Investigating the σ^B Stress Response System in Two Lineage IIIA Strains of *Listeria monocytogenes*

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ABSTRACT

Strains of *Listeria monocytogenes*, an intracellular foodborne pathogen, classify into three genetic lineages. Lineage III strains, associated most commonly with animal disease, have not been extensively studied. σ^B, a transcription factor encoded by *sigB*, has been shown to contribute to stress survival and virulence in *L. monocytogenes*. Recent studies have revealed that σ^B regulons have varied among strains, and that σ^B did not contribute to stress survival in one representative lineage IIIA strain. The purpose of this study was to determine if σ^B significantly contributes to acid and oxidative stress survival in two additional lineage IIIA strains of different serotypes. Two lineage IIIA strains, FSL F2-695 and FSL F2-525, had isogenic Δ*sigB* mutants made using allelic exchange mutagenesis. Those strains and a control lineage II lab strain, 10403S, with its Δ*sigB* mutant were subjected to acid stress at pH 2.5 and oxidative stress with 13 mM cumene hydroperoxide for 15 minutes. All three isogenic Δ*sigB* mutants had significantly higher acid stress death rates ($p < 0.001$) and oxidative stress death ($p < 0.01$) than their corresponding wild-type strains. FSL F2-525 exhibited a significantly higher acid stress death rate than the other wild-type strains. 10403S had significantly higher oxidative stress death than FSL F2-695 ($p < 0.0006$). Consistent with strains in other lineages, σ^B is a significant factor in acid and oxidative stress survival in most lineage IIIA strains of *L. monocytogenes.*

INTRODUCTION

Foodborne illness is a serious concern for the United States, as 76 million cases, 325,000 hospitalizations, and 5,000 deaths are estimated to result from foodborne illness annually (17). *Listeria monocytogenes* is a Gram positive, intracellular foodborne pathogen that is responsible for listeriosis, the symptoms of which can include: meningitis, encephalitis, materno-fetal and perinatal infections, septicemia, and febrile gastroenteritis (23). *L. monocytogenes* accounts for over one quarter of foodborne deaths, with 2,500 infections, 2,300 hospitalizations, and 500 deaths annually in the United States (17).

L. monocytogenes can survive in multiple environments like soil, water, and food processing plants (12). *L. monocytogenes*' capability to grow and survive in extreme environments also aids its ability to survive in processing plants. For example, it has been found to survive at temperatures as low as 0^{o}C and in environments at pH 4.4 (7). Its widespread presence in the environment also contributes to its ability to transfer to foods (12, 15). *L. monocytogenes* prevalence has been reported to be 12.9% in smoked seafood, 11.8% in fruit, 7.0% in raw seafood, and 1.8% in ready-to-eat deli meats (8). Common sources of *L. monocytogenes* include ready-to-eat deli meats, hot dogs, smoked and processed seafood, fruit, raw seafood, soft cheeses, and milk products (8).

Listeriosis occurs predominantly in populations with weakened immune systems, such as the elderly, pregnant, children, and the immunocompromised due to disease (6). Once intracellular, *L. monocytogenes* is an especially effective killer, killing about one in five infected individuals (17). To cause a disease, pathogenic *L. monocytogenes* must first contaminate a food that is ingested. It then must survive the acidic environment of the stomach, invade the intestine, and survive the oxidative environment of the body's immune defenses (6). Once *L. monocytogenes* has entered and spread through the intestinal cells, it travels throughout the body via the blood and lymph systems to the liver. Eventually it can cross the membrane surrounding the brain as well as the placenta (6). All of these steps of environmental proliferation, survival, and infection are partly aided by the expression of genes regulated by a sigma factor known as σ^{B} (16). σ^{B} has been shown to contribute to stress survival in Gram positive bacteria other than *L. monocytogenes* like *Staphylococcus aureus* and *Bacillus subtilis* (2, 25). Thus, it is important to understand σ^{B} stress response in order to better understand Gram positive bacteria survival and proliferation under wide ranging conditions, including infection (6).

L. monocytogenes strains can be classified into three genetic lineages. Lineage I is more prevalent in human disease isolates (13). Lineage II is more frequently observed in food and environmental isolates (13, 20), and lineage III is more commonly found in animal and diseased animal isolates (13). Despite its importance, σ^{B} stress response has only been extensively studied in lineage II strains, like 10403S (16, 22). A preliminary investigation of the σ^{B} stress response of selected lineage I, II, and III strains found that the representative lineage IIIA strain did not require σ^{B} to survive acid and oxidative stresses (21). The purpose of this study was to determine whether or not this lack of dependence on σ^{B} for acid and oxidative stress survival was strain-specific or characteristic of lineage IIIA strains in general.

MATERIALS AND METHODS

Strain selection, mutant creation, and storage. All strains used in this study are listed in Table 1. A *L. monocytogenes* laboratory strain from lineage II, 10403S, was chosen as a control for comparison to previous laboratory results (4, 9, 10). Additionally, FSL A1-254 is an isogenic Δ*sigB* mutant of 10403S, and was chosen as a control for comparison to previous studies (3, 9, 26). Two *L. monocytogenes* lineage IIIA strains different from the strain used in Oliver et al. (21), FSL F2-525 and FSL F2-695, were stored in a -80°C freezer at the Cornell Food Science Food Safety Lab. These strains were chosen because they are classified in lineage IIIA, but are both different serotypes than the strain used in Oliver et al. (21) (5, 23). FSL J2-071, the lineage IIIA strain used in Oliver et al. (21), is serotype 4c whereas F2-525 and F2-695 are serotypes 4b and 4a, respectively (5, 23). Stock cultures were stored at -80°C in brain heart infusion (BHI) broth mixed with 15% glycerol. The stock cultures were streaked onto BHI agar plates (BHI; Difco, Detroit, MI) and incubated at 37°C for 24 h. The two lineage IIIA strains, FSL F2-525 and FSL F2-695, had isogenic Δ*sigB* mutants—FSL O1-014 and FSL O1-012, respectively—made using allelic exchange mutagenesis, as described in Wiedmann et al. (26) and Horton et al. (14). Briefly, DNA segments upstream and downstream of the *sigB* gene were sequenced for FSL F2-525 and FSL F2-695. PCR was used to create two DNA fragments that flank the 5' and 3' ends of the *sigB* gene for each strain. These fragments were joined by a second PCR to create an in-frame DNA fragment that included the DNA sequence upstream and downstream of *sigB*, but excluded the majority of the *sigB* coding region. The Δ*sigB* fragments were ligated into the pkSV7 vector and transformed into electro-competent *Escherichia coli* cells to maintain and propagate the Δ*sigB* plasmids. The Δ*sigB* plasmids were amplified, harvested, and electroporated into the two *L. monocytogenes* strains. Potential Δ*sigB* mutants were screened by antibiotic sensitivity to chloramphenicol, and deletion of *sigB* was confirmed by PCR and sequencing (14, 21, 26).

Acid stress treatment. All six strains were subjected to acid and oxidative stresses. For the acid stress, strains were plated overnight from a -80°C freezer stock. 5 mL BHI broth tubes were inoculated and grown at 37°C for approximately 12 h with shaking. The resulting cultures were transferred to new 5 mL BHI tubes and grown until an optical density at 600 nm (OD_{600}) of approximately 0.400 ± 0.05. Then, 500 µL of culture was transferred into a 300 mL Nephelo flask (Bellco Glass, Vineland, NJ) containing 50 mL of pre-warmed BHI broth, and incubated for 10 h at 37°C with shaking. This step ensured that samples were at the stationary phase of growth when exposed to stress. A 0 min negative control sample was taken before the acid stress treatment. 5 mL samples of culture were then reduced to a pH of 2.5 ± 0.05 as measured with a pH meter (Beckman, Coulter Inc., Fullerton, CA) by adding 12N HCl (VBR, Westchester, PA). The 5 mL acidified samples were then vortexed and returned to the 37°C incubator with shaking. Samples were serially diluted and spiral plated on BHI agar plates at 10, 30, and 60 min. Plates were incubated at 37°C and standard plate counts were taken 24 – 27 h after treatment. At least three independent repetitions of the acid stress assay were performed (21).

Oxidative stress treatment. For the oxidative stress, samples were grown to stationary phase as done for the acid. A 0 minute negative control aliquot of the 10 h stationary phase sample was taken prior to treatment. 900 µL of the stationary phase samples were treated with cumene hydroperoxide (CHP; Signa-Aldrich) dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 13mM CHP. The stressed sample was placed back in a 37^{o}C incubator with shaking for 15 min. An equivalent volume of DMSO was added to the 0 min non-stress samples. The 0 and 15 min samples were enumerated as described above. At least three independent replicates of the oxidative stress procedure were performed.

Statistical analysis of acid and oxidative stress data. Statistical analysis of the data from the two stresses was performed using Statistical Analysis Software (SAS) 9.0 (SAS Institute, Inc., Cary, NC). Regression analysis was used to calculate the death rates of the acidified samples. Repeated measures analysis of variance (ANOVA) was used to see if there was a significant difference in the death rates between wild-type strains and their Δ*sigB* mutants. For the oxidative stress, two-sided t tests were conducted for cell death (log CFU/mL) after 15 min between wild-type and mutant strains. Additionally, ANOVA tests were conducted to detect the differences between the wild-type strains for the acid and oxidative stresses. Significance was set at $p < 0.05$ (21).

RESULTS

Acid stress death rates and oxidative stress death of Δ*sigB* mutant cells were significantly higher than their parent strains. Data regarding acid stress death rates and oxidative stress death for all of the strains used in this study is presented in Table 2. The isogenic Δ*sigB* mutants died at significantly higher rates than their corresponding wild-type strains when subjected to acid stress ($p < 0.001$). All of the isogenic Δ*sigB* mutants also exhibited significantly higher death after 15 minutes of oxidative stress than their wild-type strain ($p < 0.01$). σ^B thus significantly contributes to the survival of the two lineage IIIA strains when subjected to both acid and oxidative stresses.

Differences in the degree of σ^B contribution were noticed for acid and oxidative stresses. The largest average difference in death rate and average death after 15 minutes between a wild-type strain and its mutant for both acid and oxidative stresses was observed between FSL F2-525 and FSL O1-014 (4.12 log CFU/mL per hour for acid death rates and 1.75 log CFU/mL for oxidative death). The smallest difference in death rate for the acid stress was observed between the other lineage IIIA pair: FSL F2-695 and FSL O1-012 (2.60 log CFU/mL). However, for the oxidative stress, the lineage II controls—10403S and FSL A1-254—exhibited the smallest difference in average death (1.41 log CFU/mL).

Acid stress death rates and oxidative stress death differences were significant between wild-type strains. Among wild-type strains, F2-525 had a significantly higher acid stress death rate than both 10403S ($p = 0.0019$) and F2-695 ($p = 0.0343$). 10403S showed significantly more death after 15 minutes of oxidative stress than F2-695 ($p =$

0.0006). No other differences between two wild-type strains were observed for oxidative stress.

DISCUSSION

In all, this study demonstrated that (i) two lineage IIIA *L. monocytogenes* strains did significantly rely on σ^B for survival to acid and oxidative stresses; (ii) one of those strains (FSL F2-695) was more resistant to oxidative stress than 10403S; and (iii) the other lineage IIIA strain (FSL F2-525) was significantly more susceptible to acid stress than the other two wild-types. Given the variability in behavior among lineage IIIA strains used in this work and others (21) demonstrates the importance of strain consideration when conducting studies of *L. monocytogenes*.

σ^B –mediated acid and oxidative stress survival varies among lineage IIIA strains of *L. monocytogenes*. The lineage IIIA strains' behaviors are largely consistent with other studies that showed an isogenic Δ*sigB* mutant was significantly more susceptible to acid and oxidative stresses (3, 9, 26). This finding that the lineage IIIA strains studied at least partially depend on σ^B for acid and oxidative stress survival suggests variability across lineage IIIA strains regarding σ^B contributions to acid and oxidative stress survival in the context of Oliver et al. (21)'s finding. This is supported by the fact that σ^B was both not a significant factor (FSL J2-071; $p > 0.08$; 21) and a significant factor (FSL F2-525, FSL F2-695; $p < 0.01$) for acid and oxidative stress survival. Therefore, FSL J2-071's (21) oxidative and acid stress survival behavior with respect to σ^B may be strain-specific and cannot be generalized to lineage IIIA. These results are inconsistent with the hypothesis that lineage IIIA strains do not exhibit at least some dependence on σ^B for acid and oxidative stress survival, because one would expect the same contribution of σ^B if it was characteristic to lineage IIIA.

The relative σ^B contribution to acid stress survival varies within lineage IIIA strains. Another interesting observation was the variation of σ^B as a factor in acid stress survival between strains within lineage IIIA. Although both of the lineage IIIA strains used in this study depended significantly on σ^B for acid stress survival, the relative importance of σ^B seemed to differ. FSL F2-525 had the largest difference in average acid death rate with its Δ*sigB* mutant (4.12 log CFU/mL per hour), which suggests that σ^B is a larger factor in its acid and oxidative stress survival. Alternatively, the other lineage IIIA strain, FSL F2-695, had the smallest difference in average acid stress death rate (2.60 log CFU/mL per hour), and was less dependent on σ^B than FSL F2-525 for survival. 10403S had an average difference of 3.03 log CFU/mL with its mutant. If σ^B-mediated stress response were uniform throughout lineage IIIA, then one would expect that lineage IIIA strains would exhibit the same level of dependence on σ^B for survival. However, lineage IIIA strains had both the largest (FSL F2-525) and smallest (FSL F2-695) σ^B contribution to acid stress survival among the strains studied here. The lineage II strain was in between the two in its level of σ^B dependence. This reinforces the aforementioned finding that the lack of σ^B contribution to stress survival observed in Oliver et al. (21) is not universal among lineage IIIA strains.

Observed phenotypic differences in oxidative stress death between a *L. monocytogenes* lineage IIIA strain and 10403S suggest the existence of significant factors in addition to σ^B. This study found significant differences in oxidative stress death between 10403S and FSL F2-695. It appears that F2-695 is significantly more resistant to oxidative stress than 10403S ($p = 0.0006$). In fact, FSL F2-695 seemed to have nearly no log CFU death after 15 min of exposure to oxidative stress. The average oxidative stress death of FSL F2-695 was -0.055 ± 0.193 (log CFU/mL average ± standard deviation). σ^B also seemed to have the same contribution to oxidative stress resistance for the strains. The observed differences in average death between a wild-type and its Δ*sigB* mutant for F2-695/O1-012 (1.60 log CFU/mL) and 10403S/A1-254 (1.41 log CFU/mL) were fairly similar. This suggests that the observed phenotypic differences in oxidative stress resistance with FSL F2-695 are due to additional factors to σ^B. One possibility is that FSL F2-695 dies more rapidly at a time beyond 15 min of oxidative stress exposure, but this does not seem to be likely because 15 min of exposure was enough to produce an average death of 0.50 ± 0.35 log CFU/mL in FSL F2-525 and 1.06 ± 0.05 log CFU/mL in 10403S. Therefore, further research needs to be conducted into other factors that contribute to *L. monocytogenes* oxidative stress response survival.

The observed differences in phenotype with respect to acid stress survival in *L. monocytogenes* lineage IIIA highlight lineage IIIA's variability. A lineage IIIA strain (FSL F2-525) exhibited a significantly higher death than 10403S ($p = 0.0019$) and FSL F2-695 ($p = 0.0343$). This seems to show that FSL F2-525 is significantly more susceptible to acid than the other strains. This is interesting because FSL F2-525 was not found to be significantly more susceptible to oxidative stresses than the other wild-type strains. The fact that FSL F2-695 could be very hardy in one stress environment while the other lineage IIIA strain was significantly more susceptible in another highlights the variability among lineage IIIA strains. Multilocus sequence typing of *L. monocytogenes* has shown that lineage III strains have the ability to participate in horizontal gene transfer and recombination (18), which could contribute to this variability. For instance, FSL F2-525 was the only strain of the lineage IIIA strains mentioned that has *lmaA,* which the majority of lineage IIIA strains were not found to have (23). *lmaA* is a gene associated with virulence in *L. monocytogenes* (11). This highlights the importance of considering the strain used and its phenotype when conducting experiments with *L. monocytogenes*. Furthermore, it also highlights the necessity of considering the strain and its behavior when interpreting and generalizing the results of studies on *L. monocytogenes*.

Differences in σ^B-mediated stress survival may be attributable to differences in isolate source or serotype. In this study, both of the lineage IIIA strains used were human isolates (5, 23) and σ^B played a significant role in acid and oxidative stress survival for both strains. In Oliver et al. (21), the lineage IIIA strain for which σ^B was not a significant factor in acid or oxidative stress survival was an animal isolate (23). Because lineage III strains are underrepresented among human disease isolates, it is possible that the relative contributions of σ^B could be related to strain host specificity. Additionally, FSL F2-695 is serotype 4a and FSL F2-525 is serotype 4b (5, 23). It is possible that the lack of σ^B contribution to stress survival in FSL J2-071 (21) is specific

to serotype 4c. This latter possibility is consistent with one study that demonstrated that a serotype 4c strain did not exhibit a significant difference in death with its isogenic Δ*sigB* mutant when exposed to acid, heat, and oxidative stresses. Further, this study showed that a serotype ½a strain did have a σ^B reliance for survival of acid and oxidative environmental stresses (19). Further, Moorhead et al. (19) also proposes serotype as a potential explanation for the observed differences in σ^B-mediated stress response. However, another serotype 4c strain was not used in this study, and further research should be conducted to determine if serotype 4c may have a different σ^B stress response behavior than the other serotypes.

As can be seen by the differences in *L monocytogenes*' ability to survive stress, even purely phenotypic studies that do not directly investigate gene regulation should consider such things as genotype, isolate source, and serotype. Additionally, it may be possible that there are regulation mechanisms in addition to σ^B for acid and oxidative stress in *L. monocytogenes* lineage IIIA strains, and further study should be conducted. More study should also be conducted to see if Oliver et al. (21)'s and Moorhead et al. (19)'s finding that a serotype 4c strain lacks σ^B dependence for acid and oxidative stress survival can be generalized to the 4c serotype strains as it cannot be for lineage IIIA strains. This study suggests that serotype 4a and 4b strains were dependent on σ^B for acid and oxidative stress survival, which is consistent with other *L. monocytogenes* lineages and serotypes (3, 9, 26).

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TABLES

Table 1. *L. monocytogenes* strains and plasmids used in this study

Table 2. σ^B contributions to acid and oxidative stress survival among three *L. monocytogenes* strains

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Table 1. *L. monocytogenes* strains and plasmids used in this study.

Strain or Plasmid	Lineage	Serotype	Relevant Genotype	Reference
pMDM1	IIIA	4b	Δ*sigB* in pkSV7	this work
pMDM2	IIIA	4a	Δ*sigB* in pkSV7	this work
10403S	II	1/2a	parent strain	(1)
FSL A1-254	II	1/2a	Δ*sigB*	(9, 26)
FSL F2-525	IIIA	4b	wild-type	(5, 23)
FSL O1-014	IIIA	4b	Δ*sigB*	this work
FSL F2-695	IIIA	4a	wild-type	(5, 23)
FSL O1-012	IIIA	4a	Δ*sigB*	this work

Table 2. σ^B contributions to acid and oxidative stress survival among 3 *L. monocytogenes* strains

	Death rate (log CFU/h), pH 2.5			Death (log CFU), 13mM CHP		
Parent Strain	**Wild-type[a]**	**Δ*sigB*[b]**	**p-value[c]**	**Wild-type[d]**	**Δ*sigB*[e]**	**p-value[f]**
10403S	0.81± 0.42	3.84 ± 0.40	<0.0001	1.06 ± 0.05	2.47 ± 0.30	0.0014
F2-695	1.23 ± 0.44	3.83 ± 0.67	<0.0001	-0.05 ± 0.19	1.51 ± 0.38	0.0032
F2-525	3.16 ± 1.43	7.28 ± 1.30	0.0009	0.50 ± 0.35	2.26 ± 0.37	0.0041

[a]Average death rate (log CFU/h) of wild-type parent strain exposed to pH 2.5 for 1 hour
[b]Average death rate (log CFU/h) of isogenic Δ*sigB* strain exposed to pH 2.5 for 1 hour
[c]P-value of time*strain interaction; p-value <0.05 indicates significant difference in the average death rate between wild-type and isogenic Δ*sigB* strain
[d]Average death (log CFU) of wild-type parent strain
[e]Average death (log CFU) of isogenic Δ*sigB* strain
[f]P-value of two-sided t-test; p-value <0.05 indicates significant difference in the average death between wild-type and isogenic Δ*sigB* strains