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

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Abstract

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* Δ*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 Δ*sigB* and Δ*prfA* strains were compared. Exponential-phase cells of the Δ*sigB* and Δ*prfA* strains were similarly defective at invasion; however, stationary-phase Δ*sigB* cells were significantly less invasive than stationary-phase Δ*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.

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

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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; Wuenscher *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 upstream of *inlB* ($P2_{inlB}$) (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 $P2_{prfA}$ 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 Δ*sigB* and Δ*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 Δ*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 OD_{600} 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. Δ*inlA*Δ*sigB* (strain FSL B2-042), Δ*inlB*Δ*sigB* (strain FSL K4-008), and Δ*inlAB*Δ*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 Δ*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 % CO_2. 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, $1{\cdot}5\times10^5$ Caco-2 and $7{\cdot}5\times10^5$ 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 cover-slips. 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 10^8 c.f.u. of exponential- or stationary-phase bacteria were inoculated onto the host cell monolayer in each well. Host cells were 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 RNAprotect/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 $P<0{\cdot}05$. 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 Δ*sigB* strain in non-phagocytic cells (Kim *et al.*, 2004), of $P4_{inlA}$ as a σ^B-dependent promoter, and of $P2_{inlB}$ 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 ($P3_{inlA}$) and a confirmed σ^B-dependent promoter ($P4_{inlA}$) upstream of *inlA*, and a putative σ^B-dependent promoter ($P2_{inlB}$) upstream of *inlB* (Kazmierczak *et al.*, 2003; Lingnau *et al.*, 1995). The PrfA-regulated promoter, $P3_{inlA}$, 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 $P2_{inlB}$. 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 Δ*sigB* mutation in a Δ*inlAB* background could be interpreted as the contribution of σ^B beyond that which is mediated by InlA or InlB.

Invasion of the Δ*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 Δ*inlA* strain was more pronounced with stationary-phase than with exponential-phase bacteria (Table 3): invasion capability of the Δ*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 Δ*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 Δ*inlB* invasion defect was less severe than that of Δ*inlA* in both cell lines (Table 3). Further, in contrast to the Δ*inlA* strain, the relative invasion defect associated with the Δ*inlB* strain was similar regardless of growth phase (Table 3). Specifically, invasion of the Δ*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 Δ*inlB* strain was more defective in invasion of HepG-2 cells than that of Caco-2 cells. However, our Δ*inlB* strain was less defective at invasion than the Δ*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 Δ*inlB* strain relative to that of an EGD Δ*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 Δ*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 Δ*sigB* strain to invade was reduced 3- and 4-fold with exponential- and stationary-phase bacteria, respectively. In HepG-2 cells, invasion of the Δ*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 Δ*inlA* mutation in this host cell line.

Loss of σ^B in the Δ*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 Δ*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 Δ*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 Δ*inlB* background for *L. monocytogenes* invasion into HepG-2 cells.

Invasion capabilities of the Δ*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 Δ*prfA* strain to invade these host cells was similar to that of the Δ*sigB* strain for exponential-phase bacteria, but greater than that of the Δ*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 Δ*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 Δ*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 $P2_{prfA}$ is σ^B-dependent (Nadon *et al.*, 2002). Further, both *prfA* and *clpC* have been shown to modulate transcription of the *inlAB* locus (Dramsi *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 Δ*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 Δ*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 Δ*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 Δ*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 Δ*sigB* strains, as the $P2_{prfA}$ 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 (Dramsi *et al.*, 1993; Lingnau *et al.*, 1995). Although *prfA* expression appeared higher in the wild-type strain than in the Δ*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 $P2_{prfA}$ 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* transcripts were measured using qRT-PCR in both wild-type and Δ*sigB* backgrounds. *iap* encodes a major surface protein, p60, which is indirectly involved in invasion (Wuenscher *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 Δ*sigB* strains ($P<0{\cdot}05$). Although *clpC* and *iap* transcripts appeared to be present at higher levels in the wild-type strain than in the Δ*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 Δ*sigB* strain, and did not differ significantly between the wild-type and Δ*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 Δ*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 Δ*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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Abbreviation

qRT-PCR	quantitative reverse-transcriptase polymerase chain reaction

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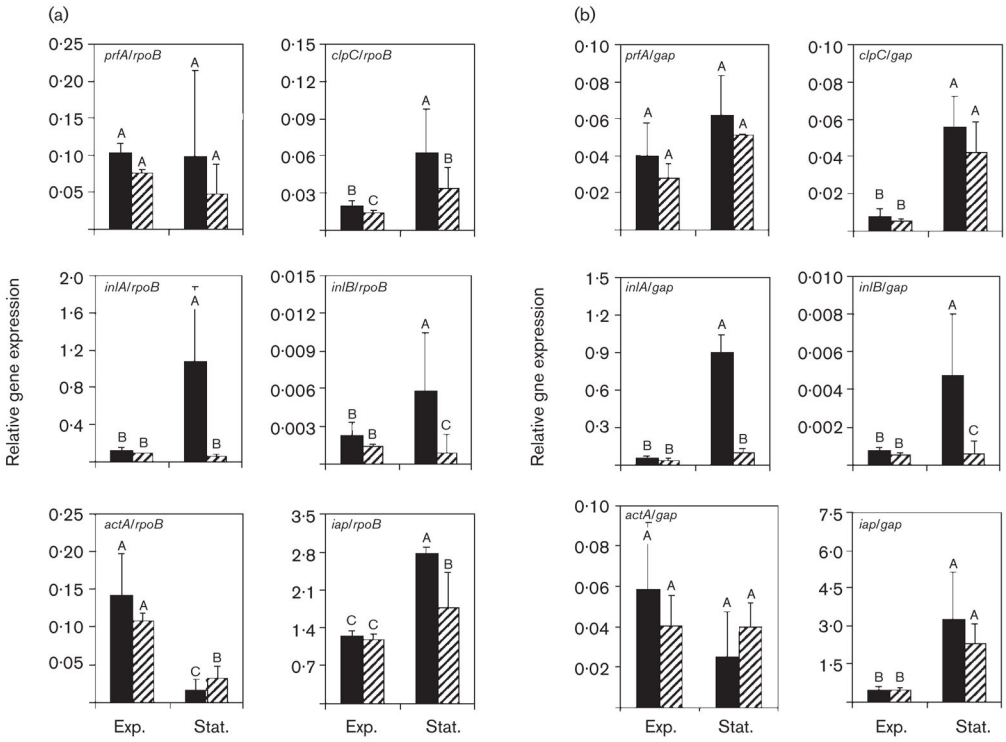

Fig. 1.

Relative expression of six virulence genes in exponential-phase (Exp.) and stationary-phase (Stat.) cells of wild-type and Δ*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 Δ*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 ($P<0{\cdot}05$) 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.

Table 1

L. monocytogenes strains

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

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Table 2

TaqMan primer and probe sequences

Gene	Forward primer (5′→3′)	Taqman probes (5′→3′)*	Reverse primer (5′→3′)
gap	AAAGCTGGCGCTAAAAAAGTTG	FAM-ATCTCCGCTCCAGCAACTGGCGATAT†	TTCATGGTTTACATTGTAAACGATTG
prfA	CAATGGGATCCACAAGAATATT-GTAT	FAM-TGTAAATTCATGATGGTCCCGTTCTC-GCT†	AATAAAGCCAGACATTATAACGAAAGC
clpC	CGGCGAAAGCTCTCTATGAACT	FAM-TCCCTCTACCTCTTGCTGCACTTTTT-CAGA†	GGTGTATATTGGATCGTCGTCACA
inlB	GCAAATTTTTCCAGATGATGCT-TT	FAM-CAGAAACAATCAAAGACAAT†-MGB‡	TGTCACTGCATCTGTCACACTTTT
actA	TGCGTGCGATGATGGTAGTT	FAM-CCAACTGCATTACGATTAACCCCGA-CATAA†	TTCGCTATCTGTCGCTGCAA
iap	AGCTGGGATTGCGGTAACAG	FAM-TGCTGCTCCAACAATCGCATCCG†	CAAAGAGTATCACCAGCTTCGACTAC

*FAM represents the reporter dye, 6-carboxyfluorescein.

†Represents the non-fluorescent quenching dye, QSY7.

‡MGB is a minor groove binder.

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 $1{\cdot}27\times10^{-4}\pm0{\cdot}30\times10^{-4}$ for Caco-2 exponential-phase; $4{\cdot}37\times10^{-4}\pm1{\cdot}27\times10^{-4}$ for Caco-2 stationary-phase; $8{\cdot}76\times10^{-5}\pm1{\cdot}42\times10^{-5}$ for HepG-2 exponential-phase; $6{\cdot}89\times10^{-4}\pm2{\cdot}12\times10^{-4}$ for HepG-2 stationary-phase.

Strain	Caco-2		HepG-2	
	Exponential-phase	Stationary-phase	Exponential-phase	Stationary-phase
Wild-type	100	100	100	100
Δ*sigB*	29·2±14·6*	26·8±6·2**	17·9±10·5**	1·7±0·7***
Δ*prfA*	30·7±3·2***	64·8±31·1	13·3±7·2**	57·8±14·1*
Δ*inlA*	5·8±1·3***	2·4±1·6***	15·6±15·3*	2·3±0·5***
Δ*inlA*Δ*sigB*	1·8±0·9***(*)	1·1±0·5***	13·7±20·0*	1·8±1·4***
Δ*inlB*	48·6±14·7*	52·4±2·9**	26·2±6·4**	20·1±5·2**
Δ*inlB*Δ*sigB*	18·5±9·1**(*)	23·4±12·9**(*)	9·9±8·9**	1·4±0·5***(**)
Δ*inlAB*	2·6±0·3***	2·3±2·3***	10·8±10·8**	0·8±0·4***
Δ*inlAB*Δ*sigB*	2·0±0·7***	1·3±1·3***	6·3±7·9**	0·8±1·0***

P values for comparison by one-sample *t* test of invasion capabilities between a strain bearing a single mutation (e.g. Δ*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 Δ*inlA*Δ*sigB* and Δ*inlA*, Δ*inlB*Δ*sigB* and Δ*inlB*, Δ*inlAB*Δ*sigB* and Δ*inlAB* (*, <0·05; **, <0·01).