σ^B contributes to *Listeria monocytogenes* invasion by controlling expression of *inlA* and *inlB*

Heesun Kim,¹ H Marquis² and Kathryn J. Boor¹

Correspondence
Kathryn J. Boor
kjb4@cornell.edu

Department of Food Science¹ and Department of Microbiology and Immunology², Cornell University, Ithaca, NY 14853, USA

The ability of *Listeria monocytogenes* to invade non-phagocytic cells is important for development of a systemic listeriosis infection. The authors previously reported that a *L. monocytogenes* $\Delta sigB$ strain is defective in invasion into human intestinal epithelial cells, in part, due to decreased expression of a major invasion gene, *inlA*. To characterize additional invasion mechanisms under the control of σ^B , mutants were generated carrying combinations of in-frame deletions in *inlA*, *inlB* and *sigB*. Quantitative assessment of bacterial invasion into the human enterocyte Caco-2 and hepatocyte HepG-2 cell lines demonstrated that σ^B contributes to both *InlA* and *InlB*-mediated invasion of *L. monocytogenes*. Previous identification of the σ^B -dependent P2_{prfA} promoter upstream of the major virulence gene regulator, positive regulatory factor A (PrfA), suggested that the contributions of σ^B to expression of various virulence genes, including *inlA*, could be at least partially mediated through PrfA. To test this hypothesis, relative invasion capabilities of $\Delta sigB$ and $\Delta prfA$ strains were compared. Exponential-phase cells of the $\Delta sigB$ and $\Delta prfA$ strains were similarly defective at invasion; however, stationary-phase $\Delta sigB$ cells were significantly less invasive than stationary-phase $\Delta prfA$ cells, suggesting that the contributions of σ^B to invasion extend beyond those mediated through PrfA in stationary-phase *L. monocytogenes*. TaqMan quantitative reverse-transcriptase PCRs further demonstrated that expression of *inlA* and *inlB* was greatly increased in a σ^B -dependent manner in stationary-phase *L. monocytogenes*. Together, results from this study provide strong biological evidence of a critical role for σ^B in *L. monocytogenes* invasion into non-phagocytic cells, primarily mediated through control of *inlA* and *inlB* expression.

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INTRODUCTION

The Gram-positive facultative intracellular food-borne pathogen *Listeria monocytogenes* is associated with serious invasive infections in humans and animals (Farber & Peterkin, 1991). Its ability to invade and multiply in a wide range of mammalian cells (Vazquez-Boland *et al.*, 2001) is essential for development of systemic listeriosis. For example, the ability of *L. monocytogenes* to invade non-phagocytic cells plays an important role in this organism's traversal of the intestinal barrier (Lecuit *et al.*, 2001; MacDonald & Carter, 1980; Racz *et al.*, 1972), and its ability to multiply in hepatocytes is essential for causing a systemic infection (Conlan & North, 1991; Gaillard *et al.*, 1996). Several bacterial factors that mediate internalization events have been identified. Critical among these are two cell-wall-anchored proteins, internalin A (*InlA*) and internalin B (*InlB*). *InlA* mediates *L. monocytogenes* entry into the Caco-2 human colon adenocarcinoma cell line (Gaillard *et al.*, 1991), while *InlB* mediates entry into hepatocytes and

several endothelial and epithelial cell lines of various human and animal origins, including HepG-2 (human hepatocyte), TIB73 (mouse hepatocyte), HUVEC (human endothelial) and Vero (African green monkey epithelial) cells (Dramsi *et al.*, 1995; Ireton *et al.*, 1996; Parida *et al.*, 1998). Expression of *inlA* and *inlB* is regulated by both positive regulatory factor A (PrfA)-dependent and -independent mechanisms (Dramsi *et al.*, 1993; Lingnau *et al.*, 1995; Sokolovic *et al.*, 1993). Other bacterial factors, including ActA, p60, FbpA, Iap, and the ClpC ATPase, also have been reported to contribute to *L. monocytogenes* invasion (Alvarez-Dominguez *et al.*, 1997; Dramsi *et al.*, 2004; Kuhn & Goebel, 1989; Nair *et al.*, 2000a; Wuenschel *et al.*, 1993).

Recently, the alternative sigma factor, σ^B , which was initially identified as responsible for general stress responses in Gram-positive bacteria (Hecker & Volker, 2001), has also been associated with invasion capabilities in *L. monocytogenes*. Specifically, a σ^B -dependent promoter has been identified upstream of *inlA* (P4_{inlA}) (Kazmierczak *et al.*, 2003). Loss of σ^B resulted in reduced *inlA* expression and *InlA* levels in stationary-phase cells (Kim *et al.*, 2004). However, the presence of a putative σ^B -dependent promoter

Abbreviation: qRT-PCR, quantitative reverse-transcriptase polymerase chain reaction.

upstream of *inlB* (P_{2inlB}) (Kazmierczak *et al.*, 2003) suggests that contributions of σ^B to *L. monocytogenes* invasion may not be solely limited to modulation of *inlA* expression.

To further study the role of σ^B in *L. monocytogenes* invasion, we analysed invasion capabilities of various mutant strains bearing combinations of in-frame deletions in *inlA*, *inlB* and *sigB* in the human enterocyte Caco-2 and hepatocyte HepG-2 cell lines. Previous identification of the σ^B -dependent P_{2prfA} promoter (Nadon *et al.*, 2002) suggested that the contributions of σ^B to *L. monocytogenes* virulence gene expression might be at least partially mediated through PrfA. To quantify the relative functional contributions of σ^B and PrfA, invasion capabilities of $\Delta sigB$ and $\Delta prfA$ strains were compared. We also measured σ^B -mediated contributions to expression of multiple genes reported to contribute to *L. monocytogenes* invasion and virulence using TaqMan quantitative reverse transcriptase polymerase chain reactions (qRT-PCR). Specifically, relative expression of *inlA*, *inlB*, *prfA*, *iap*, *act A* and *clpC* was measured in both the wild-type and $\Delta sigB$ backgrounds. Here, we present evidence that σ^B is a major contributor to *L. monocytogenes* invasion, primarily through modulation of expression of *inlA* and *inlB*.

METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. *L. monocytogenes* cells were grown overnight at 37 °C prior to use in the invasion assays to optimize PrfA-mediated gene expression (Johansson *et al.*, 2002). Specifically, stationary-phase bacteria were prepared by growth in brain heart infusion broth (BHI) overnight at 37 °C with constant shaking (250 r.p.m.). Exponential-phase bacteria were prepared by passaging overnight cultures 1:100 into BHI and then growing the resulting culture to OD₆₀₀ 0.8 under the same conditions. For invasion assays, bacteria were harvested by centrifugation, washed and diluted in PBS.

Construction of *L. monocytogenes* mutant strains. An internal in-frame deletion in the *inlAB* operon, which inactivated both genes, was generated by SOE (site-directed mutagenesis by overlap

extension) PCR (Ho *et al.*, 1989). Primers used were 5'-AAC TGC AGC TTT GGG AGT GAC ATG C-3' (*inlAB*-SOEA), 5'-TGC CCT TAA ATT AGC TGC TCT CAC TAT ATA CAC TCC-3' (*inlAB*-SOEB), 5'-GGA GTG TAT ATA GTG AGA GCA GCT AAT TTA AGG GCA-3' (*inlAB*-SOEC) and 5'-CCG GAT CCA GTG AAA TTA TTG CTG GT-3' (*inlAB*-SOED) (Dramsi *et al.*, 1995). Primers *inlAB*-SOEB and *inlAB*-SOEC are complementary, and primers *inlAB*-SOEA and *inlAB*-SOED contain *Pst*I and *Bam*HI sites, respectively. Briefly, two fragments were amplified by PCR from 10403S chromosomal DNA using either primer pair *inlAB*-SOEA and *inlAB*-SOEB, or primer pair *inlAB*-SOEC and *inlAB*-SOED. The products were gel-purified and combined in a second PCR with primers *inlAB*-SOEA and *inlAB*-SOED. The resulting product was digested with *Pst*I and *Bam*HI and ligated between the *Pst*I and *Bam*HI sites of the shuttle vector pKSV7 (Camilli *et al.*, 1993) to yield plasmid pHK2. The recombinant sequence in pHK2 was used to replace the wild-type *inlAB* sequence in the chromosome of the *L. monocytogenes* 10403S strain by allelic exchange, as previously described (Camilli *et al.*, 1993), to create strain FSL K4-009. $\Delta inlA\Delta sigB$ (strain FSL B2-042), $\Delta inlB\Delta sigB$ (strain FSL K4-008), and $\Delta inlAB\Delta sigB$ (strain FSL K4-010) mutants were generated from strains DP-L4405 (Bakardjiev *et al.*, 2004), HEL-137 (Kim *et al.*, 2004) and FSL K4-009 (this study), respectively, by replacing the chromosomal allele of *sigB* with the $\Delta sigB$ allele of pTJA-57, as previously described (Wiedmann *et al.*, 1998).

Cell culture and invasion assay. The human colorectal epithelial cell line Caco-2 (ATCC HTB-37) and human hepatic epithelial cell line HepG-2 (ATCC HB-8065) were cultivated at 37 °C in a cell culture incubator at 80–95 % relative humidity under 5 % CO₂. Caco-2 cells were cultured in EMEM (Eagle's Minimum Essential Medium with Earle's Salts) supplemented with 20 % fetal bovine serum (FBS), 1 % non-essential amino acids, 1 % sodium pyruvate, and antibiotics (penicillin G 100 units ml⁻¹; streptomycin 100 µg ml⁻¹). HepG-2 cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10 % FBS, 1 % non-essential amino acids, 1 % sodium pyruvate, and antibiotics (penicillin G 100 units ml⁻¹, streptomycin 100 µg ml⁻¹). Two days prior to infection, 1.5 × 10⁵ Caco-2 and 7.5 × 10⁵ HepG-2 cells in media without antibiotics were seeded into each of six (35 mm diameter) tissue culture plate wells that contained three 12 mm glass coverslips. Host cells were grown to confluence for 2 days. Thirty minutes before infection, the medium in each well was replaced with pre-warmed fresh medium without antibiotics. For infection, approximately 10⁸ c.f.u. of exponential- or stationary-phase bacteria were inoculated onto the host cell monolayer in each well. Host cells were

Table 1. *L. monocytogenes* strains

Strain	Genotype	Reference; source
10403S	Wild-type	Camilli <i>et al.</i> (1993); provided by D. Portnoy*
FSL A1-254	$\Delta sigB$	Wiedmann <i>et al.</i> (1998)
DP-L4137	$\Delta prfA$	Cheng & Portnoy (2003); provided by J. Miller*
DP-L4405	$\Delta inlA$	Bakardjiev <i>et al.</i> (2004); provided by J. Miller
FSL B2-042	$\Delta inlA\Delta sigB$	This study
HEL-137	$\Delta inlB$	Kim <i>et al.</i> (2004)
FSL K4-008	$\Delta inlB\Delta sigB$	This study
FSL K4-009	$\Delta inlAB$	This study
FSL K4-010	$\Delta inlAB\Delta sigB$	This study

*Dr Daniel Portnoy, Department of Molecular & Cell Biology, University of California, Berkeley, CA, USA; Dr Jeffrey Miller, Department of Microbiology, Immunology, & Molecular Genetics, UCLA, Los Angeles, CA, USA.

Table 2. TaqMan primer and probe sequences

Gene	Forward primer (5'→3')	Taqman probes (5'→3')*	Reverse primer (5'→3')
<i>gap</i>	AAAGCTGGCGCTAAAAAAGTTG	FAM-ATCTCCGCTCCAGCAACTGGCGATAT†	TTCATGGTTTACATTGTAAACGATTG
<i>prfA</i>	CAATGGGATCCACAAGAATATT-GTAT	FAM-TGTAAATTCATGATGGTCCCGTCTC-GCT†	AATAAAGCCAGACATTATAACGAAAGC
<i>clpC</i>	CGGCGAAAGCTCTCTATGAACT	FAM-TCCCTCTACCTCTTGCTGCACTTTTT-CAGA†	GGTGTATATTGGATCGTCGTCACA
<i>inlB</i>	GCAAATTTTCCAGATGATGCT-TT	FAM-CAGAAACAATCAAAGACAAT†-MGB‡	TGTCATGTCATCTGTCACACTTTT
<i>actA</i>	TGCGTGCGATGATGGTAGTT	FAM-CCAACTGCATTACGATTAACCCCGA-CATAA†	TTCGCTATCTGTCGCTGCAA
<i>iap</i>	AGCTGGGATTGCGGTAACAG	FAM-TGCTGCTCCAACAATCGCATCCG†	CAAAGAGTATCACCAGCTTCGACTAC

*FAM represents the reporter dye, 6-carboxyfluorescein.

†Represents the non-fluorescent quenching dye, QSY7.

‡MGB is a minor groove binder.

washed with PBS at 30 min post-infection and prewarmed fresh medium containing 50 µg gentamicin sulfate ml⁻¹ was added. The number of internalized bacteria per coverslip was determined at 1 h post-infection by lysing infected cells in distilled water and plating appropriate serial dilutions of lysates onto LB (Luria-Bertani) agar plates.

Total RNA preparation and TaqMan qRT-PCR. Total RNA was purified from exponential- and stationary-phase bacterial cells using the RNeasy Protect/RNeasy Midi kit (Qiagen) and treated with RNase-free DNase as described by Sue *et al.* (2004). qRT-PCR was performed as described previously (Sue *et al.*, 2004) using the ABI Prism 7000 Sequence Detection System (Applied Biosystems). TaqMan primers and probes were designed using Primer Express software (Applied Biosystems) according to the manufacturer's guidelines. The primers and probes for *rpoB* and *inlA* were reported previously (Sue *et al.*, 2004); those created for this study are listed in Table 2. All primers were tested in PCRs with 10403S genomic DNA as template and the amplification products were evaluated by gel electrophoresis. For each RNA sample, the control transcript (*rpoB* or *gap* mRNA) and target gene transcripts (*prfA*, *clpC*, *inlA*, *inlB*, *actA* or *iap* mRNAs) were transcribed in the same 96-well plate, and the resulting cDNAs were quantified by real-time PCR. Specifically, RT-PCR reactions were performed using the TaqMan One-Step RT-PCR Master Mix Reagents kit according to the manufacturer's instructions (Applied Biosystems) using 25 ng total RNA with the following reaction conditions: 1 cycle at 48 °C for 30 min, 1 cycle at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Transcript levels for each gene (i.e. cDNA copy numbers) 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. Standard curves for each gene were generated by using serial dilutions of 10403S genomic DNA template that had been prepared as described by Flamm *et al.* (1984). Absolute cDNA copy numbers, which were calculated based on genomic DNA standard curves to reflect mRNA levels for each gene present in each RNA sample, were used for subsequent analyses.

Statistical analyses. For qRT-PCR data, expression levels of targeted genes were normalized using expression levels for housekeeping genes that had been processed in parallel with the targeted genes. *rpoB*, which encodes the β subunit of RNA polymerase (Milohanic *et al.*, 2003), and *gap*, which encodes glyceraldehyde-3-phosphate dehydrogenase, were chosen as two independent

housekeeping genes for data normalization (M. Kazmierczak & M. Wiedmann, unpublished data). Target gene expression level was normalized to a housekeeping gene expression level in the same sample by dividing (target gene cDNA copy number) by (*rpoB* cDNA copy number) or (*gap* cDNA copy number). Normalized target gene expression levels were then scale-transformed using their natural logarithms (ln) to stabilize the variance to approximate normality and expressed as ln[(target gene cDNA copy number)/(*rpoB* cDNA copy number)] or ln[(target gene cDNA copy number)/(*gap* cDNA copy number)]. Relative gene expression was evaluated by analysis of variance for strain, growth phase and interaction effects. Individual comparisons were done by the Bonferroni multiple comparison test. One-sample *t* tests were used to compare bacterial invasion abilities between the wild-type 10403S and each mutant strain. For all analyses, statistical significance was declared at $P < 0.05$. All statistical analyses were done with Statistix 7 (Analytical Software).

RESULTS AND DISCUSSION

Relative contributions of InlA, InlB, σ^B and PrfA to *L. monocytogenes* invasion of Caco-2 and HepG-2 cells

L. monocytogenes invasion of human non-professional phagocytic cells is predominantly mediated by two surface proteins, InlA and InlB (Dramsi *et al.*, 1995; Drevets *et al.*, 1995; Gaillard *et al.*, 1987, 1991, 1996; Lingnau *et al.*, 1995; Mengaud *et al.*, 1996). Recognition of the invasion-defective phenotype of the $\Delta sigB$ strain in non-phagocytic cells (Kim *et al.*, 2004), of P_{4_{inlA}} as a σ^B -dependent promoter, and of P_{2_{inlB}} as a putative σ^B -dependent promoter (Kazmierczak *et al.*, 2003) identified σ^B as an important factor contributing to regulation of *inlA* and *inlB*. The *inlA* and *inlB* genes are transcribed both individually and in an operon (Lingnau *et al.*, 1995). To date, six promoters have been identified in the *inlAB* locus, which include a confirmed PrfA-regulated promoter (P_{3_{inlA}}) and a confirmed σ^B -dependent promoter (P_{4_{inlA}}) upstream of *inlA*, and a putative σ^B -dependent promoter (P_{2_{inlB}}) upstream of *inlB* (Kazmierczak *et al.*, 2003; Lingnau *et al.*, 1995). The PrfA-regulated promoter,

Table 3. Bacterial invasion

Results are reported as percentages relative to wild-type strain invasion, which was arbitrarily set to 100 %. Means and standard deviations from three independent experiments are shown. The 100 % values correspond to absolute invasiveness values of $1.27 \times 10^{-4} \pm 0.30 \times 10^{-4}$ for Caco-2 exponential-phase; $4.37 \times 10^{-4} \pm 1.27 \times 10^{-4}$ for Caco-2 stationary-phase; $8.76 \times 10^{-5} \pm 1.42 \times 10^{-5}$ for HepG-2 exponential-phase; $6.89 \times 10^{-4} \pm 2.12 \times 10^{-4}$ for HepG-2 stationary-phase.

P values for comparison by one-sample *t* test of invasion capabilities between a strain bearing a single mutation (e.g. $\Delta sigB$) and the wild-type strain are indicated by asterisks: *, <0.05; **, <0.01; ***, <0.001. In parentheses are *P* values for comparison by one-sample *t* test of invasion capabilities between $\Delta inlA \Delta sigB$ and $\Delta inlA$, $\Delta inlB \Delta sigB$ and $\Delta inlB$, $\Delta inlA \Delta sigB$ and $\Delta inlAB$ (*, <0.05; **, <0.01).

Strain	Caco-2		HepG-2	
	Exponential-phase	Stationary-phase	Exponential-phase	Stationary-phase
Wild-type	100	100	100	100
$\Delta sigB$	$29.2 \pm 14.6^*$	$26.8 \pm 6.2^{**}$	$17.9 \pm 10.5^{**}$	$1.7 \pm 0.7^{***}$
$\Delta prfA$	$30.7 \pm 3.2^{***}$	64.8 ± 31.1	$13.3 \pm 7.2^{**}$	$57.8 \pm 14.1^*$
$\Delta inlA$	$5.8 \pm 1.3^{***}$	$2.4 \pm 1.6^{***}$	$15.6 \pm 15.3^*$	$2.3 \pm 0.5^{***}$
$\Delta inlA \Delta sigB$	$1.8 \pm 0.9^{***(*)}$	$1.1 \pm 0.5^{***}$	$13.7 \pm 20.0^*$	$1.8 \pm 1.4^{***}$
$\Delta inlB$	$48.6 \pm 14.7^*$	$52.4 \pm 2.9^{**}$	$26.2 \pm 6.4^{**}$	$20.1 \pm 5.2^{**}$
$\Delta inlB \Delta sigB$	$18.5 \pm 9.1^{**(*)}$	$23.4 \pm 12.9^{**(*)}$	$9.9 \pm 8.9^{**}$	$1.4 \pm 0.5^{***(**)}$
$\Delta inlAB$	$2.6 \pm 0.3^{***}$	$2.3 \pm 2.3^{***}$	$10.8 \pm 10.8^{**}$	$0.8 \pm 0.4^{***}$
$\Delta inlAB \Delta sigB$	$2.0 \pm 0.7^{***}$	$1.3 \pm 1.3^{***}$	$6.3 \pm 7.9^{**}$	$0.8 \pm 1.0^{***}$

P_{3inlA} , is the only promoter reported to generate a bicistronic transcript (Lingnau *et al.*, 1995); therefore, we reasoned that any σ^B -mediated effects on *inlB* expression would probably occur through P_{2inlB} . To quantify contributions of σ^B to *L. monocytogenes* invasion, we analysed invasion capabilities of various mutant strains bearing combinations of in-frame deletions in *inlA*, *inlB* and *sigB* (Table 1) using Caco-2 and HepG-2 cells (Table 3). We rationalized that additional effects of a $\Delta sigB$ mutation in a $\Delta inlAB$ background could be interpreted as the contribution of σ^B beyond that which is mediated by InlA or InlB.

Invasion of the $\Delta inlA$ strain was significantly reduced in both Caco-2 and HepG-2 cells (Table 3), confirming the importance of InlA to *L. monocytogenes* invasion into both cell lines. The invasion defect of the $\Delta inlA$ strain was more pronounced with stationary-phase than with exponential-phase bacteria (Table 3): invasion capability of the $\Delta inlA$ strain was reduced by 17- and 42-fold in Caco-2 cells, and by 6- and 43-fold in HepG-2 cells, with exponential- and stationary-phase bacteria, respectively. These findings are in agreement with previous reports of the importance of InlA in *L. monocytogenes* invasion of these host cell lines (Dramsi *et al.*, 1995; Gaillard *et al.*, 1991; Lecuit *et al.*, 1999), and further demonstrate that the contributions of InlA to invasion are more critical in stationary-phase cells than in exponential-phase cells.

The $\Delta inlB$ strain was more defective in invasion of HepG-2 cells than of Caco-2 cells, as previously reported (Dramsi *et al.*, 1995) (Table 3); however, the $\Delta inlB$ invasion defect was less severe than that of $\Delta inlA$ in both cell lines (Table 3).

Further, in contrast to the $\Delta inlA$ strain, the relative invasion defect associated with the $\Delta inlB$ strain was similar regardless of growth phase (Table 3). Specifically, invasion of the $\Delta inlB$ strain was reduced 2- and 2-fold in Caco-2 cells and 4- and 5-fold in HepG-2 cells with exponential- and stationary-phase bacteria, respectively. In the absence of both *inlA* and *inlB*, *L. monocytogenes* invasion was reduced 38- and 43-fold in Caco-2 cells, and 9- and 125-fold in HepG-2 cells with exponential- and stationary-phase bacteria, respectively (Table 3). While loss of both InlA and InlB greatly reduced *L. monocytogenes* invasion of Caco-2 cells independently of growth phase, the effects of their loss on HepG-2 invasion were more pronounced with stationary-phase bacteria.

InlB is required for *L. monocytogenes* entry into hepatocytes (Dramsi *et al.*, 2004). As previously reported (Dramsi *et al.*, 1995), we also found that the *L. monocytogenes* 10403S $\Delta inlB$ strain was more defective in invasion of HepG-2 cells than that of Caco-2 cells. However, our $\Delta inlB$ strain was less defective at invasion than the $\Delta inlA$ strain in both HepG-2 and Caco-2 cells (Table 3). This observation contrasts with the results of Dramsi *et al.* (1995), who showed a threefold reduced invasion capacity for a *L. monocytogenes* EGD $\Delta inlB$ strain relative to that of an EGD $\Delta inlA$ strain in HepG-2 cells. The most likely explanations for this discrepancy are: (i) as InlA-mediated invasion is affected by bacterial growth phase (Table 3), differences in bacterial growth and harvest conditions between the experiments are likely to affect relative strain invasion capacity; and (ii) the relative roles of the internalin proteins in mediating host cell entry may differ between *L. monocytogenes* EGD and 10403S.

Invasion by the $\Delta sigB$ strain was significantly reduced compared with that of the wild-type strain (Table 3). Loss of σ^B resulted in a greater bacterial invasion defect with HepG-2 cells than with Caco-2 cells. In Caco-2 cells, the ability of the $\Delta sigB$ strain to invade was reduced 3- and 4-fold with exponential- and stationary-phase bacteria, respectively. In HepG-2 cells, invasion of the $\Delta sigB$ strain was decreased 6- and 59-fold with exponential- and stationary-phase bacteria, respectively, which is essentially equivalent to the invasion defect resulting from the $\Delta inlA$ mutation in this host cell line.

Loss of σ^B in the $\Delta inlA$ background resulted in a further reduction of exponential-phase bacterial invasion in Caco-2 cells (Table 3), while loss of σ^B in the $\Delta inlB$ background resulted in a further reduction in *L. monocytogenes* invasion in both host cell lines, regardless of bacterial growth phase (Table 3). Loss of σ^B in the $\Delta inlAB$ background did not contribute to a further reduction in *L. monocytogenes* invasion (Table 3). Taken together, these results suggest that σ^B contributes to invasion of *L. monocytogenes* into Caco-2 and HepG-2 cells predominantly by directly affecting InlA- and InlB-mediated invasion pathways rather than through indirect mechanisms, such as those that might be mediated by PrfA. Further, the invasion defects resulting from additional loss of σ^B are essentially equivalent to those resulting from loss of InlA in the $\Delta inlB$ background for *L. monocytogenes* invasion into HepG-2 cells.

Invasion capabilities of the $\Delta prfA$ strain were reduced relative to those of the wild-type strain in both Caco-2 and HepG-2 cells (3- and 2-fold decrease in Caco-2 cells, and 8- and 2-fold decrease in HepG-2 cells with exponential- and stationary-phase bacteria, respectively; Table 3). The ability of the $\Delta prfA$ strain to invade these host cells was similar to that of the $\Delta sigB$ strain for exponential-phase bacteria, but greater than that of the $\Delta sigB$ strain for stationary-phase bacteria (Table 3). These results suggest that the contribution of PrfA to *L. monocytogenes* invasion differs with growth phase, with a greater relative contribution in exponential-phase than in stationary-phase bacteria.

σ^B modulates expression of *inlA* and *inlB*

To determine the effect of σ^B on the expression of genes responsible for *L. monocytogenes* entry into non-phagocytic cells, we analysed relative expression of six selected genes (*prfA*, *clpC*, *inlA*, *inlB*, *actA* and *iap*) in the wild-type and $\Delta sigB$ strains using TaqMan qRT-PCR. To provide two independent assessments of relative gene expression patterns, mRNA collected from two different housekeeping genes, *rpoB* and *gap*, was used to normalize target gene expression data. Although expression patterns generated by normalizing target gene transcripts with those of each housekeeping gene were similar, they were not identical (Fig. 1). To provide the most conservative interpretation of the data, transcript levels representing a target gene under a given condition were only deemed different from those of the gene under a different condition (e.g. exponential- vs

stationary-phase) or in a different background (wild-type vs $\Delta sigB$) if levels were statistically significantly different by both normalizing analyses.

σ^B -dependent *inlA* expression has been reported previously (Kim *et al.*, 2004); however, several lines of evidence suggest that σ^B -mediated effects on *inlA* and *inlB* expression may be direct or indirect, and that several factors affect *inlAB* expression. For example, σ^B could indirectly contribute to *inlAB* locus transcription through its control of *prfA* expression, as transcription initiated from P_{2prfA} is σ^B -dependent (Nadon *et al.*, 2002). Further, both *prfA* and *clpC* have been shown to modulate transcription of the *inlAB* locus (Drams *et al.*, 1993; Lingnau *et al.*, 1995; Nair *et al.*, 2000a; Sokolovic *et al.*, 1993).

qRT-PCR analyses showed that in exponential-phase *L. monocytogenes*, levels of *inlA* expression were similarly low in the wild-type and the $\Delta sigB$ strains (Fig. 1). In stationary phase, however, *inlA* expression was significantly up-regulated in the wild-type strain (9–19-fold) ($P < 0.05$), but remained at a level similar to that in exponential phase in the $\Delta sigB$ strain (Fig. 1). These results show that σ^B plays a critical role for stationary-phase up-regulation of *inlA*. As with *inlA* (Fig. 1), exponential-phase *inlB* expression was similarly low in the wild-type and the $\Delta sigB$ strains (Fig. 1), and stationary-phase *inlB* expression was significantly up-regulated in the wild-type (3–6-fold) ($P < 0.05$), but not in the $\Delta sigB$ strain (Fig. 1). These findings demonstrate that σ^B contributes to *inlB* expression as well as to *inlA* expression in stationary-phase bacteria.

Relative expression of *prfA* was evaluated for the wild-type and $\Delta sigB$ strains, as the P_{2prfA} promoter has been shown to be σ^B -dependent (Nadon *et al.*, 2002) and as regulation of *inlA* and *inlB* also is influenced by a PrfA-dependent mechanism (Drams *et al.*, 1993; Lingnau *et al.*, 1995). Although *prfA* expression appeared higher in the wild-type strain than in the $\Delta sigB$ strain, both in exponential and in stationary phase (Fig. 1), the differences were not statistically significant. Further, *prfA* expression also was not statistically different in exponential- and stationary-phase cells. These results suggest that the σ^B -regulated P_{2prfA} promoter does not play a predominant role in *prfA* expression under the conditions examined in this study, and that increased transcriptional activation of *prfA* is not required for increased expression of *inlA* and *inlB* in stationary-phase *L. monocytogenes* cells. These data provide additional support for the conclusion that σ^B contributes to *L. monocytogenes* invasion primarily by directly affecting *inlA* and *inlB* expression, rather than through indirect effects mediated by PrfA.

σ^B does not make major contributions to *clpC*, *actA* or *iap* expression under the conditions examined in this study

To quantify contributions of σ^B to expression of multiple *L. monocytogenes* invasion genes, *iap*, *actA* and *clpC*

Fig. 1. Relative expression of six virulence genes in exponential-phase (Exp.) and stationary-phase (Stat.) cells of wild-type and $\Delta sigB$ backgrounds. Relative gene expression, reported as [target gene mRNA level/*rpoB* mRNA level] (a) or [target gene mRNA level/*gap* mRNA level] (b) on the y-axis for *prfA*, *clpC*, *inlA*, *inlB*, *actA* and *iap* in the wild-type (black bars) and $\Delta sigB$ (striped bars) strains. Error bars represent standard deviations from three independent experiments. Differing upper-case letters within the same graph (i.e. A, B, C) indicate statistically significant differences in relative gene expression ($P < 0.05$) by ANOVA on ln-transformed data (ln[mRNA level of gene of interest/mRNA level of housekeeping gene, *rpoB* (a) or *gap* (b)]) for strain, growth phase and interaction effect in a given gene of interest.

transcripts were measured using qRT-PCR in both wild-type and $\Delta sigB$ backgrounds. *iap* encodes a major surface protein, p60, which is indirectly involved in invasion (Wuenschel *et al.*, 1993) and *actA* also participates in *L. monocytogenes* invasion (Alvarez-Dominguez *et al.*, 1997). *clpC* reportedly contributes to *L. monocytogenes* virulence (Rouquette *et al.*, 1996, 1998) and influences expression of *inlA*, *inlB* and *actA* (Nair *et al.*, 2000a). Regulation of *clpC* appears to be very complex, involving several regulators, including CtsR, PrfA and σ^B (Nair *et al.*, 2000b; Ripio *et al.*, 1998).

Expression of *clpC* and *iap* was significantly affected by bacterial growth phase (Fig. 1). Specifically, transcripts for both genes were present at significantly higher levels in stationary-phase bacteria than in exponential-phase bacteria for both the wild-type and $\Delta sigB$ strains ($P < 0.05$). Although *clpC* and *iap* transcripts appeared to be present at higher levels in the wild-type strain than in the $\Delta sigB$

strain (Fig. 1a), the differences were not statistically significant at the 95 % confidence level when expression data were normalized by *gap* (Fig. 1b), suggesting that σ^B is not a predominant contributor to *clpC* or *iap* expression under the conditions examined in this study. In contrast, while *actA* also appeared to be affected by bacterial growth phase, *actA* transcripts were present at higher levels in exponential-phase than in stationary-phase bacteria for both strains when data were normalized by *rpoB* (Fig. 1). Transcript levels were not lower in the $\Delta sigB$ strain, and did not differ significantly between the wild-type and $\Delta sigB$ strains in data normalized by *gap*, suggesting that σ^B is not a positive regulator of *actA* expression in *L. monocytogenes*. These results suggest that any contributions of Iap, ActA and ClpC to *L. monocytogenes* invasion are predominantly independent of σ^B , providing further support for the hypothesis that σ^B -mediated invasion effects occur primarily through its regulation of expression of *inlA* and *inlB*.

Conclusions

The ability of *L. monocytogenes* to invade non-phagocytic cells allows the organism to breach host barriers, and hence is critical for systemic listeriosis. Our results demonstrate that σ^B significantly contributes to *L. monocytogenes* invasion of human enterocytes and hepatocytes, predominantly through InlA- and InlB-mediated pathways, as shown by both invasion and TaqMan qRT-PCR assay results. Specifically, we have shown that while stationary-phase expression of *inlA* and *inlB* is significantly enhanced (9–18-fold for *inlA* expression; 3–6-fold for *inlB* expression) in the wild-type strain relative to that in exponential phase (Fig. 1), stationary-phase expression of *inlA* and *inlB* does not increase in the $\Delta sigB$ strain. Further, loss of σ^B did not significantly reduce expression of *Iap*, *ActA* or *ClpC*, each of which have been associated with *L. monocytogenes* invasion. Our data support a model in which invasion defects associated with loss of σ^B result from loss of σ^B -mediated transcription of the *inlAB* locus, with relatively minor, if any, indirect effects resulting from σ^B -dependent expression of *prfA*. In support of this hypothesis, we have demonstrated dramatically reduced expression of both *inlA* and *inlB* in stationary-phase $\Delta sigB$ cells despite essentially wild-type expression levels for *prfA* (Fig. 1). However, our results do not rule out the possibility that the relative role of PrfA in invasion reflects growth-phase-dependent changes in PrfA activity to a greater extent than it reflects changes in *prfA* transcriptional activation, as PrfA is known to exist in both active and inactive forms (Renzoni *et al.*, 1997). While additional studies will be necessary to fully attribute the relative contributions of PrfA- and σ^B -mediated mechanisms to invasion, the results presented in this study clearly highlight critical contributions of σ^B to *L. monocytogenes* invasion into non-phagocytic cells.

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