Contributions of *Listeria monocytogenes* σ^B and PrfA to expression of virulence and stress response genes during extra- and intracellular growth

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Listeria monocytogenes σ^B and PrfA are pleiotropic regulators of stress response and virulence gene expression. Quantitative RT-PCR (qRT-PCR) was used to measure transcript levels of σ^B - and PrfA-dependent genes in exponential-phase *L. monocytogenes* wild-type and $\Delta sigB$ strains as well as in bacteria exposed to environmental stresses (0.3 M NaCl or growth to stationary phase) or present in the vacuole or cytosol of human intestinal epithelial cells. Stationary-phase or NaCl-exposed *L. monocytogenes* showed σ^B -dependent increases in *opuCA* (10- and 17-fold higher, respectively) and *gadA* transcript levels (77- and 14-fold higher, respectively) as compared to non-stressed, exponential-phase bacteria. While PrfA activity, as reflected by *plcA* transcript levels, was up to 95-fold higher in intracellular *L. monocytogenes* as compared to non-stressed bacteria, σ^B activity was only slightly higher in intracellular than in non-stressed bacteria. Increased *plcA* transcript levels, which were similar in both host cell vacuole and cytosol, were associated with increases in both *prfA* expression and PrfA activity. qRT-PCR assays were designed to measure expression of *prfA* from each of its three promoter regions. Under all conditions, readthrough transcription from the upstream *plcA* promoter was very low. The relative contribution to total *prfA* transcription from the σ^A -dependent P1*prfA* promoter ranged from ~17% to 30%, while the contribution of the P2*prfA* region, which appears to be transcribed by both σ^A and σ^B , ranged from ~70% to 82% of total *prfA* transcript levels. In summary (i) σ^B is primarily activated during environmental stress and does not contribute to PrfA activation in intracellular *L. monocytogenes* and (ii) the partially σ^B -dependent P2*prfA* promoter region contributes the majority of *prfA* transcripts in both intra- and extracellular bacteria.

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INTRODUCTION

Listeria monocytogenes is an intracellular human foodborne pathogen. While a number of genes contributing to its virulence have been identified, the majority of these virulence genes are arranged in a single cluster on the *L. monocytogenes* chromosome and are regulated by the transcriptional activator PrfA (Chakraborty *et al.*, 1992; Domann *et al.*, 1992). Loss-of-function mutations in *prfA* result in reduced expression of several virulence genes (Leimeister-Wachter *et al.*, 1990; Mengaud *et al.*, 1991) as well as in reduced *L. monocytogenes* virulence in animal and in tissue culture models of infection (Chakraborty *et al.*, 1992; Freitag & Portnoy, 1994). Some virulence genes (e.g. *inlA*, *bsh*) are regulated by both PrfA and σ^B (Kazmierczak *et al.*, 2003; Kim *et al.*, 2004; Nadon *et al.*, 2002; Sue *et al.*, 2003), a stress-responsive, stationary-phase sigma factor present in a number of Gram-positive bacteria. A $\Delta sigB$

strain exhibits reduced invasion of Caco-2 cells in parallel with reduced σ^B -dependent *inlA* expression (Kim *et al.*, 2004, 2005) and is virulence-attenuated in orally infected guinea pigs (Garner *et al.*, 2006). Recent evidence suggests that σ^B activates transcription of *prfA* from a promoter located in the P2*prfA* region (Nadon *et al.*, 2002; Rauch *et al.*, 2005; Schwab *et al.*, 2005). Although loss of expression from the P2*prfA* region affects *in vitro* virulence phenotypes, e.g. haemolysin production (Freitag & Portnoy, 1994; Nadon *et al.*, 2002), it does not affect *L. monocytogenes* invasion capabilities in human intestinal epithelial cell lines (Kim *et al.*, 2004, 2005). In *L. monocytogenes*, σ^B also contributes to survival under a variety of lethal environmental stresses (Ferreira *et al.*, 2001), including acidic conditions mimicking mammalian gastric fluid (Chaturongakul & Boor, 2004; Cotter *et al.*, 2001; Wiedmann *et al.*, 1998).

Interestingly, stress-responsive alternative sigma factors, including σ^B and σ^S (RpoS), have also been shown to contribute to expression of virulence genes in other pathogens

Abbreviations: qRT-PCR, quantitative RT-PCR; RACE-PCR, rapid amplification of cDNA ends PCR.

such as *Staphylococcus aureus*, *Salmonella enterica* serovar Typhimurium and *Pseudomonas aeruginosa* (Kazmierczak *et al.*, 2005). These links between environmental stress responses and virulence in bacteria suggest a central role for alternative sigma factors in the ability of bacterial pathogens to survive environmental stress conditions, to direct the expression of virulence genes, and to cause disease (Kazmierczak *et al.*, 2005). Therefore, we hypothesized that coordinated transcriptional regulation of virulence and stress response gene expression is critically important for foodborne and enteric pathogens, which are exposed to a variety of different stress conditions (e.g. reduced pH) before and during the infection process. To determine the specific contributions of the transcriptional regulators σ^B and PrfA to expression of *L. monocytogenes* virulence and stress response genes, we measured transcript levels of selected σ^B - and PrfA-dependent genes under conditions encountered during critical stages of infection in a mammalian host (e.g. bacterial presence in host cell vacuoles or cytosol). As both σ^B and PrfA can be present in either active or inactive states, measurement of transcript levels for σ^B - and PrfA-dependent genes was used to indirectly quantify the activities of these two proteins. Transcriptional reporter fusions (Klarsfeld *et al.*, 1994; Moors *et al.*, 1999) and semiquantitative PCR (Bubert *et al.*, 1999) have been used previously to provide qualitative, descriptive information on expression of selected *L. monocytogenes* virulence genes in intracellular and extracellular bacteria. In this study, we report a reverse transcriptase-PCR approach for generating simultaneous quantitative data on expression of both σ^B - and PrfA-dependent genes to yield detailed insight into the interplay between these two key *L. monocytogenes* transcriptional regulators. Quantitative transcriptional data of this nature will be needed for future development of mathematical models describing complex transcriptional regulatory networks.

METHODS

Bacterial strains and growth. All experiments were performed with *L. monocytogenes* strain 10403S or derivatives (Table 1). Transcriptional activation of targeted genes was not evaluated in the $\Delta prfA$ background due to the broad effects of this mutation on

virulence gene expression, which, for example, prevent *L. monocytogenes* escape from the host cell vacuole and thus prevent studies on virulence gene expression in the cytosol. Bacteria were grown at 37 °C either in Brain Heart Infusion (BHI) broth with shaking at 250 r.p.m. or on BHI agar plates unless otherwise noted.

Genetic manipulations. Standard allelic exchange mutagenesis procedures with the *L. monocytogenes* suicide vector pKSV7 (Wiedmann *et al.*, 1998) were used to construct three new *L. monocytogenes* mutant strains (Table 1). Strain FSL K3-017 ($\Delta actA \Delta sigB$) was constructed by introducing plasmid pEDF1 (pKSV7 containing an in-frame *actA* deletion allele; Roberts, 2004) into FSL A1-254 ($\Delta sigB$). Strains FSL K4-001 ($\Delta hly \Delta sigB$) and FSL B2-002 ($\Delta P1prfA \Delta sigB$) were constructed by introducing plasmid pTJA-57 (which contains the in-frame *sigB* deletion allele used to construct the $\Delta sigB$ strain FSL A1-254; Wiedmann *et al.*, 1998) into DP-L2161 (Δhly) or DP-L1956 ($\Delta P1prfA$). In-frame deletions within the targeted genes in the resulting mutant strains were confirmed by DNA sequencing.

Experimental growth conditions. *L. monocytogenes* 10403S or FSL A1-254 were inoculated from single colonies on BHI plates into BHI broth and grown overnight, then subcultured 1:200 in BHI pre-warmed to 37 °C, and grown to OD₆₀₀ 0.4 (representing exponential-phase cells). Exponential-phase cells and NaCl-stressed cells were prepared and collected in parallel. One culture of each strain (10403S or FSL A1-254) was divided into two 4 ml aliquots and centrifuged for 3 min at 3400 g. Cell pellets were resuspended in 2 ml prewarmed BHI broth. Two millilitres of prewarmed BHI broth or BHI plus 0.6 M NaCl was added to the cultures and each was incubated for 7 min at 37 °C with shaking, corresponding to the approximate peak of σ^B -induced transcriptional response reported in induction experiments in *Bacillus subtilis* (Völker *et al.*, 1995). For harvest of stationary-phase cells, cultures were grown to OD₆₀₀ 0.8 and then incubated for one additional hour. For RNA isolation, 4 ml of each culture (exponential-phase, stationary-phase or NaCl-stressed cells) was added to 8 ml RNA Protect Bacteria Reagent (Qiagen), vortexed briefly, incubated at room temperature for 5 min as recommended by Qiagen, and centrifuged 3 min at 3400 g. Cell pellets were frozen at -80 °C for subsequent RNA isolation as described below.

Cell-culture infection experiments. Caco-2 human intestinal epithelial cells (ATCC HTB-37) were selected for cell-culture experiments to enable exploration of bacterial gene expression under model conditions relevant to the gastrointestinal stage of *L. monocytogenes* infection. Caco-2 cells were grown at 37 °C in modified Eagle's medium with Earle's salts (Gibco) containing 20 % fetal bovine serum, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate and 0.15 % sodium bicarbonate

Table 1. Strains used in this study

Strain	Genotype	Experiments	Reference
10403S	Wild-type	Broth	Bishop & Hinrichs (1987)
FSL A1-254	10403S, $\Delta sigB$	Broth	Wiedmann <i>et al.</i> (1998)
DP-L2161	10403S, Δhly	Caco-2 vacuole	Jones & Portnoy (1994)
FSL K4-001	10403S, Δhly , $\Delta sigB$	Caco-2 vacuole	This study
FSL R3-001	10403S, $\Delta actA$	Caco-2 cytoplasm	Roberts (2004)
FSL K3-017	10403S, $\Delta actA$, $\Delta sigB$	Caco-2 cytoplasm	This study
DP-L1956	10403S, $\Delta P1prfA$	<i>prfA</i> RACE-PCR	Freitag & Portnoy (1994)
FSL B2-002	10403S, $\Delta P1prfA$, $\Delta sigB$	<i>prfA</i> RACE-PCR	This study

(MEM-E). To obtain sufficient amounts of RNA for quantitative RT-PCR (qRT-PCR) from intracellular *L. monocytogenes*, Caco-2 cells used for these infection experiments were grown to near confluency in T-75 flasks. Pairs of appropriate mutant strains were selected to enable quantification of the loss of σ^B on gene expression for *L. monocytogenes* cells in either host cell vacuoles or host cell cytosol. Specifically, Δhly or $\Delta hly \Delta sigB$ *L. monocytogenes* strains were used to harvest mRNA from bacterial cells arrested in the vacuole, and $\Delta actA$ and $\Delta actA \Delta sigB$ *L. monocytogenes* strains were used to harvest mRNA from bacterial cells arrested in the cytosol of the primary infected Caco-2 cells. *L. monocytogenes* Δhly , $\Delta hly \Delta sigB$, $\Delta actA$ and $\Delta actA \Delta sigB$ strains were prepared for inoculation as described previously (Portnoy *et al.*, 1988) and infections were performed with an m.o.i. of approximately 25.

For infection with the Δhly or $\Delta hly \Delta sigB$ strains, the medium was removed from the host cell monolayers at 30 min post-infection, and host cells were washed three times with PBS and then covered with pre-warmed MEM-E containing 50 μ g gentamicin ml⁻¹. At 1 h post-infection, cell monolayers in the flasks were washed with PBS and covered with 20 ml lysis solution consisting of 50% RNA Protect Bacteria Reagent and 1% saponin in PBS. Caco-2 cells were lysed 5 min at room temperature, then bacterial cells were collected by centrifugation for 5 min at 2200 g; pellets were frozen at -80 °C for subsequent RNA purification. Infection with $\Delta actA$ or $\Delta actA \Delta sigB$ *L. monocytogenes* strains was performed as described above, except that gentamicin was added at 1 h post-infection, and lysis and collection were performed at 4 h post-infection to allow all bacterial cells to escape from the vacuole and adapt to the cytosolic environment.

DNA and RNA purification. RNA was isolated from frozen bacterial pellets with the RNeasy kit (Qiagen) according to the manufacturer's protocol for enzymic and mechanical disruption, except that cells were sonicated on ice for three 30 s bursts at 18–21 W. Two consecutive DNase treatments were performed during purification with an on-column DNase kit (Qiagen) according to the manufacturer's protocol. Purified RNA was precipitated and stored at -20 °C. Bacterial RNA isolated from infected Caco-2 cells was further enriched from contaminating mammalian RNA with the MICROBEnrich kit (Ambion).

qRT-PCR. All TaqMan primers and probes (Table 2) were designed using Primer Express software (Applied Biosystems). Probes

with MGB quencher dye were synthesized by Applied Biosystems and probes with QSY7 quencher were synthesized by MegaBases. qRT-PCR was performed in 25 μ l reactions as previously described (Sue *et al.*, 2004). For samples containing mixed bacterial and mammalian RNA, the entire output from the MICROBEnrich kit was used in the TaqMan reactions for the eight target genes; 150–250 ng total RNA was used per reaction. All reactions were performed in duplicate on at least three independent RNA preparations. For exponential-phase and NaCl-stressed bacterial cells, *gap*, *rpoB*, *prfA*-P1 and *prfA*-CDS transcripts were quantified using two additional independent RNA preparations. For bacterial cells harvested from the Caco-2 vacuolar environment, all target genes were quantified using one additional independent RNA preparation. These additional experiments were performed due to variation observed among the data collected in the initial three replicates.

A genomic DNA standard curve was generated for each set of TaqMan reactions as previously described to allow for absolute quantification of mRNA levels (Sue *et al.*, 2004). The term 'transcript levels' is used throughout this manuscript to describe mRNA levels, although absolute numbers were calculated using a DNA standard curve (Sue *et al.*, 2004). As also previously described (Sue *et al.*, 2004), transcript levels for each gene were determined as the difference between the experimental reactions and the corresponding reverse-transcriptase-negative controls, which were used to quantify the amount of contaminating *L. monocytogenes* DNA in each reaction. If the difference between the experimental reactions and the corresponding reverse-transcriptase-negative controls was less than twice the value of the reverse-transcriptase-negative control, the transcript level was considered to be equal to that of the negative control. This 'flooring' approach is based on the ability of the ABI PRISM 7000 and the TaqMan system to detect a minimum twofold difference in mRNA levels (ABI PRISM 7000 Sequence Detection System Specifications, Publication 117SP03-04, Applied Biosystems).

RACE-PCR. Transcriptional start sites within the *prfA* promoter regions were mapped using the 5' rapid amplification of cDNA ends (RACE) system (Invitrogen) as previously described (Kazmierczak *et al.*, 2003). RNA for the procedure was isolated as described above from *L. monocytogenes* 10403S and DP-L1956 ($\Delta P1prfA$) cells grown to stationary phase.

Statistical analyses. Statistical analyses were performed with the Statistical Analysis Software (SAS). Raw transcript levels were

Table 2. TaqMan primers and probes used in this study

Target	Forward primer*	TaqMan probe†	Reverse primer	Reference
<i>gap</i>	aaagctggcgcctaaaaagttg	atctccgctccagcaactggcgatat-QSY7‡	ttcatggtttacattgtaaacgattg	Schwab <i>et al.</i> (2005)
<i>rpoB</i>	tgtaaaatattggacggcatcgt	ctgattcgcgcaaaacttctacgcg-QSY7	gctgtttgaatctcaattaagtgttg	Sue <i>et al.</i> (2004)
<i>plcA</i>	gatttatttacgacgcacattagttt	cccattaggcggaaagcatattcgc-QSY7	gagttctttattggtctattccagttatt	This study
<i>prfA</i> -P1	gcgtgacttctttcaacagcta	caattgtgttactgcctaat-MGB	cccccaatcgttttttatcg	This study
<i>prfA</i> -CDS	caatgggattccacagaattattgtat	tgtaaatcatgatgttccgttctcgct-QSY7	aataaagccagacattataacgaaagc	This study
<i>opuCA</i>	acatcgataaagagaaattgtttggt	cgttttcccaacaaccattggacgcg-QSY7	gccgggttaatcatcttcattggt	Sue <i>et al.</i> (2004)
<i>gadA</i> §	tggcgggttggcaatga	tccgattgttacgcagcgaaa-QSY7	tgccgttatatccagacctcgtt	This study
<i>plcA</i> - readthrough	cctcggaacctataactactatttca	tcttttgcgaatcaa-MGB	gaaagtcacgcctaatgatgtttttt	This study

*All sequences are presented 5'→3'.

†All probes have a 5' FAM reporter dye.

‡QSY7 is a custom dark quencher dye. MGB is a minor groove binding dark quencher dye.

§mo2434 is designated *gadA* following the nomenclature of Conte *et al.* (2002); the same gene has been designated *gadD* by Wemekamp-Kamphuis *et al.* (2004) and *gadD3* by Cotter *et al.* (2005)

normalized to the geometric mean of the two housekeeping genes, *gap* and *rpoB*. Data were log transformed to provide a normal distribution. We performed standard regression diagnostics (residual vs predicted values, Cook's distance, and leverage) to assess the validity of the model and identify outliers. The general linear model procedure was used to assess the effect of stress, $\Delta sigB$ mutation, cell collection date, RNA collection date and assay date for each gene individually. In no instance did cell collection date, RNA collection date, or assay date have a significant effect on the data. LSMEANS with Tukey's correction was then used to determine whether individual stress conditions (0.3 M NaCl, growth to stationary phase, presence in host cell vacuole, presence in host cell cytosol) had significant effects on expression of each gene. This analysis was performed on the wild-type and $\Delta sigB$ data separately. A final general linear model procedure and LSMEANS with Tukey's correction was used to determine whether the $\Delta sigB$ mutation had a significant effect on gene transcript levels under each stress condition. All results are reported as the mean log of the normalized copy numbers, unless otherwise noted.

RESULTS

Activity of σ^B -dependent genes during environmental stress and infection of human epithelial cells

RNA collected from *L. monocytogenes* wild-type and $\Delta sigB$ cells grown to (i) exponential phase, (ii) exponential phase followed by a 7 min 0.3 M NaCl exposure or (iii) early stationary phase, or isolated from (iv) the vacuole or (v) the cytosol of human intestinal epithelial cells, was used to quantify transcript levels of the σ^B -dependent genes *gadA* (*lmo2434*) and *opuCA* using TaqMan qRT-PCR (Fig. 1). To enable RNA isolation from a large population of bacteria in the same stage of intracellular infection, we used strains bearing Δhly or $\Delta actA$ mutations, which remain in the host vacuole or cytosol, respectively (Bubert *et al.*, 1999). Each mutation (Δhly or $\Delta actA$) was created in both wild-type and $\Delta sigB$ backgrounds to allow measurement of the relative contribution of σ^B to gene expression in both intracellular compartments. Microscopic examination of infected Caco-2 cell cultures verified appropriate compartmentalization of each mutant strain (data not shown).

In exponential-phase cells, *gadA* and *opuCA* transcript levels were lower in the $\Delta sigB$ strain than the wild-type, although the difference was only statistically significant for *opuCA* (Fig. 1). Upon exposure of exponential-phase wild-type cells to NaCl, *opuCA* transcript levels increased significantly (17-fold) and *gadA* transcript levels increased with borderline significance (14-fold; Fig. 1, Table 3). No increase in expression of *opuCA* or *gadA* was observed in the $\Delta sigB$ mutant. Stationary-phase wild-type cells also showed significant *gadA* and borderline significant *opuCA* transcript level increases (77- and 10-fold increase over exponential-phase cells, respectively; Fig. 1, Table 3), while no increases in transcript levels were observed in the $\Delta sigB$ strain.

For intracellular *L. monocytogenes*, absolute transcript levels for the housekeeping genes *rpoB* and *gap* were lower in the

Fig. 1. Transcript levels for σ^B -dependent genes. Transcript levels for *opuCA* (a) and *gadA* (b) were quantified by TaqMan qRT-PCR, normalized (see Methods), and represented on the y-axes as $\log_{10}$ values. Dark and light bars represent wild-type and $\Delta sigB$ strains, respectively. Error bars represent one standard deviation. An asterisk indicates that the difference between wild-type and $\Delta sigB$ transcript levels is statistically significant for that condition. Growth conditions or intracellular localizations of bacteria are indicated on the x-axes.

$\Delta sigB$ background as compared to the wild-type background, consistent with the observation that the $\Delta sigB$ strain shows reduced Caco-2 cell invasion (Kim *et al.*, 2004, 2005). Specifically, *gap* transcript levels were 0.29 and 0.72 logs lower and *rpoB* transcript levels were 0.27 and 0.97 logs lower in the $\Delta sigB$ than in the wild-type strain in the cytoplasm and vacuole, respectively. Therefore, *prfA*, *plcA*, *opuCA* and *gadA* transcript levels were normalized relative to *rpoB* and *gap* transcript levels in the same strain to enable comparison of transcript levels between wild-type and mutant strains. *opuCA* and *gadA* transcripts levels were significantly higher in the wild-type than in the $\Delta sigB$ strain in the cytosol (Fig. 1); however, relative *opuCA* and *gadA* transcript levels for *L. monocytogenes* present in either intracellular compartment were not significantly different from those in either exponential-phase extracellular bacteria or bacteria exposed to 0.3 M NaCl (Fig. 1, Table 3), implying that intracellular expression of these genes is intermediate relative to that in exponential-phase and salt-stressed extracellular bacteria. Expression of *opuCA* and *gadA* in the $\Delta sigB$ strains was at or below the detection limit in both intracellular compartments. For both wild-type and $\Delta sigB$ strains, transcript levels for *opuCA* and *gadA* were similar in *L. monocytogenes* arrested in either the vacuole or the cytosol (Fig. 1), with no significant differences in transcript levels between the bacterial cells present in the two compartments (Table 3).

Table 3. Statistical comparisons of cDNA copy numbers among selected growth conditions

Gene	Significance levels (<i>P</i> values) for expression differences*					
	Exp-NaCl	Exp-Sta	Exp-Vac	Exp-Cyt	NaCl-Vac	Vac-Cyt
<i>opuCA</i>	0.0361†	0.0941	NS	NS	NS	NS
<i>gadA</i>	0.0591	0.0025	NS	NS	NS	NS
<i>plcA</i>	NS	0.0152	<0.0001	<0.0001	<0.0001	NS
<i>prfA</i>	NS	0.0176	0.0001	<0.0001	0.0014	NS

*Results from multiple pair-wise comparisons of normalized cDNA copy numbers in wild-type bacteria among growth conditions (see Methods). Abbreviations: Exp, non-stressed exponential-phase cells; NaCl, 0.3 M NaCl; Sta, stationary-phase cells; Vac, Caco-2 vacuole; Cyt, Caco-2 cytoplasm.

†*P* values less than 0.05 were considered significant. *P* values between 0.1 and 0.05 were considered borderline significant. *P* values above 0.1 are listed as NS, not significant.

Activity of PrfA-dependent genes during environmental stress and infection of human epithelial cells

RNA collected from *L. monocytogenes* wild-type and $\Delta sigB$ cells as described above was also used to quantify transcript levels of *prfA* and the PrfA-dependent *plcA* (Fig. 2). *L. monocytogenes* cells exposed to 0.3 M NaCl showed no significant increases in *plcA* or *prfA* transcript levels over exponential-phase cells, while *plcA* and *prfA* transcript levels were both significantly higher in stationary-phase cells as compared to exponential-phase cells (Table 3). Neither *plcA* nor *prfA* transcript levels differed between wild-type and $\Delta sigB$ mutant under any of the conditions tested.

plcA transcript levels in bacteria present in the host cell vacuole were 95- and 83-fold higher as compared to transcript levels in exponential-phase and NaCl-stressed cells, respectively; these differences were highly significant ($P < 0.0001$; Table 3). *prfA* transcript levels in vacuolar bacteria were also significantly higher as compared to exponential-phase and NaCl-stressed cells (11- and 7-fold higher, respectively). *plcA* and *prfA* transcript levels did not differ between wild-type and $\Delta sigB$ strains in either intracellular compartment. Transcript levels for either *plcA* or *prfA* did not differ significantly between bacteria in the vacuole and the cytosol (Table 3).

To compare levels of active PrfA in bacteria exposed to different environmental stress conditions or present in the host cell vacuole or the host cell cytosol, we calculated PrfA activity, which we defined as the ratio of normalized PrfA-dependent *plcA* transcript levels to normalized *prfA* transcript levels. Using this approach, we showed that PrfA activity is more than 10-fold higher in bacteria found in the vacuole as compared to that in extracellular exponential-phase bacteria. The observed increase in PrfA activity is not likely due to bacterial exposure to MEM tissue culture media instead of BHI broth, as this switch from BHI to MEM was previously reported not to induce elevated PrfA levels (Renzoni *et al.*, 1999). While exposure of exponential-phase cells to 0.3 M NaCl did not increase PrfA activity,

Fig. 2. Activity of PrfA in *L. monocytogenes* grown in different conditions. Transcript levels for *plcA* (a) and *prfA* (b) were quantified by TaqMan qRT-PCR, normalized (see Methods), and represented on the y-axes as $\log_{10}$ values. Dark and light bars represent wild-type and $\Delta sigB$ strains, respectively. Error bars represent one standard deviation. In no instance was the difference between wild-type and $\Delta sigB$ transcript levels statistically significant. (c) 'PrfA activity' is defined as the ratio of normalized *plcA* transcript levels to normalized (total) *prfA* transcript levels. Growth conditions or intracellular localizations of bacteria are indicated on the x-axis.

stationary-phase bacteria showed increased PrfA activity over exponential-phase bacteria (Fig. 2).

Characterization of *prfA* promoters

The region immediately upstream of *prfA* contains two previously identified promoter regions, including the P2*prfA* region, which has been suggested to include both a σ^A - and a σ^B -dependent promoter (Nadon *et al.*, 2002; Rauch *et al.*, 2005; Schwab *et al.*, 2005), termed here P2*apr**fA* and P2*bpr**fA*, respectively. RACE-PCR identified a transcript with a 5' end corresponding to the previously described P1*prfA* promoter (Freitag *et al.*, 1993; Freitag & Portnoy, 1994) in both the wild-type and the Δ *sigB* mutant (Fig. 3a, top arrow); a σ^A consensus sequence (5'-TTGCGA-12 nt-TATAAT-3') was identified 12 bp upstream of the 5' end for this transcript (Fig. 3c), which overlaps with the 5'-TGCGAA-16 nt-TAAAAT-3' site originally proposed by Freitag & Portnoy (1994). A second 5' transcript end, which

corresponds to the previously identified P2*prfA* promoter, was identified in the wild-type strain, but not in the Δ *sigB* strain; however, the RACE-PCR band for the proposed P2*prfA* transcriptional start site was weak and diffuse (Fig. 3a, bottom arrow). Therefore, RACE-PCR was repeated using RNA from a Δ *sigB* and a wild-type strain that each also carried a deletion in the -10 region of P1*prfA* (strains FSL B2-002 and DP-L1956, respectively; Table 1) to eliminate transcription initiation from the σ^A -dependent P1*prfA* promoter. As expected, the larger RACE-PCR product, corresponding to the P1*prfA* transcript (Fig. 3a, top arrow), was not observed in either strain, confirming that elimination of the -10 region prevented initiation of transcription from the predicted σ^A -dependent P1*prfA*. The smaller product, corresponding to the P2*prfA* transcript, was present only in the Δ P1-10*prfA* strain, but not in the Δ *sigB* Δ P1-10*prfA* strain (Fig. 3b), confirming that this P2*prfA* region RACE-PCR product originates from a σ^B -dependent promoter. Sequencing of the RACE-PCR

Fig. 3. RACE-PCR of the *prfA* promoter region. (a) RNA from stationary-phase *L. monocytogenes* wild-type and Δ *sigB* strains was used to identify transcriptional start sites immediately upstream of *prfA*. Lanes 1–5: DNA marker, wild-type negative control, wild-type positive reaction, Δ *sigB* positive reaction, and Δ *sigB* negative control. The three arrows depict bands present in lane 3; only the top two bands were visible in lane 4. The faint bottom band in lane 3 corresponds to transcription initiation in the P2*prfA* region. (b) RNA from stationary-phase *L. monocytogenes* Δ P1*prfA* and Δ P1*prfA* Δ *sigB* strains was used to verify the σ^B -dependent nature of a transcriptional start site in the P2*prfA* region. Lanes 1–5: DNA marker, Δ P1*prfA* negative control, Δ P1*prfA* positive reaction, Δ P1*prfA* Δ *sigB* positive reaction, and Δ P1*prfA* Δ *sigB* negative control. The single band present in lane 3 corresponds to the bottom band in (a), lane 3. In both (a) and (b), negative controls consisted of reactions conducted without terminal transferase. Individual bands were excised, purified and sequenced. (c) DNA sequence of the *prfA* promoter region (adapted from Rauch *et al.*, 2005). Triangles indicate transcriptional start sites identified in (a) and (b) by modified RACE. The start site for the transcript corresponding to the middle arrow in (a), lane 3, which lacks an identifiable promoter, is labelled with a question mark (see text). Three different adjacent transcriptional start sites, possibly reflecting transcription from either P2*apr**fA* or P2*bpr**fA*, were identified in the P2*prfA* region; these sites are marked by triangles. The σ^A -dependent P1*prfA* promoter is in bold type and underlined once. In the P2*prfA* region, the σ^B -dependent promoter (P2*bpr**fA*) is in bold type and underlined twice; the proposed σ^A -dependent promoter (P2*apr**fA*; Rauch *et al.*, 2005) is marked with wavy lines above the sequence; and the PrfA binding box (as depicted by Sheehan *et al.*, 1995), which is immediately upstream of the P2*bpr**fA* -35 region, is marked by a broken line (---) beneath the sequence.

products identified a σ^B promoter consensus sequence (5'-GTTA-16 nt-GGGTAT-3') 9 bp upstream of the P2*prfA* 5' transcript end, coinciding with the previously described P2*prfA* promoter (Freitag & Portnoy, 1994). We also detected an unexpected third RACE-PCR band that mapped between P2*prfA* and P1*prfA* (Fig. 3a, middle arrow); however, no putative promoter site for any recognized sigma factor in *L. monocytogenes* (σ^A , σ^B , σ^H , σ^{54} or ECF) could be identified upstream of the 5' end corresponding to this RACE-PCR product. This third RACE-PCR product was only identified in the 10403S wild-type *prfA* promoter background and not in either of the Δ P1-10*prfA* strains. We propose that this signal represents either an artefact due to incomplete reverse transcription of the P1*prfA* transcript or a P1*prfA* transcript degradation product.

Expression of *prfA* from individual promoters

While RACE-PCR allows for qualitative determination of transcription start sites, it does not provide accurate quantification of transcript levels, especially when different-sized RACE-PCR products compete for amplification in a single RACE-PCR reaction, as in our experiments. To allow quantification of *prfA* transcript levels, TaqMan primer and probe sets were designed and used to measure relative transcription originating from each of the promoters upstream of *prfA* (Fig. 4a). A '*plcA*-readthrough' primer-probe set (Table 2 and Fig. 4a, probe 2) was designed to specifically detect *plcA* readthrough transcripts that originate from the *plcA* promoter but that do not terminate at the predicted transcriptional terminator downstream of *plcA* (Mengaud *et al.*, 1989). A '*prfA*-P1' primer-probe set (Table 2 and Fig. 4a, probe 3) was designed to detect transcripts originating from P1*prfA* as well as *plcA* terminator readthrough transcripts. To quantify levels of transcripts specifically originating from P1*prfA*, the *plcA*-readthrough transcript levels (detected by the '*plcA*-readthrough' primer-probe set) were subtracted from transcript levels detected by the '*prfA*-P1' primer-probe set. Transcript levels detected by the '*prfA*-CDS' primer-probe set (Table 2 and Fig. 4a, probe 4) represent all *prfA* transcripts regardless of origin; subtracting the transcript levels detected by the '*prfA*-P1' primer-probe set from the transcript levels detected by the '*prfA*-CDS' primer-probe set thus allowed us to determine transcript levels originating from P2*prfA* (Fig. 4a). Since absolute transcript levels for each probe set were determined using standard curves, differences in PCR amplification with different primer-probe combinations were accounted for in these calculations.

Overall, P1*prfA* transcript levels and *plcA* readthrough transcript levels did not differ between wild-type and Δ *sigB* strains under any of the extra- or intracellular conditions tested (Fig. 4). On the other hand, P2*prfA* transcript levels were twice as high in the wild-type as compared to the Δ *sigB* strain for cells exposed to 0.3 M NaCl stress (significant at $P=0.0276$) and in cells grown to stationary phase (not significant; Fig. 4). No difference in P2*prfA* transcript levels

between the wild-type and Δ *sigB* mutant strains were detected in bacteria arrested in the vacuole or the cytosol (Fig. 4). Transcription of P1*prfA* ranged from 16.83 % to 29.97 % of total *prfA* transcription (Table 4). Transcript levels corresponding to readthrough transcription from the *plcA* promoter were low in all conditions, ranging from 0.36 % of total *prfA* transcript levels in non-stressed, broth-grown exponential-phase cells to 2.92 % in the intravacuolar bacteria (Table 4). Transcript levels originating from the P2*prfA* region ranged from 69.67 % (in wild-type, exponential-phase cells) to 82.43 % (wild-type, NaCl-stressed cells) of total *prfA* transcript levels.

DISCUSSION

We hypothesized that σ^B , either alone or in conjunction with PrfA, plays a critical role during foodborne *L. monocytogenes* infection since (i) σ^B -dependent gene expression is activated under stress conditions simulating the gastrointestinal environment, (ii) specific genes contributing to gastrointestinal pathogenesis (e.g. *opuCA*, *bsh*, *inlA*) are at least partially σ^B -dependent (Sue *et al.*, 2004) and (iii) a *L. monocytogenes* Δ *sigB* null mutant shows reduced virulence after gastrointestinal infection, but not after intravenous infection, in guinea pigs (Garner *et al.*, 2006). To test this hypothesis, we characterized transcript levels of selected σ^B - and PrfA-dependent genes under conditions encountered during critical stages of infection in a mammalian host, including exposure to extracellular stress conditions or to the vacuole or the cytosol of infected host cells. Our results not only provide quantitative data on transcription of σ^B - and PrfA-dependent genes during infection by *L. monocytogenes*, but also reveal new insights into relationships between σ^B and PrfA activity. Specifically, our data show that (i) σ^B is primarily activated by environmental stress and does not contribute to PrfA activation in intracellular *L. monocytogenes* and (ii) the partially σ^B -dependent P2*prfA* promoter region contributes the majority of *prfA* transcript levels in both intra- and extracellular bacteria. We propose a model of *L. monocytogenes* gene expression during the gastrointestinal stage of infection, which involves a switch from predominantly σ^B -dependent expression of genes required for gastrointestinal survival to PrfA-dependent expression of genes required for intracellular survival, spread and multiplication.

σ^B is primarily activated during environmental stress and does not contribute to PrfA activation in intracellular *L. monocytogenes*

Our data show that environmental stress conditions induce σ^B activity as indicated by enhanced transcription of the σ^B -dependent genes *opuCA* and *gadA*, in concert with previous reports that have shown stress induction of σ^B activity in *L. monocytogenes* (Fraser *et al.*, 2003; Sue *et al.*, 2003, 2004). While σ^B -dependent *opuCA* transcription under salt stress has been reported previously, the current study establishes reference values for transcript levels of σ^B -dependent genes

Fig. 4. qRT-PCR of the *prfA* promoter region. (a) Location of TaqMan primers and probes. The top line represents the DNA sequence, and gene coding regions are indicated as dark boxes; the four large arrows below this line represent possible mRNA transcripts. The region is drawn to scale, except for breaks indicated by double slashes. Transcriptional start sites and a terminator are indicated by bent arrows and a stem-loop, respectively. Inverted arrows around a bar, upstream of each possible mRNA transcript, and labelled 1 through 4, indicate locations of TaqMan primers and probes. Probe 1 (labelled as '*plcA*' in Table 2) detects transcripts covering the *plcA* coding region; probe 2 ('*plcA*-readthrough') detects *plcA* readthrough transcription; probe 3 ('*prfA*-P1') detects P1*prfA* transcript including *plcA* readthrough transcripts; probe 4 ('*prfA*-CDS') detects all *prfA* coding region transcripts. To quantify transcript levels specifically originating from P1*prfA*, transcript levels obtained using the '*plcA*-readthrough' probe were subtracted from transcript levels for '*prfA*-P1' probe; to quantify transcript levels specifically originating from P2*prfA*, transcript levels obtained using the '*prfA*-P1' probe were subtracted from transcript levels for the '*prfA*-CDS' probe. (b–e) Transcript levels were quantified by TaqMan and normalized (see Methods). Bars depict levels of each *prfA* transcript from wild-type (b, c) and $\Delta sigB$ (d, e) *L. monocytogenes* grown in broth (b, d) or following infection of Caco-2 cells (c, e). Abbreviations: readthrough, *plcA* readthrough transcription; P1tx, P1*prfA*-initiated transcription; P2tx, P2*prfA*-initiated transcription. Growth conditions or intracellular localizations of bacteria are indicated on the x-axes. Note that different y-axis scales were used for broth and intracellular plots.

in extracellular *L. monocytogenes* at 37 °C, which were needed to characterize relative σ^B activity in intracellular *L. monocytogenes*. As Milohanic *et al.* (2003) showed that the σ^B -dependent *opuCA* also may be co-regulated by PrfA, we measured expression of a second σ^B -dependent gene, *gadA*, [lmo2434 (Conte *et al.*, 2002); which is also designated *gadD* (Wemekamp-Kamphuis *et al.*, 2004) and *gadD3* (Cotter *et al.*, 2005)]. σ^B activity (as determined by *gadA* and *opuCA* transcript levels) in intracellular *L. monocytogenes* arrested in either the host cell vacuole or the host cell cytosol was higher than in unstressed, exponentially growing bacteria and lower than in bacteria exposed to extracellular

environmental stresses. While these results may seem surprising, considering the well-documented occurrence in the vacuole of the types of stressful conditions that have been shown to activate σ^B or to affect survival of a $\Delta sigB$ mutant (e.g. low pH, oxidative stress) (Chaturongakul & Boor, 2004; Ferreira *et al.*, 2001; Maul *et al.*, 1995; Sue *et al.*, 2004), virulence gene expression has been shown to differ between *L. monocytogenes* present in professional and non-professional phagocytes (Bubert *et al.*, 1999). Hence, it is possible that the intravacuolar environment of professional phagocytes is a more stressful environment for intracellular bacterial pathogens than that in other cell types, which may

Table 4. Origins of *prfA* transcription and their relative contributions to total *prfA* transcript level

Condition*	Percentage of total <i>prfA</i> transcript levels		
	<i>plcA</i> readthrough†	P1 <i>prfA</i> ‡	P2 <i>prfA</i> §
Exponential	0.36	29.97	69.67
Salt	0.25	17.32	82.43
Stationary	1.52	20.99	77.49
Vacuole	2.92	16.83	80.25
Cytoplasm	2.31	21.41	76.29

*See first footnote of Table 3 for details of conditions.

†Calculated as ('*plcA*-readthrough' cDNA copy ÷ '*prfA*-CDS' cDNA copy) × 100.

‡Calculated as [('*prfA*-P1' cDNA copy – '*plcA*-readthrough' cDNA copy) ÷ '*prfA*-CDS' cDNA copy] × 100.

§Calculated as [('*prfA*-CDS' cDNA copy – '*prfA*-P1' cDNA copy) ÷ '*prfA*-CDS' cDNA copy] × 100.

explain the relatively low σ^B activity measured in Caco-2 cell vacuoles. In support of this hypothesis, and consistent with our findings, Hanawa *et al.* (1995) found that stress proteins induced by environmental stresses were absent in *L. monocytogenes* grown in J774 cells.

Even though the P2*prfA* promoter region includes a σ^B -dependent promoter site, *prfA* and *plcA* transcript levels were independent of the presence of σ^B . While neither *prfA* nor *plcA* transcript levels increased after exposure to 0.3 M NaCl, transcript levels for these two genes were higher in stationary-phase cells as compared to exponential-phase cells and were higher still in intracellular bacteria. While we found similar *plcA* transcript levels in bacteria present in the host vacuole as in the cytosol, Bubert *et al.* (1999) previously observed decreased expression of *plcA* after *L. monocytogenes* escape from the vacuole. Differences between the studies might reflect the use of different bacterial strains, different host cell lines, and different methodologies (i.e. qRT-PCR versus GFP reporter fusions). Our results not only extend findings that PrfA activation (or increased PrfA translation) and possibly increased *prfA* transcription (Bubert *et al.*, 1999; Renzoni *et al.*, 1999) contribute to the activation of PrfA-dependent virulence genes upon *L. monocytogenes* entry into host cells, but also indicate that σ^B plays a limited, if any, role in activating PrfA or in transcribing PrfA-dependent genes after *L. monocytogenes* has invaded intestinal epithelial cells.

Based on the σ^B and PrfA activity profiles observed in this and other studies (Sue *et al.*, 2004), we propose that the gastrointestinal stage of *L. monocytogenes* infection involves a switch from σ^B -mediated expression of stress response and selected virulence genes, triggered by stressful environmental conditions encountered inside the gastrointestinal tract (e.g. low pH, high osmolarity), to PrfA-mediated expression of virulence genes required for intracellular

survival and multiplication (e.g. *hly*, *plcA* and *actA*). This model is not only consistent with recent data showing that σ^B -dependent transcription in *L. monocytogenes* is activated in the murine intestine (Begley *et al.*, 2005), but also provides a functional explanation for the observation that a $\Delta sigB$ mutant shows attenuated virulence after oral infection of guinea pigs (Garner *et al.*, 2006). The switch to PrfA-dependent transcription may be stimulated by *L. monocytogenes* attachment to host epithelial cells, as Renzoni *et al.* (1999) demonstrated an increase in the amount of PrfA following *L. monocytogenes* adherence to host cells. σ^B -mediated activation of gene expression in the gastrointestinal environment appears to facilitate *L. monocytogenes* survival of gastrointestinal environmental stress conditions [e.g. by regulating expression of *gadA*, a putative acid resistance gene, *bsh*, which encodes a bile salt hydrolase, and *opuCA*, which contributes to intestinal colonization (Sleator *et al.*, 2001)] as well as invasion of human gastrointestinal epithelial cells [e.g. by regulating expression of *inlA*, which encodes a protein required for invasion of intestinal epithelial cells (Kazmierczak *et al.*, 2003; Kim *et al.*, 2004, 2005; Sue *et al.*, 2004)].

The partially σ^B -dependent P2*prfA* promoter region contributes the majority of *prfA* transcript levels in both intra- and extracellular bacteria

Our data show that the majority of *prfA* transcripts originate from the P2*prfA* promoter region, which has previously been shown to include a σ^B -dependent promoter (Rauch *et al.*, 2005; Schwab *et al.*, 2005). Overall, our data suggest that compensation for loss of σ^B -dependent *prfA* transcription involves σ^B -independent transcription at P2*aprfA*. Although independent qRT-PCR measurements of transcript levels originating from P2*aprfA* and P2*bprfA* were not possible due to the proximity of these promoter sites, our findings suggest *in vivo* relevance of previous *in vitro* transcription results, which indicated that the P2*prfA* region comprises overlapping σ^A - and σ^B -dependent promoters (Rauch *et al.*, 2005). Since a PrfA box is present upstream of P2*prfA* (Freitag & Portnoy, 1994), we propose a working model of P2*prfA* transcriptional regulation that includes at least partial σ^B -dependent transcriptional activation at P2*bprfA* under environmental stress conditions, when σ^B is active and when PrfA is not activated. When *Listeria* invades host cells, the resulting high level of active PrfA may activate P2*aprfA* at the PrfA box upstream of this promoter. While experiments using (i) transcriptional profiling in a $\Delta prfA$ and $\Delta prfA \Delta sigB$ double mutant and (ii) *in vitro* transcription systems and purified σ^A , σ^B and PrfA will be necessary to further clarify regulation of transcription at P2*prfA*, it is clear that this promoter region plays an important role in regulating *prfA* transcription at the transition between environmental and intracellular stress conditions.

Consistent with data generated using broth-grown *L. monocytogenes* reporter fusion strains (Schwab *et al.*, 2005),

readthrough transcription from *PplcA* contributed only a minor fraction of total *prfA* expression, even in intracellular bacteria. The relative contribution of *plcA* readthrough to total *prfA* transcript was greater in intravacuolar bacteria than in exponential-phase extracellular bacteria, however. While our results thus support the idea that readthrough transcription from the *plcA* promoter may aid in cell-to-cell spread (Camilli *et al.*, 1993), our quantitative estimates contrast with data suggesting that increased readthrough at the *plcA* promoter is largely responsible for the increase in *prfA* transcription observed upon infection (Renzoni *et al.*, 1999). These discrepancies may be related to methodological factors, including the use by Renzoni *et al.* (1999) of a transposon mutant in which *plcA* readthrough transcription was predicted to be abolished; this transposon insertion may disrupt or introduce unidentified regulatory elements, producing unpredictable effects on gene expression. We conclude that while increased *plcA* readthrough transcription may contribute to increased *prfA* transcription in intracellular *L. monocytogenes*, the overall contribution of readthrough transcription to *prfA* transcript levels is minimal.

Conclusions

We propose a model of *L. monocytogenes* gene expression during the gastrointestinal stage of infection that involves a switch from predominantly σ^B -dependent expression of genes required for gastrointestinal survival to PrfA-dependent expression of genes required for intracellular survival, multiplication and spread. It is possible that σ^B -dependent expression at *P2prfA* following stress exposure primes *prfA* transcription during the transition from gastrointestinal extracellular survival to host cell invasion. The resulting availability of PrfA upon *L. monocytogenes* entry into the intracellular environment could enhance PrfA-dependent expression of virulence genes required for intracellular *L. monocytogenes* survival (e.g. *hly*, *actA*). Our model groups genes contributing to *L. monocytogenes* virulence and intra-host survival into categories that are regulated by either PrfA, σ^B , or both (Fig. 5). σ^B -dependent (class B, Fig. 5) genes with potential functions during infection, including survival contributions under host-associated stress conditions such as those encountered in the gastrointestinal tract, include *hfq* (Christiansen *et al.*, 2004). Expression of virulence genes involved in intracellular survival, spread and multiplication, including *plcA*, *actA* and *hly*, is exclusively PrfA-dependent (class A genes, Fig. 5). Finally, *L. monocytogenes* genes with a role in virulence, particularly at the gastrointestinal interface, and that are regulated by both σ^B and PrfA, either simultaneously or independently, include *inlA* (Kazmierczak *et al.*, 2003; Kim *et al.*, 2004; Lingnau *et al.*, 1995), *bsh* (Dussurget *et al.*, 2002; Kazmierczak *et al.*, 2003) and *opuCA* (Sleator *et al.*, 2001; Milohanic *et al.*, 2003). Classes A and C may include genes that are directly or indirectly regulated by PrfA, as a number of PrfA-dependent genes identified by transcriptome analysis lacked an upstream PrfA-box but had an apparent

Fig. 5. Model of σ^B - and PrfA-mediated regulation of virulence genes. Class A genes are activated only by PrfA, class B genes only by σ^B , and class C genes by both. Arrows indicate σ^B activation at the *P2prfA* promoter and autoregulation by each factor. References: *plcA* (Chakraborty *et al.*, 1992), *hly* (Chakraborty *et al.*, 1992; Leimeister-Wachter *et al.*, 1990), *actA* (Domann *et al.*, 1992), *inlA* (Kazmierczak *et al.*, 2003; Lingnau *et al.*, 1995; Sue *et al.*, 2004), *bsh* (Dussurget *et al.*, 2002; Kazmierczak *et al.*, 2003; Sue *et al.*, 2003), *opuCA* (Fraser *et al.*, 2003; Kazmierczak *et al.*, 2003; Sue *et al.*, 2003; Milohanic *et al.*, 2003), *hfq* (Christiansen *et al.*, 2004).

σ^B promoter consensus sequence (Milohanic *et al.*, 2003). The autoregulatory features affecting expression of both *prfA* and *sigB* (Becker *et al.*, 1998; Freitag & Portnoy, 1994; Wiedmann *et al.*, 1998) and σ^B -dependent transcription of *prfA* further support the importance of an interactive PrfA and σ^B regulatory network in *L. monocytogenes* virulence. The quantitative data reported here provide a starting point for development of mathematical models to further probe the structure and function of this proposed regulatory network.

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