σ^B - and PrfA-Dependent Transcription of Genes Previously Classified as Putative Constituents of the *Listeria monocytogenes* PrfA Regulon

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Abstract

Mounting evidence suggests that σ^B and PrfA coregulate transcription of multiple genes in *Listeria monocytogenes*, therefore, the relative contributions of σ^B and PrfA to transcript levels of genes identified previously as differentially regulated by PrfA were measured. Group I genes are recognized virulence genes that are positively regulated by PrfA; group II genes were reported previously as negatively regulated by PrfA; and multiple group III genes were proposed to be coregulated by σ^B and PrfA. Transcript levels for selected genes were measured by quantitative reverse transcriptase polymerase chain reaction (RT-PCR) in *L. monocytogenes* 10403S as well as in otherwise isogenic $\Delta sigB$, $\Delta prfA$, and $\Delta sigB\Delta prfA$ strains grown under conditions demonstrated to induce either PrfA activity (0.2% activated charcoal) or both PrfA and σ^B activity (stationary phase). Although the Group I gene *plcA* was positively regulated by PrfA, transcript levels for the group II genes *lmo0278* and *lmo0178* were not affected by the *prfA* deletion. While the *sigB* deletion significantly affected transcript levels for the selected group III genes (i.e., *lmo0596*, *lmo0654*, *bsh*, and *opuCA*), with lower transcript levels in the $\Delta sigB$ strains under all conditions tested, transcript levels for these genes were not significantly affected by the *prfA* deletion. Our results suggest that the regulatory interactions between PrfA and σ^B contribute to PrfA's predominant role as a direct regulator of virulence genes critical for invasion and intracellular survival in *L. monocytogenes* 10403S, while σ^B regulates a wider range of virulence and stress response genes.

Introduction

THE GRAM-POSITIVE BACTERIUM *Listeria monocytogenes*, which can be found in many natural environments, can cause severe invasive disease in humans and many other animal species (Gellin and Broome, 1989; Mead *et al.*, 1999; Vazquez-Boland *et al.*, 2001; Roberts and Wiedmann, 2003). To fully understand how foodborne pathogens such as *L. monocytogenes* are transmitted through food systems, and hence, to enable development of effective control strategies for these pathogens, bacterial mechanisms that enable the switch between survival in the environment and survival in a

host must be identified and characterized. In *L. monocytogenes*, mounting evidence suggests that the transformation between saprophyte and pathogen is mediated through complex regulatory pathways that modulate the expression of multiple genes, including virulence factors, in response to environmental signals (Gray *et al.*, 2006). While initial data (Kazmierczak *et al.*, 2003; Kim *et al.*, 2005; McGann *et al.*, 2007) suggest that two regulators, positive regulatory factor A (PrfA) and the alternative sigma factor σ^B , are critical for this switch, our understanding of their interactions is still limited.

A number of *L. monocytogenes* virulence genes and their specific functions in the intracellular

infection process have been identified and characterized (Portnoy *et al.*, 1992; Vazquez-Boland *et al.*, 2001). One of these virulence genes, designated *prfA*, encodes PrfA. PrfA plays a central role in regulating the expression of many virulence genes that are critical for *L. monocytogenes* entry into host cells and its spread into neighboring cells (Leimeister-Wachter *et al.*, 1990; Chakraborty *et al.*, 1992; Ireton *et al.*, 1999). PrfA shares sequence and structural similarities with the cAMP receptor protein (CRP) in *Escherichia coli* (Lampidis *et al.*, 1994). Specifically, the helix-turn-helix DNA binding domains are highly conserved between CRP and PrfA (Sheehan *et al.*, 1996; Kreft and Vazquez-Boland, 2001). PrfA recognizes and binds to a 14 bp palindromic DNA sequence called the PrfA box, which is typically located ~41 bp upstream of the transcriptional start site of PrfA-regulated genes (Mengaud *et al.*, 1991; Freitag *et al.*, 1992, 1993; Sheehan *et al.*, 1996). Post-transcriptional modifications of the wildtype PrfA protein have been shown to affect transcriptional activity of this protein in a growth medium-dependent manner (Ripio *et al.*, 1996; Renzoni *et al.*, 1997).

A whole genome macroarray-based characterization of gene expression patterns in *L. monocytogenes* strains EGDe, P14, P14-A (which contains a *prfA** allele that encodes a constitutively active PrfA*) and their isogenic $\Delta prfA$ derivatives, grown under PrfA-inducing or -repressing conditions, resulted in identification of 73 putative PrfA-regulated genes, which were then classified into three groups (Milohanic *et al.*, 2003). Group I contains 12 genes that are positively regulated by PrfA, including 10 recognized virulence genes; transcription of group I genes was activated by charcoal and repressed by cellobiose, conditions previously reported to activate or inhibit PrfA activity, respectively (Ripio *et al.*, 1996; Renzoni *et al.*, 1997). The eight group II genes appeared to be negatively regulated by PrfA, suggesting the possibility of a transcriptional repressor function for PrfA. While the majority of the 53 group III genes were reported to be positively regulated by both PrfA and PrfA*, PrfA-dependent transcription of many group III genes was neither repressed by cellobiose nor induced by the presence of activated charcoal. Interestingly, putative σ^B -dependent promoters were identified upstream

of a number of group III genes, suggesting the possibility of σ^B -PrfA coregulation of these genes in *L. monocytogenes* (Milohanic *et al.*, 2003).

The alternative general stress response sigma factor σ^B directs transcription of genes contributing to survival under multiple environmental stress conditions in gram-positive bacteria (Hecker and Völker, 2001). In *L. monocytogenes*, expression of σ^B -dependent genes is induced by exposure to a number of conditions, including low pH and elevated osmolarity, which mimic conditions encountered during gastrointestinal infection (Sue *et al.*, 2004; Garner *et al.*, 2006). Previous studies have shown that *L. monocytogenes* σ^B also contributes to virulence (Wiedmann *et al.*, 1998; Nadon *et al.*, 2002; Kazmierczak *et al.*, 2003, 2006; Kim *et al.*, 2004) by regulating transcription of PrfA-dependent genes (Kazmierczak *et al.*, 2003, 2006; Kim *et al.*, 2004, 2005) as well as of *prfA* itself (Nadon *et al.*, 2002; Schwab *et al.*, 2005). Multiple lines of evidence thus suggest that (i) both PrfA and σ^B contribute to transcriptional regulation of numerous genes, including those contributing directly to *L. monocytogenes* virulence, and (ii) PrfA and σ^B are part of a regulatory network that is important for virulence and transmission of *L. monocytogenes* (Kazmierczak *et al.*, 2003).

While the σ^B and PrfA regulons in *L. monocytogenes* have been investigated previously using macro- and microarray-based approaches, these studies compared transcript levels only between a parent strain and either an isogenic $\Delta sigB$ strain (Kazmierczak *et al.*, 2003) or an isogenic $\Delta prfA$ strain (Milohanic *et al.*, 2003). The objective of the present study was to examine the regulatory network between PrfA and σ^B by using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) to measure relative transcript levels for genes previously classified into the PrfA regulon in isogenic $\Delta sigB$, $\Delta prfA$, and $\Delta sigB \Delta prfA$ strains grown under conditions that either activate both σ^B and PrfA or PrfA alone.

Materials and Methods

Bacterial strains

L. monocytogenes 10403S and its derivatives were used throughout this study (Table 1). Stock cultures were stored at -80°C in brain heart infusion (BHI; Difco Laboratories, Sparks, MD)

TABLE 1. STRAINS USED IN THIS STUDY

Strains	Genotype	Reference
10403S	Parent strain; serotype 1/2a	Bishop and Hinrichs, 1987
FSL A1-254	$\Delta sigB$	Wiedmann <i>et al.</i> , 1998
FSL B2-046 ^a	$\Delta prfA$	McGann <i>et al.</i> , 2007
FSL B2-068	$\Delta sigB\Delta prfA$	McGann <i>et al.</i> , 2007

^aThis strain, which was first reported by Wong and Freitag (2004), is designated herein as in McGann *et al.* (2007).

broth with 15% glycerol. Prior to each experiment, stock cultures were streaked onto BHI plates and incubated for 24 hours at 37°C to obtain isolated colonies for subsequent experiments.

Growth to log and stationary phase

For each experimental culture, an isolated colony was used to inoculate 5 mL of BHI broth, which was then incubated overnight at 37°C with shaking (220 rpm); overnight (12 hours) cultures were then inoculated 1:100 into 5 mL fresh BHI broth, followed by growth at 37°C with shaking (220 rpm) to an optical density representing log-phase cells ($OD_{600} = 0.4$). To further synchronize cells, the log-phase culture was diluted again (1:100) into a side arm flask containing 50 mL fresh BHI that had been prewarmed to 37°C. When cells reached $OD_{600} = 0.4$, 4 mL of culture were added to 8 mL of RNAProtect™ (Qiagen, Valencia, CA) to preserve cells for RNA isolation as described below. The remainder of the culture was incubated until cells reached stationary phase (defined as an $OD_{600} = 2.0$) followed by RNAProtect™ treatment as described above.

Exposure of *L. monocytogenes* to activated charcoal

Previous studies have shown that PrfA can be present in two functional states (weakly or highly active [Renzoni *et al.*, 1997]) and that exposure of *L. monocytogenes* cultures to activated charcoal results in increased transcription of PrfA-regulated virulence genes (Ripio *et al.*, 1996). Therefore, to activate PrfA-mediated transcription, in one set of experiments, *L. monocytogenes* 10403S and its isogenic mutants ($\Delta sigB$, $\Delta prfA$, and $\Delta sigB\Delta prfA$) were grown to log phase and then exposed to a final concentration of 0.2%

activated charcoal for 2 hours at 37°C as previously validated and reported (McGann *et al.*, 2007). Briefly, BHI containing 0.4% (w/v) activated charcoal was prepared by adding 160 μ L of a sterile 5% aqueous activated charcoal stock solution into 2 mL sterile BHI. Cells were grown to log phase ($OD_{600} = 0.4$) in 50 mL fresh prewarmed BHI as described above and 2 mL of log phase cultures were added to either 2 mL fresh, prewarmed BHI or to 2 mL prewarmed BHI containing 0.4% activated charcoal (to yield a final concentration of 0.2% activated charcoal). After 2 hours at 37°C with shaking, 8 mL of RNAProtect™ was added to each tube to stabilize the RNA for subsequent RNA purification, as detailed below. Quantitative RT-PCR analysis of transcript levels for *plcA*, which is positively regulated by PrfA (Mengaud *et al.*, 1991; Bohne *et al.*, 1994; Sheehan *et al.*, 1995; Lalic-Multhaler *et al.*, 2001), validated that this charcoal exposure protocol increased PrfA-dependent transcription by >1 log (Fig. 1).

RNA purification

Following addition of RNAProtect™, the culture was held for 5 minutes at room temperature, then cells were collected by centrifugation at 5000 g for 5 minutes. Total RNA was extracted as previously described (Kazmierczak *et al.*, 2006) except that contaminating DNA was removed using TurboDNase (Ambion Inc., Austin, TX) according to the manufacturer's instructions. Optical densities (OD) at 230, 260, and 280 nm were determined for the purified RNA using the NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). Purity of extracted RNA was evaluated using $OD_{260}/OD_{280\text{ nm}}$ and $OD_{260}/OD_{230\text{ nm}}$ ratios, which assess protein and organic substance contamination, respectively. RNA concentrations

FIG. 1. Transcript levels for selected group I, II, and III genes in the *L. monocytogenes* parent strain 10403S and in isogenic $\Delta sigB$, $\Delta prfA$, and $\Delta sigB \Delta prfA$ strains exposed to either (A) BHI + 0.2% activated charcoal or (B) BHI alone. Normalized log copy numbers shown on the Y-axis represent log-transformed transcript levels for a given target gene normalized to the geometric mean of the transcript levels for the housekeeping genes *rpoB* and *gap*. Genes shown on the X-axis are, from left to right, the group I gene (*plcA*), group II genes (*lmo0178*, *lmo0278*), and group III genes (*opuCA*, *bsh*, *lmo0596*, and *lmo0654*). Data represent mean values from qRT-PCR data collected from three or four independent RNA extractions (except for transcript levels for *lmo0654* and *lmo0596* from cells grown in BHI + 0.2% charcoal, which are represented by two replicates); error bars represent one standard deviation.

were calculated based on OD_{260nm} measurements. All purified RNA preparations were precipitated using sodium acetate and ethanol and stored at -80°C .

TaqMan qRT-PCR

All primers and probes used for qRT-PCR were designed using Primer Express software (Applied Biosystems, Foster City, CA; Table 2). Probes with a Minor Groove Binder (MGB) and a nonfluorescent quencher (NFQ) were synthesized by Applied Biosystems; probes with QSY7 quencher dye were synthesized by MegaBases Inc. (Evanston, IL). Quantitative RT-PCR was

performed in 25 μL reactions as described previously (Sue *et al.*, 2004). All reactions were performed in duplicate on at least three independent RNA preparations. A gene-specific genomic DNA standard curve was generated to allow for absolute quantification of mRNA levels (Sue *et al.*, 2004).

Statistical analysis

mRNA transcript level data were log₁₀ transformed to approximate normality. Prior to our final statistical analysis, absolute mRNA transcript levels for our target genes were normalized to the geometric mean of the transcript

TABLE 2. TaqMan PRIMERS AND PROBES USED IN THIS STUDY

Gene	Forward primer (5'-3')	Taqman probe (5'-3') ^a	Reverse primer (5'-3')	Reference
<i>rpoB</i>	TGTAAATATGGACGGCATCGT	CTGATTCGCGCAAAACTTCTACGCG-QSY7	GCTGTTTGAATCTCAATTAAAGTTTGG	Sue <i>et al.</i> , 2004
<i>gap</i>	AAAGCTGGCGCTAAAAAGTTG	TTCATGGTTTACATTTGTAAAGCATTTG-QSY7	ATCTCCGCTCCAGCAACTGGCGATAT	Schwab <i>et al.</i> , 2005
<i>plcA</i>	GATTTATTTACGACGCACATTAGTTT	CCCATTAGCGGGAAGCAATATTCGC-QSY7	GAGTTCTTTTATTGGCTTATTCACGTTATT	Kazmierczak <i>et al.</i> , 2006
<i>lmo0178</i>	ATTTACAGCAAGAAGGCATTA	AGGTGCCGAACAT-MGB-NFQ	GCACGCTCTGCCCTCCAGTAGAT	This study
<i>lmo0278</i>	GAATTCATCGTTTTTTGTAGGACCAT	TGGTTGTGGTAAATCTA-MGB-NFQ	CCAGCAATCATACGCAAAAGTTG	This study
<i>lmo0596</i>	TTCTGGTAATTTCCACACAATCTCTT	TTTTCAGACACAGAAAATCACAAA-MGB-NFQ	GTCAGCTAGTACCCAGCCAGAAAA	This study
<i>lmo0654</i>	TTGCCGACAAACATATCAAGAGGTAA	CAGCGAAACAAGAGAGT-MGB-NFQ	TTTGGAGTTGTGGGTCCTCA	This study
<i>opuCA</i>	ACATCGATAAAGGAGAAATTTGTTGTT	CGTTTCCCAACCACTTGGACCG-QSY7	GCCGGTTAATCATCTTCATTGTT	Sue <i>et al.</i> , 2004
<i>bsh</i>	GGCCTTAGTATGGCAGGACTCA	CCTTGTAATCCGCAATTCCTGAGAA-QSY7	CTCATTTGCTTACCTTCTGCAAA	Sue <i>et al.</i> , 2003

^aAll probes have a 6-FAM reporter dye at the 5' end. Taqman probes were synthesized with either the QSY7 dark quencher or a Minor Groove Binder (MGB) and a non-fluorescent quencher (NFQ) at the 3' end.

levels for the two housekeeping genes, *rpoB* and *gap*, as previously described (Chaturongakul and Boor, 2006; Kazmierczak *et al.*, 2006). For statistical analysis, transcript levels measured in cells grown in the presence or absence of activated charcoal were separately analyzed from transcript levels measured in cells grown to different growth phases. To assess the effects of growth condition and strain on transcript levels observed for a given gene and to measure statistical interactions among data obtained under different conditions, ANOVA was performed on the log-transformed normalized copy number of each gene with strain and growth condition as main effects using the following linear model: transcript levels = μ + strain + growth condition + strain*growth condition + RNA extraction date + ϵ (factor "strain" includes 10403S, $\Delta sigB$, $\Delta prfA$, and $\Delta sigB\Delta prfA$, factor "growth condition" includes growth with or without charcoal or growth to log or stationary phase). A second ANOVA was subsequently performed to assess the effects of (i) the *sigB* deletion, (ii) the *prfA* deletion, and (iii) the interactions between the *sigB* and the *prfA* deletions on the transcript levels for a given gene in *L. monocytogenes* grown under a given condition using the following linear model: transcript levels = μ + *sigB* + *prfA* + *sigB***prfA* + RNA extraction date + ϵ (factor "sigB" represents presence or absence of *sigB*, factor "prfA" represents presence or absence of *prfA*). RNA extraction date was included in all models to control for contributing variation. All effects

were treated as fixed effects. All statistical analyses were performed in S-Plus 6.2 (Insightful Corp., Seattle, WA).

Results and Discussion

Pathogenesis of listeriosis typically includes a gastrointestinal stage followed by a systemic stage, which is characterized by intracellular infection of a number of different host cell types. PrfA has long been recognized as a transcriptional activator of virulence genes that are important during the intracellular stage of infection, in particular the virulence genes located in the *prfA* virulence gene island (i.e., *actA*, *hly*, *plcA*, *plcB*, and *mpl*). The dual observations of coregulation of some virulence genes (i.e., *inlA*, *inlB*) by σ^B and PrfA (Kim *et al.*, 2005; McGann *et al.*, 2007) as well as the observation that σ^B also directly regulates transcription of *prfA* (Kazmierczak *et al.*, 2006; Nadon *et al.*, 2002; Rauch *et al.*, 2005) suggests that a regulatory network involving σ^B and PrfA is important for *L. monocytogenes* virulence. Therefore, the focus of this study was to quantitatively characterize relative contributions of PrfA and σ^B to transcription of genes previously characterized as members of the PrfA operon in isogenic *sigB* and *prfA* single and double mutants. We thus used qRT-PCR to measure transcript levels for genes selected for this study on the basis of their regulatory elements (Table 3) and preliminary PrfA regulon classification (Milohanic *et al.*, 2003). Transcript levels were measured in *L. mono-*

TABLE 3. GENES CHARACTERIZED IN THIS STUDY

Gene	Group ^a	Function	Regulatory Elements
<i>plcA</i>	I	Lecithinase	PrfA box ^b
<i>lmo0178</i>	II	Similar to xylose repressor	PrfA box ^c
<i>lmo0278</i>	II	Similar to sugar ABC transporter, ATP-binding protein	PrfA box ^c
<i>opuCA</i>	III	Similar to glycine/ choline ABC transporter	σ^B promoter ^d
<i>lmo0654</i>	III	Unknown	σ^B promoter ^d
<i>bsh</i>	III	Bile salt hydrolase	PrfA box ^c ; σ^B promoter ^d
<i>lmo0596</i>	III	Unknown	PrfA box ^c overlapping –35 region of σ^B promoter ^d

^aGroups I, II, and III as defined by Milohanic *et al.* (2003).

^bIdentified by primer extension (Vazquez-Boland *et al.*, 1992).

^cIdentified by sequence analysis (Glaser *et al.*, 2001).

^dIdentified by HMM; σ^B -dependent promoters for *opuCA* and *bsh* were also confirmed by 5' Rapid Amplification of cDNA ends (RACE) (Kazmierczak *et al.*, 2003).

cytogenes 10403S and isogenic $\Delta sigB$, $\Delta prfA$, and $\Delta sigB\Delta prfA$ strains grown under conditions demonstrated to induce σ^B and PrfA activity. A qRT-PCR approach was chosen rather than a microarray-based strategy to allow for absolute transcript quantification and thus, comparison of transcript levels for bacteria grown under multiple conditions. Our data indicate that (i) transcription of *L. monocytogenes* group II genes is PrfA-independent, at least under a number of conditions that induce PrfA activity, and (ii) selected group III genes show σ^B -dependent, but PrfA-independent, transcription under a number conditions that induce PrfA activity.

Transcription of group II genes lmo0178 and lmo0278 is PrfA-independent

Macroarray studies by Milohanic *et al.* (2003) identified eight group II genes (including one seven-gene operon [*lmo0178–lmo0184*] as well as a single gene [*lmo0278*]), which were negatively regulated by PrfA, but not differentially regulated by the constitutively active PrfA*. To evaluate transcriptional regulation of these group II genes, we designed qRT-PCR probes for *lmo0278* as well as for the first gene of the group II operon (i.e., *lmo0178*; Table 2). When *lmo0178* and *lmo0278* transcript levels were determined for *L. monocytogenes* grown in the presence or absence of charcoal, transcription of both genes was significantly affected by the addition of activated charcoal (ANOVA using data for all four strains; $p = 0.0013$ and 0.0103 for *lmo0178* and *lmo0278*, respectively; Suppl. Table S1 and Suppl. Fig. S1 [to view supplementary material, go to www.liebertpub.com/fpd]); both genes generally showed higher transcript levels in cells exposed to activated charcoal than in cells not exposed to charcoal. *lmo0178* and *lmo0278* transcript levels were not significantly affected by a *prfA* deletion, however (Table 4, Suppl. Fig. S1), indicating that increased transcription of these genes in cells exposed to charcoal is not mediated by PrfA. In contrast, the known PrfA-dependent *plcA* (which served as a control gene) not only showed increased transcript levels in cells grown in the presence of charcoal (Suppl. Table S1, Suppl. Fig. S2), but also clearly showed PrfA-dependent transcrip-

tion (Table 4; Fig. 1). Overall, our results on the effects of charcoal on *lmo0178* and *lmo0278* transcript levels are consistent with an increasing body of data indicating that activated charcoal not only increases PrfA activity, but may also interfere with other, not yet identified, environmental signals that affect PrfA-independent transcription (Andre *et al.*, 2003; McGann *et al.*, 2007). While *L. monocytogenes* cultures supplemented with activated charcoal have been used as a tool to increase PrfA-mediated transcription (Geoffroy *et al.*, 1989; Ripio *et al.*, 1996; Milohanic *et al.*, 2003), pleiotropic effects of charcoal thus may complicate interpretation of transcriptional data aimed at understanding PrfA-dependent gene regulation.

While it has been well documented that σ^B activity increases in *L. monocytogenes* stationary phase cells (Ferreira *et al.*, 2001), transcription of PrfA-dependent genes and PrfA activity also appear to be higher in stationary phase cells as compared to log phase cells (Ermolaeva *et al.*, 2004; Kazmierczak *et al.*, 2006). Transcript levels for the PrfA-dependent *plcA* were higher in stationary phase cells as compared to log phase cells in the experiments reported here (Table 4, Suppl. Fig. S2). Similar to the data for *L. monocytogenes* exposed to charcoal, the *prfA* deletion did not affect *lmo0178* and *lmo0278* transcript levels in bacteria grown to either stationary phase or log phase (Table 4; Fig. 2), further supporting PrfA-independent transcription of the group II genes *lmo0178* and *lmo0278* under different conditions, including conditions that yielded high transcript levels for the PrfA-dependent control gene *plcA* (Suppl. Fig. S2). While putative PrfA boxes have been identified upstream of *lmo0178* and *lmo0278* (Glaser *et al.*, 2001), these PrfA boxes differ from the PrfA consensus sequence by 2 nt (Fig. 3), a difference that has been shown to greatly reduce efficient PrfA binding (Sheehan *et al.*, 1995). These observations suggest that PrfA binding to these putative PrfA sites (thus possibly resulting in negative regulation) may only occur under specific environmental conditions and/or in the presence of high concentrations of active PrfA. Overall, however, our findings are consistent with in vitro transcription results by Rauch *et al.* (2005), which suggested PrfA-independent transcription of *lmo0178* and *lmo0278*, with

TABLE 4. RESULTS FROM STATISTICAL ANALYSES OF THE EFFECTS OF *sigB* AND *prfA* NULL MUTATIONS ON TRANSCRIPT LEVELS FOR SELECTED GROUP I, II, AND III TARGET GENES IN *L. MONOCYTOGENES* GROWN UNDER DIFFERENT CONDITIONS

Gene	Condition	Effect of <i>sigB</i> deletion		Effect of <i>prfA</i> deletion	
		Difference in marginal means (in log normalized copies) ^a	p-value ^b	Difference in marginal means (in log normalized copies) ^a	p-value ^b
<i>plcA</i>	Charcoal	—	NS	−2.02	<0.0001
	No charcoal	—	NS	−0.86	<0.0001
	Log phase	—	NS	−0.62	<0.0001
	Stationary phase	—	NS	−0.89	<0.0001
<i>lmo0178</i>	Charcoal	+0.12	0.0221	—	NS
	No charcoal	—	NS	—	NS
	Log phase	—	NS	—	NS
	Stationary phase	−0.61	0.0512	—	NS
<i>opuCA</i>	Charcoal	−1.01	<0.0001	—	NS
	No charcoal	−1.29	<0.0001	—	NS
	Log phase	−1.03	0.0012	—	NS
	Stationary phase	−0.83	0.0004	—	NS
<i>bsh</i>	Charcoal	−1.72	<0.0001	—	NS
	No charcoal	−1.29	<0.0001	—	NS
	Log phase	−0.91	0.0005	—	NS
	Stationary phase	−1.45	<0.0001	—	NS
<i>lmo0596</i>	Charcoal	−2.33	<0.0001	—	NS
	No charcoal	−3.67	0.0099	—	NS
	Log phase	−2.16	0.0009	—	NS
	Stationary phase	−2.69	<0.0001	—	NS
<i>lmo0654</i>	Charcoal	—	NS	—	NS
	No charcoal	−1.24	0.0006	—	NS
	Log phase	−0.73	0.0078	—	NS
	Stationary phase	−0.82	0.0020	—	NS

^a“Difference in marginal means” refers to the difference in log normalized transcript copies for a given gene between strains that have a given gene (e.g., for *sigB*, the parent strain 10403S and the $\Delta prfA$ strain) and the strains that lack a given gene (e.g., for *sigB*, the $\Delta sigB$ and the $\Delta sigB \Delta prfA$ strains); negative values indicate lower transcript levels in the mutant strains. Differences in marginal means are only reported for gene/condition combinations with $p < 0.1$.

^bActual p -values are given for p -values between 0.0001 and 0.10; NS represents a p -value > 0.1 ; no significant effects of either the *prfA* or the *sigB* deletion on *lmo0278* transcript levels were observed; therefore, data from this gene were not included in this table. Interactions between the *sigB* deletion and the *prfA* deletion were not statistically significant for any of the genes under any of the conditions tested.

no evidence for direct PrfA-dependent transcriptional repression of these genes, as well as with microarray findings by Marr *et al.* (2006), who reported that *lmo0178*, *lmo0182*, *lmo0183*, and *lmo0278* were not differentially regulated by PrfA.

Transcript levels for *lmo0278* and *lmo0178* were not significantly affected by the *prfA* deletion in cells exposed to activated charcoal, however, deletion of *sigB* significantly affected *lmo0178* transcript levels (ANOVA; $p = 0.0221$, Table 4), with slightly (0.12 log) higher transcript levels in the *L. monocytogenes* strains with *sigB* deletions. Interestingly, in stationary-phase cells, *lmo0178* and *lmo0278* transcripts were lower in the *sigB* deletion strains as compared to the strains with intact *sigB* (0.61 logs and 0.55

logs, respectively; Table 4 and data not shown, respectively); however, the effect of the *sigB* deletion on *lmo0178* transcript levels was only borderline significant (ANOVA; $p = 0.051$; Table 4) and the effect on *lmo0278* transcript levels was not significant. No significant interaction effects between the *sigB* and the *prfA* deletions were observed on either *lmo0178* or *lmo0278* transcript levels. Application of a Hidden Markov Model that had been trained with 33 confirmed σ^B -dependent promoter sequences (Raengpradub *et al.*, 2007) did not identify putative σ^B -dependent promoter sequences upstream of *lmo0178* or *lmo0278*, suggesting that possible effects of the *sigB* deletion on transcript levels for these genes is indirect. Additionally, neither the *lmo0178-lmo0184* operon nor *lmo0278* were

FIG. 2. Transcript levels for the selected group I, II, and III genes in the *L. monocytogenes* parent strain 10403S and in isogenic $\Delta sigB$, $\Delta prfA$, and $\Delta sigB\Delta prfA$ strains grown to either (A) log phase (OD₆₀₀ = 0.4) or (B) stationary phase (OD₆₀₀ = 2.0). Normalized log copy numbers shown on the Y-axis represent log-transformed transcript levels for a given target gene normalized to the geometric mean of the transcript levels for the housekeeping genes *rpoB* and *gap*. Genes shown on the X-axis are, from left to right, the group I gene (*plcA*), group II genes (*lmo0178*, *lmo0278*), and group III genes (*opuCA*, *bsh*, *lmo0596*, and *lmo0654*). Data represent mean values from qRT-PCR data collected from three or four independent RNA extractions; error bars represent one standard deviation. To enable comparisons between transcript levels for a given gene in a given mutant strain, the data presented in Figs. 1 and 2 were also rearranged by strain and are presented in Suppl. Figs. S1 through S3.

found to be σ^B dependent in a full genome microarray study of the *L. monocytogenes* σ^B regulon (Raengpradub *et al.*, 2007), further supporting that group II genes are only weakly or inconsistently regulated by either σ^B or PrfA.

Transcript levels for the group III genes lmo0596, lmo0654, bsh, and opuCA are σ^B -dependent and PrfA-independent, even under PrfA inducing conditions

A total of 53 of the 73 genes identified as members of the PrfA regulon had been classified as group III genes by Milohanic *et al.* (2003), who reported that transcript levels for all group III

genes were positively regulated by PrfA, with the majority of genes also positively regulated by the constitutively active PrfA*. While PrfA-dependent transcription of *L. monocytogenes* virulence genes (i.e., group I genes) appeared to increase in cells grown in the presence of activated charcoal and to decrease in cells grown in the presence of cellobiose, PrfA-dependent transcription of most group III genes was not affected by cellobiose (Milohanic *et al.*, 2003). In addition, only one of the group III genes showed evidence of PrfA-dependent transcription in *L. monocytogenes* EGD-e cells grown in the presence of charcoal (Milohanic *et al.*, 2003), suggesting that the group III genes identified by

<i>plcA</i>	5' - <u>TTAACAAATGTTAATGCCTCCACATAAAAGTAACTTTAAGAT</u> -N ₃₁ -TTG-3'
	σ^A_{-35} σ^A_{-10}
<i>actA</i>	5' - <u>TTAACAAATGTTAgAGAAAAATTAATTCTCCAAGTGATATTCT</u> -N ₁₅₈ -GTG-3'
	σ^A_{-35} σ^A_{-10}
<i>hly</i>	5' - <u>TTAACATTTGTTAATGACGTCAAAAGGATAGCAAGACTAGAAT</u> -N ₁₃₉ -ATG-3'
	σ^A_{-35} σ^A_{-10}
<i>lmo0178</i>	5' - <u>TTAAaACCTGTgAA</u> -N ₁₇₉ - <u>TGCATATTGCAAACGATTACAATATAAT</u> -N ₆₅ -ATG-3'
	σ^A_{-35} σ^A_{-10}
<i>lmo0278</i>	5' - <u>TTAcCATTTGTTcA</u> -N ₂₇₃ - <u>TgAACACAAgTTAA</u> -N ₂₃ -TTG-3'
	σ^A_{-35} σ^A_{-10}
<i>bsh</i>	5' - <u>TTAAaAATTtTTAA</u> -N ₄₈ - <u>GTTTTACTCCAAACTCCGAGGGTAC</u> -N ₃₅ -ATG-3'
	σ^B_{-35} σ^B_{-10}
<i>lmo0596</i>	5' - <u>TTAAaAGGTtTTAAATTCGTTTTTTAGGCTAT</u> -N ₆₃ -ATG-3'
	σ^B_{-35} σ^B_{-10}
<i>lmo0654</i>	5' - <u>GATTACATTTCTATTTATTGGGGAAA</u> -N ₄₀ -ATG-3'
	σ^B_{-35} σ^B_{-10}
<i>opuCA</i>	5' - <u>GTTTAAATCTATACTAGTTAGGGAAA</u> -N ₅₅ -ATG-3'
	σ^B_{-35} σ^B_{-10}

FIG. 3. *L. monocytogenes* 10403S promoter sequences of confirmed (i.e., *plcA*, *actA*, *hly*) and putative PrfA-regulated genes (i.e. *lmo0178*, *lmo0278*, *bsh*, *lmo0596*, *lmo0654*, *opuCA*). DNA sequences were obtained from the genome sequence for strain 10403S (*Listeria monocytogenes* Sequencing Project, Broad Institute of Harvard and MIT; <http://www.broad.mit.edu>). Promoter sequences are underlined and PrfA boxes are italicized. Deviations from the PrfA binding site consensus sequence are shown by lower case letters. The PrfA boxes and σ^A -dependent -10 and -35 sites for *plcA*, *hly*, and *actA* (Mengaud *et al.*, 1989; Vazquez-Boland *et al.*, 1992); the PrfA boxes for *bsh*, *lmo0178*, *lmo0278*, and *lmo0596* (Glaser *et al.*, 2001; Dussurget *et al.*, 2002); and the σ^B -dependent -10 and -35 sites for *bsh*, *lmo0596*, and *opuCA* (Kazmierczak *et al.*, 2003) have all been reported previously. For *bsh*, a putative -10 site has been described 19 nt downstream of the PrfA box (Dussurget *et al.*, 2002) however, this promoter has not been confirmed experimentally and, therefore, was not included in this figure. The putative PrfA boxes for *lmo0596*, *lmo0178*, and *lmo0278* are shown as described by Glaser *et al.* (2003). The indicated σ^A -dependent -10 promoter site for *lmo0178* was mapped by primer extension (Luo *et al.*, 2004).

Milohanic *et al.* (2003) do not follow the classical PrfA-dependent transcription patterns of group I genes. Group III genes also show diverse promoter configurations; a number of group III genes have putative σ^B -dependent and/or putative PrfA-dependent promoter sites (Milohanic *et al.*, 2003; Fig. 3). To evaluate σ^B - and PrfA-dependent transcription of different group III genes, we designed qRT-PCR primers and probes for four group III genes, including *lmo0596*, *bsh*, *opuCA*, and *lmo0654* (Table 2). All four of these genes have upstream confirmed (*bsh*, *opuCA*) (Fraser *et al.*, 2003; Kazmierczak *et al.*, 2003) or putative σ^B -dependent promoters. *bsh* also has an upstream promoter with a PrfA box that was previously mapped by primer extension (Fig. 3) (Dussurget *et al.*, 2002) and *lmo0596* has an upstream PrfA box, which overlaps with the -35 region of the putative σ^B -dependent promoter (Fig. 3). Interestingly, there was no significant effect of the *prfA* deletion on transcript levels for any of the four group III genes tested (Table 4), indicating that transcription of these genes was PrfA independent under

the conditions tested. In addition, while growth in the presence of activated charcoal led to a clear PrfA-dependent increase in transcript levels for the PrfA-regulated *plcA* (Suppl. Fig. S2), the tested group III genes did not show consistent patterns of higher transcript levels in cells grown in the presence of charcoal (Suppl. Fig. S3), consistent with the findings by Milohanic *et al.* (2003), who also found that the four group III genes tested here do not show PrfA-dependent transcript levels in EGD-e grown in the presence of charcoal. The PrfA boxes identified upstream of *lmo0596* and *bsh* differ from the PrfA binding site consensus by 2 nt; in addition, putative -10 promoter sequences were 12 and 86 nt downstream from the PrfA binding sites for *lmo0596* and *bsh*, respectively; closer and further, respectively, than the optimal 22–23 nt distance (Luo *et al.*, 2005) between a PrfA box and the promoter's -10 region (Fig. 3). Similar to our results, microarray data reported by Marr *et al.* (2006) also did not find evidence of PrfA-mediated differential regulation for *lmo0596* and *bsh*. Previous in vitro studies also reported PrfA-

independent transcription of *lmo0596* and *bsh* with no evidence for direct PrfA-dependent activation of these genes (Rauch *et al.*, 2005). Overall, these data support that group III genes are weakly or inconsistently regulated by PrfA, as proposed by Scotti *et al.* (2007).

While the *prfA* deletion had no significant effects on transcript levels for group III genes, there was a significant effect of the *sigB* deletion on transcript levels for all four group III genes tested (Figs. 1 and 2, Table 4). This significant effect was observed for bacteria grown under all four conditions (log and stationary phase; with and without charcoal) (Suppl. Fig. S3); except for *lmo0654* transcript levels, which were not significantly affected by the *sigB* deletion in cells grown in the presence of charcoal (Table 4). Interactions between the *sigB* and the *prfA* deletions did not have a significant effect on transcript levels for any of the four group III genes tested. While *bsh* and *opuCA* transcription has previously been reported to be σ^B -dependent (Fraser *et al.*, 2003; Kazmierczak *et al.*, 2003), *lmo0596* and *lmo0654* have not previously been reported as σ^B -dependent. Recent full genome microarray studies using only a parent and a $\Delta sigB$ strain have confirmed *lmo0596* and *lmo0654* as σ^B -dependent (Raengpradub *et al.*, 2007). Among all 53 group III genes, a total of 33 were initially predicted by Milohanic *et al.* (2003) to be preceded by a σ^B -dependent promoter (either directly upstream or upstream of the first gene of an operon); recent Hidden Markov Model searches identified putative σ^B -dependent promoters upstream of an additional eight genes within this group (Raengpradub *et al.*, 2007). Not only are the majority of group III genes preceded by putative (or confirmed, e.g., for *opuCA*, *bsh*) σ^B -dependent promoters, 50 of these 53 group III genes were also recently confirmed as positively regulated by σ^B in microarray experiments comparing transcript levels between the *L. monocytogenes* 10403S parent strain and an isogenic $\Delta sigB$ strain (Raengpradub *et al.*, 2007). Our data provide specific evidence that the tested *L. monocytogenes* group III genes, which were previously reported to be part of the PrfA regulon, actually appear to be predominantly regulated by σ^B with weak or inconsistent contributions by

PrfA. Although both Milohanic *et al.* (2003) and Marr *et al.* (2006) found considerable numbers of *L. monocytogenes* genes (i.e., 73 and 201, respectively) to be differentially regulated by PrfA, including genes that are up- or down-regulated by PrfA, only a few PrfA-regulated genes, representing predominantly the group I virulence genes, were identified by both groups. A number of PrfA-dependent genes, including many of those classified by Milohanic *et al.* (2003) into groups II and III, appear to be weakly or inconsistently regulated by PrfA (Scotti *et al.*, 2007). On the other hand, σ^B appears to play a major and robust role in the regulation of the group III genes identified by Milohanic *et al.* (2003).

Conclusions

While interactions between σ^B and PrfA clearly are important for transcription of a number of virulence genes, as supported by the observations that σ^B regulates *prfA* transcription (Nadon *et al.*, 2002) and that *inlA* and *inlB* transcription are affected by both σ^B and PrfA (McGann *et al.*, 2007), σ^B and PrfA coregulation of selected group III genes were not confirmed under the conditions used in this study. Our data support that PrfA has a role in regulating transcription of specific virulence genes critical for intracellular survival, while σ^B broadly contributes to transcription of stress response as well as some virulence genes. Interactions between σ^B and PrfA in a regulatory network certainly appear to be critical for appropriate expression of virulence genes important for early stages of listerial infection (e.g., *inlA*, *inlB*). It is possible that additional regulatory interactions between these two proteins may be enhanced only under conditions found within the host. If this is true, these interactions may not be easily detectable under many of the laboratory growth conditions typically used to characterize *L. monocytogenes* stress response and virulence gene expression. Further studies designed to investigate physical interactions between σ^B and PrfA at targeted promoter sites will be necessary to obtain additional insight into specific interactions between these two regulatory proteins.

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