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Functional Consequences of Genome Evolution in *Listeria monocytogenes*: the lmo0423 and lmo0422 Genes Encode σ^C and LstR, a Lineage II-Specific Heat Shock System†

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***Listeria monocytogenes* strains belonging to phylogenetic lineage II (serotypes 1/2a, 1/2c, and 3a) carry a lineage-specific genome segment encoding a putative sigma subunit of RNA polymerase (lmo0423, herein referred to as *sigC*), a gene of unknown function (lmo0422) similar to the *padR* family of regulators, and a gene that is similar to the *rodA-ftsW* family of cell wall morphology genes (lmo0421). To understand the function of this set of genes, their expression patterns and the effects of null mutations in the lineage II *L. monocytogenes* strain 10403S were examined. The data are consistent with the three genes comprising an operon (the *sigC* operon) that is highly induced by temperature upshift. The operon is transcribed from three different promoters, the proximal of which (P1) depends upon *sigC* itself. Null mutations in *sigC* or lmo0422 increase the death rate at lethal temperatures and cause loss of thermal adaptive response, whereas the lmo0421 mutation causes only a loss of the adaptive response component. Only the *sigC* mutation affects transcription from the P1 promoter, whereas ectopic expression of lmo0422 from the P_{SPAC} promoter complements the individual lmo0422 and *sigC* null mutations, showing that lmo0422 is the actual thermal resistance regulator or effector while *sigC* provides a mechanism for temperature-dependent transcription of lmo0422 from P1. Our genetic and phylogenetic analyses are consistent with lmo0422—renamed *lstR* (for lineage-specific thermal regulator)—and *sigC* comprising a system of thermal resistance that was ancestral to the genus *Listeria* and was subsequently lost during divergence of the lineage I *L. monocytogenes* population.**

Although only a relatively small number of cases of human listeriosis occur each year in the United States (estimated at 2,500 per year), the associated rates of morbidity and mortality are substantial, with nearly 30% mortality in some outbreaks (40, 58). Because the causative agent of the disease, *Listeria monocytogenes*, is ubiquitous in nature and possesses durable physiological characteristics, the organism is one of the most significant food safety problems in the food production industry.

One of the best characterized physiological attributes of *L. monocytogenes* is its ability to grow at refrigeration temperatures, in conditions of high osmolarity, and at low pH. Several different stress adaptation systems have been defined for *L. monocytogenes* that contribute to growth characteristics under these conditions (2, 13, 33, 63). These characteristics facilitate contamination of foods and subsequent transmission to humans and have also been shown to contribute to virulence (18, 34, 42, 53, 62).

The role of the general stress regulator sigma B in facilitating growth under stress conditions and in facilitating virulence has recently been of interest with *L. monocytogenes* (2, 3, 11, 34, 41, 56, 63). In addition to sigma B, there are four other putative alternative sigma subunits in the *L. monocytogenes* EGDe genome sequence (21). So far, only one of these, the *rpoN* gene encoding sigma 54, has been studied (1), and its specific physiological role remains unclear.

Based on phylogenetic analysis of genome composition, we have recently shown that a previously unknown sigma factor-like gene, lmo0423 (herein referred to as *sigC*), is part of a three gene region that is carried only by *L. monocytogenes* strains comprising phylogenetic lineage II of the species (65). Lineage II includes serotype 1/2a, one of the most commonly found in foods (43, 47, 48, 59), and it is possible that the *sigC* region contributes to physiological characteristics important to survival in the food production environment. Analysis of compositional bias in *sigC* and the adjacent lmo0422-lmo0421 region is consistent with the region being ancestral to the species and subsequently lost during the divergence of phylogenetic lineage I (65). Indeed, orthologous genes are also found in the *Listeria innocua* genome at the same relative position, further underscoring the conclusion that it is ancestral to the genus.

Sequence alignments of the *sigC*-lmo0421 region indicate that *sigC* encodes a putative member of the extracytoplasmic function (ECF) family of sigma subunits that typically modulate regulons, responding to extracytoplasmic stress and/or mediating extracytoplasmic functions (25). Lmo0422 shows significant similarity to the PadR family of transcription regulators (23), while Lmo0421 encodes a member of the RodA/FtsW family of proteins, which modulate peptidoglycan biosynthesis during the elongation (RodA) and septation (FtsW) phases of cell division (4, 5, 8, 10, 16, 27, 46, 64).

To understand the characteristics conferred upon lineage II

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype and features	Reference or source
Strains		
SM10	*E. coli* conjugation donor; F^- *thi-1 thr-1 leuB6 recA tonA21 lacY1 supE44* (Mu_c^+) λ^-[RP4-2(Tc::Mu)] Km^r Tra^+	52
RM1602	*E. coli* (*dam recA* negative)	W. Haldenwang
TOPO10	*E. coli* host strain F^- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80*lacZ* ΔM15 Δ*lacX74 recA1 deoR araD139* Δ(*ara-leu*)7697 *galU galK rpsL* (Str^r)*endA1 nupG*	Invitrogen
10403S	*L. monocytogenes* wild type	7; N. Freitag
Δ*sigC*	*L. monocytogenes* Δ*sigC* derivative of 10403S	This study
Δlmo0422	*L. monocytogenes* Δlmo0422 derivative of 10403S	This study
Δlmo0421	*L. monocytogenes* Δlmo0421 derivative of 10403S	This study
Δ*sigC*/P_{SPAC}-422E	pPD422E integrated in strain Δ*sigC*	This study
Δlmo0422/P_{SPAC}-422E	pPD422E integrated in strain Δlmo0422	This study
Plasmids		
pDEH21	P_{SPAC} vector	N. Freitag
pPD422E	P_{SPAC}-controlled lmo0422	This study
pPL2	Site-specific integration vector	38
pSKV7	Temperature-sensitive integration vector	54
pKSV7Δlmo0421	Δlmo0421 allele in the pKSV7 vector	This study
pKSV7Δlmo0422	Δlmo0422 allele in the pKSV7 vector	This study
pKSV7ΔsigC	Δ*sigC* allele in the pKSV7 vector	This study
pCR4-TOPO	Cloning vector	Invitrogen

strains by the unique *sigC*-lmo0421 region, we examined the expression and function of these genes in the lineage II serotype 1/2a strain 10403S. Our data are consistent with the three genes comprising an operon that is induced in response to different types of environmental stress, including thermal stress and antibiotics. The operon is transcribed from three different promoters, with the primary heat-inducible promoter depending upon *sigC* itself. Mutations in *sigC* and lmo0422 genes both cause significant sensitivity to high temperature, but only the *sigC* deletion effects expression of the operon. When lmo0422 expression is placed under control of the P_{SPAC} promoter, it complements both the Δlmo0422 and the Δ*sigC* deletions in an IPTG (isopropyl-β-D-thiogalactopyranoside)-dependent manner. Therefore, lmo0422 appears to be a lineage-specific regulator or effector of thermal resistance, while *sigC* comprises a mechanism for thermally regulated expression of lmo0422.

MATERIALS AND METHODS

Strains and plasmids. The bacterial strains used and generated in this study are listed in Table 1. *Listeria monocytogenes* strain 10403S was the parental strain for all of the studies described (7). *L. monocytogenes* strains were propagated in brain heart infusion (BHI) broth at 37°C unless otherwise specified. Ampicillin was added to cultures of *Escherichia coli* strains at 80 μg/ml. Antibiotic selection in *L. monocytogenes* was achieved with 10-μg/ml chloramphenicol, 50-μg/ml kanamycin, or 2-μg/ml erythromycin. The shuttle vector plasmid pKSV7 was used for allele replacements in *L. monocytogenes* (54). Plasmid pPL2 (38) was obtained from R. Calendar and used for site-specific integration of the lmo0422 gene. The P_{SPAC} cassette was derived from pDEH21, a precursor of the pLIV-1 plasmid (14), which was obtained from N. Freitag.

Generation of the Δ*sigC*, Δlmo0422, and Δlmo0421 mutants. In-frame mutations were generated in the lmo0421, lmo0422, and *sigC* genes by using splicing by overlap extension (SOEing). The Δ*sigC* allele was generated with SOEing PCR primers sigCA1 and sigCB (Table 2), which amplify a 570-bp fragment comprising the 5′ end of *sigC*, and primers sigCBC and sigCD, which amplify a 782-bp fragment comprising the 3′ end of *sigC*. Recombinant PCR using the two fragments as templates and the sigCA1 and sigCD primers produced a 224-bp in-frame deletion of *sigC* extending from +753 to +977. In-frame deletions in the lmo0422 and lmo0421 genes were also constructed by a similar approach. The SOEing primers are summarized in Table 2. The Δlmo0422 allele has a 219-bp in-frame deletion and Δlmo0421 mutant has 963-bp in-frame deletion. The deletion junctions for all three alleles were confirmed by sequencing the SOEing PCR products cloned in plasmid pCR4-TOPO (Invitrogen, Carlsbad, CA). Once confirmed, the Δ*sigC* allele was excised as a BamHI-PstI fragment, and the Δlmo0422 and Δlmo0421 alleles were excised as EcoRI-HindIII fragments from the respective pCR4-TOPO vectors and cloned into the temperature-sensitive pKSV-7 shuttle vector (54) to generate plasmids pKSV7Δ421, pKSV7Δ422, and pKSV7ΔsigC.

To recombine Δ*sigC*, Δlmo0422, and Δlmo0421 deletions onto the *L. monocytogenes* chromosome, the pKSV7Δ421, pKSV7Δ422, and pKSV7ΔsigC plasmids were electroporated into *L. monocytogenes* 10403S as previously described (2, 11). Transformants were selected on BHI plates containing 10 μg of chloramphenicol/ml. The transformants were grown at briefly at 30°C and plated at 42°C in BHI plus chloramphenicol to select for integration of the plasmid by homologous recombination. Single colonies with a chromosomal integration were then serially transferred in BHI without chloramphenicol at 30°C to allow the excision and eventual loss of the plasmid. Single colonies were picked and replica plated on BHI and BHI plus chloramphenicol to identify those having undergone excision and loss of the plasmid. Chloramphenicol-sensitive colonies were picked to confirm allelic exchange by both PCR amplification and Southern blot analysis. PCR amplification was performed using the corresponding SOEing PCR primers. Southern blot analyses were performed using a *sigC* internal probe (293 bp) generated by primers Lm423F1 and Lm423R1 and an lmo0421 internal probe (516 bp) generated by primers I421F and I421R (Table 2). The deletion junctions were further confirmed by DNA sequence analysis of the PCR products.

Construction of the Δ*sigC*/P_{SPAC}-lmo0422E and Δlmo0422/P_{SPAC}-lmo0422E strains. The lmo0422 gene was PCR amplified using primers Lm0422Ex1 (5′TGGTCTAGAGCGCTGTTATGGATTTACCCG3′; XbaI site underlined) and primer Lm0422Ex2 (5′TGCTCTAGACTCCCATTTCCTGCATCGCC3′; XbaI site underlined). The amplicon was digested with XbaI and then cloned into the XbaI site in the vector pDEH21. The pDEH21 is fundamentally the same as vector pLIV1 (14) except that a *plcB* gene was cloned in front of the *orfXYZ* locus. The entire P_{SPAC}-lmo0422E cassette was excised from the recombinant pDEH21-422E with KpnI and cloned in the KpnI site of the integration vector pPL2, leading to the generation of pPD422E. The pPD422E plasmid was then transferred to *E. coli* SM10, and the transformants were used to mate pPD422E into *L. monocytogenes* as previously described (38). *L. monocytogenes* strain 10403S was originally selected as a streptomycin-resistant variant of strain 10403 (7), allowing us to directly counterselect against the SM10 *E. coli* donor strain on BHI plates with 50 μg of streptomycin/ml. Transconjugants were isolated on BHI agar containing 50-μg/ml streptomycin and 7.5-μg/ml chloramphenicol. The transconjugants were confirmed by Southern blot analysis, PCR, and sequencing.

RNA analysis by Northern blotting and S1 nuclease analysis. Cells were grown overnight at 37°C in BHI and diluted 1:100 into fresh medium to initiate

TABLE 2. Primers used in this study

Probe	Sequence
Northern blot and S1	
Lm423F1	5′-CAACTTCCCTGTACTTTACGGG-3′
Lm423R1	5′-AGAATCGAACGACAGCGG-3′
I421F	5′-GCCATTAAAGTAGCACTGGG-3′
I421R	5′-CGAGTAGTGCAGCGTAAAC-3′
sigCB	5′-GCGTTAAATCTTCCGCTGTCG-3′
SOEing primers	
sigCA1[a,b]	5′-cgtCTGCAG-TGGTCAATCGCCAATTTAGGC-3′
sigCB	5′-GCGTTAAATCTTCCGCTGTCG-3′
sigCBC	5′-CGACAGCGGAAGATTTAACGCGCGCTGTTATGGATTTACCCG-3′
SigC3D[a,c]	5′-ggtGGATCCTCCCATTTCCTGCATCGC-3′
Lm422A[a,d]	5′-gggAAGCTT-AAAGAATCGAACGACAGCGG-3′
Lm422B	5′-TTTGCTTCGTCTTGCAGTGC-3′
Lm422BC	5′-GCACTGCAAGACGAAGCAAAGACTCTAAGCAACACGGATCACTG-3′
Lm422D[a,e]	5′-tgcGAATTCTAGGATCAAGGCAGCCGC-3′
Lm421A[a,d]	5′-gggAAGCTTACTGCAAGACGAAGCAAAGC-3′
Lm421B	5′-CGCAATAAGGAGCCAATCC-3′
Lm421BC	5′-GGATTGGCTCCTTATTGCGTTTACGCTGCACTACTCGGTC-3′
Lm421D[a,e]	5′-tgtGAATTCTTTATTGTTGCGAGCGGC-3′
P_{SPAC}-lmo0422 construct	
Lm422Ex1[a,f]	5′-tggTCTAGA-GCGCTGTTATGGATTTACCCG-3′
Lm422Ex2[a,f]	5′tgcTCTAGA-CTCCCATTTCCTGCATCGCC-3′

[a] Lowercase nucleotides are nongenomic sequences added to facilitate restriction endonuclease cleavage.
[b] Recognition site for PstI underlined.
[c] Recognition site for BamHI underlined.
[d] Recognition site for HindIII underlined.
[e] Recognition site for EcoRI underlined.
[f] Recognition site for XbaI underlined.

the experiments. The cultures were then grown to an optical density at 600 nm (OD_{600}) of ~0.4, subdivided into equal volumes, and shifted to the following conditions: 48°C for 25 min, addition of acetic acid to a pH of 4.5, addition of ethanol to a final concentration of 4%, addition of NaCl to 4%, addition of penicillin to 50 μg/ml, addition of bile salts mixture (BBL, Maryland) to 0.08%, and addition of nisin to 62.5 IU/ml. After 25 min of each treatment, the cells were harvested and resuspended in 1 ml of TRI-REAGENT (MRC, Cincinnati, OH). The cells were then disrupted by homogenization with glass beads for four 1-min intervals at 4,000 rpm with a Mini-Bead Beater. Total RNA was then extracted from the disrupted cells according to the manufacturer's directions.

Northern blot analyses were carried out using 30 μg of total RNA for each sample. The RNA was electrophoresed in 1% agarose gels with 0.66 M formaldehyde in MOPS buffer (20 mM morpholinepropanesulfonic acid [MOPS], 8 mM sodium acetate, 1 mM EDTA, pH 7.0). The RNA was then transferred to nylon membranes in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). After UV cross-linking, the membranes were prehybridized and then hybridized in solution as described by Church and Gilbert (12) at 70°C and 65°C, respectively. The 293-bp *sigC* internal probe generated by primers Lm423F1 and Lm423R1 (Table 2) and the 516-bp lmo0421 internal probe generated by primers I421F and I421R (Table 2) were used for the Northern blot analysis. Both of the probes span the deleted region of the corresponding mutants. The probes were labeled by incorporation of [γ-^{32}P]dATP. The membranes were washed twice at 65°C, each time for 45 min in 0.1 M Na_2HPO_4, and twice at 55°C, each time for 30 min in 0.2× SSC and 0.15% sodium dodecyl sulfate, before being subjected to autoradiography.

S1 nuclease reactions were performed using single-stranded probes that were synthesized by elongation of primer sigCB on a single-strand template. The primer was end labeled with [^{32}P]ATP and annealed in 1× sequenase buffer (USB, Cleveland, Ohio) to single-strand DNA prepared from an M13mp19 clone carrying a 540-bp fragment of the *sigC* region extending from +151 of the sigC coding region to −389 bp upstream of *sigC*. The sigCB primer was extended on the template with 1 U of Sequenase and 1 mM final concentration of deoxynucleoside triphosphates for 20 min at 42°C. The extended primer-template hybrids were then digested with HindIII, which cuts at −315 relative to *sigC*, giving a final single-strand fragment of 466 bp that extends from −315 to +151. The single-stranded, end-labeled probe molecules were then purified by electrophoresis in a 4.5% denaturing gel using autoradiography to locate the band. The gel slices were crushed and soaked overnight in diffusion buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA [pH 8.0], and 0.1% sodium dodecyl sulfate), followed by phenol extraction and ethanol precipitation. Precipitated probe molecules were redissolved in diethyl pyrocarbonate-treated H_2O and annealed to 50 μg of RNA by being heated to 95°C for 5 min and incubated at 65°C for 1 h. After annealing, 300 μl of S1 nuclease buffer (50 mM sodium acetate [pH 4.5], 280 mM NaCl, 4.5 mM $ZnSO_4$) and 300 U of S1 nuclease were added, and the reaction was incubated at 37°C for 30 min. The reaction products were phenol extracted, ethanol precipitated, redissolved in distilled H_2O, and electrophoresed in 6% denaturing polyacrylamide gels alongside sequencing reactions primed with the same primer.

Thermal sensitivity. Wild-type and mutant strains were compared for their ability to survive lethal temperatures by subjecting mid-logarithmic-phase cultures to sublethal and lethal temperatures and enumerating survivors over a time course. Overnight cultures were grown in BHI at 37°C and diluted 1:200 into fresh BHI. The cells were then grown to OD_{600} of ~0.4, and the culture was shifted to a 60°C shaking water bath. Samples were removed before and after temperature upshift, serially diluted, and plated onto BHI. The number of colonies from duplicate plates of each dilution was enumerated and averaged after 24 h of growth at 37°C. To test for adaptive responses to sublethal upshifts, the diluted overnight cultures were grown to an OD_{600} of ~0.4, followed by transfer of the culture to 48°C for 30 min prior to shifting to 60°C. Samples were removed and enumerated immediately before upshift to 60°C and at 15-min intervals thereafter. Each experiment was repeated at least three times, and the curves shown are representative of the trend in all three experiments.

Thermal sensitivity of P_{SPAC}-lmo0422E strains. Overnight cultures of the 10403S, Δ*sigC*, Δlmo0422, Δ*sigC*/P_{SPAC}-lmo0422E, and Δlmo0422/P_{SPAC}-lmo0422E strains were grown in BHI broth at 37°C (with 7.5-μg/ml chloramphenicol added for the Δ*sigC*/P_{SPAC}-lmo0422E and Δlmo0422/P_{SPAC}-lmo0422E strains). The overnight cultures were diluted 1:200 into fresh BHI without antibiotic and grown to mid-logarithmic phase (OD_{600}, ~0.4). The cultures were then subdivided into equal volumes, and IPTG was added to some of the cultures at concentrations ranging from 1 μM to 2.5 μM as indicated; the cells were incubated an additional 30 min at 37°C. The cultures were then transferred to a 60°C shaking water bath. Samples were removed immediately before upshift and at 30 min intervals after the upshift, diluted, and plated onto BHI plates. Colonies were enumerated from duplicate plates after overnight incubation at 37°C

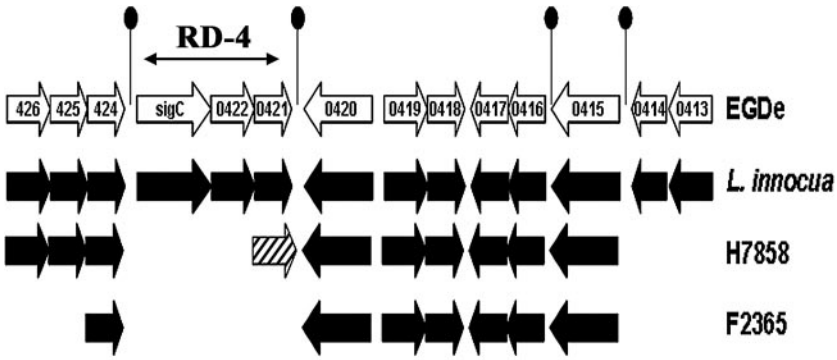

FIG. 1. Organization and synteny of the RD4 region in genomes of *L. monocytogenes* and *L. innocua* strains. Representation of the alignment between the *L. monocytogenes* strain EGDe, *L. innocua*, and *L. monocytogenes* strains H7858 (serotype 4b) and F2365 (serotype 4b) genomes in the RD-4 region is illustrated. Genes are indicated by arrows showing their relative orientations (not drawn to scale). Putative transcription terminators are indicated by a line and filled ellipse. Black arrows represent orthologous genes, stippled arrows indicate nonhomologous genes, and white space indicates absence of the orthologous genes.

and averaged. The experiments were repeated at least three times, and the results shown are representative of the trend in all experiments.

RESULTS

Organization of the *sigC* operon. The *sigC*-lmo0421region (Fig. 1) was originally identified in *Listeria monocytogenes* as a region of genome difference (RD-4) that is specific to lineage II strains (65) and is absent in all lineage I and III strains that have been examined (65; C. Zhang and and A. K. Benson, unpublished data). Comparison of the available *L. monocytogenes* and *L. innocua* genome sequences (21, 45) further reveals that this region is conserved in the *L. monocytogenes* EGDe and *L. innocua* CLIP strains, suggesting it is ancestral to the *Listeria* species and was likely lost early during divergence of lineage I strains. Indeed, genome sequences of the *L. monocytogenes* serotype 4b strains H7858 and F2365 show that the region has undergone further differentiation between different populations of lineage I strains (Fig. 1).

The genes within RD-4 encode a protein that is homologous to the ECF family of sigma subunits of RNA polymerase (Lmo0423, referred to herein as sigma C), a PadR-like protein (Lmo0422), and a protein belonging to the RodA-FtsW family (Lmo0421). In the strain EGD genome sequence, these genes appear to comprise an operon based on two characteristics. First, they are positioned close together; *sigC* and lmo0422 are separated by 2 bp, and the lmo0422 and lmo0421 open reading frames overlap by 4 bp. Second, putative transcription terminators are positioned downstream of lmo0421 (the terminal gene of the operon) and upstream of the initial gene of the operon, *sigC* (Fig. 1). The latter terminator likely terminates upstream transcription from the lmo0424 transcription unit.

To determine experimentally if *sigC*, lmo0422, and lmo0421 comprise an operon and to determine the conditions under which these genes are expressed, the accumulation pattern and size distribution of transcripts containing *sigC* and lmo0421 genes in *L. monocytogenes* strain 10403S were examined by Northern blotting. Probes for these experiments were generated by PCR amplification of internal segments from the lmo0421 and *sigC* open reading frames. RNA was prepared from logarithmically growing cells (OD_{600}, ~0.4) grown at 37°C, as well as logarithmically growing cells that had been subjected to temperature upshift or downshift, osmotic upshift, addition of bile, addition of ethanol, addition of nisin, or addition of penicillin G. As shown in Fig. 2, transcripts were detected under each of the conditions except for the logarithmically growing cells. By visual inspection of the band intensities, the most significant accumulation of RNA occurred in cells that had been subjected to temperature upshift. The lmo0421 and *sigC* probes both detected bands of 2.3 kb each, and the blots showed identical patterns of accumulation under the different environmental conditions (data not shown). Along with the organization of these genes in the genome, the Northern hybridization experiments support the conclusion that *sigC*, lmo0422, and lmo0421 comprise a stress-induced operon.

Sensitivity of the Δ*sigC*, Δlmo0422, and Δlmo0421 mutants to high temperature. Our discovery that the *sigC* operon is expressed under several conditions of environmental stress prompted us to test whether the *sigC*, lmo0422, and lmo0421 genes contribute to survival under different environmental stress conditions. To measure their contribution, in-frame deletions were introduced into the *sigC*, lmo0422, and lmo0421 coding regions; the resulting Δ*sigC*, Δlmo0422, and Δlmo0421 mutants were then subjected to several different stress conditions. In the first set of experiments, the parental and mutant strains were grown at 37°C to mid-logarithmic phase and then

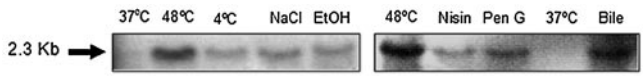

FIG. 2. Northern blot analysis of RNA from the *sigC* operon. RNA was prepared from *L. monocytogenes* strain 10403S grown to mid-logarithmic phase at 37°C in brain heart infusion and 30 min after temperature upshift or downshift (48°C or 4°C), addition of NaCl (to 4%), ethanol (to 4%), nisin (to 62.5 U/ml), penicillin G (50 μg/ml), or bile (0.08%). Total RNA was extracted from the treated cells and 50-μg samples of RNA were loaded in the appropriate lanes. The blots are derived from independent experiments with 37°C and 48°C included on both blots as a point of reference. Both blots shown were probed with a 293-bp segment of the *sigC* gene amplified using the Lm423F1 and Lm423R1 primers (the amplicon extends from +121 to +414 of the lmo0423 coding region).

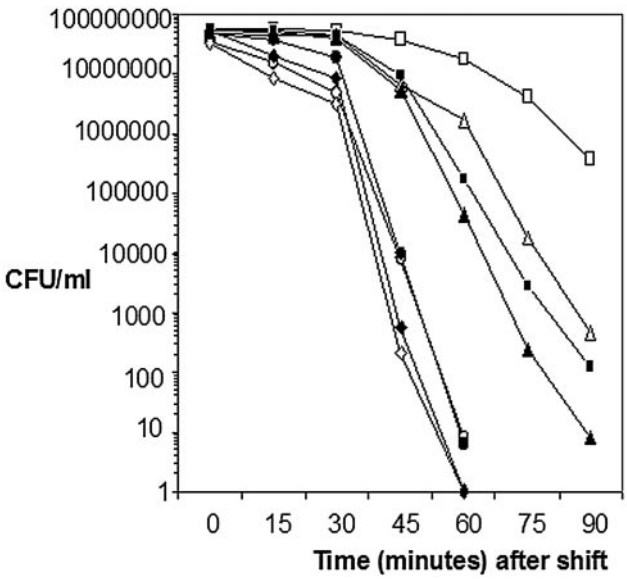

FIG. 3. Survival of *sigC* operon mutants at 60°C. Overnight cultures of the parental strain 10403S and mutant derivatives Δlmo0421, Δlmo0422, and Δ*sigC* were grown in BHI and inoculated 1:200 into fresh BHI and grown to mid-log phase (OD_{600}, ~0.4) at 37°C. Each mid-log phase culture was then divided in half, with one-half remaining at 37°C and the other half transferred to a 48°C water bath (preadaptation) for 20 min. After the 20-min period, both halves were then shifted to a 60°C water bath. Immediately before the shift to 60°C (time zero) and at intervals after the shift, samples from the cultures were withdrawn, serially diluted, and enumerated by plating onto BHI agar. The results shown here are representative of three independent experiments. Symbols: ■, 10403S; □, 10403S preadapted; ▲, Δlmo0421; △, Δlmo0421 preadapted; ●, Δlmo0422; ○, Δlmo0422 preadapted; ◆, Δ*sigC*; ◇, Δ*sigC* preadapted.

shifted to medium containing 10% ethanol, 4% NaCl, or 0.1% bile salts or to medium with a pH of 4.5. Additional cultures of the strains were also grown at 37°C to mod-logarithmic phase and subjected to temperature downshift to 4°C or upshift to 60°C. Survival of the different strains was monitored by enumeration of viable cells at various times after each of the shifts. The parental (wild-type) strain and the Δ*sigC*, Δlmo0422, and Δlmo0421 derivatives all showed similar susceptibilities to high osmolarity, low pH, temperature downshift, ethanol, and bile salt treatments (data not shown).

In contrast to osmolarity, pH, ethanol, or bile, the Δ*sigC* and Δlmo0422 strains showed substantially enhanced death rates when mid-logarithmic cells were upshifted from 37°C to the lethal temperature of 60°C. As shown in Fig. 3, viability of the parental strain assumes a logarithmic decline that began about 45 min after upshift from 37°C to 60°C. If cells of the parental strain were preadapted by a shift to 48°C prior to the 60°C upshift, the parental strain displayed a characteristic adaptive response, assuming a much slower decline in viability and achieving a nearly 5,000-fold enhancement of survival at the later timepoints. Unlike the wild-type strain, viability of the Δlmo0422 and Δ*sigC* strains decreased at a much more rapid rate after the upshift to 60°C. Moreover, these strains also showed no adaptive response when the cells were preadapted to 48°C prior to the upshift to 60°C. The Δlmo0421 mutant showed a nearly indistinguishable death rate from the wild type when upshifted from 37°C to 60°C, but it lost a portion of the adaptive response when the cells were preadapted at 48°C prior to the upshift to 60°C. The level of adaptation of the Δlmo0421 mutant was only 100 fold compared to the nearly 5,000-fold adaptation achieved with the wild type.

Based on the temperature-induced expression of the *sigC* operon, the thermal sensitivity of the Δ*sigC* and Δlmo0422 mutants, and the similarity of *sigC* and lmo0422 to the ECF sigma factors and the *padR* family of transcription regulators (23), respectively, we conclude that *sigC* and lmo0422 likely contribute to regulation of a unique heat shock regulon(s). Because the Δlmo0421 mutation only affects the adaptive response, its role must be less critical than that of *sigC* or lmo0422, at least under the conditions tested.

The *sigC* operon is autoregulated in *L. monocytogenes*. Our discovery that the *sigC* operon is thermally regulated and that *sigC*, lmo0422, and lmo0421 each contribute to thermal resistance prompted us to more closely examine transcription of the *sigC* operon. Alternative sigma factors, particularly members belonging to the ECF family, are often autoregulatory and positioned adjacent to genes that control their function (25). To determine if *sigC* contributes to expression of the operon under conditions of temperature upshift, Northern blots were performed to measure transcript accumulation in the mutant backgrounds before and after upshift to 48°C. Probes for the experiment were prepared from both lmo0421 and *sigC* genes, since transcripts from the operon in the Δlmo0421 mutant will not hybridize to a lmo0421-derived probe and vice versa for *sigC*.

As shown in Fig. 4, transcripts from the *sigC* operon were detected in all of the mutant strains after thermal upshift. The hybridizing bands in the mutant strains are shorter in length, due to the deletions. However, inspection of the band intensity in the Δ*sigC* strain (probed with lmo0421) showed substantially reduced signal after upshift to 48°C. The reduced intensity is particularly notable compared to the signal generated by stripping and reprobing the blot with the *dapE* gene probe, which is not known to be heat shock regulated (39). The reduced transcript accumulation observed in the Δ*sigC* background is consistent with the operon being transcribed from multiple promoters, at least one of which is σ^C dependent and accumulates after temperature upshift.

The transcription start site(s) of the *sigC* operon were next mapped using S1 nuclease assays to identify the 5′ end of transcripts originating upstream of the *sigC* gene under inducing (48°C) and noninducing (37°C) conditions. As shown in Fig. 5A, S1 nuclease protection assays detected transcripts with start sites positioned at −19, −58, and −92 relative to the *sigC* start codon (termed P1, P2, and P3, respectively). Alignment of the sequences upstream of these start sites (Fig. 5E) did not reveal similarity to any known promoter elements of *L. monocytogenes*. To determine if any of the transcripts depend on *sigC*, lmo0422, or lmo0421, S1 nuclease protection assays were used to measure the relative concentrations of transcripts from the wild type and the mutant strain before and after upshift to 48°C. As shown in Fig. 5B, the Δ*sigC* mutation eliminates only the proximal transcript at −19, indicating that this transcript is σ^C dependent. The other two transcripts remained intact with the Δ*sigC* mutant, indicating that only the proximal P1 promoter depends on *sigC*. These results are consistent with the

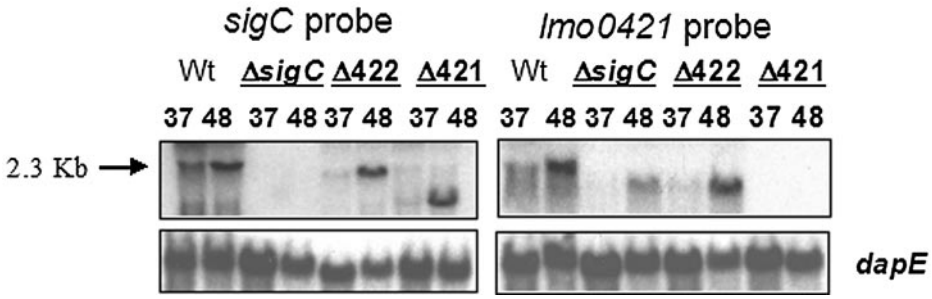

FIG. 4. Northern blot analysis of *sigC* operon RNA from the wild type and Δlmo0421, Δlmo0422, and Δ*sigC* mutants. Overnight cultures of the wild-type 10403S and derived Δlmo0421, Δlmo0422, and Δ*sigC* mutants were diluted 1:200 into fresh BHI, grown to mid-log phase (OD_{600}, ~0.4), and shifted to 48°C. RNA was extracted from samples harvested before (37) and 30 min after (48) the shift, and 50 μg of RNA was electrophoresed in formaldehyde-agarose gels and transferred to nylon membranes. The membranes were probed with labeled PCR products derived from internal segments of the *sigC* (+121 to +414 of the lmo0423 coding region) or lmo0421 (+735 to +1191 of the lmo0421 coding region) genes. The observed hybridizing transcripts are shorter in length in the mutant strains due to the length of the individual deletions (*sigC* deletion = 224 bp; lmo0422 deletion = 219 bp; lmo0421 deletion = 963 bp). To confirm equal loading of RNA, the blots were stripped and reprobed with a probe from a temperature-independent housekeeping gene, lmo0265 (*dapE*) gene (extending from +190 to +319), which encodes succinyl-diaminopimelate desuccinylase (39).

Northern blot data of Fig. 4 and show that the *sigC* operon is transcribed from multiple promoters, one of which is σ^C dependent.

In contrast to the Δ*sigC* mutation, the Δlmo0422 and Δlmo0421 deletions did not negatively effect accumulation of the *sigC*-dependent transcript from the P1 promoter (Fig. 5C). This finding was somewhat surprising, given the similarity in phenotypes of the Δ*sigC* and Δlmo0422 mutants. Thus, if σ^C is directly responsible for recognition of the P1 promoter, then σ^C activity itself is not substantially negatively affected by the Δlmo0422 or Δlmo0421 mutation.

Ectopic expression of lmo0422 can complement the *sigC* thermal resistance phenotype. The fact that the temperature-inducible, *sigC*-dependent transcript from P1 accumulates normally in the Δlmo0422 mutant implies that *sigC* function is intact in the lmo0422 mutant background. Therefore, a simple explanation for the similarity in thermal death phenotypes of the Δ*sigC* and Δlmo0422 mutants is that lmo0422 is the actual

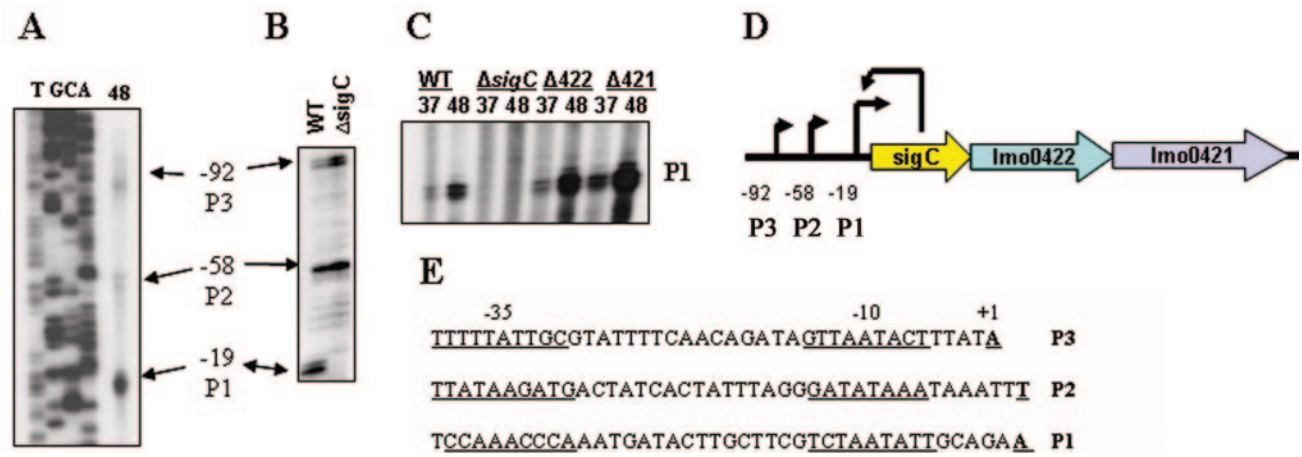

FIG. 5. S1 nuclease protection mapping of transcripts from the *sigC* operon. (A to C) A single-stranded S1 nuclease probe was generated by elongation of the CEX 1 primer (+172 to +151 of *sigC*) that had been end labeled with [γ-^{32}P]ATP using T4 kinase. The primer was extended on a single-strand template of an M13 mp19 clone carrying the promoter region using Sequenase. The extension products were digested with HindIII at position −316 relative to the transcription start site, and the end-labeled single-stranded probe molecules (extending from +172 to −316 relative to the *sigC* start codon) were purified by electrophoresis in a denaturing gel. The purified probe was then mixed with 50 μg of RNA from wild-type or mutant cells, heated to 95°C, annealed at 65°C, and treated with S1 nuclease as previously described (35). The digestion products were dissolved in loading buffer and electrophoresed alongside a sequencing ladder primed with the CEX1 primer. (A) RNA was derived from mid-logarithmic-phase wild-type cells that had been shifted from 37°C to 48°C for 20 min. The positions of the three prominent bands relative to the start codon of the *sigC* gene are indicated to the right. (B) S1 nuclease reactions were performed on RNA extracted from mid-logarithmic-phase cells of the wild-type and Δ*sigC* strains that had been shifted from 37°C to 48°C for 20 min. The lengths of the three transcripts are indicated to the left of the image. (C) RNA was extracted from cultures of mid-logarithmic-phase wild-type, Δlmo0421, Δlmo0422, and Δ*sigC* cells at 37°C (37) and 20 min after upshift to 48°C (48). Only the P1 transcript is shown. (D) Relative map of the operon with the three transcription start sites indicated upstream of the *sigC* gene. The map is not to scale. (E) Putative promoter sequences upstream of the transcription start sites. Putative −10 and −35 region sequences are underlined.

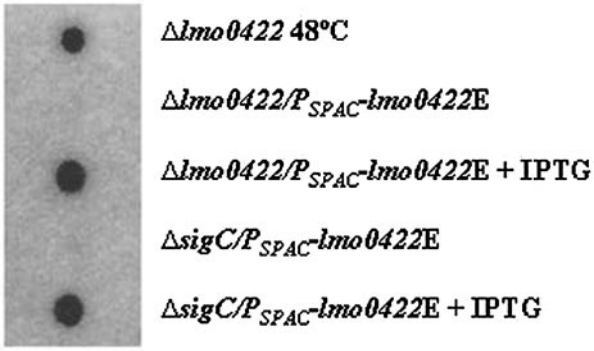

FIG. 6. Dot blot analysis of lmo0422 transcripts from P_{SPAC}-422E strains. Cultures of the Δlmo0422/P_{SPAC}-422E and Δ*sigC*/P_{SPAC}-422E were grown at 37°C to mid-logarithmic phase (OD_{600}, ~0.4), and RNA was extracted from cells harvested prior to or 30 min after addition of IPTG to a final concentration of 1 mM. As a control, RNA was also extracted from mid-logarithmic-phase cells of the Δlmo0422 strain 30 min after upshift of the culture to 48°C. Samples of 20 μg of RNA (each) were then spotted onto nylon, cross-linked by UV light, and then hybridized with a ^{32}P-labeled PCR product derived from the lmo0422 coding region, which includes 180 bp of the 3′ end of *sigC*, the entire lmo0422 coding region, and 182 bp of the 5′ end of lmo0421.

thermal resistance effector-regulator and that *sigC* is only necessary because it is required for induction of lmo0422 under conditions of temperature upshift. To test this hypothesis, we introduced an IPTG-inducible copy of lmo0422 into the Δ*sigC* and Δlmo0422 mutants to determine if ectopic expression of lmo0422 could complement the temperature sensitivity of the Δ*sigC* mutant. An intact copy of the lmo0422 gene was placed under the control of the IPTG-inducible P_{SPAC} promoter as described in Materials and Methods and inserted at a unique location in the genome using the pPL2 system of Lauer et al. (38). Dot blot analysis, shown in Fig. 6, confirmed that expression of lmo0422 in the Δlmo0422/P_{SPAC}-lmo0422E and the Δ*sigC*/P_{SPAC}-lmo0422E strains was IPTG inducible.

When the thermal resistance profiles of the Δlmo0422 and Δlmo0422/P_{SPAC}-lmo0422E strains were compared, we observed that, as expected, addition of IPTG allowed the P_{SPAC}-lmo0422E construct to complement the Δlmo0422 deletion (Fig. 7A). Importantly, titration experiments showed that full complementation only occurred at low IPTG concentrations (1 μM), implying that tightly coordinated expression of lmo0422 is important for its function. As with the Δlmo0422/P_{SPAC}-lmo0422E strain, we also observed that IPTG-dependent induction of lmo0422 expression in the Δ*sigC*/P_{SPAC}-lmo0422 strain restored its thermal resistance phenotype to wild-type levels (Fig. 7B). Moreover, complementation of the Δ*sigC* thermal resistance phenotype was also dependent on IPTG concentration; full complementation was only observed at 2.5 μM and was not complete at concentrations of >5 μM or <2.5 μM.

Because IPTG-dependent expression of lmo0422 can overcome the temperature sensitivity of the Δ*sigC* mutation, the role of *sigC* in thermal resistance must be to provide temperature-inducible expression of lmo0422. The fact that overexpression of either *sigC* or lmo0422 was detrimental to the thermal resistance phenotype underscores the importance of

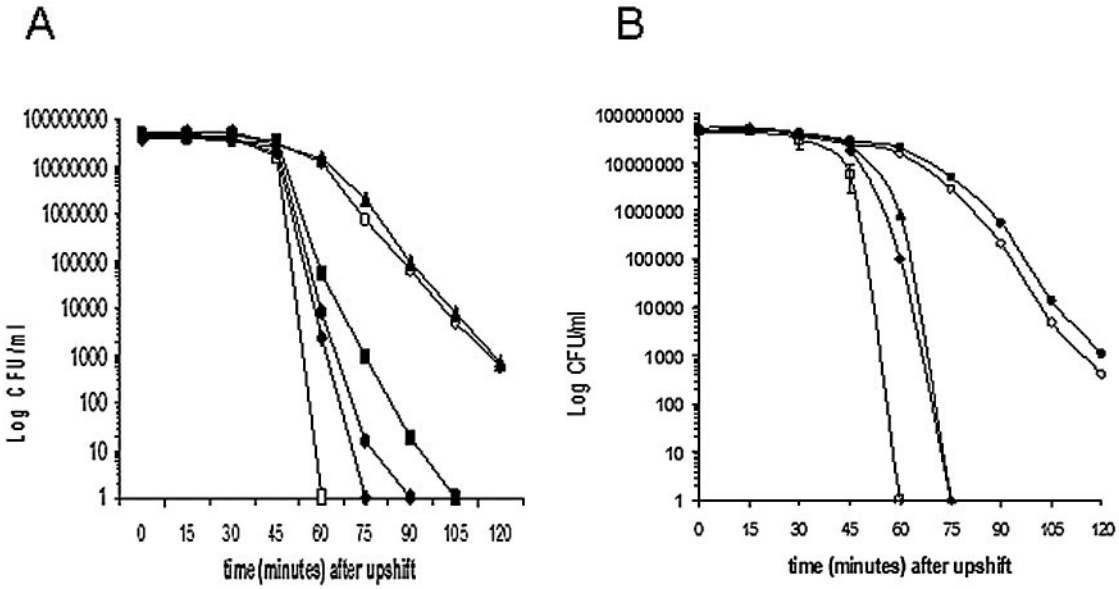

FIG. 7. Ectopic expression of lmo0422 rescues the Δlmo0422 and Δ*sigC* thermal sensitivity phenotypes. Cells of the wild-type, Δlmo0422, Δ*sigC*, Δlmo0422/P_{SPAC}-422E, and Δ*sigC*/P_{SPAC}-422E strains were grown overnight in BHI, diluted 1:200 in fresh BHI, and grown to mid-logarithmic phase (OD_{600}, ~0.4). The cultures were then subdivided into several equal portions, and IPTG was then added at 0, 0.5 μM, 1 μM, 2.5 μM, and 5 μM concentrations to the cultures of the P_{SPAC}-lmo0422E strains. After 30 min, samples of the cultures were removed and enumerated by serial dilution and plating. The remaining portions of the cultures were then transferred to 60°C. Samples were removed at various times after temperature upshift and enumerated by serial dilution and plating. (A) ○, 10403S; □, Δlmo0422; ◆, Δlmo0422/P_{SPAC}-422E; ■, Δlmo0422/P_{SPAC}-422E plus 0.5 μM IPTG; ▲, Δlmo0422/P_{SPAC}-422E plus 1 μM IPTG; ●, Δlmo0422/P_{SPAC}-422E plus 2.5 μM IPTG. (B) ○, 10403S; □, Δ*sigC*; ◆, Δ*sigC*/P_{SPAC}-422E; ▲, Δ*sigC*/P_{SPAC}-422E plus 1 μM IPTG; ●, Δ*sigC*/P_{SPAC}-422E plus 2.5 μM IPTG. Data shown are representative of three or more independent experiments.

appropriate control of lmo0422 expression by *sigC*. Thus, we conclude that lmo0422 is the actual effector or regulator of the heat resistance regulon and we therefore propose to name this gene *lstR* (for lineage-specific thermal regulator).

DISCUSSION

Several independent studies have observed that *L. monocytogenes* strains from the three phylogenetic lineages are isolated at different frequencies from food, environmental, and clinical samples (9, 19, 22, 32, 61, 66). These findings have been regarded as evidence that the three evolutionary lineages (and indeed the serotypes which are distributed in a monophyletic fashion among the lineages) possess unique virulence or transmissibility characteristics. Recent studies of genome diversity among populations of *Listeria monocytogenes* have further shown that transcription factors and genes associated with the cell surface (encoding cell surface proteins or biosynthetic pathways of extracellular material) represent the largest classes of lineage-specific genes (17, 28, 31, 45, 65), implying that these genes could be responsible for lineage-specific traits.

In this study, we have shown that the lineage II-specific *sigC*, *lstR*, and lmo0421 genes comprise an operon and that *lstR* is important for thermal resistance characteristics of the lineage II serotype 1/2a strain 10403S. Thermal resistance characteristics are primarily conferred by temperature-inducible sets of genes, collectively referred to as heat shock genes. There are three major heat shock regulatory pathways that appear to be conserved in *L. monocytogenes* and the related species *Bacillus subtilis*. The primary pathway is controlled by HrcA. The *hrcA* gene is positioned as the promoter-proximal member of the *dnaK* operon in *B. subtilis* and *L. monocytogenes* (24, 50), and HrcA functions to reduce transcription of heat shock genes at moderate growth temperatures by binding to CIRCRE elements in the promoter regions of its target genes (50). A secondary pathway (referred to as class II genes) is controlled by an alternative sigma factor, σ^B, which, like the putative σ^C, is induced under a wide variety of environmental and physical stress conditions including heat shock (2, 6, 34, 60, 63). The third pathway is controlled by CtsR, the proximal gene of the *clp* operon in *B. subtilis* and *L. monocytogenes* (33, 44). Like HrcA, CtsR is a DNA-binding protein that acts to negatively regulate expression of its target genes in a temperature-dependent manner (36). In both *L. monocytogenes* and *B. subtilis*, at least one additional class of thermally regulated genes exist, exemplified by the *lon* protease gene, for which no regulatory system has been identified (51).

Our results from this report have now clearly defined yet another regulatory system, σ^C-LstR, which participates in thermally regulated gene expression in phylogenetic lineage II *L. monocytogenes* populations. The *lstR* gene encodes a PadR-like protein whose function is necessary for full thermal resistance, presumably by controlling transcription of a fifth class of heat shock genes. *lstR* is embedded within an operon that carries a positive regulator of its transcription (*sigC*) and a gene whose function contributes to the adaptive phase of the response (lmo0421). Although σ^C is the primary positive regulator of the operon via the thermally regulated P1 promoter, the *sigC* gene appears to be dispensable for thermal resistance apart from its role in *lstR* expression. Therefore, σ^C may comprise a specialized device for controlling *lstR* expression. As shown by ectopic expression of *lstR* (Fig. 7), appropriate expression of *lstR* is absolutely critical for its function. Thus, σ^C activity is likely to be tightly governed in the cell.

The contribution of σ^C-LstR to thermal resistance in the lineage II strain 10403S background is substantial. We are in the process of measuring the relative contribution of the different heat shock responses by comparing the thermal sensitivity of isogenic strains carrying deletions in *lstR*, *sigC*, and *sigB* and strains carrying IPTG-inducible versions of *hrcA* and *ctsR* in the 10403S background. Preliminary studies of the Δ*sigC* and Δ*sigB* single and double mutants indicate that Δ*sigC* mutants are much more susceptible to thermal death than *sigB*:Km^r mutants (Zhang and Benson, unpublished). Moreover, Δ*sigC sigB*:Km^r double mutants are even more sensitive than either single mutant, underscoring that the σ^C-LstR and σ^B pathways are independent. No formal, systematic comparisons of thermal resistance characteristics and pathways of lineage I and lineage II strains have been reported. Therefore, it remains to be determined how lineage I strains mount adaptive responses to high temperatures.

Distribution of *lstR* and the *sigC* operon in *Listeria* and the bacilli. LstR is a member of a family of proteins sharing similarity to PadR. Originally discovered in *Lactobacillus plantarum*, PadR negatively controls transcription of genes involved in utilization of phenolic acids, presumably by acting directly at *cis*-acting sequences (23). Although several paralogous members of the PadR family can be found in the genomes of *Listeria* and *Bacillus* species, only lineage II *Listeria monocytogenes*, *Listeria innocua*, *Bacillus cereus*, *Bacillus anthracis*, *Bacillus thuringiensis*, and *Bacillus halodurans* strains carry an orthologous *lstR* gene. In each instance, *lstR* is positioned between *sigC* and lmo0421 orthologues, indicating that the operon is a conserved element.

In the *L. monocytogenes* and *L. innocua* genomes, the *sigC* operon is positioned between a glucose permease and a conserved hypothetical protein with hydrolase-like domains. The synteny implies that the operon was likely ancestral to the genus *Listeria*, while absence of the operon in lineage I and lineage III *L. monocytogenes* strains is a consequence of gene loss during divergence of these populations. In the sensu lato members of the *B. cereus* subgroup (*B. cereus*, *B. anthracis*, and *B. thuringiensis*), the operon is positioned adjacent to the *glpD* and *glpK* genes while in *B. halodurans*, the operon is positioned in a third genomic region, suggesting that it has undergone rearrangement in the genome or that it was acquired independently in *Listeria* and the two different *Bacillus* lineages. It is interesting to note that like the situation with *L. monocytogenes* phylogenetic lineages, the *sigC* region in the *B. cereus* ATCC14579 strain has apparently been lost, but it is conserved in the *B. cereus* ATCC10987 and ZK strains, suggesting that there may be some propensity for the region to be lost in certain subpopulations of a species.

Lmo0421 and lineage-specific expansion of *rodA-ftsW* genes. The function of lmo0421, the distal gene of the *sigC* operon, remains enigmatic. The Δlmo0421 mutant had a normal death rate after direct upshift to lethal temperature but had lost a portion of its adaptive response when first upshifted to a sublethal temperature (Fig. 3). This phenotype is consistent with Lmo0421 representing a unique pathway to controlling *lstR*

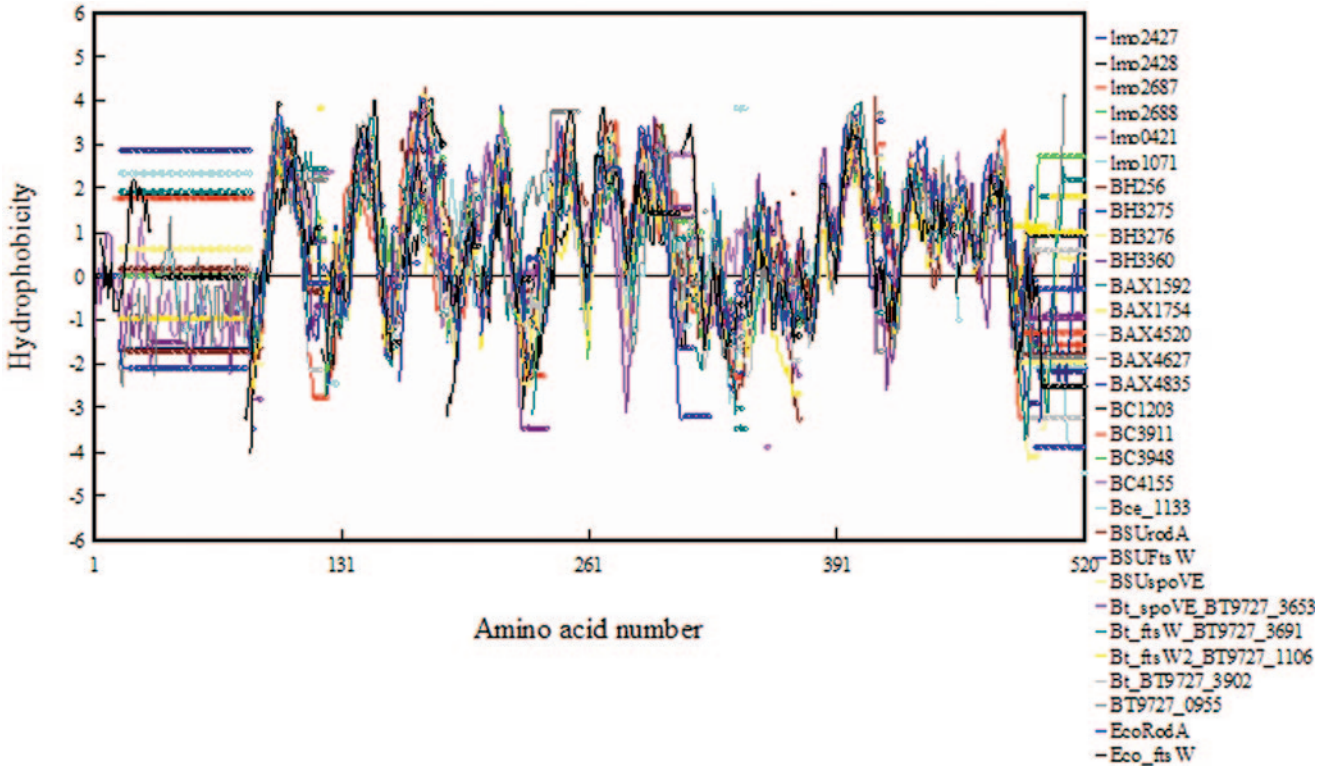

FIG. 8. Multiple hydropathy alignment of RodA-FtsW-like proteins from *L. monocytogenes* and related species. Proteins belonging to the RodA-FtsW family were identified from the genomes of *L. monocytogenes* strain EGDe (Lmo), *Bacillus subtilis* 168 (BSU), *Bacillus cereus* ATCC10987 (BC), *Bacillus anthracis* Sterne (BAX), *Bacillus thuringiensis* serovar konkukian strain 97-27 (BT), *Bacillus halodurans* C-125 (BH), and *Escherichia coli* MG1655 (ECO). Multiple alignment was performed using CLUSTAL W (57) using a Blosum scoring matrix, and multiple hydrophobicity plots based on the alignment were generated based on the methods of Hopp and Woods (29, 37) as implemented in the DNAman package. The plot shown was developed using a window size of eight amino acid residues. Each colored plot and the corresponding protein are shown to the right of the plot. Circles in the plot indicate gaps in the alignment or start-stop positions.

expression or function. In addition to its unique effect on temperature resistance, the Lmo0421 protein has some rather remarkable structural characteristics. Lmo0421 shares substantial similarity to the RodA-FtsW family of proteins, which modulates cell wall biosynthesis during the elongation phase (RodA) and the septation phase (FtsW) of cell division (4, 5, 15, 27). Because of the similarity to this family, we initially examined the Δlmo0421 strain for morphological defects but were unable to observe defects under any of the conditions tested (room temperature, 37°C, or 45°C). One reason for the absence of morphological phenotype could be the redundancy of the RodA-FtsW family of proteins in *L. monocytogenes*. Most rod-shaped species carry a single copy of the *rodA* and *ftsW* genes, and they are essential for normal morphology in *E. coli* and *B. subtilis* (27, 55). *B subtilis* carries an additional member of this family, *spoVE*, which appears to be dedicated to wall biosynthesis in the developing endospore (26). BLAST analyses of the *L. monocytogenes* genome sequences show that Lineage II *L. monocytogenes* and *L. innocua* carry six paralogus members of the RodA-FtsW family (including Lmo0421 and its orthologue in *L. innocua*, Lin0441). Remarkably, *B. anthracis*, *B. cereus*, *B. thuringiensis*, *B. halodurans*, and *Bacillus licheniformis* also share this phenomenon of carrying multiple copies of RodA-FtsW genes. With the exception of *B. licheniformis*, these are also the only genomes which carry orthologous *sigC* operons, begging the question of whether functional linkage exists between these systems. Although *B. licheniformis* does not contain a *sigC* operon orthologue, it is interesting to note that it carries a cell wall peptidase-like gene adjacent to the *sigX* operon and that *sigX* is known to confer thermal resistance (30).

Is Lmo0421 a unique member of the RodA-FtsW family that somehow links gene expression and cell wall biosynthesis? Multiple alignment of the individual hydrophobicity plots of the RodA-FtsW proteins from *Listeria* and *Bacillus* species reveals that indeed Lmo0421 and all of the additional RodA-FtsW proteins share striking topological characteristics with the known *B. subtilis* and *E. coli* RodA and FtsW proteins (Fig. 8). Moreover, cluster analysis (Fig. 9) shows that Lmo0421 and the orthologous proteins Lin0441, BH3360, BA1592, BT0955, and BC1133 (derived from the *sigC* operons of these organisms) comprise a single cluster supported by a significant bootstrap score. Thus, the structural characteristics of Lmo0421 are consistent with it being a unique member of the RodA-FtsW family. The phenotype, operon organization, and structural characteristics of Lmo0421 make it tempting to speculate that the Lmo0421 provides a unique mechanism for linking cell wall biosynthesis to gene expression, perhaps through σ^C and LstR. It will be important to further characterize function of σ^C, LstR, and Lmo0421 and to understand the characteristics this

FIG. 9. Phylogenetic analysis of Lmo0421 and other RodA-FtsW-like proteins from *L. monocytogenes* and related species. Proteins belonging to the RodA-FtsW family were identified from the genomes of *L. monocytogenes* strain EGDe (Lmo), *Bacillus subtilis* 168 (BSU), *Bacillus cereus* ATCC10987 (BC), *Bacillus anthracis* Sterne (BAX), *Bacillus thuringiensis* serovar konkukian strain 97-27 (BT), and *Bacillus halodurans* C-125 (BH) using BLAST analyses. The set of proteins was then subjected to bootstrap analysis (20) using a neighbor-joining search (49). Significant bootstrap values (>50%) from 10,000 repetitions are indicated on the branches of the relevant nodes. Proteins from the genome of each species are filled with the same color. Nodes corresponding to the *B. subtilis* RodA (purple), *B. subtilis* SpoVE (green), and the *L. monocytogenes* Lmo0421 proteins (red) are colored to highlight putatively functionally similar proteins.

specialized set of proteins confers on the phylogenetic lineage II populations of *Listeria monocytogenes*.

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