. monocytogenes* parent strain 10403S (PAR.) compared to Δ*BCHL* (yellow) and lower expression in PAR. compared to Δ*BCHL* (orange). Role category numbers correspond to: (1) Amino acid biosynthesis; (2) Biosynthesis of cofactors, prosthetic groups, and carriers; (3) Cell envelope; (4) Cellular processes; (5) Central intermediary metabolism; (6) Energy metabolism; (7) Fatty acid and phospholipid metabolism; (8) Hypothetical proteins; (9) Protein fate; (10) Protein synthesis; (11) Purines, pyrimidines, nucleosides, and nucleotides; (12) Regulatory functions; (13) Transcription; (14) Transport and binding proteins; (15) Unclassified; (16) Unknown function; (17) Viral functions. One protein may be classified into more than one role category. Statistical analysis of contingency tables for regulons with >10 proteins (i.e., proteins positively regulated by σ^H; proteins negatively regulated by σ^L; proteins with higher or lower expression level in the parent strain) found that role categories were not randomly distributed among proteins negatively regulated by σ^L and proteins with lower expression levels in the parent strain.

Table 4.1: Proteins found to be differentially regulated by σ^H, as determined by a proteomic comparison between *L. monocytogenes* 10403S Δ*BCL* and Δ*BCHL*

Protein[a]	Fold Change Δ*BCL*/Δ*BCHL*	Description	Gene Name	Role Category[b]	Sub-Role Category[b]	Promoter[d]	Sigma Factor
Proteins with positive fold change (≥ 1.5) and $p < 0.05$ (indicating positive regulation by σ^H)							
Lmo0027	1.55	beta-glucoside-specificPTS system IIABC component	*lmo0027*	Transport and binding proteins	Carbohydrates, organic alcohols, and acids	aggacacgtgtatgcgtggagtcctcgaatga	SigmaH
				Amino acid biosynthesis	Aromatic amino acid family		
				Energy metabolism	Pyruvate dehydrogenase		
Lmo0096	3.39	mannose-specific PTS system IIAB component ManL	*mptA*	Energy metabolism	Pyruvate dehydrogenase	tggcacagaacttgca	SigmaL
				Amino acid biosynthesis	Aromatic amino acid family		
				Transport and binding proteins	Carbohydrates, organic alcohols, and acids		
Lmo0239	1.82	cysteinyl-tRNA synthetase	*cysS*	Protein synthesis	tRNA aminoacylation	ttgcaaggaattttattgctgttataatag	SigmaA
Lmo0319	1.77	beta-glucosidase	*bglA*	Energy metabolism	Sugars	N/A	N/A
Lmo0356	2.16	YhhX family oxidoreductase	*lmo0356*	Energy metabolism	Fermentation	tggctaagtacagcgctagtgtagtactat	SigmaA
				Energy metabolism	Electron transport		
				Central intermediary metabolism	Other		
Lmo1001	1.65	hypothetical protein	*lmo1001*	Unclassified	Role category not yet assigned	N/A	N/A
Lmo1070	2.18	similar to B. subtilis YlaN protein	*lmo1070*	Hypothetical proteins	Conserved	ttgcgtggcaaataaattatgctatact	SigmaA
Lmo1255	1.60	trehalose-specific PTS system IIBC component	*lmo1255*	Energy metabolism	Pyruvate dehydrogenase	ttgcgctttcaactgatttatagtatagt	SigmaA
				Amino acid biosynthesis	Aromatic amino acid family		
				Transport and binding proteins	Carbohydrates, organic alcohols, and acids		
Lmo1439	1.66	superoxide dismutase	*sodA*	Cellular processes	Detoxification	ttgcaagcatttagggagcatggtaggct	SigmaA
						gtttaacttttgagtttcagggaaa	SigmaB
Lmo1454[c]	1.85	RNA polymerase sigma factor RpoD	*rpoD*	Transcription	Transcription factors	gttttaaaaccgctaaatgatggtat	SigmaB
						aggacttttgcttttttgtggcgaatat	SigmaH
						ttgacttttagcaaaaatacagtatctt	SigmaA
Lmo2006	1.60	acetolactate synthase catabolic	*alsS*	Amino acid biosynthesis	Aspartate family	ttgcaataattcttttgagtagtataaat	SigmaA
				Amino acid biosynthesis	Pyruvate family		
Lmo2064	2.01	large conductance mechanosensitive channel protein	*mscL*	Cellular processes	Adaptations to atypical conditions	tttcacatcgcagttagatgttttatact	SigmaA
Lmo2487	1.65	hypothetical protein	*lmo2487*	Hypothetical proteins	Conserved	N/A	N/A
Lmo2614	2.05	50S ribosomal protein L30	*rpmD*	Protein synthesis	Ribosomal proteins: synthesis and modification	ttgattactacccctaacccgtgtataat	SigmaA
Lmo2621	1.63	50S ribosomal protein L24	*rplX*	Protein synthesis	Ribosomal proteins: synthesis and modification	ttgattactacccctaacccgtgtataat	SigmaA
Proteins with negative fold change (≤ 1.5) and $p < 0.05$ (indicating negative regulation by σ^H)							
Lmo1877	-1.61	formate-tetrahydrofolate ligase	*fhs*	Amino acid biosynthesis	Aspartate family		
				Protein synthesis	tRNA aminoacylation		
				Amino acid biosynthesis	Histidine family		
				Purines, pyrimidines, nucleosides, and nucleotides	Purine ribonucleotide biosynthesis		
				Biosynthesis of cofactors, prosthetic groups, and carriers	Pantothenate and coenzyme A		
Lmo2094	-7.35	hypothetical protein	*lmo2094*	Energy metabolism	Sugars		
Lmo2097	-3.17	galactitol-specific PTS system IIB component	*lmo2097*	Energy metabolism	Pyruvate dehydrogenase		
				Amino acid biosynthesis	Aromatic amino acid family		
				Transport and binding proteins	Carbohydrates, organic alcohols, and acids		
Lmo2098	-2.33	galactitol-specific PTS system IIA component	*lmo2098*	Energy metabolism	Pyruvate dehydrogenase		
				Amino acid biosynthesis	Aromatic amino acid family		
				Transport and binding proteins	Carbohydrates, organic alcohols, and acids		

[a]Protein names are based on the *L. monocytogenes* EGD-e locus.

[b]Role Categories and Sub-Role categories are based on JCVI classification [32]

[c]Reported as positively and directly regulated by σ^H in Chaturongakul *et al*., 2011 [7]

[d]Promoters were identified based on RNA-Seq data (Orsi *et al*., unpublished) or previously published data. -10 and -35 (σ^A, σ^B, σ^H) and -12 and -24 (σ^L) regions are underlined. N/A indicates that a promoter was not identified.

σ^L appears to contribute to negative regulation of a number of proteins.

Our proteomic comparison identified only 2 proteins (Lmo0096 and Lmo2006) as positively regulated by σ^L, as supported by higher protein expression levels (FC $\geq$ 1.5; $p^c < 0.05$) in *L. monocytogenes* Δ*BCH* as compared to the Δ*BCHL* strain (Table 4.2). Both of these proteins also showed higher expression levels in the parent strain (which expresses all 4 alternative σ factors) as compared to the quadruple mutant. Lmo0096 (MptA) is annotated as the mannose-specific PTS system IIAB component, while Lmo2006 (AlsS) is annotated as an acetolactate synthase. Both lmo0096 and lmo2006 have previously been reported to be positively regulated by σ^L at the transcriptomic level [18]. Lmo0096 was also reported as showing lower expression levels in a *L. monocytogenes* EGD-e *rpoN* (σ^L) mutant in a 2-DE based proteomic analysis [22] and the lmo0096 gene was found to be preceded by a putative σ^L consensus promoter in the same study, further supporting positive regulation of the gene encoding this protein by σ^L.

A total of 56 proteins showed lower expression levels in the presence of σ^L (in the comparison between the Δ*BCH* and the Δ*BCHL* strain), suggesting negative regulation of these proteins by σ^L (Table 4.2); two of the genes encoding these proteins had previously been shown to have higher transcript levels in a Δ*sigL* null mutant as compared to a parent strain, further supporting negative regulation by σ^L [7]. Twenty-one of the proteins with evidence for negative regulation by σ^L also showed lower protein levels in the parent strain as compared to the Δ*BCHL* strain (Appendix 4.2), further supporting their negative regulation. Four of these 21 proteins as well as three other proteins found to be negatively regulated by σ^L in this study were also

reported as showing higher transcript levels in a *L. monocytogenes* EGD-e *rpoN* (σ^L) mutant [22] (Table 4.2), supporting their negative regulation by σ^L. Overall, the 56 proteins identified here as negatively regulated by σ^L represented 13 role categories (e.g., energy metabolism, transport and binding proteins, central intermediary metabolism), including 31 proteins in the energy metabolism role category; statistical analyses showed overrepresentation of the role category "energy metabolism" ($p < 0.01$; Odds Ratio = 5.6) among these 56 proteins. Specific proteins identified as negatively regulated by σ^L included flagellin (FlaA), chemotaxis protein CheA, and a glutamate-γ-aminobutyric acid (GABA) antiporter (Lmo2362, GadC), which have known roles in stress adaptation or virulence in *L. monocytogenes* [1, 25].

Table 4.2: Proteins found to be differentially regulated by σ^L, as determined by a proteomic comparison between *L. monocytogenes* 10403S Δ*BCH* and Δ*BCHL*

Protein[a]	Fold Change Δ*BCH*/Δ*BCHL*	Description	Gene Name	Role Category[b]	Sub-Role Category[b]
Proteins with positive fold change (≥ 1.5) and $p < 0.05$ (indicating positive regulation by σ^L)					
Lmo0096[d,f]	64.16	mannose-specific PTS system IIAB component ManL	*mptA*	Energy metabolism	Pyruvate dehydrogenase
				Amino acid biosynthesis	Aromatic amino acid family
				Transport and binding proteins	Carbohydrates, organic alcohols, and acids
Lmo2006[g]	3.41	acetolactate synthase catabolic	*alsS*	Amino acid biosynthesis	Aspartate family
				Amino acid biosynthesis	Pyruvate family
Proteins with negative fold change (≤ 1.5) and $p < 0.05$ (indicating negative regulation by σ^L)					
Lmo0027[c,e]	-3.62	beta-glucoside-specific PTS system IIABC component	*lmo0027*	Transport and binding proteins	Carbohydrates, organic alcohols, and acids
				Amino acid biosynthesis	Aromatic amino acid family
				Energy metabolism	Pyruvate dehydrogenase
Lmo0130	-3.64	hypothetical protein	*lmo0130*	Unclassified	Role category not yet assigned
Lmo0178	-2.07	hypothetical protein	*lmo0178*	Regulatory functions	Other
Lmo0181	-3.25	multiple sugar transport system substrate-binding protein	*lmo0181*	Transport and binding proteins	Unknown substrate
Lmo0260	-1.68	hydrolase	*lmo0260*	Hypothetical proteins	Conserved
Lmo0278	-1.67	maltose/maltodextrin transport system ATP-binding protein	*lmo0278*	Transport and binding proteins	Carbohydrates, organic alcohols, and acids
Lmo0319[c,e]	-2.96	beta-glucosidase	*bglA*	Energy metabolism	Sugars
Lmo0343	-3.94	transaldolase	*tal2*	Energy metabolism	Pentose phosphate pathway
Lmo0344	-4.69	short chain dehydrogenase	*lmo0344*	Energy metabolism	Biosynthesis and degradation of polysaccharides
Lmo0345	-6.04	ribose 5-phosphate isomerase B	*lmo0345*	Energy metabolism	Pentose phosphate pathway
Lmo0346	-2.74	triosephosphate isomerase	*tpiA2*	Energy metabolism	Glycolysis/gluconeogenesis
Lmo0348	-2.41	dihydroxyacetone kinase	*lmo0348*	Fatty acid and phospholipid metabolism	Biosynthesis
				Energy metabolism	Sugars
Lmo0391	-1.67	hypothetical protein	*lmo0391*		
Lmo0401	-2.16	alpha-mannosidase	*lmo0401*	Unclassified	Role category not yet assigned
Lmo0517[e]	-3.21	phosphoglycerate mutase	*lmo0517*	Energy metabolism	Glycolysis/gluconeogenesis
Lmo0521	-2.23	6-phospho-beta-glucosidase	*lmo0521*	Energy metabolism	Sugars
Lmo0536	-1.97	6-phospho-beta-glucosidase	*lmo0536*	Central intermediary metabolism	Other
Lmo0574	-1.65	6-phospho-beta-glucosidase GmuD	*lmo0574*	Central intermediary metabolism	Other
Lmo0640	-1.78	oxidoreductase	*lmo0640*	Energy metabolism	Fermentation
				Central intermediary metabolism	Other
				Energy metabolism	Electron transport
Lmo0643	-2.61	transaldolase	*lmo0643*	Energy metabolism	Pentose phosphate pathway
Lmo0689	-1.71	chemotaxis protein CheV	*lmo0689*	Cellular processes	Chemotaxis and motility
Lmo0690	-2.44	flagellin	*flaA*	Cellular processes	Chemotaxis and motility
Lmo0692	-1.66	chemotaxis protein CheA	*cheA*	Cellular processes	Chemotaxis and motility
Lmo0813	-2.04	fructokinase	*lmo0813*	Energy metabolism	Sugars
Lmo0930	-1.88	hypothetical protein	*lmo0930*	Unclassified	Role category not yet assigned
Lmo1242	-1.59	hypothetical protein	*lmo1242*	Hypothetical proteins	Conserved
Lmo1254	-2.10	alpha-phosphotrehalase	*lmo1254*	Energy metabolism	Biosynthesis and degradation of polysaccharides
Lmo1348	-2.42	glycine cleavage system T protein	*gcvT*	Energy metabolism	Amino acids and amines
Lmo1349	-2.68	glycine cleavage system P-protein	*gcvPA*	Energy metabolism	Amino acids and amines
				Central intermediary metabolism	Other
Lmo1350[e]	-2.11	glycine dehydrogenase subunit 2	*gcvPB*	Central intermediary metabolism	Other
				Energy metabolism	Amino acids and amines
Lmo1388[e]	-2.02	ABC transport system	*tcsA*	Unclassified	Role category not yet assigned
Lmo1389	-2.32	simple sugar transport system ATP-binding protein	*lmo1389*	Transport and binding proteins	Carbohydrates, organic alcohols, and acids
Lmo1538[e]	-1.89	glycerol kinase	*glpK*	Energy metabolism	Other
Lmo1699	-1.92	Methyl-accepting chemotaxis protein	*lmo1699*	Cellular processes	Chemotaxis and motility
Lmo1730	-2.55	lactose/L-arabinose transport system substrate-binding protein	*lmo1730*	Transport and binding proteins	Carbohydrates, organic alcohols, and acids
Lmo1791	-1.75	hypothetical protein	*lmo1791*		
Lmo1812	-1.70	L-serine dehydratase iron-sulfur-dependent alpha subunit	*lmo1812*	Energy metabolism	Amino acids and amines
				Energy metabolism	Glycolysis/gluconeogenesis
Lmo1856	-1.65	purine nucleoside phosphorylase	*deoD*	Purines, pyrimidines, nucleosides, and nucleotides	Salvage of nucleosides and nucleotides
Lmo1860	-1.64	peptide-methionine (S)-S-oxide reductase	*msrA*	Protein fate	Protein modification and repair
Lmo1877	-2.14	formate-tetrahydrofolate ligase	*fhs*	Amino acid biosynthesis	Aspartate family
				Protein synthesis	tRNA aminoacylation
				Amino acid biosynthesis	Histidine family
				Purines, pyrimidines, nucleosides, and nucleotides	Purine ribonucleotide biosynthesis
				Biosynthesis of cofactors, prosthetic groups, and carriers	Pantothenate and coenzyme A
Lmo1954[e]	-1.97	phosphopentomutase	*deoB*	Purines, pyrimidines, nucleosides, and nucleotides	Salvage of nucleosides and nucleotides
Lmo1993	-1.81	pyrimidine-nucleoside phosphorylase	*pdp*	Purines, pyrimidines, nucleosides, and nucleotides	Salvage of nucleosides and nucleotides
Lmo2094	-28.99	hypothetical protein	*lmo2094*	Energy metabolism	Sugars
Lmo2097	-12.12	galactitol-specific PTS system IIB component	*lmo2097*	Energy metabolism	Pyruvate dehydrogenase
				Amino acid biosynthesis	Aromatic amino acid family
				Transport and binding proteins	Carbohydrates, organic alcohols, and acids
Lmo2098	-3.96	galactitol-specific PTS system IIA component	*lmo2098*	Energy metabolism	Pyruvate dehydrogenase
				Amino acid biosynthesis	Aromatic amino acid family
				Transport and binding proteins	Carbohydrates, organic alcohols, and acids
Lmo2160	-2.37	sugar phosphate isomerase/epimerase	*lmo2160*	Hypothetical proteins	Conserved
Lmo2161	-2.58	hypothetical protein	*lmo2161*	Hypothetical proteins	Conserved
Lmo2362	-1.87	glutamate/gamma-aminobutyrate antiporter	*lmo2362*	Transport and binding proteins	Amino acids, peptides and amines
Lmo2425	-1.59	glycine cleavage system H protein	*gcvH*	Energy metabolism	Amino acids and amines
Lmo2481	-1.52	pyrophosphatase PpaX	*ppaX*	Central intermediary metabolism	Other
Lmo2529	-1.72	ATP synthase F1 beta subunit	*atpD2*	Energy metabolism	ATP-proton motive force interconversion
Lmo2648	-2.50	hypothetical protein	*lmo2648*	Unclassified	Role category not yet assigned
Lmo2664	-1.72	L-iditol 2-dehydrogenase	*lmo2664*	Central intermediary metabolism	Other
				Energy metabolism	Glycolysis/gluconeogenesis
				Energy metabolism	Electron transport
				Energy metabolism	TCA cycle
				Energy metabolism	Fermentation
Lmo2696	-2.68	dihydroxyacetone kinase L subunit	*lmo2696*	Energy metabolism	Sugars
				Fatty acid and phospholipid metabolism	Biosynthesis
Lmo2697	-3.10	dihydroxyacetone kinase	*lmo2697*	Hypothetical proteins	Conserved
Lmo2743	-2.71	transaldolase	*tal1*	Energy metabolism	Pentose phosphate pathway

[a]Protein names are based on the *L. monocytogenes* EGD-e locus.

[b]Role Categories and Sub-Role categories are based on JCVI classification [32]

[c]Reported as negatively regulated by σ^L in Chaturongakul *et al*., 2011 [7]

[d]Reported as downregulated in a *rpoN* (σ^L) mutant compared to wildtype *L. monocytogenes* EGD-e in Arous *et al*., 2004 [22]

[e]Reported as upregulated in a *rpoN* (σ^L) mutant compared to wildtype *L. monocytogenes* EGD-e in Arous *et al*., 2004 [22]

[f]Preceded by a putative σ^L promoter; tggcacagaacttgca; -12 and -24 regions are underlined.

[g]Preceded by a putative σ^A promoter; ttgcaataattcttttgagtagtataat; -10 and -35 regions are underlined.

σ^C regulates a small number of proteins.

Previous studies indicated a role for σ^C in *L. monocytogenes* thermal adaptive response as well as in cold adaptation [3, 13], however only a few genes have been identified as part of the σ^C regulon [7]. Similarly, we were only able to identify one protein, Lmo0096 that showed higher protein expression levels (FC $\geq$ 1.5; $p^c < 0.05$) in the presence of σ^C (i.e., the comparison between the Δ*BHL* and the Δ*BCHL* strain; Table 4.3). Lmo0096 has been previously reported to be induced under cold stress in *L. monocytogenes* [26], supporting a role of σ^C in response to temperature stress in the bacterium. By comparison, the transcriptomic study by Chaturongakul *et al*., 2011 only identified lmo0422, which is in the same operon as *sigC* (lmo0423), as positively regulated by σ^C [7].

We also identified two proteins, Lmo2094 and Lmo1902, that showed higher protein expression levels in the absence of σ^C, suggesting negative regulation of these proteins by σ^C (Table 4.3). By comparison, the transcriptomic study by Chaturongakul *et al*. (2011) identified three different genes, representing two operons (lmo1854; lmo2185, and lmo2186) that showed lower expression in the parent strain compared to the Δ*sigC* mutant, suggesting negative regulation by σ^C [7]. While our data are consistent with previous findings of a limited σ^C regulon in *L. monocytogenes* 10403S, it is possible that the σ^C dependent gene regulation only occurs under specific conditions (e.g., heat stress [3]) and that more complete identification of the σ^C regulon requires transcriptomic and proteomic studies under specific conditions that remain to be defined.

Table 4.3: Proteins found to be differentially regulated by σ^C, as determined by a proteomic comparison between *L. monocytogenes* 10403S Δ*BHL* and Δ*BCHL*

Protein[a]	Fold Change Δ*BHL*/Δ*BCHL*	Description	Gene Name	Role Category[b]	Sub-Role Category[b]
Proteins with positive fold change (≥ 1.5) and $p < 0.05$ (indicating positive regulation by σ^C)					
Lmo0096[c]	3.19	mannose-specific PTS system IIAB component ManL	*mptA*	Energy metabolism	Pyruvate dehydrogenase
				Amino acid biosynthesis	Aromatic amino acid family
				Transport and binding proteins	Carbohydrates, organic alcohols, and acids
Proteins with negative fold change (≤ 1.5) and $p < 0.05$ (indicating negative regulation by σ^C)					
Lmo2094	-1.82	hypothetical protein	*lmo2094*	Energy metabolism	Sugars
Lmo1902	-1.61	3-methyl-2-oxobutanoate hydroxymethyltransferase	*panB*	Biosynthesis of cofactors, prosthetic groups, and carriers	Pantothenate and coenzyme A

[a]Protein names are based on the *L. monocytogenes* EGD-e locus.

[b]Role Categories and Sub-Role categories are based on JCVI classification [32]

[c]Preceded by a putative σ^L promoter; tggcacagaacttgca; -12 and -24 regions are underlined.

Proteins regulated by multiple alternative σ factors include MptA, which has a potential role in regulation of PrfA.

Our data reported here also provide an opportunity to gather further insight into genes and proteins that are co-regulated by multiple σ factors and, consequently, into regulatory networks among different alternative σ factors. To facilitate these analyses, we also compared the protein expression levels between the *L. monocytogenes* parent strain and the Δ*BCHL* strain (which does not express any alternative σ factors). This analysis identified (i) 33 proteins that showed significantly higher expression (FC ≥ 1.5; $p^c < 0.05$) in the parent strain as compared to the Δ*BCHL* strain (Appendix 4.2) and (ii) 44 proteins that show lower expression in the parent as compared to the Δ*BCHL* mutant (Appendix 4.2). Approximately 40% of the proteins are involved in energy metabolism and transport and binding functions (Fig. 4.1). Among the 44 proteins with lower expression levels in the parent strain, statistical analyses showed overrepresentation of three role categories, including (i) "energy metabolism" ($p < 0.01$; Odds Ratio = 3.02), (ii) "biosynthesis of cofactors, prosthetic groups, and carriers" ($p = 0.04$; Odds Ratio = 2.72), and (iii) "purines, pyrimidines,

nucleosides, and nucleotides" (p = 0.04; Odds Ratio = 3.29), as well as underrepresentation of the role category "hypothetical proteins" (p = 0.01; Odds Ratio = 0.208).

Among the 33 proteins with higher expression levels in the parent strain, (i) 2 were also found to be positively regulated by σ^H; (ii) one was also positively regulated by σ^H and σ^L, and (iii) one was also positively regulated by σ^H, σ^L and σ^C (Fig. 4.2; Table 4.4). In addition, 12 of the 29 proteins that were found to be positively regulated in the parent strain, were also found to be positively regulated by σ^B in a recent proteomics study, which compared *L. monocytogenes* parent strain 10403S and Δ*sigB* mutant grown to stationary phase under the same conditions as used here (Mujahid *et al*., unpublished). While these 12 proteins likely represent proteins that are positively regulated by σ^B, the other 17 proteins that show high expression levels in the parent strain as compared to the Δ*BCHL* strain, but were not identified as positively regulated by any of the alternative σ factors, represent candidate proteins for redundant co-regulation by multiple alternative σ factors. Regulatory redundancy among multiple alternative σ factors has also previously been demonstrated through the analyses of *Bacillus subtilis* alternative σ factor mutants; in particular, certain phenotypes displayed by a *B. subtilis* triple alternative σ factor deletion mutant were not found among single or double mutants of each of the three alternative σ factors, suggesting regulatory overlaps [27]. Among the 44 genes that showed lower expression levels in the parent strain as compared to the Δ*BCHL* mutant (Appendix 4.2), (i) two also showed evidence for negative regulation by σ^H and σ^L (Lmo2097 and Lmo1877); (ii)

one also showed evidence for negative regulation by σ^H, σ^L, and σ^C (Lmo2094; located in the same operon as lmo2097).

Overall, our data provide additional evidence that a number of genes and proteins are co-regulated by more than one σ factor. This is consistent with previous microarray studies [7] that have reported considerable overlaps between σ factor regulons in *L. monocytogenes*, in particular between the σ^H and the σ^B regulon. We also identified some proteins with particularly striking patterns of co-regulation, including (i) members of the lmo2093-lmo2099 operon, specifically Lmo2094, which was found to be negatively regulated by σ^H, σ^L, and σ^C and Lmo2097 and Lmo2098, which were found to be negatively regulated by σ^H and σ^L (Table 4.4) and (ii) MptA (Lmo0096), which was found to be positively regulated by σ^H, σ^L, and σ^C (Table 4.4).

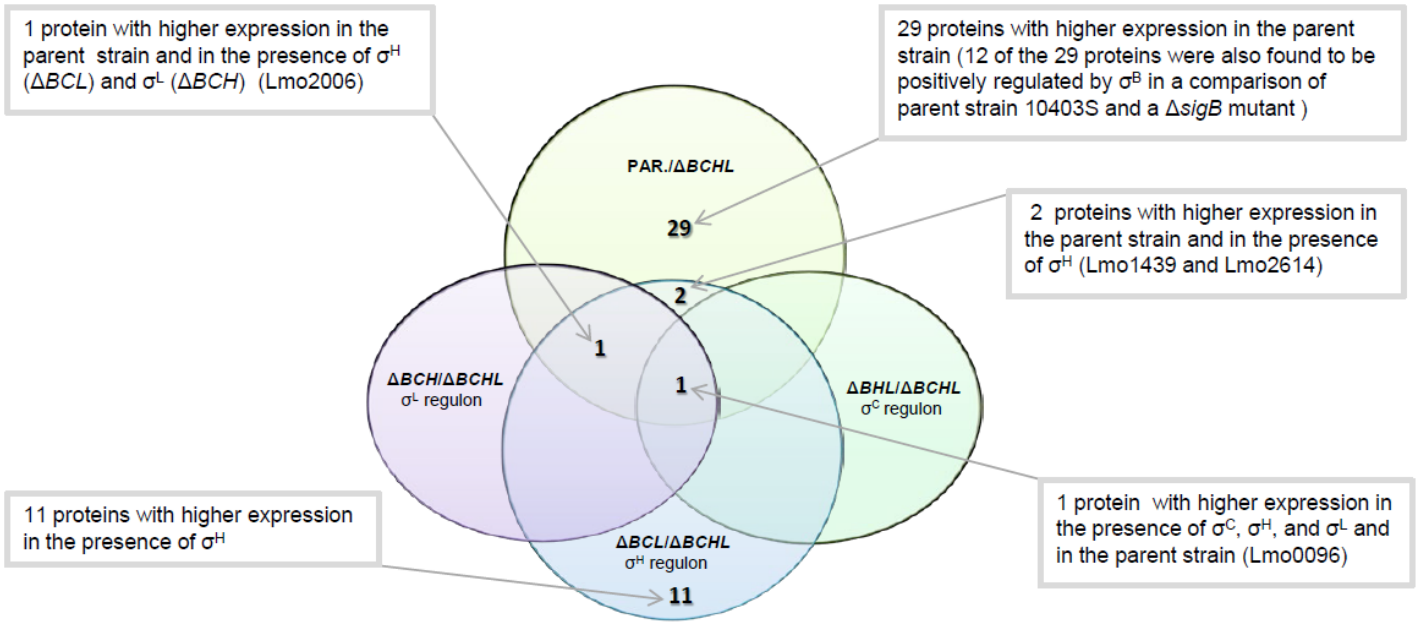

Figure 4.2. Venn diagram of proteins identified as showing higher expression levels in comparisons of (i) *L. monocytogenes* parent strain 10403S (PAR.) and Δ*BCHL*; (ii) Δ*BCH* and Δ*BCHL* (identifying genes positively regulated by σ^L); Δ*BCL* and Δ*BCHL* (identifying genes positively regulated by σ^H); and Δ*BHL* and Δ*BCHL* (identifying genes positively regulated by σ^C). Twelve of the 29 proteins that were found to be positively regulated in the parent strain were also found to be positively regulated by σ^B in a recent proteomics study, which compared *L. monocytogenes* parent strain 10403S and a Δ*sigB* mutant (Mujahid *et al*., unpublished); these proteins include Lmo2748, Lmo2213, Lmo2158, Lmo2047, Lmo1830, Lmo0913, Lmo0796, Lmo0794, Lmo0722, Lmo0654, Lmo0539, and Lmo0265. The 17 proteins that show high expression levels in the parent strain as compared to the Δ*BCHL* strain, but were not identified as positively regulated by any of the alternative σ factors include Lmo1540, Lmo2610, Lmo1422, Lmo1421, Lmo1602, Lmo1426, Lmo1428, Lmo2205, Lmo2398, Lmo1601, Lmo0554, Lmo1634, Lmo0110, Lmo2558, Lmo0783, Lmo0134, and Lmo0098.

Lmo2094 shows particularly striking negative regulation of protein expression by σ^H, σ^L, and σ^C with respective fold changes of -7.35, -28.99, and -1.82. Although Lmo2094 is annotated as a fuculose-phosphate aldolase, it is expressed within an operon in which most of the other genes with assigned functions are annotated as being involved in the galactitol degradation pathway. Specifically, the lmo2093 to lmo2099 operon encodes components of a putative PTS galactitol family permease [28], including the PTS system galactitol-specific enzyme IIC (Lmo2096), IIB (Lmo2097), and IIA (Lmo2098) components, as well as a transcription antiterminator (Lmo2099), tagatose-6-phosphate kinase/1-phosphofructokinase (Lmo2095), L-fuculose-phosphate aldolase (Lmo2094), and a hypothetical protein (Lmo2093). Therefore, it is possible that Lmo2094 is also involved in this pathway functioning as a tagatose-1,6-biphosphate aldolase. This enzyme converts tagatose-1,6,-biphosphate into glyceraldehyde 3-phosphate and dihydroxyacetone phosphate, which allows both tagatose and galactitol to be used as energy sources for glycolysis [29].

MptA, the component of a permease of the PTS mannose–fructose–sorbose family, which is another one of the seven PTS families of *L. monocytogenes* [28], showed the highest fold change in the Δ*BCH* strain as compared to the Δ*BCHL* strain, supporting σ^L dependent protein levels (FC = 64.16); fold changes supporting σ^H and σ^C dependent protein levels were 3.39 and 3.19, respectively. MptA is encoded by a gene that is part of a 3-gene operon (*mptACD* [30], which also has been reported as *manLMN* [24]); these three genes encode a mannose-specific PTS system IIAB component, a mannose-specific PTS system IIC component, and a mannose-specific PTS system IID component, respectively [24, 30]. Recently, it was suggested that

during glucose uptake, MptA dephosphorylates, which directly, or indirectly, inhibits PrfA, the major positive regulator of *L. monocytogenes* virulence genes [24]. These findings thus provide for a hypothesis that redundant upregulation of MptA, through multiple alternative σ factors, may provide a critical initial step towards inactivation of PrfA.

Table 4.4: Proteins found to be differentially regulated by at least two of the three alternative sigma factors studied here

Protein[#]	Regulation by[*]			Regulation by σ^{B}[^]	Differential expression in comparison between parent and Δ*sigBCHL*
	σ^{H}	σ^{L}	σ^{C}		
Lmo0027	+	-	NDE	NDE	-
Lmo0096 (MptA)	+	+	+	NDE	+
Lmo0319 (BglA)	+	-	NDE	NDE	-
Lmo1877 (Fhs)	-	-	NDE	NDE	-
Lmo2006 (AlsS)	+	+	NDE	NDE	+
Lmo2094	-	-	-	NDE	-
Lmo2097	-	-	NDE	NDE	-
Lmo2098	-	-	NDE	NDE	NDE

[#]Where available, protein name is shown in parenthesis

[*]Proteins that were identified here as positively (+) or negatively (-) regulated (FC $\geq$ 1.5; $p < 0.05$) by a given σ factor are shown; NDE ("not differentially expressed") indicates that a protein was not found to be differentially expressed between strains with and without a given alternative σ factor.

[^]Data for proteins differentially expressed by σ^{B} were obtained from Mujahid *et al*. (unpublished); this study compared protein levels between the 10403S parent strain and an isogenic Δ*sigB* strain.

CONCLUSIONS

Transcriptional regulation through the interplay between alternative σ factors represents an important component of *L. monocytogenes* stress response systems and the ability of this pathogen to regulate gene expression during infection. In addition to transcriptional regulation, alternative σ factors may also regulate gene expression post-transcriptionally and/or post-translationally. To allow for further insights into the roles of different alternative σ factors in *L. monocytogenes*, we thus completed a global evaluation of alternative σ factor-dependent protein expression patterns in *L. monocytogenes* stationary phase cells. In concert with previous transcriptomic studies, our data not only provide a further refinement of our understanding of the alternative σ factor regulons in this important pathogen, but also provide clear evidence for co-regulation, by multiple σ factors, of different PTS systems, including one PTS system that has been suggested to be linked to regulation of PrfA.

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CHAPTER 5

CONCLUSIONS

Alternative sigma factor dependent expression of genes, non-coding RNAs (ncRNA), and proteins represents an important and complex mechanism used by *L. monocytogenes* to overcome a diverse range of stress conditions and cause infection. σ^B is the general stress response alternative sigma factor of *L. monocytogenes* and the most extensively characterized among the four alternative sigma factors. Although previous studies have explored σ^B-dependent gene and protein regulation independently, we conducted a combined analysis of transcriptomic and proteomic data to gain a better understanding of regulation by σ^B at both levels. We were thus able to develop the most comprehensive definition of the σ^B regulon to date with a total of 149 genes and proteins, which were found to be positively regulated by σ^B at either or both the transcript and protein level and are involved in a wide range of cellular functions. Along with direct regulation of gene transcription by σ^B, our data suggest indirect mechanisms of regulation at the transcriptional and post-transcriptional level, for example through σ^B-dependent regulation of ncRNAs that are involved in metabolic pathways.

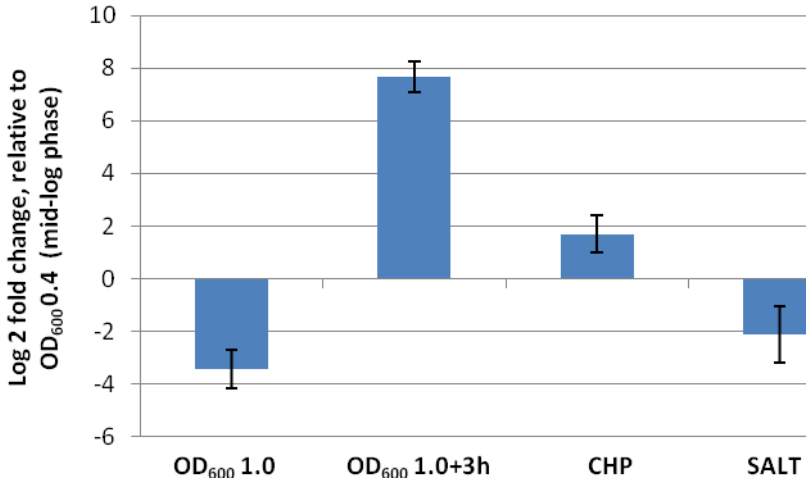
Studies further characterizing alternative sigma factor dependent regulation of ncRNAs in *L. monocytogenes* will provide essential information on the intricate regulation mechanisms of σ^B, σ^C, σ^H, and σ^L in response to environmental stress conditions. To this end, our exploration of the role of the σ^B-dependent ncRNA, SbrE, suggests that SbrE represents a conserved mechanism employed by *L. monocytogenes*

to fine tune gene expression and overcome specific environmental stress conditions, such as the low nutrient conditions of stationary phase and oxidative stress. Correlation of SbrE expression with σ^B-dependent gene and protein expression under specific environmental stress conditions will provide some of the missing links in the understanding of the σ^B regulon.

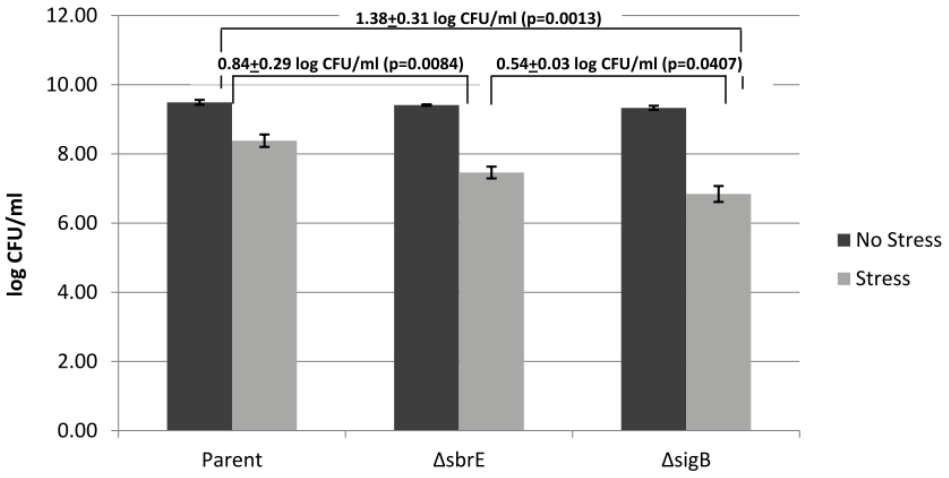
In contrast to σ^B, there is limited or no information available on regulation by σ^C, σ^H, and σ^L at the protein and ncRNA level in *L. monocytogenes*, although information on regulation at the transcriptomic level is available. In order to gain more knowledge about the *L. monocytogenes* σ^C, σ^H, and σ^L regulons at the protein level, we conducted quantitative proteomics comparisons of *L. monocytogenes* quadruple and triple alternative sigma factor mutant strains. We also determined the protein expression profile of *L. monocytogenes* in the absence of all four alternative sigma factors. Overlaps identified among each of the independent alternative sigma factor regulons suggest co-regulation and a high level of complexity in the regulatory network of *L. monocytogenes* alternative sigma factors. This is further demonstrated by the protein expression profile of *L. monocytogenes* in the absence of all four alternative sigma factors, which shows some but not entire overlap with the independent alternative sigma factor regulons. The identified proteins in each regulon are involved in a wide range of cellular functions including energy metabolism, transport and binding, and cellular processes. Proteins identified as being regulated by multiple alternative sigma factors include components of PTS systems, including a protein with a potential role in regulation of the major positive regulator of *L. monocytogenes* virulence genes, PrfA. Comparison of our protein data on the σ^C, σ^H,

and σ^L regulons with published transcriptomic data found a few genes to be regulated at both levels. Further analyses and comparisons at the proteomic and transcriptomic levels using similar strains and growth conditions is thus necessary to provide more insight into the transcriptional and post-transcriptional regulation of genes and proteins and functional redundancies of *L. monocytogenes* alternative sigma factors.

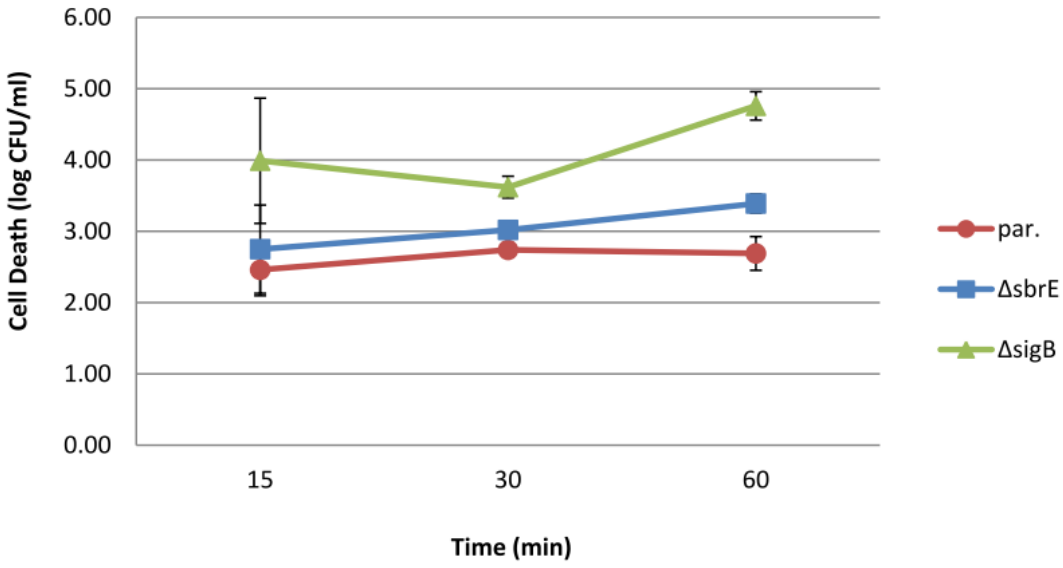
Overall, this work illustrates that the alternative sigma factors of *L. monocytogenes* regulate diverse cellular functions at the transcript and protein level and exhibit some functional redundancies. Exploration of the connections between σ^B, σ^C, σ^H, and σ^L dependent gene, protein, and ncRNA expression is critical for gaining a better understanding of the mechanisms employed by *L. monocytogenes* to survive in the environment and cause infection; and consequently, facilitating the development of effective strategies for the prevention and treatment of listeriosis.



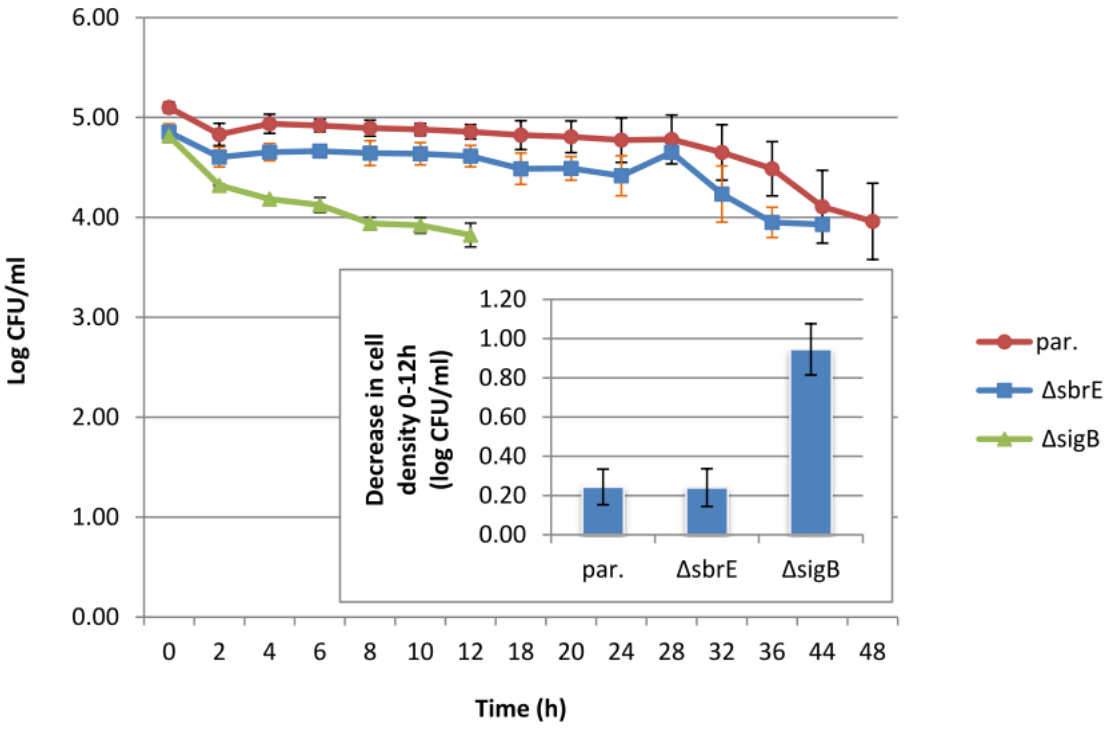
Appendix 2.1. Growth phase and environmental stress dependent expression of SbrE. Mean log2 transcript levels of SbrE at late log phase (OD 1.0), early stationary phase (OD 1.0 + 3 h), as well as after exposure of mid-log phase cells to CHP (13 mM cumene hydroperoxide, 15 min) or salt (10% NaCl, 15 min) stress relative to mid-log phase (OD 0.4) are indicated. Values are means from three independent qRT-PCR experiments; error bars indicate standard deviation.



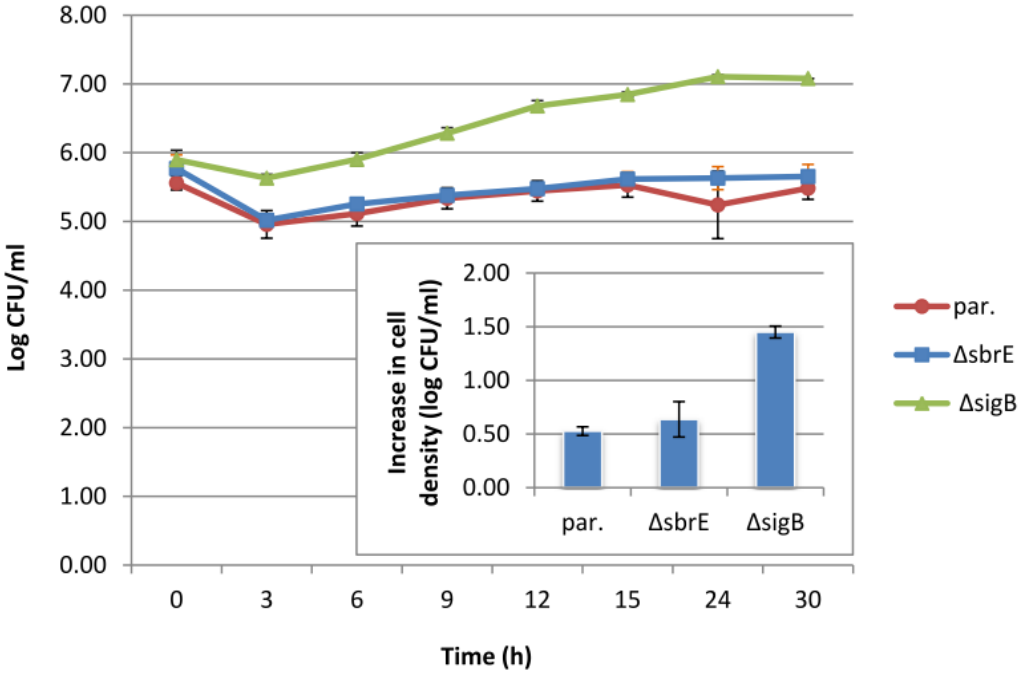
Appendix 2.2. Oxidative stress survival of parent, Δ*sigB*, and Δ*sbrE* strains. Bacterial numbers in log Colony Forming Units/mL before and after exposure to 13 mM CHP stress for 15 min are shown. Survival was expressed as log reduction in viable cells, which was calculated by subtracting bacterial numbers of stressed cells from non-stressed controls. The difference in cell death (in log Colony Forming Units/mL) between strains is indicated. Values are means from three independent experiments; error bars indicate standard deviation.



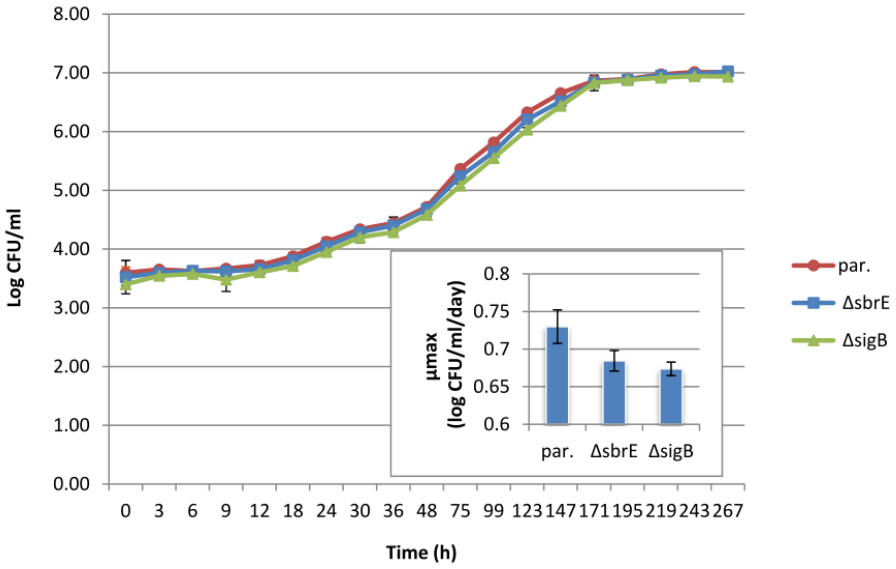
Appendix 2.3. Oxidative stress survival of parent (par., circle), Δ*sbrE* (square), and Δ*sigB* (triangle) strains over 60 min. Reduction in cell numbers in log CFU/ml after exposure to 13 mM CHP for 15 min, 30 min, and 60 min is shown. Values are means from at least three independent experiments; error bars indicate standard deviation.



Appendix 2.4. Salt stress survival of parent strain (par., circle), Δ*sbrE* (square), and Δ*sigB* (triangle) strains. Bacterial numbers in log Colony Forming Units/mL over 48 h of growth in BHI supplemented with 1.75 M NaCl are plotted. The inset shows the decrease in cell density of each strain from 0–12 h. Values are means from three independent experiments; error bars indicate standard deviation.



Appendix 2.5. Growth of parent strain (par., circle), Δ*sbrE* (square), and Δ*sigB* (triangle) strains under energy stress. Bacterial numbers in log Colony Forming Units/mL over 30 h of growth in glucose-limiting DM are plotted. The inset shows the increase in cell density of each strain from 3–30 h. Values are means from three independent experiments; error bars indicate standard deviation.



Appendix 2.6. Growth of parent strain (par., circle), Δ*sbrE* (square), and Δ*sigB* (triangle) under cold stress (7 °C). Bacterial numbers in log Colony Forming Units/mL over 12 days of growth are plotted. The inset shows the average growth rate (μmax) of each strain in log Colony Forming Units/mL/day. Values are means from three independent experiments; error bars indicate standard deviation.

Appendix 2.7. Primers and probes used in this study.

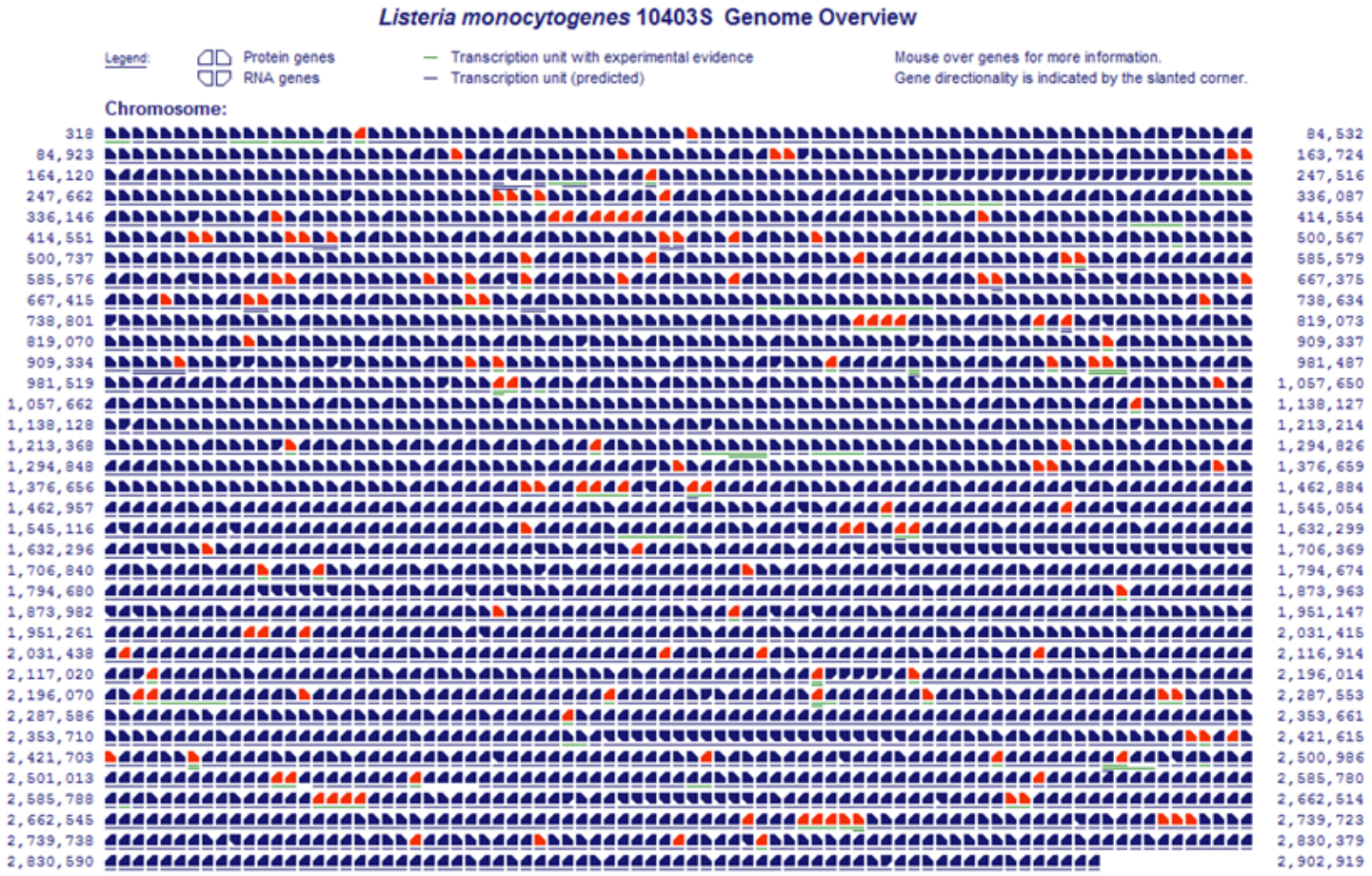
Primer	Sequence (5'→3')
SOE-A [a]	CGTCTAGAGCAGAAATTGATATCGCTGTGC
SOE-B	GCATCACCTTTTATTTGTTCGTTGTAC
SOE-C [b]	GTACAACGAACAAATAAAAGGTGATGCGGCAAGACAAGCTCATCCG
SOE-D [c]	CGAAGCTTACTAGCTGCTCGAGAGCATG
SbrE-XF	ACTTAAAAGTCCGCCCGG
SbrE-XR	ACAAAAACTACAAGAACAAGACGCAG
SbrE-Fwd	CAGGAGGAAGGCGAGGAGTATA
SbrE-Rev	CGATACTTTATTCGCTTATTTACCAATG
SbrE-probe	CGG AAT TTC GTT ACG TCG C
rpoB-Fwd	CCGGACGTCACGGTAACAA
rpoB-Rev	CAGGTGTTCCGTCTGGCATA
rpoB-probe [d]	TTATCTCCCGTATTTTACC
lmo0636-Fwd	ACCCTAAAAACCACAGCGAAAG
lmo0636-Rev	CCTTATTCATCACTTCGCCAATC
lmo0636-probe[d]	CAGCCGCACTGCT

[a] The *Xba*I restriction site incorporated into this primer to facilitate cloning is underlined; [b] The overhang complementary to SOE-B is underlined; [c] The *Hind*III restriction site incorporated into this primer to facilitate cloning is underlined; [d] TaqMan probes were designed with FAM-5' and MGB-3' ends.

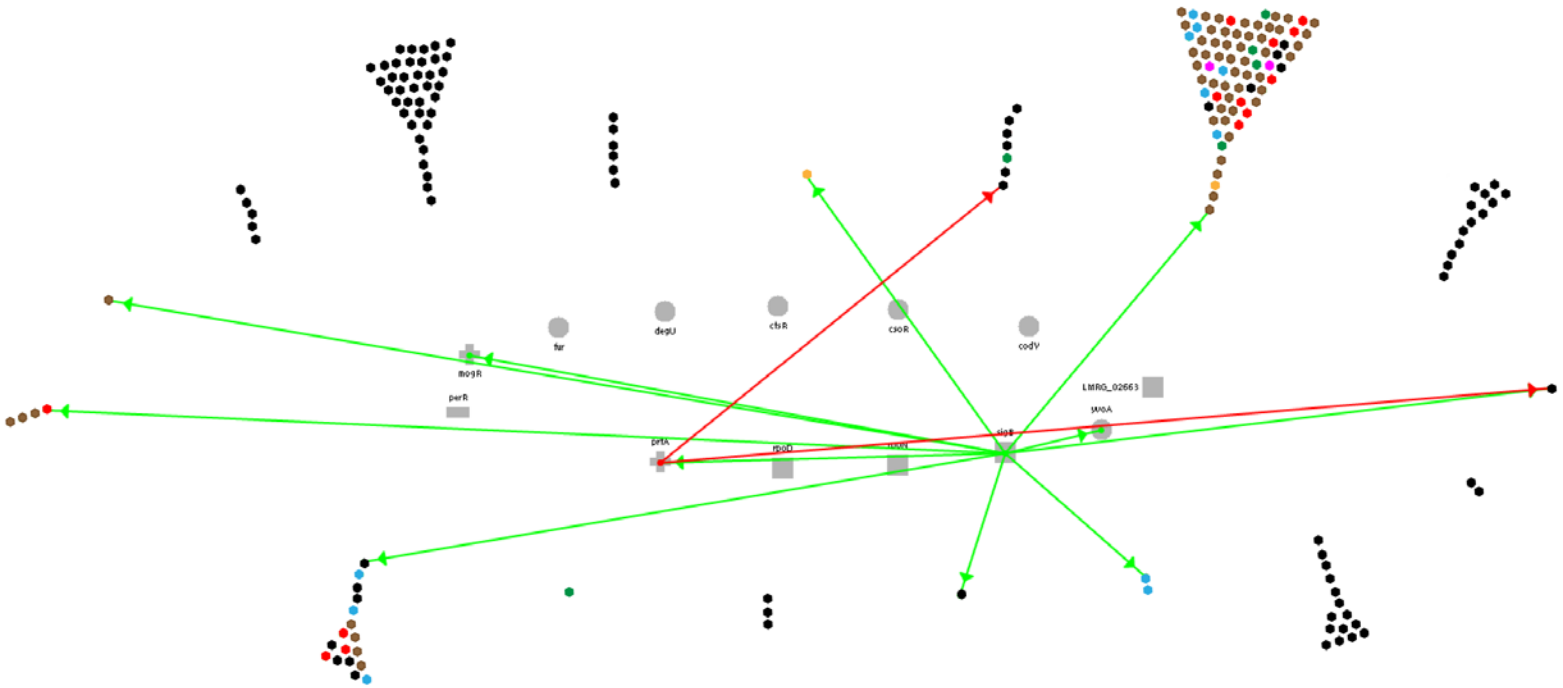
Appendix 2.8. Comparison of plaque formation in *L. monocytogenes* 10403S, Δ*sbrE*, Δ*sigB*, and MACK after infection with phages.

Phage	10403S [a]	Δ*sigB* [a]	Δ*sbrE* [a]	MACK [a]
LP-047	-	-	-	-
LP-106	+++	+++	+++	+++
LP-044	+++	+++	+++	+++
LP-034	+++	+++	+++	++
LP-095	+++	+++	+++	+++
LP-038	++	++	+++	+++
LP-054	-	-	-	-
LP-103	+++	+++	+++	+++
LP-110	+++	+++	+++	+++
LP-049	++	++	++	+++
LP-048	+++	+++	+++	+++
LP-083	NA	NA	NA	NA
LP-101	-	-	-	+++
LP-014	-	-	-	-
LP-017	+	+	+	+
LP-020	-	-	-	-
LP-021	-	-	-	-
LP-030	-	-	-	-
LP-090	+++	+++	+++	+++
LP-099	+++	+++	+++	+++
LP-109	+++	+++	+++	+++
LP-114	+++	+++	+++	+++

[a] Plaque formation: - indicates no plaque; + indicates turbid plaque; ++ indicates diffuse plaque (incomplete lysis); +++ indicates clear plaque (confluent lysis).



Appendix 3.1. *L. monocytogenes* 10403S genome overview generated by Pathway Tools (version 15.0) showing genes or proteins identified as positively regulated by σ^B through microarray, RNA-Sequencing, or proteomics. The 149 genes described in this study are indicated in red.



Appendix 3.2. Transcriptional regulatory overview of *L. monocytogenes* 10403S generated by Pathway Tools (version 15.0). The overview includes 91 of the 149 genes listed in Appendix 3.3 as positively regulated by σ^B. Genes shown as circles are color coded based on their presence in Group A (Red), B (Blue), C (Green), E (Orange), F (Pink) or G (Brown) in Appendix 3.3. No genes from Group D are part of the overview.

Appendix 3.3. Genes or proteins identified as positively regulated by σ^B through microarray, RNA-Sequencing, or proteomics

Gene[a]	Description	Role Category[b]	Sub-Role Category[b]
Group A. Genes or proteins identified as positively regulated by σ^B through microarray, proteomics, and RNA-Sequencing (15)			
lmo0134[21]	hypothetical protein, similar to E. coli YjdJ protein	Central intermediary metabolism	Other
lmo0539	tagatose 1,6-diphosphate aldolase, lacD	Energy metabolism	Biosynthesis and degradation of polysaccharides
lmo0554[89]	hypothetical protein, similar to NADH-dependent butanol dehydrogenase	Unknown function	General
		Central intermediary metabolism	Other
lmo0654	hypothetical protein		
lmo0722	pyruvate oxidase	Energy metabolism	Sugars
lmo0783[124]	manse-specific PTS system IIB component	Transport and binding proteins	Carbohydrates, organic alcohols, and acids
lmo0794	hypothetical protein, similar to B. subtilis YwnB protein	Hypothetical proteins	Conserved
lmo0796	YceI like family protein	Hypothetical proteins	Conserved
lmo0913	succinate-semialdehyde dehydrogenase	Energy metabolism	Other
lmo1602[272]	hypothetical protein	Hypothetical proteins	Conserved
lmo1830	short chain dehydrogenase	Hypothetical proteins	Conserved
lmo2158	stress response protein, similar to B. subtilis YwmG protein	Unclassified	Role category not yet assigned
lmo2213	similar to antibiotic biosynthesis mooxygenase subfamily	Hypothetical proteins	Conserved
lmo2398[427]	phosphatidylglycerophosphatase A, ltrC, low temperature requirement C protein, also similar to B. subtilis YutG protein	Protein fate	Degradation of proteins, peptides, and glycopeptides
		Cellular processes	Adaptations to atypical conditions
		Protein fate	Protein and peptide secretion and trafficking
lmo2748	general stress protein 26, similar to B. subtilis stress protein YdaG	Hypothetical proteins	Conserved
Group B. Genes identified as positively regulated by σ^B through microarray only (35)			
lmo0210	L-lactate dehydrogenase, ldh1	Energy metabolism	Aerobic
		Energy metabolism	Glycolysis/gluconeogenesis
lmo0342[58]	transketolase	Energy metabolism	Pentose phosphate pathway
lmo0343[58]	transaldolase, tal2	Energy metabolism	Pentose phosphate pathway
lmo0345[58]	ribose 5-phosphate isomerase B, similar to sugar-phosphate isomerase	Energy metabolism	Pentose phosphate pathway
lmo0346[58]	triosephosphate isomerase, tpiA2	Energy metabolism	Glycolysis/gluconeogenesis
lmo0347[58]	dihydroxyacetone kinase L subunit	Fatty acid and phospholipid metabolism	Biosynthesis
		Energy metabolism	Sugars
lmo0348[58]	dihydroxyacetone kinase	Fatty acid and phospholipid metabolism	Biosynthesis
		Energy metabolism	Sugars
lmo0406[70]	lactoylglutathione lyase, similar to B. subtilis YyaH protein	Energy metabolism	Other
lmo0408[70]	hypothetical protein	Hypothetical proteins	Conserved
lmo0524	hypothetical protein, similar to putative sulfate transporter	Transport and binding proteins	Anions
lmo0579[94]	hypothetical protein	Hypothetical proteins	Conserved
lmo0580[94]	hypothetical protein, weakly similar to carboxylesterase	Unclassified	Role category not yet assigned
lmo0590[95]	hypothetical protein, similar to a fusion of two types of conserved hypothetical protein	Unknown function	General
lmo0648[105]	magnesium and cobalt transporter CorA, similar to membrane proteins	Transport and binding proteins	Unknown substrate
		Transport and binding proteins	Amino acids, peptides and amines
lmo0896[136]	sigma-B negative regulator, rsbX, Indirect negative regulation of sigma B dependant gene expression (serine phosphatase)	Unclassified	Role category not yet assigned
lmo0956[150]	N-acetylglucosamine-6-phosphate deacetylase	Central intermediary metabolism	Amino sugars
lmo0957[150]	glucosamine-6-phosphate isomerase, nagB, Glucosamine-6-phosphate deaminase	Central intermediary metabolism	Amino sugars
lmo0995	YkrP protein	Hypothetical proteins	Conserved
lmo1261	hypothetical protein	Hypothetical proteins	Conserved
lmo1376	6-phosphogluconate dehydrogenase, decarboxylating	Energy metabolism	Pentose phosphate pathway
lmo1388	ABC transport system, tcsA, CD4+ T cell-stimulating antigen, lipoprotein	Unclassified	Role category not yet assigned
lmo1432[235]	hypothetical protein		
lmo1580	universal stress protein	Cellular processes	Adaptations to atypical conditions
lmo1605[273]	UDP-N-acetylmuramate-alanine ligase, murC	Cell envelope	Biosynthesis and degradation of murein sacculus and peptidoglycan
lmo1636[281]	ABC-2 type transport system ATP-binding protein	Transport and binding proteins	Unknown substrate
lmo1666[288]	Listeria adhesion protein B, LapB, peptidoglycan linked protein (LPxTG)	Protein fate	Degradation of proteins, peptides, and glycopeptides

lmo1929[347]	nucleoside diphosphate kinase, ndk	Purines, pyrimidines, nucleosides, and nucleotides	Purine ribonucleotide biosynthesis
		Purines, pyrimidines, nucleosides, and nucleotides	Nucleotide and nucleoside interconversions
lmo1930[347]	heptaprenyl diphosphate synthase component II (menaquinone biosynthesis)	Biosynthesis of cofactors, prosthetic groups, and carriers	Other
lmo1933[347]	GTP cyclohydrolase I, folE	Biosynthesis of cofactors, prosthetic groups, and carriers	Folic acid
lmo2041[368]	methylase MraW, S-adenosyl-L-methionine-dependent methyltransferase mraW	Hypothetical proteins	Conserved
lmo2169[395]	hypothetical protein		
lmo2191	arsenate reductase, spxA, Regulatory protein spx	Hypothetical proteins	Conserved
lmo2386	hypothetical protein, similar to B. subtilis YuiD protein	Unclassified	Role category not yet assigned
lmo2389	hypothetical protein, similar to NADH dehydrogenase	Central intermediary metabolism	Other
lmo2539	serine hydroxymethyltransferase, glyA, highly similar to glycine hydroxymethyltransferase	Amino acid biosynthesis	Serine family
		Biosynthesis of cofactors, prosthetic groups, and carriers	Folic acid

Group C. Genes identified as positively regulated by σ^B through RNA-Sequencing only (13)			
lmo0122[20]	hypothetical protein, similar to phage proteins	Viral functions	General
lmo0133[21]	hypothetical protein, similar to E. coli YjdI protein	Hypothetical proteins	Conserved
lmo0274	hypothetical protein		
lmo0372[64]	beta-glucosidase	Central intermediary metabolism	Other
lmo0405[70]	PiT family inorganic phosphate transporter, similar to phosphate transport protein	Transport and binding proteins	Amino acids, peptides and amines
		Transport and binding proteins	Unknown substrate
lmo0433[77]	internalin A, inlA	Cellular processes	Pathogenesis
lmo0434[77]	internalin B, inlB	Cellular processes	Pathogenesis
lmo0439	hypothetical protein, weakly similar to a module of peptide synthetase	Hypothetical proteins	Conserved
lmo1421[232]	osmoprotectant transport system ATP-binding protein, bilEA, similar to glycine betaine/carnitine/choline ABC transporter (ATP-binding protein)	Transport and binding proteins	Amino acids, peptides and amines
lmo1866[331]	phosphotransferase	Hypothetical proteins	Conserved
lmo2003[361]	hypothetical protein, similar to transcription regulator GntR family	Regulatory functions	Other
lmo2572[461]	riboflavin biosynthesis protein RibD domain-containing protein, similar to Chain A, Dihydrofolate Reductase	Hypothetical proteins	Conserved
lmo2733[494]	hypothetical protein, similar to PTS system, fructose-specific IIABC component	Transport and binding proteins	Carbohydrates, organic alcohols, and acids

Group D. Proteins identified as positively regulated by σ^B through proteomics only (15)			
lmo0398[68]	fructose-specific PTS system IIA component	Transport and binding proteins	Carbohydrates, organic alcohols, and acids
lmo0399[68]	fructose-specific PTS system IIB component	Transport and binding proteins	Carbohydrates, organic alcohols, and acids
lmo0643	transaldolase	Energy metabolism	Pentose phosphate pathway
lmo1046[168]	molybdenum cofactor biosynthesis protein C, moaC	Biosynthesis of cofactors, prosthetic groups, and carriers	Molybdopterin
lmo1349[213]	glycine cleavage system P-protein, gcvPA, similar to glycine dehydrogenase (decarboxylating) subunit 1	Energy metabolism	Amino acids and amines
		Central intermediary metabolism	Other
lmo1422[232]	hypothetical protein, similar to glycine betaine/carnitine/choline ABC transporter (membrane protein)	Transport and binding proteins	Amino acids, peptides and amines
lmo2047	50S ribosomal protein L32, rpmF2	Protein synthesis	Ribosomal proteins: synthesis and modification
lmo1539[258]	glycerol uptake facilitator protein	Transport and binding proteins	Other
lmo2743[496; 497]	transaldolase, tal1	Energy metabolism	Pentose phosphate pathway
lmo2697[485]	dihydroxyacetone kinase	Hypothetical proteins	Conserved
lmo2696[485]	dihydroxyacetone kinase L subunit	Energy metabolism	Sugars
		Fatty acid and phospholipid metabolism	Biosynthesis
lmo2695[485]	dihydroxyacetone kinase DhaK subunit	Energy metabolism	Sugars
		Fatty acid and phospholipid metabolism	Biosynthesis
lmo2666[477]	galactitol-specific PTS system IIB component	Transport and binding proteins	Carbohydrates, organic alcohols, and acids
		Amino acid biosynthesis	Aromatic amino acid family
		Energy metabolism	Pyruvate dehydrogenase
lmo0110[18]	esterase/lipase	Fatty acid and phospholipid metabolism	Degradation
lmo1730[302]	lactose/L-arabinose transport system substrate-binding protein, similar to sugar ABC transporter binding protein	Transport and binding proteins	Carbohydrates, organic alcohols, and acids

Group E. Genes or proteins identified as positively regulated by σ^B through microarray and proteomics and not RNA-Sequencing (3)			
lmo1601[272]	hypothetical protein, similar to general stress protein	Cellular processes	Adaptations to atypical conditions
lmo2205[400]	phosphoglycerate mutase, gpmA, 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase	Energy metabolism	Glycolysis/gluconeogenesis
lmo1428[234]	osmoprotectant transport system ATP-binding protein, opuCA, similar to glycine betaine/carnitine/choline ABC transporter (ATP-binding protein)	Transport and binding proteins	Amino acids, peptides and amines

Group F. Genes or proteins identified as positively regulated by σ^B through RNA-Sequencing and proteomics and not microarray (3)			
lmo1426[234]	osmoprotectant transport system substrate-binding protein, opuCC, similar to glyc	Transport and binding proteins	Amino acids, peptides and amines
lmo0819[128]	hypothetical protein		
lmo0265	succinyl-diamipimelate desuccinylase, dapE	Unclassified	Role category not yet assigned

Group G. Genes identified as positively regulated by σ^B through RNA-Sequencing and microarray and not proteomics (65)			
lmo0169[25]	sugar uptake protein, similar to a glucose uptake protein	Transport and binding proteins	Unknown substrate
lmo0170[25]	hypothetical protein	Hypothetical proteins	Conserved
lmo0263	internalin C2, inlC2	Cellular processes	Pathogenesis
LMRG_02851			
lmo0321	hypothetical protein	Cellular processes	Toxin production and resistance
lmo0445	putative M protein trans-acting positive regulator, similar to transcription regulator	Regulatory functions	Other
lmo0515	universal stress protein	Cellular processes	Adaptations to atypical conditions
lmo0555[89]	Di/tripeptide permease DtpT, similar to di-tripeptide transporter	Unclassified	Role category not yet assigned
lmo0593	formate/nitrite transporter	Unclassified	Role category not yet assigned
lmo0596	membrane protein	Hypothetical proteins	Conserved
lmo0602	hypothetical protein, weakly similar to transcription regulator	Unkwn function	General
lmo0610	internalin, putative peptidoglycan bound protein (LPXTG motif)	Cell envelope	Other
lmo0628[103]	hypothetical protein		
lmo0629[103]	hypothetical protein	Biosynthesis of cofactors, prosthetic groups, and carriers	Other
lmo0655	serine/threonine protein phosphatase 1, similar to phosphoprotein phosphatases	Protein fate	Protein modification and repair
		Regulatory functions	Other
lmo0669[109]	uncharacterized oxidoreductase	Cellular processes	Adaptations to atypical conditions
lmo0670[109]	hypothetical protein	Hypothetical proteins	Conserved
lmo0781[123]	manse-specific PTS system IID component	Energy metabolism	Pyruvate dehydrogenase
		Amino acid biosynthesis	Aromatic amino acid family
		Transport and binding proteins	Carbohydrates, organic alcohols, and acids
lmo0782[123]	manse-specific PTS system IIC component	Transport and binding proteins	Carbohydrates, organic alcohols, and acids
lmo0784[124]	manse-specific PTS system IIA component	Transport and binding proteins	Carbohydrates, organic alcohols, and acids
lmo0880	peptidoglycan bound protein, similar to wall associated protein precursor (LPXTG motif)	Cell envelope	Other
lmo0911	hypothetical protein	Hypothetical proteins	Conserved
lmo0937	predicted protein		
lmo0953	hypothetical protein		
lmo0994	hypothetical protein		
lmo1140	hypothetical protein	Hypothetical proteins	Conserved
lmo1241	hypothetical protein	Hypothetical proteins	Conserved
lmo1295	host factor-I protein, hfq	Mobile and extrachromosomal element functions	Prophage functions
		Regulatory functions	Other
lmo1375	peptidase T-like protein, similar to aminotripeptidase	Protein fate	Degradation of proteins, peptides, and glycopeptides
lmo1425[234]	osmoprotectant transport system permease, opuCD, similar to betaine/carnitine/choline ABC transporter	Transport and binding proteins	Amino acids, peptides and amines
lmo1433[235]	glutathione reductase	Cellular processes	Detoxification
		Biosynthesis of cofactors, prosthetic groups, and carriers	Glutathione and analogs
lmo1526	hypothetical protein	Hypothetical proteins	Conserved
lmo1606[273]	DNA segregation ATPase FtsK/SpoIIIE, similar to DNA translocase	Unclassified	Role category not yet assigned
lmo1694	hypothetical protein, similar to CDP-abequose synthase	Cellular processes	Cell division
lmo1698	ribosomal-protein-alanine N-acetyltransferase	Protein synthesis	Ribosomal proteins: synthesis and modification
lmo1883	chitinase	Energy metabolism	Biosynthesis and degradation of polysaccharides
lmo2067	choloylglycine hydrolase with bile hydrolase activity	Unclassified	Role category not yet assigned
lmo2085	peptidoglycan binding protein (LPXTG motif)	Regulatory functions	Other
lmo2130	hypothetical protein	Cell envelope	Other
lmo2132	hypothetical protein		
lmo2157	involved in septum formation, sepA	Protein fate	Protein and peptide secretion and trafficking
lmo2230	arsenate reductase	Cellular processes	Toxin production and resistance
		Cellular processes	Detoxification

lmo2231	cation efflux family protein (putative arsenite-transporting ATPase)	DNA metabolism	DNA replication, recombination, and repair
lmo2269[413]	hypothetical protein	Unclassified	Role category not yet assigned
lmo2387	hypothetical protein	Hypothetical proteins	Conserved
lmo2391	hypothetical protein, similar to B. subtilis YhfK protein	Hypothetical proteins	Conserved
lmo2434	glutamate decarboxylase, Probable glutamate decarboxylase gamma	Energy metabolism	Amino acids and amines
		Central intermediary metabolism	Other
lmo2454[434]	hypothetical protein		
lmo2463[436]	hypothetical protein, similar to transport protein	Cell envelope	Other
lmo2484[441]	membrane protein, similar to B. subtilis YvlD protein	Cell envelope	Other
lmo2485[441]	PspC domain-containing protein, similar to B. subtilis yvlC protein	Mobile and extrachromosomal element functions	Prophage functions
lmo2494	phosphate transport system regulatory protein PhoU, similar to negative regulator of phosphate regulon	Energy metabolism	Glycolysis/gluconeogenesis
		Energy metabolism	Photosynthesis
lmo2570[461]	hypothetical protein	Hypothetical proteins	Conserved
lmo2571[461]	nicotinamidase	Biosynthesis of cofactors, prosthetic groups, and carriers	Pyridine nucleotides
lmo2573[461]	NADPH2:quinone reductase, similar to zinc-binding dehydrogenase	Central intermediary metabolism	Other
		Energy metabolism	Fermentation
lmo2602[469]	Mg2+ transporter-C family protein	Unclassified	Role category not yet assigned
lmo2603[469]	amidase	Hypothetical proteins	Conserved
lmo2670[478]	hypothetical protein	Unclassified	Role category not yet assigned
lmo2671[478]	hypothetical protein	Unclassified	Role category not yet assigned
lmo2672[478]	hypothetical protein, weakly similar to transcription regulator	Regulatory functions	Other
lmo2673[479]	universal stress protein	Cellular processes	Adaptations to atypical conditions
lmo2674[479]	ribose 5-phosphate isomerase B, similar to ribose 5-phosphate epimerase	Energy metabolism	Pentose phosphate pathway
lmo2724[491]	DNA binding 3-demethylubiquine-9 3-methyltransferase domain-containing protein	Energy metabolism	Pyruvate dehydrogenase
		Energy metabolism	Other
lmo0019	transmembrane protein	Hypothetical proteins	Conserved
lmo0043	arginine deiminase, arcA	Energy metabolism	Amino acids and amines

[a]Gene names are based on the *L. monocytogenes* EGD-e locus. Genes in bold are preceded by a putative σ^{a}-dependent promoter. Operon numbers are listed as superscripts next to the gene name, with boxed genes part of an operon with more than one member in the table. Operon numbers are based on the classification by Toledo-Arana *et al.* , 2009.

[b]Role Categories and Sub-Role categories are based on JCVI classification (http://cmr.jcvi.org)

Appendix 3.4. σ^B-dependent genes and proteins among JCVI role categories

Role Category [#]	σ^B-dependent genes or proteins [*]	Fisher's Exact Test *p*-value [†]	Odds Ratio [‡]
Amino acid biosynthesis (139)	3	0.1169556	0.39
Biosynthesis of cofactors, prosthetic groups, and carriers (101)	7	0.3652719	1.37
Cell envelope (119)	6	1	0.97
Cellular processes (165)	14	0.06841697	1.75
Central intermediary metabolism (172)	9	1	1.01
DNA metabolism (102)	1	0.06406374	0.18
Energy metabolism (380)	**30**	**0.01877902**	**1.68**
Fatty acid and phospholipid metabolism (60)	5	0.2385335	1.68
Hypothetical proteins (430)	29	0.129604	1.38
Mobile and extrachromosomal element functions (23)	2	0.3374415	1.75
NULL (276)	12	0.6700001	0.82
Protein fate (100)	6	0.6463248	1.17
Protein synthesis (142)	**2**	**0.03240542**	**0.25**
Purines, pyrimidines, nucleosides, and nucleotides (67)	2	0.5814206	0.56
Regulatory functions (226)	6	0.08612031	0.48
Signal transduction (6)	0	1	NA
Transcription (43)	0	0.1690481	NA
Transport and binding proteins (383)	22	0.6239414	1.13
Unclassified (258)	14	0.8835201	1.05
Unknown function (109)	3	0.3752867	0.51
Viral functions (49)	1	0.5163073	0.38

[#]Role categories for *L. monocytogenes* EGD-e, with the total number of annotated genes in each role category indicated in parentheses.

[*]Number of σ^B-dependent genes/proteins in each role category; analysis was based on 149 σ^B-dependent genes and proteins, as some genes and proteins were assigned more than one role category the total of this column is > 149.

[†]Fisher's Exact Test *p*-values < 0.05 indicate a significant association between σ^B-dependent genes/proteins and the total number of genes in a particular role category.

[‡]Odds ratios (OR) were calculated to describe the association between σ^B-dependent genes/proteins and role category.

Appendix 4.1. Strains used in this study

Strain	Genotype
10403S	Parent strain (serotype 1/2a)
FSL C3-135	10403S, Δ*BCHL*
FSL C3-137	10403S, Δ*BCL*
FSL C3-128	10403S, Δ*BCH*
FSL C3-138	10403S, Δ*BHL*

Appendix 4.2. Proteins found to be differentially expressed between *L. monocytogenes* parent strain 10403S and Δ*BCHL*

Protein[a]	Fold Change PAR./Δ*BCHL*	Description	Gene Name	Role Category[b]	Sub-Role Category[b]
Proteins with higher expression levels in the parent strain					
Lmo1439[c]	1.63	superoxide dismutase	*sodA*	Cellular processes	Detoxification
Lmo2006	1.64	acetolactate synthase catabolic	*alsS*	Amino acid biosynthesis	Aspartate family
				Amino acid biosynthesis	Pyruvate family
Lmo1540	1.66	50S ribosomal protein L27	*rpmA*	Protein synthesis	Ribosomal proteins: synthesis and modification
Lmo2610	1.75	translation initiation factor IF-1	*infA*	Protein synthesis	Translation factors
Lmo1422	1.81	hypothetical protein	*lmo1422*	Transport and binding proteins	Amino acids, peptides and amines
Lmo2614	1.91	50S ribosomal protein L30	*rpmD*	Protein synthesis	Ribosomal proteins: synthesis and modification
Lmo1421	1.92	osmoprotectant transport system ATP-binding protein	*bilEA*	Transport and binding proteins	Amino acids, peptides and amines
Lmo1602	1.94	hypothetical protein	*lmo1602*	Hypothetical proteins	Conserved
Lmo1426	1.97	osmoprotectant transport system substrate-binding protein	*opuCC*	Transport and binding proteins	Amino acids, peptides and amines
Lmo1428	1.98	osmoprotectant transport system ATP-binding protein	*opuCA*	Transport and binding proteins	Amino acids, peptides and amines
Lmo0913	2.10	succinate-semialdehyde dehydrogenase	*lmo0913*	Energy metabolism	Other
Lmo2205	2.13	phosphoglycerate mutase	*gpmA*	Energy metabolism	Glycolysis/gluconeogenesis
Lmo2398	2.16	phosphatidylglycerophosphatase A	*ltrC*	Protein fate	Degradation of proteins, peptides, and glycopeptides
				Cellular processes	Adaptations to atypical conditions
				Protein fate	Protein and peptide secretion and trafficking
Lmo1601	2.20	hypothetical protein	*lmo1601*	Cellular processes	Adaptations to atypical conditions
Lmo0554	2.25	hypothetical protein	*lmo0554*	Unknown function	General
				Central intermediary metabolism	Other
Lmo1634	2.26	bifunctional acetaldehyde-CoA/alcohol dehydrogenase	*lmo1634*	Energy metabolism	Fermentation
Lmo2047	2.61	50S ribosomal protein L32	*rpmF2*	Protein synthesis	Ribosomal proteins: synthesis and modification
Lmo0796	2.67	YceI like family protein	*lmo0796*	Hypothetical proteins	Conserved
Lmo0265	2.87	succinyl-diaminopimelate desuccinylase	*dapE*	Unclassified	Role category not yet assigned
Lmo2213	2.91	similar to antibiotic biosynthesis monooxygenase subfamily	*lmo2213*	Hypothetical proteins	Conserved
Lmo0110	2.94	esterase/lipase	*lmo0110*	Fatty acid and phospholipid metabolism	Degradation
Lmo2558	3.12	N-acetylmuramoyl-L-alanine amidase	*ami*	Transport and binding proteins	Unknown substrate
Lmo0722	3.18	pyruvate oxidase	*lmo0722*	Energy metabolism	Sugars
Lmo2158	3.19	stress response protein	*lmo2158*	Unclassified	Role category not yet assigned
Lmo0539	3.22	tagatose 1,6-diphosphate aldolase	*lacD*	Energy metabolism	Biosynthesis and degradation of polysaccharides
Lmo0783	3.31	mannose-specific PTS system IIB component	*lmo0783*	Transport and binding proteins	Carbohydrates, organic alcohols, and acids
Lmo2748	3.59	general stress protein 26	*lmo2748*	Hypothetical proteins	Conserved
Lmo0794	3.68	hypothetical protein	*lmo0794*	Hypothetical proteins	Conserved
Lmo0096[d]	3.88	mannose-specific PTS system IIAB component ManL	*mptA*	Energy metabolism	Pyruvate dehydrogenase
				Amino acid biosynthesis	Aromatic amino acid family
				Transport and binding proteins	Carbohydrates, organic alcohols, and acids
Lmo0134	4.25	hypothetical protein	*lmo0134*	Central intermediary metabolism	Other
Lmo0098[d]	4.26	mannose-specific PTS system IID component	*mptD*	Energy metabolism	Pyruvate dehydrogenase
				Transport and binding proteins	Carbohydrates, organic alcohols, and acids
				Amino acid biosynthesis	Aromatic amino acid family
Lmo1830	4.83	short chain dehydrogenase	*lmo1830*	Hypothetical proteins	Conserved
Lmo0654	5.65	hypothetical protein	*lmo0654*		
Proteins with lower expression levels in the parent strain					
Lmo0018	-2.04	6-phospho-beta-glucosidase	*lmo0018*	Central intermediary metabolism	Other
Lmo0027	-2.21	beta-glucoside-specificPTS system IIABC component	*lmo0027*	Transport and binding proteins	Carbohydrates, organic alcohols, and acids
				Amino acid biosynthesis	Aromatic amino acid family
				Energy metabolism	Pyruvate dehydrogenase
Lmo0181	-1.97	multiple sugar transport system substrate-binding protein	*lmo0181*	Transport and binding proteins	Unknown substrate
Lmo0261	-1.80	beta-glucosidase	*lmo0261*	Energy metabolism	Sugars
Lmo0271	-1.67	beta-glucosidase	*lmo0271*	Central intermediary metabolism	Other
Lmo0319	-2.08	beta-glucosidase	*bglA*	Energy metabolism	Sugars
Lmo0343	-2.15	transaldolase	*tal2*	Energy metabolism	Pentose phosphate pathway
Lmo0344	-2.19	short chain dehydrogenase	*lmo0344*	Energy metabolism	Biosynthesis and degradation of polysaccharides
Lmo0345	-3.02	ribose 5-phosphate isomerase B	*lmo0345*	Energy metabolism	Pentose phosphate pathway
Lmo0377	-1.75	hypothetical protein	*lmo0377*		
Lmo0536	-1.92	6-phospho-beta-glucosidase	*lmo0536*	Central intermediary metabolism	Other
Lmo0690	-2.13	flagellin	*flaA*	Cellular processes	Chemotaxis and motility
Lmo0692	-1.56	chemotaxis protein CheA	*cheA*	Cellular processes	Chemotaxis and motility
Lmo0702	-1.66	hypothetical protein	*lmo0702*		
Lmo0723	-1.77	methyl-accepting chemotaxis protein	*lmo0723*	Cellular processes	Toxin production and resistance
Lmo0768	-1.61	multiple sugar transport system substrate-binding protein	*lmo0768*	Unclassified	Role category not yet assigned
Lmo0813	-1.68	fructokinase	*lmo0813*	Energy metabolism	Sugars
Lmo0859	-1.97	multiple sugar transport system substrate-binding protein	*lmo0859*	Transport and binding proteins	Carbohydrates, organic alcohols, and acids
Lmo1349	-1.71	glycine cleavage system P-protein	*gcvPA*	Energy metabolism	Amino acids and amines
				Central intermediary metabolism	Other
Lmo1388	-2.30	ABC transport system	*tcsA*	Unclassified	Role category not yet assigned
Lmo1556	-1.61	porphobilinogen deaminase	*hemC*	Biosynthesis of cofactors, prosthetic groups, and carriers	Heme, porphyrin, and cobalamin
Lmo1699	-1.62	Methyl-accepting chemotaxis protein	*lmo1699*	Cellular processes	Chemotaxis and motility
Lmo1856	-1.58	purine nucleoside phosphorylase	*deoD*	Purines, pyrimidines, nucleosides, and nucleotides	Salvage of nucleosides and nucleotides
Lmo1877	-2.67	formate-tetrahydrofolate ligase	*fhs*	Amino acid biosynthesis	Aspartate family
				Protein synthesis	tRNA aminoacylation
				Amino acid biosynthesis	Histidine family
				Purines, pyrimidines, nucleosides, and nucleotides	Purine ribonucleotide biosynthesis
				Biosynthesis of cofactors, prosthetic groups, and carriers	Pantothenate and coenzyme A
Lmo1879	-2.93	cold shock protein	*cspD*	Cellular processes	Adaptations to atypical conditions
Lmo1954	-1.80	phosphopentomutase	*deoB*	Purines, pyrimidines, nucleosides, and nucleotides	Salvage of nucleosides and nucleotides
Lmo2057	-2.08	protoheme IX farnesyltransferase	*ctaB*	Energy metabolism	Electron transport
Lmo2094	-9.66	hypothetical protein	*lmo2094*	Energy metabolism	Sugars
Lmo2097	-4.08	galactitol-specific PTS system IIB component	*lmo2097*	Energy metabolism	Pyruvate dehydrogenase
				Amino acid biosynthesis	Aromatic amino acid family
				Transport and binding proteins	Carbohydrates, organic alcohols, and acids
Lmo2101	-1.71	pyridoxine biosynthesis protein	*pdxS*	Biosynthesis of cofactors, prosthetic groups, and carriers	Pyridoxine
Lmo2125	-2.54	maltose/maltodextrin transport system substrate-binding protein	*lmo2125*	Unclassified	Role category not yet assigned
Lmo2160	-2.17	sugar phosphate isomerase/epimerase	*lmo2160*	Hypothetical proteins	Conserved
Lmo2175	-2.32	3-oxoacyl-[acyl-carrier protein] reductase	*lmo2175*	Fatty acid and phospholipid metabolism	Biosynthesis
				Fatty acid and phospholipid metabolism	Degradation
Lmo2341	-1.84	hypothetical protein	*lmo2341*	Energy metabolism	Sugars
Lmo2362	-1.95	glutamate/gamma-aminobutyrate antiporter	*lmo2362*	Transport and binding proteins	Amino acids, peptides and amines
Lmo2363	-2.47	glutamate decarboxylase	*gadB*	Energy metabolism	Amino acids and amines
				Biosynthesis of cofactors, prosthetic groups, and carriers	Heme, porphyrin, and cobalamin
				Central intermediary metabolism	Polyamine biosynthesis
				Purines, pyrimidines, nucleosides, and nucleotides	Pyrimidine ribonucleotide biosynthesis
				Central intermediary metabolism	Other
Lmo2421	-1.78	cephalosporin sensitivity histidine protein kinase	*lmo2421*	Regulatory functions	Other

Lmo2505	-1.89	D-glutamyl-L-m-Dpm peptidase P45	*spl*	Viral functions	General
				Protein fate	Degradation of proteins, peptides, and glycopeptides
Lmo2539	-1.75	serine hydroxymethyltransferase	*glyA*	Amino acid biosynthesis	Serine family
				Biosynthesis of cofactors, prosthetic groups, and carriers	Folic acid
Lmo2585	-2.24	hypothetical protein	*lmo2585*	Hypothetical proteins	Conserved
Lmo2637	-1.69	pheromone lipoprotein	*lmo2637*	Cell envelope	Other
Lmo2648	-1.59	hypothetical protein	*lmo2648*	Unclassified	Role category not yet assigned
Lmo2663	-2.84	L-iditol 2-dehydrogenase	*lmo2663*	Central intermediary metabolism	Other
				Energy metabolism	Electron transport
				Energy metabolism	Glycolysis/gluconeogenesis
				Energy metabolism	TCA cycle
				Energy metabolism	Fermentation
Lmo2666	-2.03	galactitol-specific PTS system IIB component	*lmo2666*	Transport and binding proteins	Carbohydrates, organic alcohols, and acids
				Amino acid biosynthesis	Aromatic amino acid family
				Energy metabolism	Pyruvate dehydrogenase

[a]Protein names are based on the *L. monocytogenes* EGD-e locus.

[b]Role Categories and Sub-Role categories are based on JCVI classification [32]

[c]Preceded by a putative σ^A promoter (ttgcaagcatttagggagcatggtaggct) and σ^B promoter (gtttaacttttgagtttcagggaaa); -10 and -35 regions are underlined.

[d]Preceded by a putative σ^L promoter; tggcacagaacttgca; -12 and -24 regions are underlined.