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Original Article

σ^B and σ^L Contribute to *Listeria monocytogenes* 10403S Response to the Antimicrobial Peptides SdpC and Nisin

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Abstract

The ability of the foodborne pathogen *Listeria monocytogenes* to survive antimicrobial treatments is a public health concern; therefore, this study was designed to investigate genetic mechanisms contributing to antimicrobial response in *L. monocytogenes*. In previous studies, the putative bacteriocin immunity gene *lmo2570* was predicted to be regulated by the stress responsive alternative sigma factor, σ^B. As the alternative sigma factor σ^L controls expression of genes important for resistance to some antimicrobial peptides, we hypothesized roles for *lmo2570*, σ^B, and σ^L in *L. monocytogenes* antimicrobial response. Results from phenotypic characterization of a *L. monocytogenes lmo2570* null mutant suggested that this gene does not contribute to resistance to nisin or to SdpC, an antimicrobial peptide produced by some strains of *Bacillus subtilis*. While *lmo2570* transcript levels were confirmed to be σ^B dependent, they were σ^L independent and were not affected by the presence of nisin under the conditions used in this study. In spot-on-lawn assays with the SdpC-producing *B. subtilis* EG351, the *L. monocytogenes* Δ*sigB*, Δ*sigL*, and Δ*sigB*/Δ*sigL* strains all showed increased sensitivity to SdpC, indicating that both σ^B and σ^L regulate genes contributing to SdpC resistance. Nisin survival assays showed that σ^B and σ^L both affect *L. monocytogenes* sensitivity to nisin in broth survival assays; that is, a *sigB* null mutant is more resistant than the parent strain to nisin, while a *sigB* null mutation in Δ*sigL* background leads to reduced nisin resistance. In summary, while the σ^B-dependent *lmo2570* does not contribute to resistance of *L. monocytogenes* to nisin or SdpC, both σ^B and σ^L contribute to the *L. monocytogenes* antimicrobial response.

Introduction

The Gram-positive, facultative intracellular foodborne pathogen *Listeria monocytogenes* is the causative agent of listeriosis, which has a human case-fatality rate >20% in the United States (Mead *et al*., 1999). The vast majority of human listeriosis cases have been reported to occur via consumption of contaminated foods (Mead *et al*., 1999); therefore, development of more effective methods for controlling the presence of *L. monocytogenes* in foods is a desirable goal. To that end, various antimicrobial peptides have been investigated as a potential means for inhibiting growth of *L. monocytogenes* in foods (Muriana, 1996; Cleveland *et al*., 2001).

Bacteriocins are bacterially produced antimicrobial peptides that are generally most effective against other bacteria that are genetically similar and present in similar ecological niches. To enhance producer strain self-preservation, bacteriocin production is frequently coupled with production of cognate bacteriocin immunity proteins (Venema *et al*., 1995; Cleveland *et al*., 2001; Eijsink *et al*., 2002). For example, NisI, which provides immunity to nisin, is encoded downstream of the nisin biosynthesis genes in *Lactococcus lacti*s (Engelke *et al*., 1994). Previous studies have reported that bacteriocin production can be influenced by bacterial environmental stress response pathways such as the RecA-dependent SOS response and the ppGpp-dependent stringent response (de los Santos *et al*., 2005). Bacteriocin production and immunity to antimicrobials are hypothesized to enhance the ability of producer bacteria to vie for limited nutrients in the presence of competitors (Nissen-Meyer and Nes, 1997).

Lactic acid bacteria are recognized as producers of various bacteriocins (Klaenhammer, 1993; Jack *et al*., 1995). Lactic acid bacteria are commonly present in human foods; therefore, the bacteriocins that they produce, such as pediocin PA-1/AcH, enterocins, and/or sakacins, also may be present in foods (Cleveland *et al*., 2001). Currently, only nisin, a class I lantibiotic bacteriocin produced by the lactic acid bacterium *L. lactis*, has generally recognized as safe status for intentional application as an antimicrobial in the U.S. food industry (Jack *et al*., 1995). Nisin creates membrane-spanning pores in the

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bacterial cell wall, which enable dissipation of the cell's proton motive force (Bruno *et al*., 1992; Bonnet *et al*., 2006). Although, in general, nisin has been demonstrated as an effective antilisterial peptide (Benkerroum and Sandine, 1988), some strains of *L. monocytogenes* have developed resistance to both nisin and pediocin PA-1 (Gravesen *et al*., 2002a, 2002b; Gandhi and Chikindas, 2007). *L. monocytogenes* resistance to nisin is a concern to the segments of the food industry (e.g., dairy and poultry) that currently use this peptide to control pathogen growth (Gandhi and Chikindas, 2007). A better understanding of the molecular mechanisms contributing to antimicrobial resistance in foodborne pathogens could lead to development of improved food safety intervention strategies. One means to that end is to identify and examine putative bacteriocin immunity genes and their physiological roles in protecting the producer strain against either endogenously or exogenously produced antimicrobial peptides.

In a previous study, Kazmierczak *et al*. (2003) identified *lmo2570* as a putative σ^B-dependent gene with 45% similarity to the *Bacillus subtilis* bacteriocin immunity gene *sdpI* (*yvaZ*) (cmr.jcvi.org), which encodes SdpI. SdpI is a membrane protein conferring resistance to the endogenously produced antimicrobial peptide SdpC (Butcher and Helmann, 2006; Ellermeier *et al*., 2006). Butcher and Helmann (2006) found that while SdpI has a predominant role in conferring resistance to SdpC, the *B. subtilis* regulon controlled by σ^W, an extracytoplasmic function sigma factor, provides secondary immunity to this antimicrobial peptide. Taken together, these data indicate the importance in antimicrobial resistance of both immunity genes and transcriptional level regulatory mechanisms as mediated by alternative sigma factors.

We hypothesized that σ^B and σ^L contribute to antimicrobial response in *L. monocytogenes*. σ^B has been shown to regulate response to antimicrobial peptides in other Gram-positive bacteria. To illustrate, the *B. subtilis* σ^B regulon is upregulated after treatment with either bacitracin or vancomycin (Mascher *et al*., 2003). In a collection of teicoplanin-resistant *Staphylococcus aureus* mutants, the majority of the mutations responsible for antimicrobial resistance mapped to *rsbW*, which encodes the RsbW anti-sigma factor that sequesters σ^B to prevent it from interacting with RNA polymerase. The teicoplanin-resistant strains with mutations in *rsbW* showed increased σ^B activity relative to their parent strain (the MB33 *rsbU* mutant strain) or to other strains carrying the *rsbU* wild-type allele (Bischoff and Berger-Bachi, 2001), providing evidence of a link between σ^B activity and antimicrobial resistance. σ^B also has been shown to contribute to bacterial stress response regulation in *S. aureus* (Chan *et al*., 1998). *L. monocytogenes* alternative sigma factor σ^L regulates expression of genes that mediate sensitivity to antimicrobials such as the class IIa bacteriocin, mesentericin Y105 (Robichon *et al*., 1997); hence, σ^L also has been associated with antimicrobial response. Therefore, in the studies described below, phenotypic and genotypic assessments were used to determine the contributions of σ^B, σ^L, and Lmo2570 to the *L. monocytogenes* response to SdpC and nisin.

Materials and Methods

Bacterial strains and growth conditions

L. monocytogenes parent strain 10403S (serotype 1/2a) and otherwise isogenic *sigB* and *sigL* single and double null mutants (Δ*sigB*, FSL A1-254; Δ*sigL*, FSL B2-124; Δ*sigB*/Δ*sigL*, FSL B2-127) were used in this study. *Listeria innocua* FSL C2-008 (Woodling and Moraru, 2005), *Listeria ivanovii* FSL C2-010, *Listeria welshimeri* FSL N1-064, and *Listeria seeligeri* FSL N1-067 (Table 1) were used to assess intragenus competition with the *L. monocytogenes* parent and mutant strains. To examine the susceptibility of the *L. monocytogenes* parent and mutant strains to a closely related bacterium that produces an antimicrobial peptide, we used strains of *B. subtilis* that produce SdpC, the bacteriocin whose cognate immunity gene is predicted by sequence similarity to be homologous to *L. monocytogenes lmo2570*. These strains included *B. subtilis* prototroph (PY49) (Youngman *et al*., 1984) and its mutant EG351 (PY79 P_{spac}-hy-*sdpABC*) (gift from Dr. J. Helmann, Department of Microbiology, Cornell University), which expresses SdpC under control of an inducible promoter.

L. monocytogenes strains were grown in brain heart infusion (BHI) broth (Difco, Sparks, MD) at 37°C with shaking (250 rpm) overnight (16–18 h), then were subcultured (1:100) and grown as described below for each experiment. *B. subtilis* strains were grown in Luria-Bertani broth as described for *L. monocytogenes*, unless otherwise stated. The Δ*sigB*/Δ*sigL* strain grew more slowly than the 10403S, Δ*sigB*, and Δ*sigL*

Table 1. Strains Used in This Study

Strain	*Characteristics*	*Reference or source*
Listeria monocytogenes 10403S	Laboratory parent strain	Bishop and Hinrichs (1987)
L. monocytogenes FSL A1-254	10403S Δ*sigB*	Wiedmann *et al*. (1998)
L. monocytogenes FSL P1-002	10403S Δ*lmo2570*	This study
L. monocytogenes FSL B2-124	10403S Δ*sigL*	Chaturongakul, unpublished
L. monocytogenes FSL B2-127	10403S Δ*sigB*/Δ*sigL*	Chaturongakul, unpublished
Listeria innocua FSL C2-008		Woodling and Moraru (2005)
Listeria ivanovii FSL C2-010		Wiedmann, unpublished[a]
Listeria welshimeri FSL N1-064		Fish processing plant environment
Listeria seeligeri FSL N1-067		Fish processing plant environment
Bacillus subtilis PY79	Prototroph, parent strain	Youngman *et al*. (1984)
B. subtilis EG351	PY79 $_{Pspac}$-hy-*sdpABC*	Butcher and Helmann (2006)

[a]Isolate kindly provided (as USDA 2717) by I. Wesley, USDA-ARS.
USDA-ARS, U.S. Department of Agriculture's Agricultural Research Service.

strains, requiring an additional incubation time of ~30 min to reach the same OD_{600}.

Mutant construction

An in-frame 543 base pair deletion within *lmo2570* was created in *L. monocytogenes* 10403S using splicing-by-overlap extension (SOE) polymerase chain reaction (PCR) and allelic exchange mutagenesis (Ho *et al*., 1989). Primers used were 5′-GGA AGC TTT AAG GCA CTG TGA GCC TGG-3′ (*lmo2570* SOEA), 5′-TCA TAC TAG GAA ATA TAC CAA C-3′ (*lmo2570* SOEB), 5′-GTT GGT ATA TTT CCT AGT ATG ATT ATT GTT GTT G-3′ (*lmo2570* SOEC), and 5′-GGG GTA CCT CAG GTT CAC TGG CAG CTA G-3′ (*lmo2570* SOED). Primers were synthesized by IDT Technologies (Coralville, IA). Allelic exchange mutagenesis was confirmed through PCR and subsequent DNA sequencing, the latter of which was performed by the Cornell BioResource Center (Ithaca, NY). The Δ*lmo2570* mutation did not affect growth rate of the mutant strain relative to the 10403S parent strain when both were grown in BHI at 37°C with shaking at 250 rpm (data not shown).

Spot-on-lawn assays

Spot-on-lawn assays were performed in triplicate as described by Butcher and Helmann (2006). Briefly, to create lawns, 100 μL of a given strain (i.e., 10403S, Δ*lmo2570*, Δ*sigB*, Δ*sigL*, or Δ*sigB*/Δ*sigL*) that had been grown to an optical density of $OD_{600} = 0.4$ was inoculated into 2 mL of 0.7% Luria-Bertani soft agar that had been tempered at 50°C. To induce P_{spac}-regulated transcription of *sdpABC* when EG351 was used as the spotting strain, 1 mM isopropyl-β-D-thiogalactopyranoside also was added to the tempered agar that had been inoculated with bacteria. Each mixture was then poured into one well in an eight-well rectangular multidish (26×33 mm; Nunc, Rochester, NY). The plates were then dried in a laminar hood for 30 min. Subsequently, 4 μL of the strain being assessed for bacteriocin production (e.g., 10403S, PY79, or EG351), which had been grown to an $OD_{600} = 0.6$, was spotted on the agar in the middle of each well. The plates were covered with lids and incubated in a moist container at 37°C for 22–24 h. In addition to the 10403S, PY79, or EG351 test strains, isolates representing five *Listeria* species also were used as spotting strains to determine if the lawn strains would demonstrate sensitivity to bacteria representing different species within the same genus (Table 1). Sensitivity of lawns to potential bacteriocin producer strains was assessed by measuring the zone of inhibition (zoi) around the growth of the spotted strain. Radii of zoi were determined by measuring the diameters of both the spotted colony and the surrounding zoi in pixels (px). The diameter of the spotted colony was then subtracted from the diameter of the zoi, and the resulting product was divided by 2 (to yield a radius). Measurements were performed using Adobe® Photoshop® CS (Adobe Systems Incorporated, Mountain View, CA).

Radii of zoi produced on the various lawn strains were initially compared to zoi produced on the 10403S reference lawn using one-way analysis of variance (ANOVA) with Dunnett's *t*-test, using SAS® 9.0 (SAS Institute, Cary, NC). To determine if there were statistically significant interaction effects between the *sigB* and the *sigL* deletions, a two-way ANOVA (with Tukey's adjustment for multiple comparisons) was performed. In this model, the dependent variable was zoi radius, and the independent variables included sigB + sigL + sigB*sigL + replicate. The factors sigB and sigL in the model indicate the presence or absence of that gene in the strains tested. An adjusted $p < 0.05$ was considered significant in this and all other statistical analyses.

Nisin minimum inhibitory concentration determination

The objective of this experiment was to determine a minimal inhibitory concentration of nisin for log-phase *L. monocytogenes* to enable selection of an appropriate sublethal concentration for subsequent quantitative reverse-transcriptase (qRT)–PCR experiments. Nisin's solubility and activity are optimal at pH 3 and 3.5, respectively (Abee and Delves-Broughton, 2003); therefore, nisin is typically dissolved in an acidified solution before use (Liu and Hansen, 1990; Huot *et al*., 1996). As σ^B expression and activity are induced at low pH (Becker *et al*., 1998; Sue *et al*., 2004), we predicted that addition of nisin in an acidified solution to the various cultures would upregulate expression of the σ^B regulon, thus conferring a survival advantage to the wild type over the Δ*sigB* strain (Wiedmann *et al*., 1998; Ferreira *et al*., 2001, 2003) and, hence, confounding interpretation of our experimental results. Therefore, to avoid induction of σ^B activity, nisin was dissolved in sterile distilled water (1000 AU/mL), and the pH of the final solution was adjusted to 7.0 using 0.01 N sodium hydroxide. Nisin solutions at pH 7.0 were used throughout these experiments. The nisin solutions were filter sterilized with a 0.2 μm, 25 mm syringe filter (NALGENE®; Thermo Fisher Scientific, Waltham, MA) and diluted to the test concentrations. The minimum inhibitory concentration of nisin (Sigma, St. Louis, MO) was determined for all strains of *L. monocytogenes* by measuring absorbance at OD_{600} using a Fusion™ Universal Microplate Analyzer (PerkinElmer, Shelton, CT). Strains were grown overnight, subcultured 1:100, and grown to $OD_{600} = 0.4$. Strains were inoculated to a final concentration of 1×10^4 CFU/well into 96-well round bottom microplates (Costar, Corning, NY), and the edges were sealed with Parafilm® (Alcan Packaging, Neenah, WI) to prevent evaporation. OD_{600} measurements were taken after 24 h incubation at 37°C with shaking. The lowest concentration that inhibited growth for all strains after a 24 h incubation in a 96-well plate format was 100 AU nisin/mL, as determined in three replicate trials. Therefore, a sublethal concentration of 75 AU nisin/mL was used for TaqMan qRT-PCR assays.

Total RNA isolation

L. monocytogenes 10403S, Δ*sigL*, and Δ*sigB* were grown to logarithmic phase ($OD_{600} = 0.4$) and collected after (i) incubation for 10 min after addition of nisin in sterile distilled water to yield a final concentration of 75 AU/mL nisin, (ii) incubation for 10 min after addition of an equivalent volume of sterile distilled water without nisin, and (iii) incubation for 10 min without any addition. RNA isolation and purification was performed as previously described (Sue *et al*., 2004; Raengpradub *et al*., 2008), except that DNase treatments were performed using TURBO™ DNase (Ambion, Austin, TX) following the manufacturer's instructions. Total nucleic acid concentrations and purity were estimated using absorbance readings (260 nm/280 nm) on a NanoDrop™ ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE).

qRT-PCR

Transcript levels of *lmo2570*, as well as of two housekeeping genes, *rpoB* and *gap*, were quantified using TaqMan primers and probes and the ABI Prism® 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) as previously described (Kazmierczak *et al*., 2006). Data were analyzed using the ABI Prism 7000 Sequence Detection System software (Applied Biosystems) as previously described by Sue *et al*. (2004). Primer Express® 1.0 software (Applied Biosystems) was used to design oligonucleotide primers and TaqMan probes for *lmo2570*: forward primer (5′-AAG TGG CGG TGC ATT TCG-3′), reverse primer (5′-TAA GCC AAG CCA CTT TTG CAT-3′), and probe (6FAM 5′-ACG GAC TTC TCC CCA GAT-3′ MGB-NFQ). Primers and probes for *gap* and *rpoB* were previously described (Sue *et al*., 2004; Schwab *et al*., 2005), respectively.

Transcript levels of *lmo2570*, as determined by qRT-PCR, were $\log_{10}$ transformed and then normalized to the geometric mean of transcript levels from the housekeeping genes *rpoB* and *gap* as previously described (Kazmierczak *et al*., 2006). Statistical analyses of normalized *lmo2570* transcript levels were performed using one-way ANOVA and Tukey's studentized range (honestly significant difference or HSD) test, performed in SAS 9.0 (SAS Institute).

Nisin survival assay

The objective of this assay was to measure relative survival characteristics of stationary phase *L. monocytogenes* 10403S, Δ*sigB*, Δ*sigL*, Δ*sigB*/Δ*sigL*, and Δ*lmo2570* strains in the presence of an initially lethal concentration of nisin (150 AU/mL nisin). Strains were grown in BHI at 37°C with shaking overnight, followed by a 1% subculture and growth to logarithmic phase ($OD_{600} = 0.4$), followed by a second subculture and growth to stationary phase ($OD_{600} = 1.0 + 3$ h), followed by a third 1% subculture (0.5 into 50 mL, final concentration of $\sim 2\times10^7$ CFU/mL) in a 300 mL flask (Bellco, Vineland, NJ). Nisin (150 AU/mL, pH 7.0) was added to the BHI, and cultures were incubated at 37°C with shaking for an additional 9 h. Bacterial numbers were determined before and after the addition of nisin. Specifically, samples were taken at 0, 0.5, 1, 2, 3, 4, 5, 6, and 9 h postaddition and spiral plated on BHI agar using a Spiral Biotech Autoplate® 4000 (Spiral Biotech, Norwood, MA). Colonies were enumerated with a QCount™ (Spiral Biotech) after 24 h incubation at 37°C. Colony counts were transformed to $\log_{10}$ CFU/mL.

Data from the nisin survival assay were used to calculate two parameters: (i) bacterial reduction after 0.5 h of nisin exposure and (ii) growth rate during recovery and re-growth (between 1 and 9 h after nisin exposure). Linear regression was used to determine the slope representing the change in bacterial numbers for each strain from 1 to 9 h (i.e., the period when viable cell numbers were increasing [re-growth]); this value represents the bacterial growth rate in $\log_{10}$ growth/h. Statistical analyses were then performed on both parameters. First, a one-way ANOVA (with Dunett's *t*-test or Tukey's studentized range [HSD] test) was performed to determine if (i) bacterial reduction or (ii) growth rate differed between the mutant strains and the parent strain. To determine if there were statistically significant interaction effects between the *sigB* and the *sigL* deletions, a two-way ANOVA (with Tukey's adjustment for multiple comparisons) was performed. In this model, the dependent variable was either (i) bacterial reduction after nisin exposure or (ii) growth rate during re-growth; the independent variables included sigB + sigL + sigB* sigL + replicate. The factors sigB and sigL in the model indicate the presence or absence of that gene in the strains tested.

Results

No intragenus competition was evident between *L. monocytogenes* and the other *Listeria* strains tested (Table 1). Specifically, no zoi occurred between any of the listerial species that were used as spotting strains (*L. innocua*, *L. ivanovii*, *L. welshimeri*, or *L. seeligeri*) and any of the *L. monocytogenes* lawn strains (10403S, Δ*sigB*, Δ*sigL*, or Δ*sigB*/Δ*sigL*; data not shown).

Lmo2570 is σ^B, but not σ^L dependent and does not contribute to resistance to nisin or SdpC

qRT-PCR was initially used to determine whether either σ^B or σ^L contributes to transcription of *lmo2570*, a putative bacteriocin immunity gene (Fig. 1). *lmo2570* transcript levels were consistently and significantly lower in the Δ*sigB* strain as compared to the 10403S parent strain ($p < 0.05$; Fig. 1), indicating σ^B-dependent transcription of *lmo2570*. While *lmo2570* transcript levels were consistently higher in the Δ*sigL* strain as compared to the parent strain, this difference was not significant ($p > 0.05$; Fig. 1). The presence of nisin at a sublethal concentration (75 AU/mL) did not affect *lmo2570* transcript levels (Fig. 1). Normalized *lmo2570* transcript levels in 10403S were low, ranging from 0.007 to 0.034. To put these low *lmo2570* transcript levels into biological context, in 10403S, ~0.02 transcripts of *lmo2570* were present relative to the mean transcript levels of the highly expressed housekeeping genes, *rpoB* and *gap*, as indicated in Fig. 1. The *lmo2570* transcript levels observed in the present study are on the same order of magnitude as those reported previously for other σ^B-dependent genes [e.g., *opuCA* and *bsh* (Chan *et al*., 2007)].

Spot-on-lawn assays were used to compare the sensitivities of *L. monocytogenes* 10403S and Δ*lmo2570* to SdpC, the antimicrobial peptide whose cognate immunity gene shares amino acid similarity with Lmo2570. Specifically, *B. subtilis* PY79 (which naturally produces SdpC) and *B. subtilis* EG351 (which overexpresses SdpC in the presence of isopropyl-β-D-thiogalactopyranoside) were spotted on lawns of either 10403S or Δ*lmo2570*. Zoi for 10403S and Δ*lmo2570* (Table 2) did not differ significantly ($p > 0.05$), indicating that *lmo2570* does not contribute to SdpC resistance. Neither 10403S nor Δ*lmo2570* showed inhibition by 10403S (Table 2) or by any other *Listeria* species (data not shown), indicating absence of intragenus inhibition, at least among the strains tested.

To characterize the responses of stationary phase *L. monocytogenes* 10403S and Δ*lmo2570* to nisin, we evaluated survival of $\sim 2\times10^7$ CFU/mL 10403S or Δ*lmo2570* in BHI in the presence of 150 AU nisin/mL (Fig. 2). Exposure to nisin for 30 min led to 4.0 and 3.9 log reductions in bacterial numbers for 10403S and Δ*lmo2570*, respectively (Fig. 2), indicating no difference in nisin susceptibility between these strains. After the initial killing by nisin, bacterial numbers increased between 1 and 9 h post-nisin exposure, reflecting growth of cells that had survived nisin exposure. Specifically, after 9 h, bacterial numbers were 6.9 and 6.6 log for the parent strain and

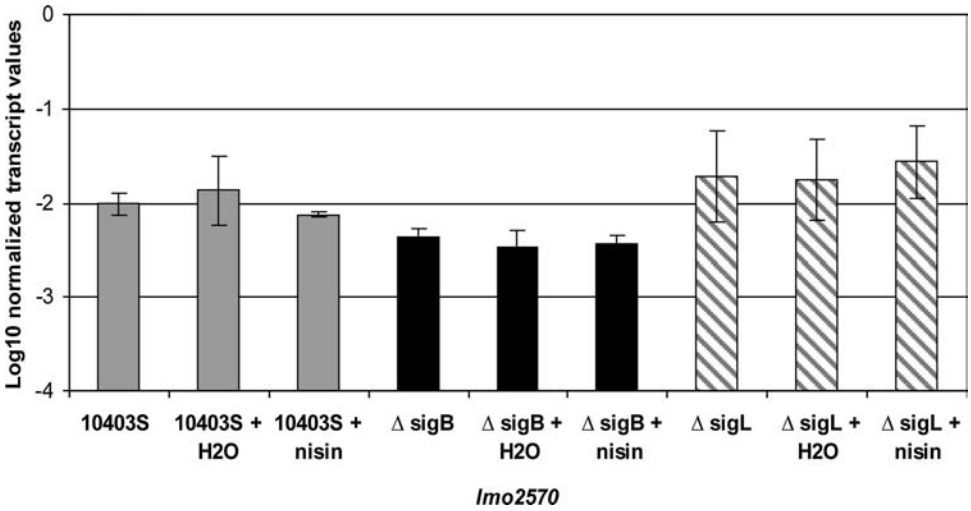

FIG. 1. Normalized log-transformed *lmo2570* transcript levels for *Listeria monocytogenes* 10403S (gray bars), Δ*sigB* (black bars), and Δ*sigL* (hatched bars). Transcript levels were determined by quantitative reverse-transcriptase–polymerase chain reaction using RNA isolated from logarithmic phase ($OD_{600} = 0.4$) *L. monocytogenes* that had been (i) incubated for 10 min after addition of nisin in sterile distilled water to yield a final concentration of 75 AU/mL nisin; (ii) incubated for 10 min after addition of an equivalent volume of sterile distilled water without nisin; or (iii) incubated for 10 min without any addition. Transcript levels were log transformed and normalized to the geometric mean of the transcript levels for the housekeeping genes *rpoB* and *gap*. Values represent mean transcript levels from three independent RNA collections; error bars indicate one standard deviation from each mean. Overall ANOVA showed a significant effect of the factor strain, but no effect of the factor condition (i.e., no addition, addition of water, or addition of nisin). Tukey's test showed significantly lower transcript levels for *lmo2570* in the Δ*sigB* strain as compared to the parent strain; transcript levels did not differ significantly between the Δ*sigL* strain and the parent strain.

Δ*lmo2570*, respectively, further supporting that Δ*lmo2570* and 10403S susceptibilities to nisin do not differ.

σ^B and σ^L both contribute to resistance to the SdpC antimicrobial peptide produced by B. subtilis

Spot-on-lawn assays were used to compare the sensitivities of *L. monocytogenes* 10403S, Δ*sigB*, Δ*sigL*, and Δ*sigB*/Δ*sigL* to the antimicrobial peptide SdpC. The zoi produced by *B. subtilis* PY79 did not differ between *L. monocytogenes* 10403S and Δ*sigB* or Δ*sigL*; the zoi produced by PY79 on the Δ*sigB*/Δ*sigL* lawn was significantly larger than the zoi produced on 10403S ($p < 0.05$; Table 2), suggesting the possibility that σ^B and σ^L contribute to SdpC resistance in an additive fashion. *B. subtilis* EG351 produced significantly larger zoi on Δ*sigB* (28.2 ± 1.6 px), Δ*sigL* (20.7 ± 5.3 px), and Δ*sigB*/Δ*sigL* lawns (32.0 ± 2.6 px), as compared to the zoi produced on 10403S (13.5 ± 3.9; Table 2). Two-way ANOVA analyses of zoi data showed no significant "sigB*sigL" interaction effect on SdpC sensitivity ($p > 0.05$), further supporting the notion of additive (as compared to multiplicative) contributions of σ^B and σ^L to SdpC resistance. Overall, results from this assay indicate that alternative sigma factors σ^B and σ^L both contribute to resistance to SdpC.

σ^B and σ^L both contribute to response to the bacteriocin nisin

A 30 min exposure to nisin (150 AU/mL) resulted in a 4.0 log reduction in bacterial numbers for stationary phase 10403S. By comparison, reduction of Δ*sigB* bacterial numbers was significantly less (3.0 log reduction; $p < 0.05$; Fig. 2), indicating increased nisin resistance of this strain relative to that of 10403S. Bacterial numbers for the Δ*sigL* and Δ*sigB*/Δ*sigL*

Table 2. Spot-on-Lawn Assay Results

	Mean zoi radii (SD) for L. monocytogenes *strains*[b]				
Strain spotted[a]	10403S	Δlmo2570	ΔsigB	ΔsigL	ΔsigB/ΔsigL
L. monocytogenes 10403S	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
B. subtilis PY79	11.8 (1.6)	10.0 (2.2)	11.5 (4.9)	16.7 (0.6)	20.5[c] (2.3)
B. subtilis EG351 (IPTG)	13.5 (3.9)	8.3 (2.9)	28.2[c] (1.6)	20.7[c] (5.3)	32.0[c] (2.6)

[a]Strains spotted on the lawns are listed in left column; the average zoi radius around each spot is shown for each lawn, with SD in parentheses.

[b]Radii were determined from three independent experiments by measuring diameters of the zoi in pixels using Adobe® Photoshop® CS.

[c]Values that are significantly different ($p < 0.05$; one-way ANOVA with Dunnett's *t*-test) from the zoi produced on the *L. monocytogenes* 10403S lawn.

IPTG, isopropyl-β-D-thiogalactopyranoside; zoi, zone of inhibition; SD, standard deviations; ANOVA, analysis of variance.

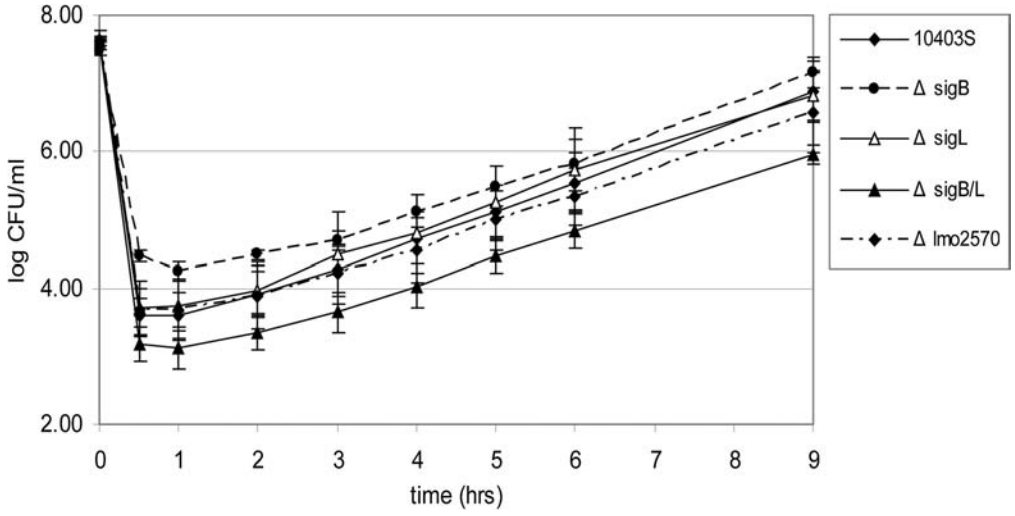

FIG. 2. Viable numbers of stationary-phase *L. monocytogenes* 10403S, Δ*lmo2570*, Δ*sigB*, Δ*sigL*, and Δ*sigB*/Δ*sigL* at various time points after exposure to 150 AU/mL nisin. Values are reported as log CFU/mL. Data shown represent the average of four independent experiments; error bars represent one standard deviation from each mean.

strains were reduced by 3.9 and 4.5 log; these reductions were not significantly different from that of 10403S ($p > 0.05$; Dunett's t-test). Interestingly, log reduction for the Δ*sigB*/Δ*sigL* strain was significantly ($p > 0.05$; Tukey's HSD) greater (4.5 log) as compared to the Δ*sigB* strain (3.0 log reduction), indicating a significant effect of the *sigL* deletion on nisin killing in a Δ*sigB* background. Based on the reductions in bacterial numbers between 0 and 0.5 h, two-way ANOVA analyses found a significant sigL*sigB interaction effect on survival after nisin exposure, indicating that the effect of one sigma factor on survival differs depending on the presence or absence of the other sigma factor.

After initial killing by nisin, all strains showed re-growth between 1 and 9 h post-nisin exposure. Rates of re-growth, as represented by the slopes of the graphs between 1 and 9 h postexposure, were compared among the strains. While the growth rate for the Δ*sigL* strain (0.39 log CFU/h) was not significantly different from that of the parent strain (0.41 log CFU/h), both the Δ*sigB* and the Δ*sigB*/Δ*sigL* strains showed significantly slower growth rates (0.36 log CFU/h for both; $p < 0.05$, Dunett's t-test) as compared to the parent strain. Although no significant sigB*sigL interaction effect on nisin survival was identified by two-way ANOVA analysis, the *sigB* deletion had a significant effect on growth rate, with s*igB* deletion strains (i.e., ΔsigB and Δ*sigB*Δ*sigL*) showing slower growth rates as compared to the corresponding strains with intact *sigB* genes.

Discussion

We hypothesized that alternative sigma factors σ^B and σ^L and the hypothetical bacteriocin immunity gene, *lmo2570*, contribute to *L. monocytogenes* antimicrobial response. This hypothesis was based on previous observations, including (i) σ^B and its homolog σ^F contribute to antimicrobial response in other Gram-positive organisms (Bischoff and Berger-Bachi, 2001; Mascher *et al*., 2003; Michele *et al*., 1999), (ii) *L. monocytogenes* σ^L controls sensitivity to class IIa bacteriocins, mesentericin Y105, pediocin PA-1, and enterocin A, and (iii) the putative σ^B-dependent *lmo2570* has sequence homology to the *B. subtilis* bacteriocin immunity gene, *sdpI*. To test our hypothesis, we assessed the sensitivities of strains with null mutations in *sigB*, *sigL*, *sigB*/*sigL*, or *lmo2570* to the antimicrobial peptides SdpC and nisin. We also characterized transcription of *lmo2570* in 10403S, Δ*sigB*, and Δ*sigL* strains exposed to a subminimal inhibitory concentration of nisin. Our results show that (i) while *lmo2570* is σ^B dependent, it does not contribute to resistance to SdpC or nisin and (ii) both σ^B and σ^L contribute to resistance to the antimicrobial peptide SdpC, as shown by results from spot-on-lawn assays. In addition, both σ^B and σ^L affect *L. monocytogenes* sensitivity to nisin in broth survival assays. Specifically, while loss of only *sigB* renders the resulting strain more resistant to nisin than the parent strain, loss of *sigB* in a Δ*sigL* background leads to reduced nisin resistance relative to the original parent strain.

The effects of antimicrobial peptides on *L. monocytogenes* appear to differ depending on the class of peptide, the strain, initial number of bacteria, growth phase, and the assay used for evaluation. To illustrate, σ^B was reported previously to contribute to *L. monocytogenes* tolerance to nisin or lacticin 3147 in broth assays (Begley *et al*., 2006), but not to nisin, lacticin 3147, or sakacin A resistance in agar overlay assays (Moorhead and Dykes, 2003; Begley *et al*., 2006). Moorhead and Dykes (2003) showed that an *L. monocytogenes* serotype 1/2a wild-type strain was less resistant to nisin than a serotype 4c wild-type strain, suggesting differences in antimicrobial sensitivities among strains. It is also likely that other environmental stresses (in addition to the presence of the antimicrobial peptide) imposed upon the cells also evoke differential phenotypic responses from the cells (e.g., exposure to low pH induces σ^B activity in *L. monocytogenes*), which may provide cross-resistance to multiple stresses (Ferreira *et al*., 2003).

Lmo2570 *is* σ^B *dependent, but does not contribute to antimicrobial resistance*

L. monocytogenes Lmo2570 is 45% similar at the amino acid level to the *B. subtilis* immunity protein SdpI, which confers

immunity against SdpC (Butcher and Helmann, 2006; Ellermeier *et al*., 2006); therefore, we hypothesized that *lmo2570* may play a role in antimicrobial immunity in *L. monocytogenes*. *lmo2570* was predicted as σ^B dependent in previous microarray experiments (Kazmierczak *et al*., 2003). As previous reports have shown that bacteriocin immunity genes can contribute to resistance to multiple antimicrobials (Matsumoto-Nakano and Kuramitsu, 2006), in addition to examining its role in SdpC resistance, we also tested the contributions of *lmo2570* to resistance to the commercially available bacteriocin, nisin. The Δ*lmo2570* strain did not show reduced sensitivity to either SdpC or nisin. Exposure to nisin did not induce transcription of *lmo2570* in either the wild-type or any of the mutant strains. *lmo2570* thus does not appear to be important for SdpC or nisin resistance in *L. monocytogenes*. A role for this gene in resistance to other bacteriocins or in contributing to nisin and SdpC resistance under environmental conditions not tested here cannot be excluded by our data, however. Our confirmation of *lmo2570* as σ^B dependent suggests a role for *lmo2570* in *L. monocytogenes* survival or growth under conditions that remain to be defined.

σ^B and σ^L both contribute to L. monocytogenes *response to SdpC and nisin*

We found clear evidence that alternative sigma factors σ^L and σ^B both contribute to SdpC resistance. Specifically, as determined in a spot-on-lawn assay, we showed that both the Δ*sigB* and the Δ*sigL* strains were significantly more susceptible to the bactericidal effect of the antimicrobial peptide SdpC produced by *B. subtilis* EG351 than the otherwise isogenic 10403S parent strain. Characterization of a Δ*sigB*/Δ*sigL* double-mutant strain suggested that deletion of both genes had an additive, but not an interactive, effect on SdpC resistance. However, deletions of both Δ*sigB* and Δ*sigL* had an interactive effect on *L. monocytogenes* resistance to nisin. Specifically, while the Δ*sigB*/Δ*sigL* strain showed decreased resistance to nisin as compared to the Δ*sigB* strain, the Δ*sigB* strain showed increased resistance to nisin as compared to the parent strain, which has both *sigB* and *sigL* intact. The interactive effect observed after the loss of both *sigB* and *sigL* may indicate that at least some genes important for recovery and re-growth after nisin exposure are coregulated, either directly or indirectly, by these alternative sigma factors. We also found that re-growth of both the Δ*sigB* and the Δ*sigB*/Δ*sigL* strains after nisin exposure was slower than that of the parent strain, consistent with previous observation that σ^B is important for *B. subtilis* recovery after rifampin treatment (Bandow *et al*., 2002). The overall observation that a deletion of the gene encoding σ^L (i.e., a single deletion) does not affect *L. monocytogenes* resistance to nisin is consistent with observations by Dalet *et al*. (2000), who reported that σ^L (which has also been designated as RpoN) is not involved in *L. monocytogenes* nisin resistance. Relative to its otherwise isogenic parent, a Δ*rpoN L. monocytogenes* strain (i.e., a strain lacking σ^L) has previously shown increased resistance to the class IIa nonlantibiotic bacteriocins mesentericin Y105, pediocin PA-1, and enterocin A (Robichon *et al*., 1997; Dalet *et al*., 2000), consistent with our finding that σ^L contributes to resistance to some bacteriocins (i.e., SdpC). Our findings, as well as previous findings by others (e.g., Dalet *et al*., 2000), thus support that different regulatory elements are critical for the ability of *L. monocytogenes*, and other bacteria, to respond to different bacteriocins, a notion consistent with the diverse nature of this group of antibacterial compounds.

Overall, our data indicate that σ^B and σ^L both contribute to the ability of *L. monocytogenes* to respond to antimicrobials. Regulatory interactions among multiple alternative sigma factors also have been shown to contribute to antibiotic resistance in *B. subtilis*. Specifically, three (σ^M, σ^W, and σ^X) of the seven *B. subtilis* extracytoplasmic function alternative sigma factors have overlapping regulons that contribute to antibiotic resistance, as demonstrated by the greatly enhanced sensitivity of a triple MWX mutant to various antimicrobials, including nisin (Mascher *et al*., 2007). Strains bearing single or double mutations in the genes encoding these alternative sigma factors displayed considerably less antimicrobial sensitivity than the strain with the triple mutation (Mascher *et al*., 2007). Thus, in combination with previous studies, our data support a model in which multiple alternative sigma factors contribute to regulatory networks important for fine-tuning transcriptional regulation of gene expression to help optimize bacterial cell resistance to antimicrobial peptides.

Conclusions

Alternative sigma factors have been shown to regulate genes and operons critical for resistance to antimicrobials in various bacteria, including *B. subtilis*, *L. monocytogenes*, *Salmonella enterica* serovar Typhimurium, *S. aureus*, and *Vibrio cholerae* (Robichon *et al*., 1997; Crouch *et al*., 2005; Zhang *et al*., 2005; Butcher and Helmann, 2006; Mathur *et al*., 2007). Our data indicate that σ^B and σ^L, as well as the simultaneous presence of both σ^B and σ^L, contribute to antimicrobial response in *L. monocytogenes* in a manner that is dependent on the antimicrobial that is present. The results reported in this study provide further evidence of the importance of regulatory networks for fine-tuning *L. monocytogenes* responses to changing environmental conditions (Chaturongakul *et al*., 2008).

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Disclosure Statement

No competing financial interests exist.

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