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 ΔsigB 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 P1prfA promoter ranged from ~17 % to 30 %, while the contribution of the P2prfA 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 P2prfA promoter region contributes the majority of prfA transcripts in both intra- and extracellular bacteria.

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 ΔsigB 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 P2prfA region (Nadon et al., 2002; Rauch et al., 2005; Schwab et al., 2005). Although loss of expression from the P2prfA 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 $\sigma^B$ and PrfA to expression of *L. monocytogenes* virulence and stress response genes, we measured transcript levels of selected $\sigma^B$- 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 $\sigma^B$ and PrfA can be present in either active or inactive states, measurement of transcript levels for $\sigma^B$- 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 $\sigma^B$- 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 ΔprfA 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 ($\Delta PrfA\Delta sigB$) were constructed by introducing plasmid pTJA-57 (which contains in the $\Delta sigB$ deletion allele used to construct the $\Delta sigB$ strain FSL A1-254; Wiedmann et al., 1998) into DP-L2161 ($\Delta hly$) or DP-L1956 ($\Delta PrfA$). 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 OD$_{600}$ 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 $\sigma^B$-induced transcriptional response reported in induction experiments in *Bacillus subtilis* (Völker et al., 1995). For harvest of stationary-phase cells, cultures were grown to OD$_{600}$ 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 Eagles’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.

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<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Experiments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>10403S</td>
<td>Wild-type</td>
<td>Broth</td>
<td>Bishop &amp; Hinrichs (1987)</td>
</tr>
<tr>
<td>DP-L2161</td>
<td>10403S, Δhly</td>
<td>Caco-2 vacuole</td>
<td>Jones &amp; Portnoy (1994)</td>
</tr>
<tr>
<td>FSL K4-001</td>
<td>10403S, Δhly, ΔsigB</td>
<td>Caco-2 vacuole</td>
<td>This study</td>
</tr>
<tr>
<td>FSL R3-001</td>
<td>10403S, ΔactA</td>
<td>Caco-2 cytoplasm</td>
<td>Roberts (2004)</td>
</tr>
<tr>
<td>FSL K3-017</td>
<td>10403S, ΔactA, ΔsigB</td>
<td>Caco-2 cytoplasm</td>
<td>This study</td>
</tr>
<tr>
<td>FSL B2-002</td>
<td>10403S, ΔPrfA, ΔsigB</td>
<td>prfA RACE-PCR</td>
<td>This study</td>
</tr>
</tbody>
</table>
(MEM-E). To obtain sufficient amounts of RNA for quantitative RT-PCR (qRT-PCR) from intracellular \textit{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 \( \sigma^b \) on gene expression for \textit{L. monocytogenes} cells in either host cell vacuoles or host cell cytosol. Specifically, \textit{Ahly} or \textit{Ahly AsigB} \textit{L. monocytogenes} strains were used to harvest mRNA from bacterial cells arrested in the vacuole, and \textit{DactA} and \textit{DactA AsigB} \textit{L. monocytogenes} strains were used to harvest mRNA from bacterial cells arrested in the cytosol of the primary infected Caco-2 cells. \textit{L. monocytogenes} \textit{Ahly}, \textit{Ahly AsigB}, \textit{DactA} and \textit{DactA AsigB} 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 \textit{Ahly} or \textit{Ahly AsigB} 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 \( \mu \)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 \( \times g \). Pellets were frozen at \(-80^\circ \text{C}\) for subsequent RNA purification. Infection with \textit{DactA} or \textit{DactA AsigB} \textit{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 \(-20^\circ \text{C}\). 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 QS7 quencher were synthesized by MegaBases. qRT-PCR was performed in 25 \( \mu \)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, \textit{gap}, \textit{rpoB}, \textit{prfA-P1} and \textit{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 \textit{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 \textit{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 \textit{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

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward primer*</th>
<th>TaqMan probe†</th>
<th>Reverse primer</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{gap}</td>
<td>aaagctgccgctaaagaagtgg</td>
<td>atctccgcttccgaaatggcqat-QSY7‡</td>
<td>ttcatgttagttaatgtaaagctgg</td>
<td>Schwab et al. (2005)</td>
</tr>
<tr>
<td>\textit{rpoB}</td>
<td>tggaaatatgtgacggctatgg</td>
<td>cggattcgcgcaaaactctcagc-g-QSY7</td>
<td>gcgtgtagtaacctcaatgttg</td>
<td>Sue et al. (2004)</td>
</tr>
<tr>
<td>\textit{plcA}</td>
<td>gatttttttacgcacatattgttg</td>
<td>ccattaggcccggaaacaccatatgctgc-QSY7</td>
<td>gaggctctattgggctggtttcattttttccctg</td>
<td>This study</td>
</tr>
<tr>
<td>\textit{prfA-P1}</td>
<td>gcgcgtttttaaccatacga</td>
<td>caatttgtgttagtcaatctcaga-GBG</td>
<td>ccccaagtcttcttcct</td>
<td>This study</td>
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<tr>
<td>\textit{prfA-CDS}</td>
<td>caatgcgctaccaacagagatctgttg</td>
<td>tgttaattcatatgcggctccgctcctg-QSY7</td>
<td>tttcttttcacacaccaccttcgag-g-QSY7</td>
<td>This study</td>
</tr>
<tr>
<td>\textit{opuCA}</td>
<td>acatcataaagaagattgttgttg</td>
<td>cttgttcacacacacttggcagc-QSY7</td>
<td>ggcgttataatcatctcttggt</td>
<td>Sue et al. (2004)</td>
</tr>
<tr>
<td>\textit{gadD}</td>
<td>tgggctgttggcactga</td>
<td>ttcgctgctgcgctgcagaaa-QSY7</td>
<td>tgccctgatgcctgcaacgctgcgcttgcga</td>
<td>This study</td>
</tr>
<tr>
<td>\textit{plcA- readthrough}</td>
<td>ctcctggaacactaataacacttcttcca</td>
<td>tctttgctgctgctgcagaaa-MGB</td>
<td>gaagctcagcttaaatgatagt</td>
<td>This study</td>
</tr>
</tbody>
</table>

*All sequences are presented 5’→3’.
†All probes have a 5’ FAM reporter dye.
‡\textit{QS7} is a custom dark quencher dye. MGB is a minor groove binding dark quencher dye.
§\textit{lmo2434} is designated \textit{gadA} following the nomenclature of Conte et al. (2002); the same gene has been designated \textit{gadD} by Wemekamp-Kamphuis et al. (2004) and \textit{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, ΔsigB 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 ΔsigB data separately. A final general linear model procedure and LSMEANS with Tukey’s correction was used to determine whether the ΔsigB 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 ΔsigB 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 ΔactA mutations, which remain in the host vacuole or cytosol, respectively (Bubert et al., 1999). Each mutation (Δhly or ΔactA) was created in both wild-type and ΔsigB 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 ΔsigB 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 ΔsigB 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 ΔsigB strain.

For intracellular L. monocytogenes, absolute transcript levels for the housekeeping genes rpoB and gap were lower in the ΔsigB background as compared to the wild-type background, consistent with the observation that the ΔsigB 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 ΔsigB 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 ΔsigB 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 ΔsigB strains was at or below the detection limit in both intracellular compartments. For both wild-type and ΔsigB 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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Significance levels (P values) for expression differences*</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Exp-NaCl</td>
</tr>
<tr>
<td>opuCA</td>
<td>0.0361†</td>
</tr>
<tr>
<td>gsdA</td>
<td>0.0591</td>
</tr>
<tr>
<td>plcA</td>
<td>NS</td>
</tr>
<tr>
<td>prfA</td>
<td>NS</td>
</tr>
</tbody>
</table>

*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 ΔsigB 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 than compared to exponential-phase cells (Table 3). Neither plcA nor prfA transcript levels differed between wild-type and ΔsigB 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 ΔsigB 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.](http://mic.sgmjournals.org)

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 ΔsigB strains, respectively. Error bars represent one standard deviation. In no instance was the difference between wild-type and ΔsigB 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 region, which has been suggested to include both a $\sigma^A$- and a $\sigma^B$-dependent promoter (Nadon et al., 2002; Rauch et al., 2005; Schwab et al., 2005), termed here P2aprfa and P2bprfa, respectively. RACE-PCR identified a transcript with a 5’ end corresponding to the previously described P1prfa promoter (Freitag et al., 1993; Freitag & Portnoy, 1994) in both the wild-type and the $\Delta\sigB$ mutant (Fig. 3a, top arrow); a $\sigma^A$ 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’-TGGCAA-16 nt-TAAAT-3’ site originally proposed by Freitag & Portnoy (1994). A second 5’ transcript end, which corresponds to the previously identified P2prfa promoter, was identified in the wild-type strain, but not in the $\Delta\sigB$ strain; however, the RACE-PCR band for the proposed P2prfa transcriptional start site was weak and diffuse (Fig. 3a, bottom arrow). Therefore, RACE-PCR was repeated using RNA from a $\Delta\sigB$ and a wild-type strain that each also carried a deletion in the −10 region of P1prfa (strains FSL B2-002 and DP-L1956, respectively; Table 1) to eliminate transcription initiation from the $\sigma^A$-dependent P1prfa promoter. As expected, the larger RACE-PCR product, corresponding to the P1prfa 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 $\sigma^A$-dependent P1prfa. The smaller product, corresponding to the P2prfa transcript, was present only in the ΔP1-10prfa strain, but not in the $\Delta\sigB$ ΔP1-10prfa strain (Fig. 3b), confirming that this P2prfa region RACE-PCR product originates from a $\sigma^B$-dependent promoter. Sequencing of the RACE-PCR

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![Fig. 3. RACE-PCR of the prfA promoter region. (a) RNA from stationary-phase L. monocytogenes wild-type and $\Delta\sigB$ 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, $\Delta\sigB$ positive reaction, and $\Delta\sigB$ 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 P2prfa region. (b) RNA from stationary-phase L. monocytogenes $\Delta\sigB$ prfA and $\Delta\sigB$ P1prfa $\Delta\sigB$ strains was used to verify the $\sigma^B$-dependent nature of a transcriptional start site in the P2prfa region. Lanes 1–5: DNA marker, $\Delta\sigB$ negative control, $\Delta\sigB$ positive reaction, $\Delta\sigB$ $\Delta\sigB$ positive reaction, and $\Delta\sigB$ $\Delta\sigB$ 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 P2aprfa or P2bprfa, were identified in the P2prfa region; these sites are marked by triangles. The $\sigma^B$-dependent P1prfa promoter is in bold type and underlined twice. In the P2prfa region, the $\sigma^B$-dependent promoter (P2bprfa) is in bold type and underlined twice; the proposed $\sigma^A$-dependent promoter (P2aprfa; 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 P2bprfa −35 region, is marked by a broken line (---) beneath the sequence.](image-url)
products identified a $\sigma^B$ promoter consensus sequence (5'-GTGA-16 nt-GGAT-3') 9 bp upstream of the P2prfA 5' transcript end, coinciding with the previously described P2prfA promoter (Freitag & Portnoy, 1994). We also detected an unexpected third RACE-PCR band that mapped between P2prfA and P1prfA (Fig. 3a, middle arrow); however, no putative promoter site for any recognized sigma factor in L. monocytogenes ($\sigma^A$, $\sigma^B$, $\sigma^H$, $\sigma^4$ 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 $\Delta P1$-10prfA strains. We propose that this signal represents either an artefact due to incomplete reverse transcription of the P1prfA transcript or a P1prfA 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 'pAC-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 P1prfA as well as plcA terminator readthrough transcripts. To quantify levels of transcripts specifically originating from P1prfA, the plcA-readthrough transcript levels (detected by the 'pAC-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 P2prfA (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, P1prfA transcript levels and plcA readthrough transcript levels did not differ between wild-type and $\Delta\sigma^{B}\sigma$ strains under any of the extra- or intracellular conditions tested (Fig. 4). On the other hand, P2prfA transcript levels were twice as high in the wild-type as compared to the $\Delta\sigma^{B}\sigma$ 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 P2prfA transcript levels between the wild-type and $\Delta\sigma^{B}\sigma$ mutant strains were detected in bacteria arrested in the vacuole or the cytosol (Fig. 4). Trancription of P1prfA 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 intra-vacuolar bacteria (Table 4). Transcript levels originating from the P2prfA 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 $\sigma^B$, either alone or in conjunction with PrfA, plays a critical role during foodborne L. monocytogenes infection since (i) $\sigma^B$-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 $\sigma^B$-dependent (Sue et al., 2004) and (iii) a L. monocytogenes $\Delta\sigma^{B}\sigma$ 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 $\sigma^B$- 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 $\sigma^B$- and PrfA-dependent genes during infection by L. monocytogenes, but also reveal new insights into relationships between $\sigma^B$ and PrfA activity. Specifically, our data show that (i) $\sigma^B$ is primarily activated by environmental stress and does not contribute to PrfA activation in intracellular L. monocytogenes and (ii) the partially $\sigma^B$-dependent P2prfA 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 $\sigma^B$-dependent expression of genes required for gastrointestinal survival to PrfA-dependent expression of genes required for intracellular survival, spread and multiplication.

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

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**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 P1prfA, transcript levels obtained using the ‘*plcA*-readthrough’ probe were subtracted from transcript levels for ‘prfA-P1’ probe; to quantify transcript levels specifically originating from P2prfA, 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 ΔsigB (d, e) *L. monocytogenes* grown in broth (b, d) or following infection of Caco-2 cells (c, e). Abbreviations: readthrough, *plcA* readthrough transcription; P1tx, P1prfA-initiated transcription; P2tx, P2prfA-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.
Table 4. Origins of prfA transcription and their relative contributions to total prfA transcript level

<table>
<thead>
<tr>
<th>Condition*</th>
<th>Percentage of total prfA transcript levels</th>
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<tbody>
<tr>
<td></td>
<td>plcA readthrough†</td>
</tr>
<tr>
<td>Exponential</td>
<td>0.36</td>
</tr>
<tr>
<td>Salt</td>
<td>0.25</td>
</tr>
<tr>
<td>Stationary</td>
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<tr>
<td>Vacuole</td>
<td>2.92</td>
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<tr>
<td>Cytoplasm</td>
<td>2.31</td>
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*See first footnote of Table 3 for details of conditions.
†Calculated as (‘plcA-readthrough’ cDNA copy ÷ ‘prfA-CDS’ cDNA copy) × 100.

Explain the relatively low σB 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 P2prfA promoter region includes a σB-dependent promoter site, prfA and plcA transcript levels were independent of the presence of σB. 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 σB 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 σB 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 σB-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 σB-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 ΔsigB 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. σB-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 σB-dependent P2prfA 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 P2prfA promoter region, which has previously been shown to include a σB-dependent promoter (Rauch et al., 2005; Schwab et al., 2005). Overall, our data suggest that compensation for loss of σB-dependent prfA transcription involves σB-independent transcription at P2aprfA. Although independent qRT-PCR measurements of transcript levels originating from P2aprfA and P2bprfA 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 P2prfA region comprises overlapping σA- and σB-dependent promoters (Rauch et al., 2005). Since a PrfA box is present upstream of P2prfA (Freitag & Portnoy, 1994), we propose a working model of P2prfA transcriptional regulation that includes at least partial σB-dependent transcriptional activation at P2bprfA under environmental stress conditions, when σB is active and when PrfA is not activated. When Listeria invades host cells, the resulting high level of active PrfA may activate P2aprfA at the PrfA box upstream of this promoter. While experiments using (i) transcriptional profiling in a ΔprfA and ΔprfA ΔsigB double mutant and (ii) in vitro transcription systems and purified σA, σB and PrfA will be necessary to further clarify regulation of transcription at P2prfA, 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 PpLA 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 $\sigma^B$-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 $\sigma^B$-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, $\sigma^B$, or both (Fig. 5). $\sigma^B$-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 $\sigma^B$ 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 $\sigma^B$ 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 $\sigma^B$-dependent transcription of prfA further support the importance of an interactive PrfA and $\sigma^B$ 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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**REFERENCES**


gastrointestinal persistence and bile tolerance of *Listeria monocytogenes*. Infect Immun 73, 894–904.


Renzoni, A., Cossart, P. & Dramsi, S. (1999). PrfA, the transcriptional activator of virulence genes, is upregulated during interaction...


