Contributions of σB and PrfA to *Listeria monocytogenes* salt stress under food relevant conditions

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Abstract

Listeria monocytogenes is well known to survive and grow under several stress conditions, including salt stress, which is important for growth in certain foods as well as for host infection. To characterize the contributions, to salt stress response, of transcriptional regulators important for stress response and virulence (i.e., σB and PrfA), we analyzed three *L. monocytogenes* parent strains and isogenic mutants (*sigB*, *prfA*, and *sigB prfA*), representing different serotypes and lineages, for their ability to grow, at 25 °C, in BHI with 1.9 M NaCl. With regard to growth rate, only the lineage IV strain presented a significant difference between the parent strain and both of its respective mutants lacking *prfA* (*prfA* and *sigB prfA*). Conversely, the lineage I and II parent strains showed significantly shorter lag phase in comparison to their respective *sigB* mutant strains. Intestinal epithelial cell invasion assay and hemolytic activity assays showed a significant role for σB in the former and for PrfA in the latter. To explore the mechanism that may contribute to the extended lag phase in the *sigB* mutant strain and survival and growth of the parent strain upon salt shock, whole genome transcription profiling was performed to compare transcript levels between the lineage I, serotype 1/2b, parent strain and its isogenic *sigB* mutant after 30 min of lag phase growth at 25 °C in the presence of 1.9 M NaCl (salt shock) without aeration. Microarray data showed significantly higher transcript levels for 173 genes in the parent strain as compared to the *sigB* strain. Overall, 102 of the 173 σB up-regulated genes had been identified in previous studies, indicating that 71 genes were newly identified as being up-regulated by σB in this study. We hypothesize that, among these genes newly identified as σB up-regulated, four genes (lmo2174, lmo0530, lmo0527 and lmo0529) may play a major role in response to salt stress. Lmo2174 contains domains that facilitate sensing and producing a transduction signal in the form of cyclic di-GMP, which may activate the enzymes Lmo0527, Lmo0529 and Lmo0530, which encode proteins similar to those responsible for synthesis of exopolysaccharides that may protect the cell by changing the cell wall structure during salt stress. Overall, our data showed that σB, but not PrfA, contributes to growth under salt stress. Moreover, we show that the σB regulon

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of a *L. monocytogenes* lineage I strain challenged with salt shock includes salt stress-specific as well as previously unidentified σ^B up-regulated genes.

Keywords

Listeria monocytogenes; Salt stress; Salt shock; Sigma B; PrfA; Microarray

1. Introduction

Listeria monocytogenes is found in a wide range of food products such as dairy products, deli meats, salads, and seafood (Ryser and Marth, 2007). Food safety relies primarily on the use of multiple hurdle technology to prevent microbial growth in foods (Leistner, 2000). Among the well-established barriers applied to prevent microbial growth, acid and osmotic stresses are most commonly applied during food processing (e.g., by acidification or addition of NaCl), or naturally encountered in foods (e.g., low pH of fruits and desiccation). These two factors normally contribute to the preservation of food products, but *L. monocytogenes* is capable of surviving and growing in environments where these factors are present, resulting in long-term adaptation of *L. monocytogenes* to sublethal environmental stress conditions (Rogga et al., 2007; Ryser and Marth, 2007). Furthermore, *L. monocytogenes* can be challenged with adverse conditions not only in a food environment, but also during the infection of an animal host. While the empty human stomach can be highly acidic, with documented pH measurements as low as 2.0 (Chowduhry et al., 1996), bacteria encounter a mildly acidic environment with an elevated osmolarity (pH 4.5–6.5, 0.3 M NaCl) in the small intestine (Chowduhry et al., 1996; Davenport, 1982).

L. monocytogenes strains can be grouped into at least four phylogenetic lineages, which have been reported to vary in virulence potential and stress resistance (Orsi et al., 2011). Several studies have shown differential distribution of lineage I and II isolates among human clinical isolates (Hong et al., 2007; Parihar et al., 2008) and food isolates (Meloni et al., 2009; Orsi et al., 2008) as well as an apparent overrepresentation, among animal clinical isolates, of lineage III strains (Bundrant et al., 2011; Liu et al., 2006), while lineage IV isolates are very rare and have been isolated from food, animal and environmental sources (Ward et al., 2008). In addition, it has been noted that certain serotypes (4b, 1/2a, and 1/2b) are more frequently associated with human disease than others (Orsi et al., 2011). Differences between lineages and serotypes with respect to resistance to osmotic stress (Oliver et al., 2010), acid stress (Dykes and Moorhead, 2000), bacteriocins, heat (Buncic et al., 2001), and starvation (Avery and Buncic, 1997) have been observed. For example, Bergholz et al. (2010) reported that lineage II strains had an impaired ability to adapt to salt stress at 37 °C compared to both lineage I and lineage III strains.

The tolerance of *L. monocytogenes* to a variety of harsh environmental conditions, such as salt stress is at least partly attributed to genes under the control of the alternative sigma (σ) factor, σ^B (Abram et al., 2008a). Different studies have identified about 170 *L. monocytogenes* genes that are expressed in a σ^B-dependent manner and the functional categories represented by these genes include stress tolerance, carbon metabolism, transport,

cell envelope, and virulence (Kazmierczak et al., 2003; Raengpradub et al., 2008). On the other hand, genes known to be up-regulated by PrfA, the positive regulator of *L. monocytogenes* virulence genes, were shown to be down-regulated under salt stress conditions (Raengpradub et al., 2008), suggesting that exposure to salt stress mediates a decrease in PrfA activity, by acting either directly on the PrfA protein or on some PrfA-regulating element. Also, interactions between σ^B and PrfA in a regulatory network certainly appear to be critical for appropriate expression of virulence genes important for early stages of listerial infection (e.g., *inlA*, *inlB*) (Ollinger et al., 2008). The importance of this network has been investigated under stationary phase growth conditions, but the interactions of these regulators have not been investigated under salt stress conditions.

Identifying interactions between stress response and virulence, as well as understanding the global behavior of the transcriptome in response to salt stress, may provide insights into how *L. monocytogenes* survives and adapts to adverse conditions, leading to the development of new strategies for intervention and control. In this study, we compared the growth parameters of three parent strains representing three different *L. monocytogenes* lineages and serotypes and their respective isogenic mutants with null mutations in *sigB* and or *prfA* under a condition of high salt stress (11% NaCl). We also assessed the hemolytic activity and invasion efficiency in epithelial cells for the lineage I, II and IV *L. monocytogenes* strains studied here, and compared, using microarrays, the genome-wide expression of genes under salt stress for the lineage I serotype 1/2b strain. Our results suggest that σ^B plays a major role in invasion of epithelial cells in all strains studied while PrfA plays a major role in hemolytic activity in the same strains. We have also for the first time characterized, by microarray, the σ^B regulon under lag phase growth salt stress at 25 °C; this represents a salt stress condition more likely encountered by *L. monocytogenes* in the food chain than exposure to salt stress at 37 °C. For example, *L. monocytogenes* may be exposed to salt stress at temperatures above refrigeration in processing environments, certain processing stages, and in high salt foods (e.g., certain cheeses) that may be stored or transported at ambient temperatures. We have also identified 71 genes not previously described as being up-regulated by σ^B; these genes may be σ^B-up-regulated only under specific conditions (e.g., salt stress).

2. Materials and methods

2.1. Strains

The strains selected for this study represented *L. monocytogenes* lineages I (serotype 1/2b), II (serotype 1/2a), and IV (serotype 4a) and their respective isogenic *sigB* and *prfA* null mutants (Table 1). FSL C6–001, an FSL J1–194 isogenic *sigB* mutant, FSL A1–254, a 10403S isogenic *sigB* mutant, FSL B2–046, a 10403S isogenic *prfA* mutant, FSL-B2–068, a 10403S isogenic *sigB prfA* mutant and FSL O1–005, an FSL J1–208 *sigB* isogenic mutant have been reported previously (McGann et al., 2007; Oliver et al., 2010; Wiedmann et al., 1998; Wong and Freitag, 2004). FSL C6–004, an FSL J1–194 isogenic *prfA* mutant, FSL C6–005, an FSL J1–194 isogenic *sigB prfA* mutant, FSL C6–009, an FSL J1–208 isogenic *prfA* mutant and FSL C6–007, an FSL J1–208 isogenic *sigB prfA* mutants were created using allelic exchange mutagenesis, as previously described (Horton et

al., 1990; Wiedmann et al., 1998). All null mutants represent non-polar in-frame deletion mutants (hence, no complemented mutants were created). Stock cultures were stored at −80 °C in brain heart infusion (BHI) containing 15% glycerol. Cultures were streaked onto BHI agar (Difco, Detroit, MI) and incubated at 37 °C for 24 h to obtain isolated colonies for inoculation of overnight cultures.

2.2. Growth conditions and salt stress survival assays

Initially, all strains were grown to early stationary phase in BHI at 37 °C as described previously (Oliver et al., 2010). Briefly, strains were first grown in 5 ml of BHI broth at 37 °C with shaking (230 rpm) for 12 h. A 1% inoculum was transferred to 5 ml of pre-warmed BHI broth and grown to an optical density at 600 nm (OD_{600}) of 0.4; 50 μl of this culture was transferred to another 5 ml of pre-warmed (37 °C) BHI broth, followed by incubation at 37 °C with shaking (230 rpm) until bacteria reached early stationary phase (defined as growth to an OD_{600} of 1.0, followed by incubation for an additional 3 h). Serial dilutions of this early stationary phase culture (representing approx. 10^9 CFU/ml) were made in BHI broth and 1 ml of a dilution with 10^6 CFU/ml of *L. monocytogenes* was transferred to 99 ml BHI plus 1.9 M NaCl (11%) (for an inoculum of ~10^4 CFU/ml), followed by incubation at 25 °C, without aeration. Cultures were incubated for 20 days and plated onto BHI agar in 12 different time points (1, 2, 3, 4, 5, 7, 10, 12, 14, 16, 18 and 20 days post-incubation). Plates were incubated at 37 °C overnight and colonies enumerated. Three independent replicates were performed.

2.3. Growth parameter data analysis

Growth parameters for each strain (for growth in BHI or BHI plus 1.9 M NaCl at 25 °C) were estimated using the Baranyi model (Baranyi and Roberts, 1994) implemented in the NLStools v.0.0–4 package in R v.2.5.1. Cell densities (CFU/ml) for each strain at every time point were log-transformed and used to estimate the lag phase duration and maximum growth rate. The statistical significance of differences in the growth parameters between the *L. monocytogenes* strains was evaluated using one-way analysis of variance (one-way ANOVA) at $\alpha = 0.05$ significance level with the Tukey's HSD multiple correction procedure.

2.4. RNA extraction

For RNA extraction, 1 ml of early stationary phase cells (prepared as detailed in Section 2.2) was transferred to 99 ml BHI with 1.9 M NaCl (to reach ~10^7 CFU/ml), followed by incubation at 25 °C, without aeration for 30 min. RNA Protect bacterial reagent (Qiagen, Valencia, CA) was added to these 100 ml cultures, according to the manufacturer's instruction, to stabilize the mRNA followed by centrifugation at 18,000 *g* for 20 min; pellets were stored at −80 °C prior to RNA isolation. RNA was extracted from cultures using TriReagent (Ambion). Total RNA was incubated with RNasin (Promega, Madison, WI) to inhibit RNases and with RQ1 DNase (Promega) to remove contaminating DNA, according to the manufacturer's instructions. After phenol–chloroform extraction, UV spectrophotometry (Nanodrop, Wilmington, DE) was used to quantify and assess the purity of the RNA (Oliver et al., 2010). RNA quality was assessed on a Bioanalyzer (Agilent), and only samples with RNA Integrity Numbers (RIN) > 8.0 were used for subsequent analyses.

Purified RNA was stored in RNase-free water at −80 °C prior to reverse transcription (RT), which was performed as previously described (Ollinger et al., 2009).

2.5. Microarray, cDNA labeling, and microarray hybridization

The Pathogen Functional Genomics Resource Center (PFGRC)/J. Craig Venter Institute (JCVI) *L. monocytogenes* microarray (version 2) was used to identify mRNA transcript level differences between the lineage I, serotype 1/2b, parent strain FSL J1–194 and its respective isogenic *sigB* mutant both grown to lag phase with 1.9 M NaCl at 25 °C without aeration for 30 min. cDNA labeling and microarray hybridization steps were performed as previously described (Oliver et al., 2010). Three biological replicates were performed.

2.6. Microarray image processing, replicates, and statistical analysis

Image processing and analyses were performed as previously described (Raengpradub et al., 2008). Data preprocessing and statistical analyses were performed using the LIMMA package available from the BioConductor software project for the R programming environment (Gentleman et al., 2004; Smyth, 2005). Background correction was performed using the "normexp" method to produce more robust ratios for low-intensity spots, and print tip normalization was used to correct for spatial effects and dye intensity bias (Smyth and Speed, 2003). The empirical Bayes approach was used to assess differential expression (Smyth, 2004). For each probe, fold changes, moderated t-statistics, and p-values (adjusted for multiple tests by controlling for the false discovery rate) were calculated. Genes whose transcript levels were higher in the parent strain than in the *sigB* isogenic mutant (1.5 fold change) and whose adjusted p-values were <0.05 were considered positively regulated by σ^B (σ^B-up-regulated). Genes whose transcript levels were higher in the *sigB* isogenic mutant than in the parent strain (1.5 fold change) and whose adjusted p-values were <0.05 were considered σ^B-down-regulated. Raw and normalized microarray data have been deposited in the NCBI Gene Expression Omnibus (GEO) database under accession number GSE49056.

2.7. GSEA analysis

Gene set enrichment analysis (GSEA) (Subramanian et al., 2005) was used to identify role categories that were significantly overrepresented among genes up- or down-regulated by σ^B under the salt stress condition. GSEA was run on the ranked list of $\log_2$ expression ratios obtained from the fitted normalized data in LIMMA with 1000 permutations and exclusion of gene sets with fewer than 5 or greater than 500 members. Genes were classified into sets based on the TIGR Comprehensive Microbial Resource (http://cmr.tigr.org) subrole categories for *L. monocytogenes* EGD-e. False discovery rate q-values less than 0.05 were considered significant.

2.8. TaqMan quantitative RT-PCR (qRT-PCR)

Quantitative Real-Time PCR (qRT-PCR) was used to confirm σ^B-dependent transcript levels for selected genes (*inlD*, *fri*, *gltX*) that showed higher and lower transcript levels in cells grown at 25 °C in the microarray analyses (see Results section). Primers and TaqMan minor groove binder probes have been previously reported (Chan et al., 2007; Mcgann et al., 2007; Ollinger et al., 2009). qRT-PCR was performed as previously described (Kazmierczak et al.,

2006). All qRT-PCR experiments were performed using the three different RNA preparations that were also used for the microarray experiments. Relative expression (i.e. lineage I parent strain transcript levels/ *sigB* mutant strain transcript levels) was determined using the method described by Pfaffl (2001), and normalized to the geometric means for the housekeeping gene *rpoB* as previously described (Bergholz et al., 2010). Statistical significance of the relative expressions was assessed using the one-sample t-test implemented in R with $\mu = 1.0$.

2.9. Hemolysin assay

For the hemolysin assay, bacteria were grown in BHI broth (without additional salt) overnight at 37 °C with agitation. The enzymatic activity of hemolysin (LLO) from each strain was measured as described previously (Portnoy et al., 1988), except that 0.5 mM dithiothreitol was used as a reducing agent instead of cysteine. Lysis of sheep red blood cells was measured as hemoglobin release at 420 nm in a Multiskan FC microplate analyzer (Thermo Fisher, Waltham, MA). A hemolytic unit was defined as the reciprocal of the supernatant dilution at which 50% of the sheep red blood cells were lysed. The parent level, represented by *L. monocytogenes* 10403S, was set at 100% hemolysis. All comparisons were evaluated by one-way analysis of variance (one-way ANOVA). Statistical significance was established at *p*-value < 0.05. Statistical analyses were performed in Minitab (Statistical Software, State College, Pa.).

2.10. Invasion assay

The human colorectal adenocarcinoma epithelial cell line Caco-2 (ATCC HTB-37) was cultured, and invasion assays were performed as described by Garner et al. (2006) with minor modifications. In addition to the 12 strains used in this study (3 parent strains and 9 mutants), the laboratory strain Scott A was included in this experiment as a positive control. Briefly, 5.0×10^4 Caco-2 cells were seeded into 24-well plates (Corning Incorporated, Corning, NY) 48 h prior to infection. For infection, the Caco-2 cells were inoculated with approximately 5.0×10^6 *L. monocytogenes* cells grown to early stationary phase (in BHI without added salt) at 37 °C without agitation. An additional aliquot of the *L. monocytogenes* inoculum was serially diluted and plated onto BHI agar to ensure that the number of bacteria that had been used for infection was as expected, based on previous enumeration of the frozen aliquots. At 30 min postinfection, pre-warmed BHI media containing 50 μg/ml gentamicin (Invitrogen Ltd.) was added to the plates to kill extracellular bacteria. Intracellular *L. monocytogenes* numbers were determined 90 min postinfection. The invasion efficiency (expressed as a percentage) was calculated by using the ratio of the number of bacterial cells that survived the gentamicin assay to the total number of bacterial cells added initially to each well. Invasion efficiency values did not follow a normal distribution and were thus log-transformed to satisfy this assumption. Invasion efficiencies were analyzed using a one-way analysis of variance (one-way ANOVA, $\alpha = 0.05$); Tukey's HSD was used to assess the significance of differences between strains.

3. Results

3.1. L. monocytogenes strains differ in the relative contributions of σ^B and PrfA to growth under salt stress conditions

Three *L. monocytogenes* parent strains representing lineages I (serotype 1/2b), II (serotype 1/2a), and IV (serotype 4a) were grown for 20 days in BHI broth with 1.9 M (11%) NaCl at 25 °C without aeration. The parent strains showed significant differences in growth differential ($\log_{10}$[final population] − $\log_{10}$[initial population]) (p-value = 0.044, one-way ANOVA), lag phase (p-value = 0.001, one-way ANOVA) and maximum growth rate (p-value = 0.003, one-way ANOVA). Specifically, the lineage I parent strain showed a significantly shorter lag phase as compared to the lineage II and IV parent strains (p-value = 0.002 for both, Tukey's HSD). The lineage IV parent strain showed a significantly lower maximum growth rate as compared to the lineage I (p-value = 0.004, Tukey's HSD) and lineage II (p-value = 0.007, Tukey's HSD) parent strains.

Differences, in growth parameters under salt stress conditions, between the lineage I, II and IV parent strains and their isogenic mutants were also observed (Table 2). Specifically, lineage I and II strains that lacked *sigB* (∆*sigB* and ∆*sigB* ∆*prfA*) showed significantly longer lag phase as compared to their respective parent strains (p-value < 0.02 for all comparisons; Tukey's HSD); conversely, the lag phase duration of all three lineage IV mutant strains did not differ statistically (p-value > 0.05, Tukey's HSD) from that observed for their respective parent strain, indicating possible σ^B- and PrfA-independent mechanisms of salt stress adaptation during lag phase. In addition, the lineage II ∆*sigB* ∆*prfA* strain (p-value < 0.001, Tukey's HSD) and the lineage IV ∆*prfA* (p-value = 0.028; Tukey's HSD) and ∆*sigB* ∆*prfA* (p-value = 0.032; Tukey's HSD) strains showed significantly higher growth rates than their respective parent strains (Fig. 1B and C, Table 2). The lineage I ∆*sigB* ∆*prfA* strain showed significantly higher final populations than its parent strain (p-value = 0.013; Tukey's HSD). Conversely, the lineage II ∆*sigB* ∆*prfA* strain showed a significantly lower final population than its parent strain (p-value = 0.001; Tukey's HSD). Moreover, the two lineage II strains with *sigB* deletions (i.e., ∆*sigB* and ∆*sigB* ∆*prfA* mutant strains) seemed to be entering death phase after two days of exponential growth without entering an apparent stationary phase (see Fig. 1).

3.2. Contributions of σ^B and PrfA to invasion of Caco-2 cells and hemolytic activity by L. monocytogenes cells are similar among the tested strains representing different lineages

Contributions of σ^B to virulence-associated phenotypes were characterized in vitro by using hemolysis and tissue culture invasion assays. The lineage I parent strain showed significantly higher invasion efficiency (p-value < 0.003 for both comparisons against lineage II and IV parent strains; Tukey's HSD) as compared to the other two parent strains (Fig. 2). Lineage I, II, and IV parent strains had significantly (p-value < 0.02 for all comparisons; Tukey's HSD) higher invasion efficiencies in Caco-2 epithelial cells than their isogenic ∆*sigB* and ∆*sigB* ∆*prfA* mutants (Fig. 2). However, no significant difference in invasion efficiency was found between the lineage I, II and IV parent strains and their respective ∆*prfA* mutants. Overall, σ^B significantly contributed to invasion of Caco-2 cells by *L. monocytogenes*

lineage I, II, and IV parent strains while PrfA did not appear to contribute to invasion by these three strains under the same conditions.

A hemolysis assay using sheep red blood cells showed no significant difference in hemolytic activity (p-value > 0.05) among parent strains (Table 3). Significantly reduced hemolytic activities (p-value < 0.05; Tukey's HSD) were observed for $prfA$ and $sigB$ $prfA$ strains as compared to their respective parent strains across all three lineages (Table 3), but no difference was observed between the parent strains and their respective $sigB$ mutants. These results suggest that PrfA has a positive effect on hemolytic activity across different lineages in *L. monocytogenes* while σ^B apparently does not play an important role in this activity, at least under the condition tested.

3.3.   Transcriptional analysis of potential σ^B-dependent genes under high salt stress conditions

In order to explore the mechanism that may contribute to the extended lag phase observed for the $sigB$ mutant during growth in media with 1.9 M NaCl and no aeration at 25 °C, microarray was performed at 30 min after inoculation (i.e., salt shock) to compare transcript levels between the lineage I, serotype 1/2b, parent strain and its isogenic $sigB$ mutant strain. An adjusted p-value <0.05 and a fold change (FC)   1.5 in gene expression between the parent strain and the $sigB$ mutant strain or between the $sigB$ mutant strain and the parent strain were used as the cutoff criteria to consider a gene significantly up- or down-regulated by σ^B, respectively. Using these criteria, we identified 135 σ^B down-regulated and 173 σ^B up-regulated genes (Tables S1 and S2, respectively). Overall, 102 of the 173 σ^B up-regulated genes had previously been found as being σ^B-dependent in microarray, RNA-Seq, and proteomics studies using other growth conditions and/or strains (Abram et al., 2008a, 2008b; Hain et al., 2008; Mujahid et al., 2013; Oliver et al., 2009, 2010; Ollinger et al., 2009; Raengpradub et al., 2008). Therefore, 71 new σ^B up-regulated genes were identified in this study suggesting that these genes may only be transcribed in a σ^B-dependent fashion under certain conditions, including during lag phase salt shock (without aeration) at 25 °C. These 71 genes newly identified as being up-regulated by σ^B are listed in Supplemental Table 2 along with all the other σ^B up-regulated genes identified in this study. Among the 71 newly identified genes up-regulated by σ^B, genes presenting a FC   2.00 (17 genes) as well as genes also previously identified by Raengpradub et al. (2008) with FC   1.5 (6 genes, discussed below) are shown in Table 4. Among the newly identified σ^B-dependent genes are *cspL* (lmo1364, encoding a cold shock protein, FC = 2.63), *lmo0903* (encoding a putative osmotically inducible protein involved in stress response, similar to OsmC, FC = 2.41), and *lmo2174* (encoding a GGDEF domain protein, involved in signal transduction by synthesizing cyclic di-GMP, FC = 4.49). The six genes that were so far only identified as σ^B-up-regulated in this study and in another genome-wide microarray study (Raengpradub et al., 2008) that also applied salt shock to cells (Table 4) include lmo2256 (encoding a putative intracellular protease similar to a general stress protein from *Bacillus subtilis*, FC = 3.71), and *sigH* (lmo0243, encoding the alternative σ factor σ^H, FC = 1.78).

Other noteworthy genes that were previously identified as being positively regulated by σ^B and also showed significantly higher transcript levels in the parent strain as compared to the

sigB mutant strain in our study include *inlD* (LMOf2365_0282, encoding internalin D, FC = 8.07), *glpK* (lmo1538, encoding a glycerol kinase, FC = 6.96), *resD* (lmo1948, encoding a protein similar to the two-component response regulator ResD, FC = 2.05) and *gabD* (lmo0913, encoding a succinic semialdehyde dehydrogenase SSDH, GabD, FC = 14.62). Genes previously identified as being down-regulated by σ^B and that also showed significantly lower transcript levels in the parent strain as compared to the *sigB* mutant strain in this study include *dnaK* (lmo0348, encoding a dihydroxyacetone kinase, FC = −10.98), *groES* (lmo2069, encoding a class I heat-shock protein GroES, FC = −3.86) and *grpE* (lmo1474, encoding a co-chaperone GrpE, FC = −1.94). Surprisingly, *fri* (lmo0943, encoding a non-heme iron-binding ferritin, FC = −2.36), which had been shown to be positively regulated by σ^B in a lineage II strain under salt shock (0.3 M NaCl) at 37 °C with aeration (Raengpradub et al., 2008), showed σ^B-dependent down-regulation in our study (1.9 M NaCl, no aeration, and incubation at 25 °C), suggesting a lineage-specific regulation by σ^B or a salt concentration-, aeration- and/or temperature-dependent regulation of this gene by σ^B.

GSEA identified one role category, "Transport and binding proteins: Carbohydrates, organic alcohols and acids", as overrepresented among the σ^B down-regulated genes (FDR < 0.034) (Supplemental Table 3). GSEA was not able to identify overrepresented role categories among σ^B up-regulated genes (Supplemental Table 4).

3.4. Confirmation of select differentially expressed genes in lineage I by TaqMan qRT-PCR

We used TaqMan qRT-PCR to confirm differences in transcript levels between the parent and *sigB* mutant strains for select genes that were σ^B-dependent in the lineage I strain based on microarray analyses. As previous studies (Chan et al., 2007; McGann et al., 2007; Ollinger et al., 2009) showed that transcription of *gltX* and *inlD* is up-regulated by σ^B in a lineage II strain, we used TaqMan qRT-PCR to confirm that these genes were also up-regulated by σ^B in the lineage I strain used in this study. We also assessed the transcript levels of the *fri* gene in both the parent and *sigB* mutant strain since this gene had previously been shown to be positively regulated by σ^B under salt shock in a lineage II strain (Raengpradub et al., 2008). We confirmed that the *gltX* and *inlD* transcript copy numbers were numerically higher in the parent strain than in the isogenic *sigB* mutant (*p*-value = 0.098 and *p*-value =0.006, respectively; one-sided one-sample t-test). We also confirmed that *fri* transcript copy numbers were numerically lower in the parent strain than in the isogenic *sigB* mutant strain (*p*-value = 0.009; one-sided one-sample t-test), suggesting negative regulation by σ^B in the lineage I strain under the conditions used in our study (Supplemental Table 5).

4. Discussion

In this study we challenged three *L. monocytogenes* strains representing three distinct lineages (i.e., I, II and IV) to a stress condition (growth with 1.9 M NaCl with no aeration at 25 °C) that may more closely than previous studies reflect salt stress conditions. *L. monocytogenes* can be experienced in certain foods or food associated environments. We also assessed the contributions of two important transcriptional regulators (i.e., σ^B and PrfA)

to two major aspects of virulence, invasion of intestinal epithelial cells and hemolytic activity. Finally, we used microarrays to assess the transcriptional profiles of a parent strain and a *sigB* isogenic mutant strain representing lineage I, serotype 1/2b, to investigate the σ^B regulon in this strain under these food-relevant stress conditions.

4.1.   Heterogeneity in the contributions of σ^B in strains from different lineages during growth with high salt stress

Strains were shifted from growth to early stationary phase in BHI broth with aeration at 37 °C to growth in BHI broth with 1.9 M NaCl and incubated with no aeration at 25 °C for a period of 480 h. The lineage I strain presented a shorter lag phase after the condition shift as compared to the lineage II and IV strains. A previous study showed that lineage I isolates, belonging to serotype 4b, also showed shorter lag phase, as compared to lineage II isolates belonging to serotype 1/2a, when the strains were shifted to growth at 37 °C after being grown for four weeks at 4 °C (Buncic et al., 2001). Similarly, Bergholz et al. (2010) also observed a numerically shorter lag phase duration for lineage I strains grown in BHI with 6% NaCl at 7 °C as compared to lineage II strains. Another study, however, found that lineage I isolates had longer lag phases than lineage II isolates when the strains were shifted from growth in BHI broth at 37 °C for 24 h to growth in BHI broth adjusted to pH 6.5 with 0.1 M lactate at 5 °C (De Jesús and Whiting, 2003). As a shorter lag phase after environmental shifts suggests that the isolate can adapt faster to the environmental change, it is possible that the ability of lineage I isolates to more readily adapt to some environmental changes such as body temperature and high osmolarity, as represented by the shorter lag phases observed upon shifts to these conditions, could be associated with the higher virulence potential observed for lineage I isolates as compared to lineage II isolates (Chen et al., 2006). On the other hand, it is also possible that lineage II isolates have adapted to rapidly respond to environmental changes usually found during food processing and storage such as low temperature, which may be associated with the higher prevalence, of isolates belonging to this lineage, in foods (Orsi et al., 2011). Similarly, the lower growth rate observed for the lineage IV strain in comparison to the other two strains may help explain why isolates belonging to lineage IV are rarely isolated from foods (Orsi et al., 2011).

Differences in terms of σ^B contribution to growth in high osmolarity with no aeration at 25 °C were also observed for the different strains tested here. Our results suggest that in the lineage I and lineage II strains tested, σ^B contributes to adaptation of *L. monocytogenes* after shift from growth in rich media (i.e., BHI broth) with aeration at 37 °C to salt stress (1.9 M NaCl) with no aeration at 25 °C. Other studies have also shown that σ^B is highly active during the initial period after a salt-shock and up-regulates the expression of several genes involved in the general stress response (Raengpradub et al., 2008; Ringus et al., 2012). Interestingly, in our study, the lineage I parent strain presented a lower final population than its respective *sigB* mutant when grown in the presence of 1.9 M NaCl at 25 °C, suggesting that expression of σ^B and the σ^B regulon may represent a significant energy cost to the cells after exponential phase is reached. The two lineage II mutant strains that did not express *sigB* (i.e., *sigB* and *sigB prfA* strains) both entered an apparent death phase after two days of exponential growth without entering an apparent stationary phase. This illustrates the importance of σ^B for the lineage II strain in comparison to the lineage I and IV strains,

which appear to enter into stationary phase normally even when not expressing *sigB* (i.e. in the *sigB* mutant strains). Similarly, in a previous study using the same strains used in this study, σ^B showed the highest contributions in survival to acid and oxidative stresses in the lineage II strain as compared to the lineage I and lineage IV strains (Oliver et al., 2010). PrfA apparently has no influence on adaptation to salt stress in *L. monocytogenes* strains since *prfA* strains showed similar behavior compared to their respective parent strains, especially for lineages I and II. Additional studies using other strains of these lineages of *L. monocytogenes* need to be conducted in order to confirm whether these differences regarding growth behavior are specific to the strains used in this study or represent broader differences between *L. monocytogenes* lineages. Complementation of the deleted genes (i.e., *sigB* or *prfA*) might be necessary in these additional studies if strain-specific differences are observed.

4.2. PrfA, but not σ^B contributes to L. monocytogenes hemolytic activity while σ^B, but not PrfA, contribute to intestinal epithelial cell invasion

The lineage I strain presented a higher invasion efficiency than the other two parent strains, which is in agreement with previous studies that showed that lineage I strains have a higher pathogenic potential than strains from other lineages (Buncic et al., 2001; Chen et al., 2006; Gray et al., 2004; Weis and Seeliger, 1975; Wiedmann et al., 1997) and a higher invasion efficiency than strains from other lineages (Jacquet et al., 2004; Nightingale et al., 2008; van Stelten et al., 2010). However, this difference has been attributed mostly to a higher prevalence, in lineage II strains, of premature stop codons in *inlA*, which encodes the internalin protein necessary for efficient invasion into epithelial cells (Jacquet et al., 2004; Nightingale et al., 2008; van Stelten et al., 2010). Since none of the strains used in this study present a premature stop codon in *inlA*, another feature is probably involved in the higher invasion efficiency observed for the lineage I strain in this study. Only σ^B showed a significant contribution to invasion in all three strains. Although the contribution of σ^B to epithelial cell invasion has been documented in a number of studies (Garner et al., 2006; Kim et al., 2004, 2005), other studies have also found PrfA to contribute to host cell invasion, although PrfA contributions to invasion were typically lower than the σ^B contributions (Garner et al., 2006; Ivy et al., 2010). As it is well established that transcription of *inlA*, which encodes a key protein involved in invasion of human cells expressing E-cadherin, is regulated by both σ^B and PrfA (Kim et al., 2005; Lingnau et al., 1995; McGann et al., 2008), these observation likely reflect the fact that σ^B and PrfA are activated by different conditions.

All three parent strains presented similar hemolytic activities. Similarly, previous studies have found similar plaquing efficiencies of lineage III and IV strains compared to lineage II strains (Roberts et al., 2006), while another study observed higher plaquing efficiency among lineage III and IV strains compared to lineage II strains (Wiedmann et al., 1997). Although Raengpradub et al. (2008) observed a lower expression of the gene *hly* in a lineage II parent strain in comparison to its isogenic *sigB* mutant strain when a salt shock was applied to the culture (with 0.3 M NaCl, incubation with aeration at 37 °C), no contribution of σ^B to the hemolytic activities of the three strains was observed, at 25 °C, in the study reported here (1.9 M NaCl, incubated with no aeration), possibly again reflecting that σ^B

and PrfA potentially show temperature-, salt concentration- and/or aeration-dependent transcription and/or activation (temperature-dependent *prfA* translation has clearly been established (Johansson et al., 2002)). It has been suggested that a σ^B-dependent promoter exists upstream of *hly* (Orsi et al., 2011) however, transcriptional activity from that promoter has not been shown to date. Reduced hemolytic activities were only observed for *prfA* mutants consistent with the PrfA-dependent nature of *hly* transcription observed in previous studies (Bohne et al., 1994; Nadon et al., 2002; Ollinger et al., 2009; Sheehan et al., 1995).

4.3.   Transcription profile of the lineage I serotype 1/2b strain after salt shock at 25 °C with no aeration reveals new σ^B-dependent up-regulated genes and salt-shock-specific σ^B-dependent up-regulated genes

Although several studies have investigated the σ^B regulon using microarray (Hain et al., 2008; Oliver et al., 2010; Ollinger et al., 2009; Raengpradub et al., 2008), RNA sequencing (Oliver et al., 2009), and proteomics (Abram et al., 2008a, 2008b; Mujahid et al., 2013), the majority of these studies have only used lineage II strains (i.e., 10403S and EGD-e). Moreover, only one other transcriptomic study (Raengpradub et al., 2008) analyzed the genome-wide contributions of σ^B to transcription during salt-shock stress, but with conditions different than those used in this study (i.e., growth with 0.3 M NaCl with aeration at 37 °C versus growth with 1.9 M NaCl without aeration at 25 °C used here). The conditions used here permitted the identification of new σ^B-dependent genes and, in conjunction with prior work by Raengpradub et al. (2008), who applied salt-shock to *L. monocytogenes* at 37 °C, permitted initial identification of possible salt-shock-specific σ^B-dependent genes.

Among the total of 71 genes newly identified as being up-regulated by σ^B, a gene (lmo2174) encoding a GGDEF domain protein presented the highest fold change (FC = 4.49). Proteins with this domain are involved in signal transduction by synthesizing cyclic di-GMP, an intracellular signaling molecule. Proteins with this domain have been involved in processes such as exopolysaccharide synthesis, biofilm formation, motility and cell differentiation. Further Hidden Markov Model analyses using the EMBL-EBI-based InterProScan Signature Recognition Search Against the Integrated Resources of Protein Domains and Functional Sites (http://www.ebi.ac.uk/Tools/pfa/iprscan/) revealed two other domains in this protein. One domain belongs to guanylate cyclases (IPR001054), which catalyze the formation of cyclic GMP from GTP. Lmo2174 also presents a transmembrane region (IPR11620) commonly found among signal transduction histidine kinases of the LytS-YhcK type, which probably regulate genes involved in cell wall metabolism. Interestingly, *L. monocytogenes* cells challenged with 6% NaCl were previously shown to form thicker sections in the cell envelope at the septum or other areas of the cell (Zaika and Fanelli, 2003). In *Escherichia coli*, the *yjbEFGH* operon, involved in production of an unknown exopolysaccharide was shown to be induced by osmotic stress. This operon was shown to be negatively regulated by the general stress response σ factor RpoS, the analog of σ^B, although expression of this operon in the presence of RpoS was also observed (Ionescu and Belkin, 2009). Increased levels of exopolysaccharides have also been observed in response to osmotic stress in *Pseudomonas* spp. (Sandhya et al., 2010) and exopolysaccharides were also reported to protect *Synechocystis* cells against salt stress (Jittawuttipoka et al., 2013). A visual search in

the region upstream lmo2174 identified a putative σ^B-dependent promoter region 31 nt upstream the start codon of this gene. The putative promoter sequence (GAAT-N16-GGGTAT) greatly resembles the consensus sequence (GTTT-N16-GGGTAT) for σ^B-dependent promoters in *L. monocytogenes* (Oliver et al., 2009). Moreover, among the newly identified σ^B-dependent up-regulated genes is a gene (lmo0530) encoding a putative cellulose synthase subunit B (IPR018513), which is activated by cyclic di-GMP, the product of Lmo2174. In *Acetobacter xylinum*, this gene, *bcsB*, is part of a four-gene operon, *bscABCD*, with all genes in the operon required for maximum bacterial cellulose synthesis (Wong et al., 1990). In *L. monocytogenes*, lmo0530 is part of a five-gene operon (lmo0527–lmo0531). Although two other genes in this operon, lmo0527 and lmo0529, were also found to be up-regulated by σ^B in this study, none of the genes in this operon have defined function. While three genes in this operon encode proteins with a transmembrane region (lmo0527, lmo0528 and lmo0530), one (lmo0529) encodes a protein with a transmembrane domain and a glycosyl transferase domain and one (lmo0531) encodes a protein with GGDEF and guanylyl cyclase domains and, therefore, may be involved with signal transduction through synthesis of cyclic di-GMP. In *L. monocytogenes* strain 10403S, this operon seems to be co-regulated by σ^A, the housekeeping σ factor, and σ^B. A σ^B-dependent promoter 137 nt upstream the start codon of lmo0527 was identified by transcriptional start site mapping using RNA sequencing (Orsi et al., in preparation). The σ^A-dependent promoter is upstream this σ^B-dependent promoter and transcription initiation from both promoters was observed in stationary phase cells incubated at 37 °C (Orsi et al., in preparation). Therefore, we are suggesting a model in which salt-shock induces transcription of lmo2174 and the lmo0527–lmo0531 operon leading to production of (i) a cyclic di-GMP synthase/histidine kinase double functional enzyme (Lmo2174) that senses the osmolarity change and synthesizes cyclic di-GMP, and (ii) a cyclic di-GMP-activated complex (encoded by one or more genes in the lmo0527–lmo0531 operon). These gene expression changes may allow for enhanced synthesis of exopolysaccharides in response to osmolarity changes, facilitating bacterial survival under these stress conditions. Further studies will be required to investigate the function of these genes and to test the model presented above.

Other genes newly identified as up-regulated by σ^B include lmo0903 and *cspL*. lmo0903 encodes a peroxiredoxin, OsmC/Ohr-like protein. In *E. coli*, this protein is osmotically induced although it can also be induced under other conditions (Gordia and Gutierrez, 1996). *cspL* encodes a cold shock protein shown to be up-regulated during growth at 4 °C in *L. monocytogenes* (Chan et al., 2007). Six genes were found to be up-regulated by σ^B only in this study and in another transcriptomic study that also applied salt-shock to cells (Raengpradub et al., 2008). Although three of these six genes encode hypothetical proteins, one gene (lmo2256) encodes an intracellular cysteine peptidase similar to a general stress protein 18, GSP18 from *B. subtilis* (IPR06286) and one (lmo0137) encodes an oligopeptide ABC transporter. Interestingly, a third gene identified as σ^B-up-regulated both here and in the previous salt stress study by Raengpradub et al. (2008) is *sigH* (lmo0243), which encodes the alternative σ factor σ^H. While to date, no role in salt stress survival and growth has been attributed to σ^H in *L. monocytogenes*, the *L. monocytogenes* σ^H and σ^B regulons have been shown, in a previous study, to overlap (Chaturongakul et al., 2011), consistent with our findings here. None of the six genes identified as being salt-shock-stress specific in

these two transcriptomic studies (this study; Raengpradub et al., 2008) were identified as being σ^B-dependent in two previous studies that assessed the σ^B regulon under salt stress using proteomics (Abram et al., 2008a, 2008b). These discrepancies between the microarray and proteomics studies could either reflect the nature of the proteomics approach (e.g., lower detection sensitivity than microarrays, identification of genes that show different protein levels but not different transcript levels) or differences in growth conditions such as media or salt concentration used in each study. Although only six genes showed overlap between this study and the study carried out by Raengpradub et al. (2008), we cannot rule out the possibility that other genes among the 71 genes newly identified as being σ^B-dependent are also only differentially expressed under salt stress. Additional microarray studies using corresponding parent strains and isogenic *sigB* knock-out mutant, not exposed to salt stress, will be needed to definitely determine whether some of the newly identified σ^B up-regulated genes are induced only in salt stress condition.

Gene Set Enrichment Analysis (GSEA) showed that genes involved in transport and binding of carbohydrate, organic alcohols and acids were enriched for σ^B down-regulated genes. This may suggest that once the salt shock is applied using rich BHI media, which happened 30 min before RNA extraction, the cells seem to prioritize the response to the salt stress over acquisition of carbohydrates and other energy sources as previously observed (Bergholz et al., 2012). This shows how *L. monocytogenes* can rapidly regulate gene expression turning specific sets of genes on and off in response to a rapidly changing environment.

5. Conclusions

In order to survive adverse conditions, foodborne pathogens must adapt and alter their gene expression and metabolism to overcome stress conditions encountered during food processing, food storage, and gastrointestinal passage. Here we studied the *L. monocytogenes* response to salt stress under conditions that may be encountered during food processing as well as storage of some food products (e.g., certain cheeses and cheese products). However, *L. monocytogenes* is also exposed to salt stress during infection as the human small intestine presents a concentration of 0.3 M NaCl (Chowduhry et al., 1996; Davenport, 1982). Overall, our results indicate that (i) *L. monocytogenes* strains representing different phylogenetic lineages differ in the relative contributions of σ^B and PrfA to growth under salt stress conditions, (ii) *L. monocytogenes* σ^B, but not PrfA, contributes to epithelial cell invasion while PrfA, but not σ^B, contributes to hemolytic activity, and (iii) although the σ^B regulon has been extensively studied, studies using different environmental conditions may still identify new σ^B-dependent genes that are specifically regulated under those conditions. We have identified a new set of σ^B-dependent up-regulated genes that we hypothesize may be specifically used to overcome high osmolarity stress by altering the cell wall structure, which may involve σ^B-upregulated biosynthesis of exopolysaccharides. Future studies, will be needed to test this hypothesis. In addition, our data indicate that future studies applying other conditions may continue to increase our knowledge of the number of genes that are part of the *L. monocytogenes* σ^B regulon, furthering our understanding of stress response systems in *L. monocytogenes* and their contributions to the transmission of this important foodborne pathogen.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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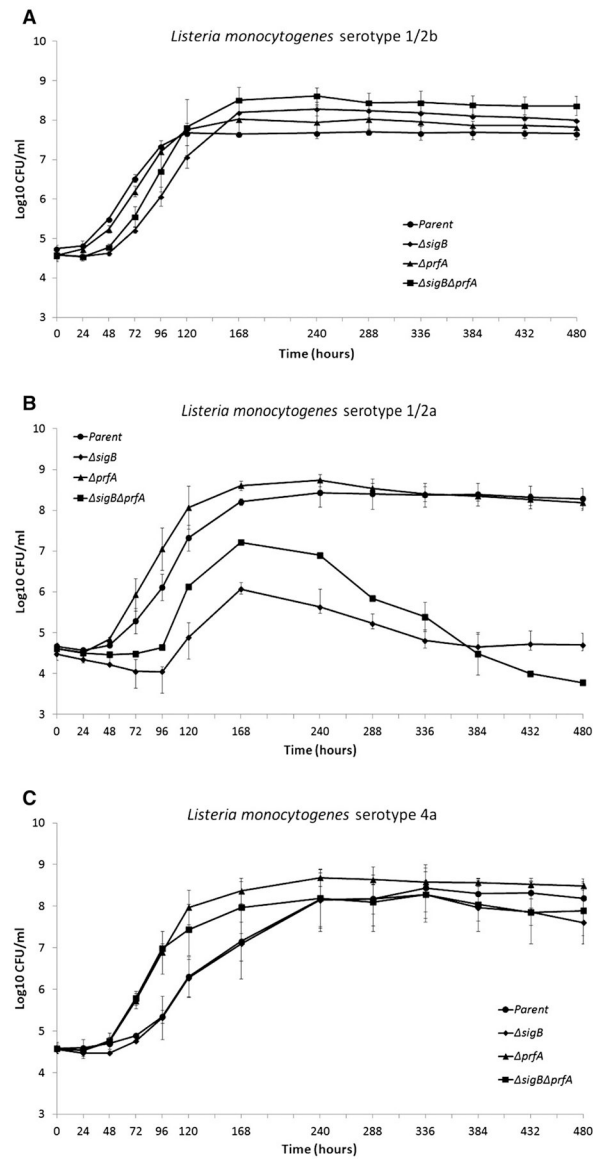
Fig. 1.
Growth patterns at 25 °C and in the presence of 11% salt for (A) *L. monocytogenes* FSL J1–194 (serotype 1/2b and lineage I) and its isogenic Δ*sigB*, Δ*prfA*, and Δ*sigB*Δ*prfA* strains; (B) *L. monocytogenes* FSL X1–001 (serotype 1/2a and lineage II) and its isogenic Δ*sigB*, Δ*prfA*, and Δ*sigB*Δ*prfA* strains; and (C) *L. monocytogenes* FSL J1–208 (serotype 4a and lineage IV) and its isogenic Δ*sigB*, Δ*prfA*, and Δ*sigB*Δ*prfA* strains. Data represent the mean of at least three biological replicates. Error bars represent standard deviations (SD).
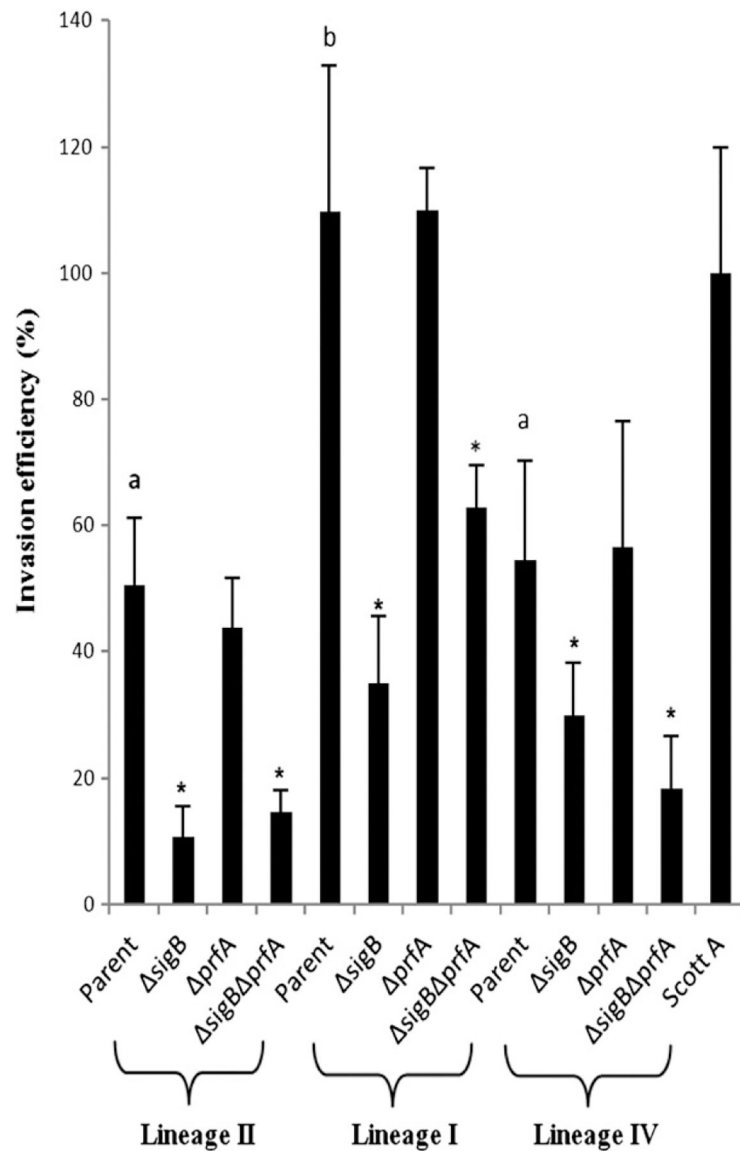
Fig. 2.
Caco-2 invasion efficiencies of *Listeria monocytogenes* parent and mutant strains. Bacteria were grown to early stationary phase (growth to $OD_{600} = 1.0$, followed by 3 h of incubation) with aeration (i.e., shaking at 220 rpm) at 37 °C. Invasion assays were performed at 37 °C. For the graphical representation of the invasion assay results, the invasion efficiency of strain Scott A (last strain at the right side) was set to 100% and the invasion efficiency of all other strains is represented as percentages relative to that of strain Scott A. Data represent the mean of at least three biological replicates. Error bars represent SD. * indicates a *p*-value < 0.05 (mutants in comparison with their parent strains; calculated based on log-transformed absolute, non-normalized numbers). Different letters on top of parent strain bars represent significant differences in invasion among parent strains.

Table 1

Listeria monocytogenes strains used in this study.

Isolate designation	Serotype	Source or parent strain	Genotype	Reference
Lineage I strains				
FSL J1–194	1/2b	Human, sporadic	Parent	Sauders et al. (2003)
FSL C6–001	1/2b	FSL J1–194	*sigB*	Oliver et al. (2010)
FSL C6–004	1/2b	FSL J1–194	*prfA*	This study
FSL C6–005	1/2b	FSL J1–194	*sigB prfA*	This study
Lineage II strains				
FSL X1–001	1/2a	Human, skin lesion	Parent	Bishop and Hinrichs (1987)
FSL A1–254	1/2a	10403S	sigB	Wiedmann et al. (1998)
FSL B2–046	1/2a	10403S	*prfA*	Wong and Freitag (2004)
FSL B2–068	1/2a	10403S	*sigB prfA*	McGann et al. (2007)
Lineage IV strains				
FSL J1–208	4a	Animal	Parent	Roberts et al. (2006)
FSL O1–005	4a	FSL J1–208	sigB	Oliver et al. (2010)
FSL C6–009	4a	FSL J1–208	*prfA*	This study
FSL C6–007	4a	FSL J1–208	*sigB prfA*	This study

Table 2

Growth parameters of *L. monocytogenes* 1/2b, 1/2a and 4a in BHI broth with 11% NaCl at 25 °C.

Strain	Genotype	Lag phase (h)[a]	Growth rate (log CFU/ml * day)[a]	Population increase[a,b] (log CFU/ml)
Lineage I, serotype 1/2b				
FSL J1–194	Parent	33.4 ± 3.49	0.108 ± 0.01	2.95 ± 0.07
FSL C6–001	*sigB*	60.5 ± 0.63[c]	0.099 ± 0.02	3.60 ± 0.16
FSL C6–004	*prfA*	36.0 ± 1.64	0.103 ± 0	3.33 ± 0.39
FSL C6–005	*sigB prfA*	54.0 ± 6.24[c]	0.117 ± 0.02	3.90 ± 0.34[c]
Lineage II, serotype 1/2a				
FSL X1–001	Parent	60.9 ± 6.80	0.104 ± 0.01	3.75 ± 0.09
FSL A1–254	*sigB*	115.5 ±23.62[c]	ND[d]	ND[d]
FSL B2–046	*prfA*	48.21 ± 6.86	0.122 ± 0.02	3.90 ± 0.21
FSL B2–068	*sigB prfA*	101.5 ± 1.85[c]	0.208 ± 0.02[c]	2.54 ± 0.01[c]
Lineage IV, serotype 4a				
FSL J1–208	Parent	62.78 ± 5.95	0.059 ± 0.02	3.74 ± 0.51
FSL O1–005	*sigB*	67.38 ± 10.59	0.065 ± 0.02	3.57 ± 0.56
FSL C6–009	*prfA*	50.44 ± 0.64	0.119 ± 0.03[c]	4.00 ± 0.10
FSL C6–007	*sigB prfA*	47.33 ± 8.24	0.118 ± 0.02[c]	3.51 ± 0.73

[a] Data represent the average and standard deviations for three independent biological replicates.

[b] Difference between the inoculum and final population.

[c] Indicates if the value for a mutant differs statistically significantly from the value for the parent strain (*p*-value < 0.05).

[d] ND—not determined; growth rate and population increase for FSL A1–254 were not calculated due to the short exponential phase (yielding insufficient time points to calculate the growth rate) and due to the rapid entry into death phase.

Table 3

Hemolytic activity of whole-cell suspension of *L. monocytogenes* lineages I, II and IV strains.

Strain	Genotype	Avg. no. of hemolytic units[a]
Lineage I, serotype 1/2b		
FSL J1–194	Parent	24.0 ± 13.0
FSL C6–001	*sigB*	21.9 ± 9.0
FSL C6–004	*prfA*	<2.0 ± 0.0[b]
FSL C6–005	*sigB prfA*	<2.0 ± 0.0[b]
Lineage II, serotype 1/2a		
FSL X1–001	Parent	21.4 ± 9.0
FSL A1–254	*sigB*	16.0 ± 2.0
FSL B2–046	*prfA*	<2.0 ± 1.0[b]
FSL B2–068	*sigB prfA*	<2.0 ± 0.0[b]
Lineage IV, serotype 4a		
FSL J1–208	Parent	16.0 ± 2.0
FSL O1–005	*sigB*	12.0 ± 2.0
FSL C6–009	*prfA*	<2.0 ± 0.0[b]
FSL C6–007	*sigB prfA*	<2.0 ± 1.0[b]

[a] Hemolytic unit is defined as the reciprocal of the dilution at which 50% lysis of sheep red blood cells occurred; data represent the average and standard deviations for three independent biological replicates.

[b] p-Value < 0.05 when data are compared with those from the parent using Tukey's HSD.

Table 4

Selected genes newly identified as σ^B-up-regulated and genes identified as σ^B-up-regulated only during salt stress. [a]

Locus	Function	Fold change	p-Value [b]
Genes newly identified as σ^B-up-regulated with FC > 2.0 [c]			
Lmo2174 [d]	GGDEF domain protein putatively involved in signal transduction and cell differentiation; function is to synthesize cyclic di-GMP	4.49	0.002
LMOf2365_0800	PTS system, IIAB component	3.85	0.001
Lmo0530 [d]	Putative cellulose synthase; this enzyme is activated by cyclic di-GMP	3.51	0.017
LMOh7858_1020	Glucosamine-6-phosphate isomerase	3.49	0.001
Lmo0197	Similar to *B. subtilis* SpoVG protein	3.25	<0.001
Lmo1364 (*cspL*)	Cold shock protein	2.63	0.002
Lmo0527 [d]	Putative membrane protein	2.54	0.001
Lmo0903	OsmC/Ohr family protein	2.41	<0.001
Lmo0186	*B. subtilis* YabE protein homolog lmo0186	2.37	0.012
Lmo0486 (*rpmF*)	Ribosomal protein L32	2.24	0.042
Lmo0223 (*cysK*)	Cysteine synthase A	2.19	0.005
Lmo0245 (*secE*)	Highly similar to preprotein SecE translocase subunit	2.16	0.001
Lmo0393	Conserved hypothetical protein	2.10	0.001
LMOh7858_2006	Chitinase	2.09	0.001
Lmo0242	Conserved hypothetical protein	2.07	0.009
Lmo2037 (*mraY*)	Phospho-N-acetylmuramoyl-pentapeptide-transferase	2.06	0.001
Lmo2795	Phosphosugar-binding transcriptional regulator, RpiR family	2.00	0.005
σ^B-Dependent genes only up-regulated under salt stress [e]			
Lmo2256	Intracellular protease, PfpI family	3.71	0.016
Lmo0653	Conserved hypothetical protein	1.93	0.026
Lmo2490	Conserved hypothetical protein	1.87	0.007
Lmo0243	RNA polymerase alternative σ factor, σ^H	1.78	0.001
Lmo0137	Oligopeptide ABC transporter, permease protein	1.66	0.049
Lmo1027	Similar to conserved hypothetical proteins	1.50	0.001

[a] Genes found to be up-regulated by σ^B in this study were compared to σ^B up-regulated genes identified by Raengpradub et al. (2008), Ollinger et al. (2008), Oliver et al. (2009), Oliver et al. (2010), Mujahid et al. (2013), Abram et al. (2008a, 2008b) and Hain et al. (2008). A minimum fold change (FC) 1.5 and an adjusted p-value < 0.05 were considered significant for microarray and proteomics studies. For the RNA-seq study (Oliver et al., 2009), the criteria used was the same as those used in the original study. Genes only identified here were considered "genes newly identified as σ^B-up-regulated", while genes identified here and by Raengpradub et al. (2008) (the only other study using transcriptomics who defined the σ^B regulon under salt stress conditions) were considered "σ^B-up-regulated only during salt stress".

[b] Adjusted p-values.

[c] Only genes with FC 2.0 are shown here; for a complete list (which includes genes with an FC between 1.5 and 2.0), see Supplemental Table 2.

[d] Genes with σ^B-dependent promoters identified upstream the transcription unit based on RNA-Seq data (Orsi et al., unpublished data).

[e] The genes under this subheader were identified as σ^B-up-regulated with FC 1.5 and significant p-value (adjusted p-value < 0.05) only in this work and in the salt-stress experiment carried out by Raengpradub et al. (2008).