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# Growth Temperature-Dependent Contributions of Response Regulators, $\sigma^B$, PrfA, and Motility Factors to *Listeria monocytogenes* Invasion of Caco-2 Cells

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## Abstract

Foodborne pathogens encounter rapidly changing environmental conditions during transmission, including exposure to temperatures below 37°C. The goal of this study was to develop a better understanding of the effects of growth temperatures and temperature shifts on regulation of invasion phenotypes and invasion-associated genes in *Listeria monocytogenes*. We specifically characterized the effects of *L. monocytogenes* growth at different temperatures (30°C vs. 37°C) on (i) the contributions to Caco-2 invasion of different regulators (including $\sigma^B$, PrfA, and 14 response regulators [RRs]) and invasion proteins (i.e., InlA and FlaA), and on (ii) *gadA*, *plcA*, *inlA*, and *flaA* transcript levels and their regulation. Overall, Caco-2 invasion efficiency was higher for *L. monocytogenes* grown at 30°C than for bacteria grown at 37°C ( $p = 0.0051$ for the effect of temperature on invasion efficiency; analysis of variance); the increased invasion efficiency of the parent strain 10403S (serotype 1/2a) observed after growth at 30°C persisted for 2.5 h exposure to 37°C. For *L. monocytogenes* grown at 30°C, the motility RRs DegU and CheY and $\sigma^B$, but not PrfA, significantly contributed to Caco-2 invasion efficiency. For *L. monocytogenes* grown at 37°C, none of the 14 RRs tested significantly contributed to Caco-2 invasion, whereas $\sigma^B$ and PrfA contributed synergistically to invasion efficiency. At both growth temperatures there was significant synergism between the contributions to invasion of FlaA and InlA; this synergism was more pronounced after growth at 30°C than at 37°C. Our data show that growth temperature affects invasion efficiency and regulation of virulence-associated genes in *L. monocytogenes*. These data support increasing evidence that a number of environmental conditions can modulate virulence-associated phenotypes of foodborne bacterial pathogens, including *L. monocytogenes*.

## Introduction

L*isteria monocytogenes* is a Gram-positive, non-spore-forming rod that is capable of causing disease in humans and animals. *L. monocytogenes* can enter intestinal epithelia via an internalization process initiated by the interaction of InlA, expressed on the surface of invading *L. monocytogenes*, and E-cadherin, expressed on the epithelial cell surface. Although the organism is widespread in nature, 99% of *L. monocytogenes* infections are foodborne (Mead *et al.*, 1999). *L. monocytogenes* therefore encounter a variety of different environments and associated stress conditions during transmission from the environment through foods to humans, including a wide range of temperature, pH, and osmotic stress conditions.

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The alternative sigma factor, $\sigma^B$, the pleiotropic transcriptional regulator PrfA, and two-component regulatory systems (TCS) have all been shown to regulate key processes important for *L. monocytogenes* stress response and virulence (Kallipolitis and Ingmer, 2001; Kazmierczak *et al.*, 2003; Williams *et al.*, 2005a; Scortti *et al.*, 2007). In addition to a large stress response regulon, $\sigma^B$ specifically regulates the transcription of genes involved in responses to stresses encountered during passage through the gastrointestinal system such as osmotic stress and acid stress. $\sigma^B$ also regulates transcription of genes encoding different internalins that are involved in entry into host cells (Raffelsbauer *et al.*, 1998; Kazmierczak *et al.*, 2003; Kim *et al.*, 2005; McGann *et al.*, 2007b). PrfA regulates the *L. monocytogenes* core virulence genes *plcA*, *plcB*, *hly*, *mpl*, and *actA*, which are important for escape from the vacuole and cell-to-cell spread (Scortti *et al.*, 2007) and also co-regulates, with $\sigma^B$, other virulence genes, including *inlA* (McGann *et al.*, 2008). Among the 16 putative or confirmed TCS in *L. monocytogenes*, a number of them have been shown to regulate bacterial adaptation (Autret *et al.*, 2003; Brondsted *et al.*, 2003; Kallipolitis *et al.*, 2003; Dons *et al.*, 2004; Mandin *et al.*, 2005; Sleator and Hill, 2005; Williams *et al.*, 2005b; Larsen *et al.*, 2006), including VirR, DegU and CheY, which have been implicated in regulating mechanisms contributing to host cell invasion (Dons *et al.*, 2004; Knudsen *et al.*, 2004; Mandin *et al.*, 2005).

Growth temperature has been shown to have a profound effect on activities of key *L. monocytogenes* regulators (Leimeister-Wachter *et al.*, 1992; Liu *et al.*, 2002; Dons *et al.*, 2004; Chan *et al.*, 2007b; McGann *et al.*, 2007a; van der Veen *et al.*, 2007). For example, expression levels of the genes comprising the PrfA-regulated *Listeria* pathogenicity island I, which are important in escape from the host cell vacuole and cell-to-cell spread, are maximal at 37°C due to a *prfA* mRNA thermosensor that represses PrfA translation at lower temperatures (Johansson *et al.*, 2002). Also, certain $\sigma^B$-dependent genes have been shown to be involved in adaptation to cold temperatures (Becker *et al.*, 2000; Chan *et al.*, 2007a), and $\sigma^B$-dependent internalin genes, including *inlC2* and *inlD*, have been shown to be expressed at higher levels at temperatures ≤30°C than at 37°C (McGann *et al.*, 2007a). Finally, *L. monocytogenes* motility, which has been shown to contribute to host cell invasion (Dons *et al.*, 2004) and increased virulence in a mouse model (O'Neil and Marquis, 2006), is temperature dependent. At 37°C, the transcription of *flaA* (the gene encoding the flagellin structural protein) and other motility-associated genes is repressed by MogR (Grundling *et al.*, 2004; Shen and Higgins, 2006), though the stringency of this repression may vary among different strains of *L. monocytogenes*. For example, repression of motility genes and associated motility phenotypes has been shown to be less stringent in *L. monocytogenes* 10403S than in other strains (Grundling *et al.*, 2004; Way *et al.*, 2004).

Although *L. monocytogenes* will have likely been adapted to a temperature below 37°C before ingestion, many studies on *L. monocytogenes* host cell invasion have been done only on *L. monocytogenes* cells grown at 37°C and have only determined the effects of single gene mutations on invasion (Cotter *et al.*, 1999; Kallipolitis and Ingmer, 2001; Autret *et al.*, 2003; Williams *et al.*, 2005a). Therefore, a more comprehensive evaluation of the effect of growth temperature on the contributions of response regulators (RRs), transcriptional regulators, and motility genes as well as functional synergisms between select genes to host cell invasion is necessary to better understand how adaptation to environments outside a mammalian host affects *L. monocytogenes* virulence. The objective of this study was, thus, to characterize the effects of growth temperature (30°C vs. 37°C) on (i) the contributions of $\sigma^B$ and PrfA, 14 RRs, and invasion proteins InlA and FlaA to Caco-2 invasion, and on (ii) transcript levels of *inlA*, *flaA*, *gadA*, and *plcA*. Growth temperatures of 30°C and 37°C were chosen because *L. monocytogenes* grown at 30°C and 37°C show very similar lag phase durations and growth rates, whereas *L. monocytogenes* grown at lower temperatures (e.g. 22.5°C; [Pal *et al.*, 2008]) show considerably longer lag phase durations and slower growth

rates. As Caco-2 invasion efficiency has been shown to vary with bacterial growth phase (Garner *et al.*, 2006a), it is critical to use bacteria grown with similar growth parameters and synchronized to similar growth phases to ensure that differences in invasion efficiency represent a temperature effect rather than differences in growth phase. In addition, *L. monocytogenes* grown at 30°C have previously been shown to display differential expression of key virulence-associated characteristics, including (i) downregulation of PrfA activity (Johansson *et al.*, 2002) and (ii) upregulation of motility (O'Neil and Marquis, 2006). Consistent with our choice of 30°C as a growth temperature that allows for appropriate comparison between *L. monocytogenes* grown at typical mammalian body temperature and bacteria grown under environmental conditions, others (Toledo-Arana *et al.*, 2009) have recently used 30°C as a growth temperature representing the saprophytic stage of the life of *L. monocytogenes*. Despite these key reasons for using growth temperatures of 30°C and 37°C to study temperature-dependent phenotypes of *L. monocytogenes*, we appreciate that these temperatures represent an experimental model that does not necessarily reflect natural transmission where foodborne bacteria are more likely exposed to refrigeration or room temperatures or heat shock conditions before ingestion.

## Materials and Methods

### Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table 1. For invasion assays, *L. monocytogenes* 10403S (Bishop and Hinrichs, 1987) and associated mutant strains were grown to early stationary phase as previously described (McGann *et al.*, 2007a). Briefly, a 12 to 18 h culture grown at 37°C with aeration (i.e., shaking at 220 rpm) in brain heart infusion broth (BHI) was diluted 1:100 into 5 mL of fresh BHI and grown at 37°C with aeration to $OD_{600}$ = 0.4. This culture was diluted 1:100 into another 5 mL of fresh BHI and grown at 30°C or 37°C with aeration to early stationary phase (defined as growth to $OD_{600}$ = 1.0, followed by an additional 3 h incubation). For temperature shift experiments, a 1 mL aliquot of the early stationary phase culture was centrifuged at 12,000 rpm for 10 min, resuspended in phosphate-buffered saline (PBS; 0.14 M NaCl, 2.7 mM KCl, 10 mM $Na_2PO_4$, and 1.8 mM $KH_2PO_4$; pH 7.2), and incubated statically at 30°C or 37°C for 0, 2.5, or 5 h.

### Mutant construction

Internal, in-frame, nonpolar deletion mutant alleles in 14 RR genes (Table 1) were created using splicing overlap extension polymerase chain reaction (PCR) (see Supplemental Table S1, available online at www.liebertonline.com for primers; all supplemental material is located at http://author.cals.cornell.edu/cals/foodsci/research/labs/wiedmann/links/ivy2010-1.cfm) and cloned into the temperature-sensitive suicide shuttle vector pKSV7 (Smith and Youngman, 1992) as previously detailed by our group (Wiedmann *et al.*, 1998). Mutant alleles were introduced into *L. monocytogenes* 10403S using standard allelic exchange mutagenesis procedures (Camilli *et al.*, 1993). Mutant strains were confirmed with sequencing. *lmo0287* is likely to be essential, as null mutants in this gene could not be constructed here, consistent with similar studies by others (Kallipolitis and Ingmer, 2001;Williams *et al.*, 2005a).

### Caco-2 invasion assays

The Caco-2 cell-line (ATCC HTB-37) was maintained at 37°C (4%–6% $CO_2$ and 85% humidity) in a Caco-2 medium, which was Dulbecco's minimal essential medium with Earle's salts, 1% sodium pyruvate, 20% fetal bovine serum, 1.0% nonessential amino acids, 1.5 g/L sodium bicarbonate, and, when appropriate, penicillin G and streptomycin (each at 100 $\mu$g/mL) (all reagents were obtained from Gibco, a subsidiary of Invitrogen). All

invasion assays were performed at 37°C as previously described (Garner *et al.*, 2006b) with minor modifications. Briefly, 48 h before the assay, Caco-2 cells were seeded (from a culture passaged no >60 times) into 24-well tissue culture plates (Corning Inc.) at a density of $5\times10^4$ cells/well in the Caco-2 medium without antibiotics. For infection, ~$2\times10^7$ CFU *L. monocytogenes* were added to each well (representing a multiplicity of infection of ~200). All inocula were enumerated on BHI agar plates. Thirty minutes postinfection, the Caco-2 monolayers were washed three times with PBS to remove any unassociated *L. monocytogenes*, and the medium was replaced with a fresh Caco-2 medium. Forty-five minutes after infection, the medium was replaced with the Caco-2 medium plus 150 $\mu$g/mL gentamycin to kill any extracellular *L. monocytogenes*. At 90 min postinfection, Caco-2 cells were washed three times with PBS and lysed with ice-cold distilled water. Intracellular *L. monocytogenes* were enumerated by plating the appropriate dilutions of the Caco-2 lysate on BHI agar, using a spiral plater (Spiral Biotech). At least three independent trials of the invasion assays were performed with duplicate wells tested for each treatment in each replicate.

### Quantitative reverse transcriptase-PCR (qRT-PCR)

Transcript levels of *inlA*, *flaA*, *plcA*, *gadA*, *rpoB*, *sigB*, and *prfA* were quantified for select strains grown to early stationary phase at 30°C or 37°C using TaqMan probes and primers and the ABI Prism 7000 Sequence Detection System as previously described (Sue *et al.*, 2003; Chaturongakul and Boor, 2006; McGann *et al.*, 2007a) with one exception: copy numbers for each gene were normalized to *rpoB* levels. Primers and probes for *inlA*, *plcA*, *gadA*, *sigB*, *rpoB*, and *prfA* have been previously described (Sue *et al.*, 2004; Kim *et al.*, 2005; Kazmierczak *et al.*, 2006). *flaA* Taqman primers ( *flaA*-F: 5′-TCGTAAAAATAACGAAGGCATGAC-3′; flaA-R: AGA ACTGTTAATACGTTTACCAGATGCT-3′) and the *flaA* MGB probe (FAM-5′-CAAGCGCAAGAAC-3′NFQ) were designed using Primer Express 1.0 (Applied Biosystems).

### Statistical analyses

All statistical analyses were performed in JMP 7.0 (SAS Institute Inc.). Invasion efficiencies were initially analyzed using a one-way analysis of variance (ANOVA; $\alpha = 0.05$). Strain and, where appropriate, date of experiment were included as variables in the model. For each ANOVA, data were log-transformed to ensure that the data set satisfied ANOVA assumptions of normality of residuals and equality of variances. To determine whether a particular mutant strain differed from the parent strain (e.g., in invasion efficiency), a *post hoc* Dunnett's many to one test was used (Shun *et al.*, 2003). To compare invasion efficiencies for a given strain exposed to multiple conditions (i.e., as shown in Table 2) or among strains (in cases where a strain with a double mutation was included in the comparison), a *post hoc* Tukey honestly significantly different (HSD) test was used. For qRT-PCR, normalized log copy numbers for a given gene were compared among strains by using one-way ANOVA, followed by Tukey HSD. To measure whether two gene deletions showed an effect on invasion efficiency or transcript level that is more than additive (which indicates synergism), the parent strain, single mutants, and double mutants were assigned unique allelic states by coding dummy variables (e.g., "gene1" and "gene2") for each allelic state with 1 meaning the gene is present and 0 meaning the gene is absent. A two-way ANOVA was performed to determine the effect of each allelic state on invasion efficiency or transcript level in these analyses. "Date of experiment," "gene1," "gene2," and "gene1*gene2" were included as effects in the two-way ANOVA model. A significant effect of gene1*gene2 in the model (i.e., $p < 0.05$) indicates a more than additive effect of the double mutation (i.e., deletion of both *gene1* and *gene2* in the same genetic background has a greater effect than the sum of the effects of deleting *gene1* alone and *gene2* alone).

## Results

### Contributions of RRs to Caco-2 invasion

Overall ANOVA analysis showed a highly significant effect of temperature on invasion efficiency ( $p = 0.0051$), and all RR mutants (except Δ*degU* and Δ*cheY*) and 10403S had numerically higher mean Caco-2 invasion efficiencies after growth to early stationary phase in BHI at 30°C than growth at 37°C. For several strains, including 10403S, Δ*resD*, Δ*lmo1022*, Δ*phoP*, Δ*cesR*, and Δ*kdpE*, this difference was statistically significant ( $p < 0.05$; *t*-test; Fig. 1).

For the *L. monocytogenes* parent strain and the 14 RR mutants grown at 30°C, ANOVA showed a significant effect of strain on Caco-2 invasion efficiency ( $p < 0.0001$). Specifically, Δ*degU* and Δ*cheY* both had ~50-fold lower mean invasion efficiencies than the parent strain ( $p < 0.0001$ for each; Dunett's test) when grown at 30°C (Fig. 1). These results confirm the findings of other studies that have shown a role for CheY in host cell invasion (Dons *et al.*, 2004). Unlike all other strains tested, the Δ*cheY* and Δ*degU* strains showed numerically higher invasion efficiency after growth at 37°C than at 30°C (Fig. 1).

For *L. monocytogenes* parent strain 10403S and the 14 RR mutants grown to early stationary phase at 37°C, the effect of the factor "strain" on Caco-2 invasion efficiencies was not statistically significant ( $p = 0.3900$; ANOVA). Therefore, the appropriate conservative statistical test (i.e., ANOVA) found no statistically significant reduction in Caco-2 invasion for any of the 14 RR mutants grown at 37°C.

As *L. monocytogenes* motility has been reported in other *L. monocytogenes* strains to be regulated by DegU and CheY at temperatures ≤30°C (Dons *et al.*, 2004; Knudsen *et al.*, 2004; Williams *et al.*, 2005a; Mauder *et al.*, 2008), we conducted swarming assays to evaluate motility of the *L. monocytogenes* 10403S, Δ*degU*, Δ*cheY*, and Δ*flaA* strains. While 10403S was clearly motile when grown at 30°C, Δ*degU*, Δ*cheY*, and Δ*flaA* showed reduced swarming (6%, 6%, and 1%, respectively, of the swarming area for 10403S grown at 30°C; see Supplemental Fig. S1, available online at www.liebertonline.com). When grown at 37°C, the parent strain showed limited and considerably reduced swarming compared to 30°C (Supplemental Fig. S1), and the three mutant strains showed no detectable swarming at this temperature. The finding that 10403S exhibited some swarming at 37°C is consistent with previous reports that *L. monocytogenes* 10403S (the parent strain used here) shows limited *flaA* expression even at 37°C (Grundling *et al.*, 2004; Way *et al.*, 2004).

### Effects of temperature shifts and holds on growth temperature-dependent phenotypes

On the basis of the data reported above, we evaluated whether the increased invasion efficiency of 10403S at 30°C is maintained after a shift to 37°C. While *L. monocytogenes* 10403S grown at 30°C and held (in PBS) at 37°C for 2.5 h (30°C–37°C) showed numerically (about twofold) reduced invasion efficiency compared to bacteria either before the shift or bacteria shifted to 30°C for 2.5 h (Table 2), the invasion efficiency of the "30°C–37°C" treatment group was not significantly different from the control groups grown and held at 30°C (30°C–30°C in Table 2) ( $p > 0.05$; Tukey HSD) but was significantly higher than control groups grown and held at 37°C (37°C–37°C) ( $p < 0.05$; Tukey HSD). These results suggest that increased invasion efficiency of *L. monocytogenes* grown at 30°C is largely maintained during a 2.5 h hold at 37°C in PBS. When *L. monocytogenes* 10403S was grown at 30°C, and shifted to and held at 37°C for 5 h (in PBS), the invasion efficiency was reduced (0.15% invasion efficiency) and virtually identical to that of *L. monocytogenes* grown at 37°C (0.14% invasion efficiency) with no significant difference ( $p > 0.05$; Tukey HSD). This indicates that prolonged exposure of *L. monocytogenes* grown at 30°C to 37°C (in PBS) reduces invasion efficiencies to values typical for bacteria grown at 37°C. Growth

and *de novo* protein synthesis (which are unlikely to occur in PBS) thus do not seem to be required for reduced invasiveness observed at 37°C.

When the *L. monocytogenes* parent strain grown at 37°C was switched to 30°C and held at this temperature for 2.5 h, bacteria showed no changes in invasion efficiency and maintained lower invasion efficiency typical for bacteria grown at 37°C ( $p < 0.05$; Tukey HSD; Table 2), suggesting that the increased invasion phenotype cannot be induced by simply switching the organism to 30°C but requires growth at 30°C. Controls included in these experiments showed that exposure to PBS for 2.5 h had no effect on invasion efficiency; bacteria grown at 30°C and exposed to PBS at 30°C for 2.5 h as well as bacteria grown at 37°C and exposed to PBS at 37°C for 2.5 h did not differ in their invasion efficiencies from bacteria grown at 30°C or 37°C, respectively, without subsequent exposure to PBS ( $p > 0.05$; Tukey HSD; Table 2).

As *L. monocytogenes* Δ*degU*, Δ*cheY*, and Δ*flaA* showed lower invasion than the parent strain after growth to early stationary phase at 30°C, we also tested whether this invasion defect was maintained after a shift from 30°C to 37°C, followed by a 2.5 h hold at this temperature (in PBS). All three strains (Δ*degU*, Δ*cheY*, and Δ*flaA*) maintained lower invasion efficiencies than the parent strain even after bacteria grown at 30°C were held for 2.5 h at 37°C (Table 2). Invasion efficiencies for these strains grown at 30°C and shifted to 37°C for 2.5 h were not significantly different ( $p > 0.05$; Tukey HSD; Table 2) from invasion efficiencies for (i) strains grown at 30°C (before shift) or (ii) strains grown at 30°C and subsequently held at 30°C in PBS for 2.5 h (Table 2). The invasion efficiencies for Δ*degU*, Δ*cheY*, and Δ*flaA* grown at 30°C and held for 2.5 h at 37°C also continued to be significantly lower than the invasion efficiencies for the same strains grown at 37°C ( $p < 0.05$; Tukey HSD); invasion efficiencies for these three strains in the "30°C–37°C" treatment group were ~20 to 30-fold lower than the same strains grown at 37°C (Table 2). These three mutant strains thus maintained their invasion deficient phenotype even after 2.5 h exposure to 37°C. To test the effect of prolonged exposure to 37°C on the reduced invasion efficiency of motility-deficient mutant strains, invasion efficiency of the Δ*flaA* strain was also evaluated for bacteria grown at 30°C and shifted to 37°C with a hold in PBS at 37°C for 5 h. Even after a hold at 37°C for 5 h, the Δ*flaA* strain maintained reduced invasion efficiency (0.006%) compared to the invasion efficiency of either the parent strain grown at 37°C (0.14%) or Δ*flaA* grown at 37°C (0.14%; both comparisons had $p < 0.05$; Tukey HSD), suggesting that increased invasion efficiency at 37°C, of a Δ*flaA* mutant, requires growth at 37°C and probably *de novo* protein synthesis (which is unlikely to occur in PBS). When the Δ*degU*, Δ*cheY*, and Δ*flaA* strains were grown at 37°C and shifted to 30°C with a hold at 30°C for 2.5 h, all three strains maintained the higher invasion efficiency as they displayed when grown at 37°C (Table 2).

### Temperature-dependent contributions of InlA and FlaA to Caco-2 invasion

In initial experiments, the *L. monocytogenes* Δ*inlA* strain showed 4.4-fold higher invasion efficiency when grown at 30°C than at 37°C (Fig. 1). In subsequent experiments, when grown at 37°C, the Δ*inlA* strain showed, on average, greater than 100-fold lower invasion efficiency than either the parent strain or the Δ*degU*, Δ*cheY*, or Δ*flaA* strains grown at 37°C (Table 2). However, when grown at 30°C, the Δ*inlA* strain showed only a 26-fold lower average invasion efficiency than the parent strain (Table 2). These experiments also confirmed a significantly higher invasion efficiency for the Δ*inlA* grown at 30°C than at 37°C ( $p < 0.05$; Tukey HSD; Table 2). Temperature shifts (i.e., 30°C–37°C, 2.5 h or 37°C–30°C, 2.5 h) did not significantly affect these statistical differences (Table 2). These results indicate an InlA-independent increase in Caco-2 invasion for *L. monocytogenes* grown at 30°C (as compared to 37°C), which is maintained after a shift to 37°C.

To further investigate the contributions of InlA and flagellin to Caco-2 invasion after growth at 30°C and 37°C and to specifically determine whether there is synergism between InlA and flagellin in facilitating invasion, 10403S, Δ*flaA*, Δ*inlA*, and Δ*inlA*Δ*flaA* were grown at 30°C or 37°C and tested for their Caco-2 invasion efficiencies (Fig. 2). After growth at 30°C, Δ*flaA* showed ~30-fold lower invasion efficiency than the parent strain (Fig. 2). At both 30°C and 37°C, the invasion efficiency of the Δ*inlA*Δ*flaA* strain was lower ( $p < 0.05$; Tukey HSD) than that of the parent strain (0.007% vs. 0.680% for 37°C and 0.003% vs. 2.064% for 30°C, respectively). Further, two-way ANOVA analysis showed a significant "flaA*inlA" interaction effect on Caco-2 invasion for *L. monocytogenes* grown at both 30°C ( $p = 0.0015$) and 37°C ( $p = 0.0269$). Interestingly, the F-ratio of the interaction effect for bacteria grown at 30°C ($F = 20.15$) was higher than that of bacteria grown at 37°C ($F = 6.97$), suggesting a greater contribution of the interaction effect to the observed variance at 30°C than at 37°C. These statistical findings indicate that the effects of deleting *inlA* and *flaA* on Caco-2 invasion are more than additive, indicating a synergism between InlA and FlaA in facilitating Caco-2 invasion for bacteria grown at either 30°C or 37°C.

## Temperature-dependent contributions of transcriptional regulators $\sigma^B$ and PrfA to Caco-2 invasion

To determine growth temperature effects on the contributions of $\sigma^B$ and PrfA to Caco-2 invasion, Δ*prfA*, Δ*sigB*, and Δ*sigB*Δ*prfA* strains grown to early stationary phase at 30°C or 37°C were used for Caco-2 invasion assays (performed at 37°C). After growth at 30°C, the invasion efficiency of Δ*prfA* was 2.737%, which was not significantly different ( $p > 0.05$; Tukey HSD) than the parent strain (2.064% invasion efficiency); the Δ*sigB* (0.376% invasion) and Δ*sigB*Δ*prfA* (0.231% invasion) strains were significantly less invasive ( $p < 0.05$; Tukey HSD) than the parent strain (Fig. 3). These results indicate that only $\sigma^B$, not PrfA, is involved in invasion when *L. monocytogenes* is grown at 30°C. When the bacteria were grown at 37°C, Δ*prfA* (0.322%), Δ*sigB* (0.060%), and Δ*sigB*Δ*prfA* (0.018%) had significantly lower invasion efficiencies ( $p < 0.05$; Tukey HSD) than the parent strain (0.680%) (Fig. 3); the Δ*sigB*Δ*prfA* strain also showed lower invasion efficiency than the parent strain and the Δ*prfA* strain ( $p < 0.05$; Tukey HSD). A two-way ANOVA also showed a significant "sigB*prfA" interaction effect, indicating a synergism between $\sigma^B$ and PrfA in regulating Caco-2 invasion for *L. monocytogenes* grown at 37°C (Table 3).

## Temperature-dependent regulation of genes involved in Caco-2 invasion

To determine the effects of growth temperature on $\sigma^B$ and PrfA-dependent regulation of *L. monocytogenes* genes involved in invasion, we determined transcript levels for *inlA*, *flaA*, *plcA*, *gadA*, *sigB*, *prfA*, and *rpoB* in 10403S, and Δ*sigB*, Δ*prfA*, and Δ*sigB*Δ*prfA* grown to early stationary phase at 30°C or 37°C. Neither *prfA* nor *sigB* transcript levels differed between the parent strain grown at 30°C and 37°C ( $p > 0.05$, *t*-test). The transcript levels for the $\sigma^B$-dependent *gadA* and the PrfA-dependent *plcA* (measured as indicators of $\sigma^B$ and PrfA activity, respectively) (Fig. 4) also did not differ significantly between bacteria grown at 30°C and 37°C ( $p > 0.05$; *t*-test), suggesting no differences in PrfA and $\sigma^B$ activity between *L. monocytogenes* 10403S grown to early stationary phase in BHI at these two temperatures.

For bacteria grown to early stationary phase at 30°C, *inlA* transcript levels were 10 times lower in the Δ*sigB* strain than the parent strain ( $p < 0.05$; Tukey HSD; Fig. 4), whereas there was no significant effect of the *prfA* deletion on *inlA* transcript levels ( $p = 0.1282$; two-way ANOVA) (Supplemental Table S2, available online at www.liebertonline.com). These data indicate that *inlA* transcription is $\sigma^B$ dependent, but PrfA independent under these conditions. For *L. monocytogenes* grown at 37°C, *inlA* transcript levels were significantly lower in Δ*sigB* than in the parent strain ( $p < 0.05$; ANOVA; Tukey HSD; Fig.

4), whereas *inlA* transcript levels were not significantly different in Δ*prfA* compared to the parent strain ( $p > 0.05$; Tukey HSD; Fig. 4). However, the sigB*prfA interaction effect on *inlA* transcript levels was borderline significant ( $p = 0.0799$; two-way ANOVA; Supplemental Table S2), suggesting contributions of both $\sigma^B$ and PrfA to regulation of *inlA* transcription in *L. monocytogenes* grown at 37°C.

In the *L. monocytogenes* parent strain, *flaA* transcript levels were higher after growth at 30°C (1.26; standard deviation = 0.07) than at 37°C (1.104; standard deviation = 0.05) ( $p = 0.0014$, *t*-test; data shown in Fig. 4). Although the difference is small, these results indicate at least some temperature-dependent regulation of *flaA* transcription in 10403S. *flaA* transcript levels were not significantly different between Δ*prfA* and the parent strain for either growth temperature ( $p > 0.05$; Tukey HSD; Fig. 4). In *L. monocytogenes* grown at 37°C, *flaA* transcript levels were not different between the Δ*sigB* strain and the parent strain ( $p > 0.05$; Tukey HSD; Fig. 4). However, at 30°C *flaA* transcription was significantly higher in Δ*sigB* and Δ*sigB*Δ*prfA* than in the parent strain ( $p < 0.05$; ANOVA, Tukey HSD; Fig. 4), and two-way ANOVA showed a significant “sigB effect” on *flaA* transcript levels ( $p = 0.0003$; Supplemental Table S2). These results are consistent with other studies that suggest a role of $\sigma^B$ in down-regulating chemotaxis genes (Raengpradub *et al.*, 2008;Toledo-Arana *et al.*, 2009).

## Discussion

### *L. monocytogenes* grown at 30 °C shows a higher Caco-2 invasion efficiency than bacteria grown at 37 °C

Out data showed that, with the exception of mutants with deletions of motility-related genes, all *L. monocytogenes* strains showed higher Caco-2 invasion efficiency when grown at 30°C than at 37°C. This finding supports previous work (Dons *et al.*, 2004) reporting that a different *L. monocytogenes* strain (12067) showed about 10-fold increased association with Caco-2 cells and twofold increased invasion of Caco-2 cells for bacteria grown at 24°C (where 12067 was motile) compared to 37°C (where this strain was not motile); this previous study did not include a statistical evaluation of these differences in invasion efficiencies (Dons *et al.*, 2004). As most *L. monocytogenes*, including strain 10403S, show increased flagellar motility when grown at 30°C, a trait that contributes to Caco-2 cell invasion (Dons *et al.*, 2004) and intestinal colonization in mice (O’Neil and Marquis, 2006), expression of motility genes at ≤30°C may explain the increased Caco-2 invasion of *L. monocytogenes* grown at ≤30°C compared to bacteria grown at 37°C.

Interestingly, other bacterial pathogens have also been shown to differ in their invasiveness and virulence depending on growth temperature (Konkel and Tilly, 2000). While in a number of bacterial pathogens virulence genes have been found to be more highly expressed at 37°C than at lower temperatures (Maurelli, 1989), some pathogens have been shown to express invasion factors at higher levels when grown at temperatures below 37°C. For example, *Yersinia pseudotuberculosis invA*, which encodes an invasin required for host cell invasion, appears to be expressed at higher levels in bacteria grown at 28°C than at 37°C (based on visual examination of Western blot data) (Isberg *et al.*, 1988). *Yersinia enterocolitica* has also been shown to express a motility phenotype when grown below 37°C, whereas motility, which may play a role in initiation of host cell invasion, is down-regulated in bacteria grown at 37°C (Young *et al.*, 2000). Therefore, adaptation to environments outside the host may increase the virulence potential of *L. monocytogenes* and other bacterial pathogens.

Enteric pathogens transmitted to humans from food and environmental sources often experience a sudden change in environmental temperature when they are ingested. Our

results show that invasion phenotypes of the parent strain and the motility mutants grown at 30°C persisted for 2.5 h after the bacteria were switched to 37°C. As 70% to 90% of human stomach contents are emptied after 2 h (Bennink *et al.*, 1999), our findings, in combination with other studies (Dons *et al.*, 2004; O'Neil and Marquis, 2006), suggest that *L. monocytogenes* grown at temperatures that permit motility [i.e., 12°C–30°C (Di Bonaventura *et al.*, 2008)] may have increased invasion potential that could be maintained during gastric passage. As regulation of motility appears to differ considerably between *L. monocytogenes* strains (Grundling *et al.*, 2004; Way *et al.*, 2004), further studies will need to use different strains to validate our findings on growth temperature dependence of Caco-2 invasion efficiency.

In addition to growth temperature, other studies (Garner *et al.*, 2006a; Andersen *et al.*, 2007) have shown that exposure to other environmental conditions (e.g., organic acids, anaerobic conditions) appear to affect the virulence potential of *L. monocytogenes*. For example *L. monocytogenes* grown in an oxygen-limited environment displayed 100-fold increased invasion efficiency in Caco-2 cells as well as increased virulence in a guinea pig model, including 10 to 100-fold higher *L. monocytogenes* fecal shedding levels, compared to numbers for bacteria grown under aerobic conditions (Andersen *et al.*, 2007). Further, *L. monocytogenes* grown in the presence of sodium lactate or NaCl showed an about 10-fold higher invasion efficiency than bacteria grown without these compounds (Garner *et al.*, 2006a). Overall, growth temperatures as well as other preinvasion environmental conditions (e.g., anaerobiosis) thus appear to affect the regulation of genes with roles in host attachment and invasion across different environmentally transmitted pathogens, including *L. monocytogenes*.

### CheY and DegU significantly contribute to invasion in *L. monocytogenes* grown ≤30°C

Our data, in conjunction with other reports (e.g., Dons *et al.*, 2004), clearly show that DegU and CheY play a temperature-dependent role in enhancing invasion of host cells across different *L. monocytogenes* strain backgrounds. For example, Δ*cheA*, Δ*cheY*, and Δ*cheYA* mutants in an *L. monocytogenes* 12067 background all showed about 100-fold reduced Caco-2 cell invasion as well as reduced motility as compared to their parent strain after growth at 24°C (Dons *et al.*, 2004), whereas Williams *et al.* (2005a) found that *L. monocytogenes* EGD Δ*degU* and Δ*cheY* strains, grown at 37°C, did not show reduced invasion efficiency of Cos-1 fibroblast cells. Previous studies have shown increased swarming and flagellar motility of *L. monocytogenes* grown at 24°C compared to *L. monocytogenes* grown at 37°C (Dons *et al.*, 2004; Knudsen *et al.*, 2004; Shen and Higgins, 2006), due to repression of *L. monocytogenes* motility genes at 37°C (Grundling *et al.*, 2004; Shen and Higgins, 2006). Therefore, contributions of motility genes to virulence phenotypes are generally apparent only when *L. monocytogenes* is grown at temperatures where the organism is typically motile (i.e., ≤30°) (Dons *et al.*, 2004; Knudsen *et al.*, 2004; Williams *et al.*, 2005b; O'Neil and Marquis, 2006). While DegU has also been shown to contribute to motility in *L. monocytogenes* grown at 24°C and to virulence in a mouse model (Williams *et al.*, 2005b), we are not aware of any previous studies reporting a specific effect of a *degU* null mutation on invasion of human intestinal epithelial cells.

While we found no contributions of other RRs, besides DegU and CheY, to invasion of Caco-2 cells, some studies have shown contributions of other RRs to virulence phenotypes in *L. monocytogenes* strains other than 10403S and in other invasion models. For example, Williams *et al.* (2005a) reported that RRs Lmo1507 and LisR are involved in invasion of Cos-1 cells. A deletion of *lisK* (encoding the LisR-associated sensory kinase) in *L. monocytogenes* LO28 was also found to affect virulence in mice (Cotter *et al.*, 1999), further supporting contributions of LisRK to *L. monocytogenes* virulence in some models. Similarly, *L. monocytogenes* EGD with a deletion of the RR VirR, grown at 37°C, has also

been reported to be deficient in Caco-2 cell invasion (Mandin *et al.*, 2005), even though the *L. monocytogenes* 10403S Δ*virR* strain used in our current study did not show evidence for reduced invasion, again possibly reflecting strain differences. Overall, different TCS thus appears to contribute to *L. monocytogenes* virulence and virulence-associated characteristics, even though contributions appear to differ based on growth conditions, strain backgrounds, and assays used.

### While InlA and FlaA show significant synergism in their contributions to Caco-2 invasion, synergism is less pronounced after growth at 37 °C

While flagellin has been identified as a critical part of the motility machinery involved in Caco-2 invasion in *L. monocytogenes* grown at temperatures that allow motility gene expression (i.e., ≤30°C) (Dons *et al.*, 2004; O'Neil and Marquis, 2006), we found flagellin and InlA contribute synergistically to Caco-2 cell invasion in *L. monocytogenes* 10403S grown at 30°C and 37°C. While studies in other *L. monocytogenes* strains (e.g., 12067, EDGe) have shown that contributions of motility factors to host cell invasion typically are only apparent in *L. monocytogenes* grown at 30°C or less (Dons *et al.*, 2004; Shen and Higgins, 2006), some studies (Grundling *et al.*, 2004; Way *et al.*, 2004) have shown increased flagellar motility at 37°C for strain 10403S as compared to other strains. For example, *flaA* repression by MogR was shown to be less stringent in 10403S than in EGDe (Grundling *et al.*, 2004), and 10403S activated a flagellum-dependent innate immune response even after growth at 37°C (Way *et al.*, 2004). Critical contributions of InlA to invasion of human intestinal epithelial cells (Dramsi *et al.*, 1993; Lingnau *et al.*, 1995) and virulence after oral infection (Lecuit *et al.*, 1999; Garner *et al.*, 2006b) have been well established for host species carrying the E-cadherin allotype that allows for InlA binding (e.g., humans and guinea pigs). Although we observed a synergism between the contributions of FlaA and InlA to Caco-2 invasion in *L. monocytogenes* grown at both 30°C and 37°C, we also found that the Δ*inlA* strain shows increased invasion efficiency when grown at 30°C compared to 37°C, suggesting InlA-independent contributions of flagellar motility to Caco-2 cell invasion. This observation further supports the importance of flagellar motility in *L. monocytogenes* virulence.

### $\sigma^B$, but not PrfA, contributes to invasion of bacteria grown at 30 °C, whereas $\sigma^B$ and PrfA show synergistic contributions to invasion if bacteria are grown at 37 °C

Our data showed that (i) $\sigma^B$ contributes to Caco-2 invasion for *L. monocytogenes* grown at both 30°C and 37°C and (ii) $\sigma^B$ positively regulates *inlA* transcription at both of these temperatures. The observation that $\sigma^B$ contributes to Caco-2 invasion in bacteria grown at both temperatures is consistent with a number of studies that have shown that $\sigma^B$ is critical for invasion of intestinal cells *in vitro* (Kim *et al.*, 2004; Garner *et al.*, 2006b) and in a guinea pig model of listeriosis (Garner *et al.*, 2006b). While McGann *et al.* (2007a) found higher transcript levels in *L. monocytogenes* grown at 30°C than bacteria grown at 37°C, for certain $\sigma^B$-dependent internalin genes (i.e., *inlC2*, *inlD*, *lmo331*, and *lmo0610*) and *opuCA*, which has been shown to be regulated by both $\sigma^A$ and $\sigma^B$ (Cetin *et al.*, 2004; Chan *et al.*, 2007a), the same study also found that *inlA* transcript levels were similar in *L. monocytogenes* grown at 30°C and 37°C (McGann *et al.*, 2007a). Another study also reported that genes found to be $\sigma^B$ dependent in the intestine were generally not differentially expressed at 30°C and 37°C (Toledo-Arana *et al.*, 2009). Overall, these data suggest that $\sigma^B$ activity and $\sigma^B$-dependent regulation of invasion is similar in *L. monocytogenes* grown at 30°C and 37°C, even though some $\sigma^B$-dependent genes (e.g., *opuCA* and *inlC2D*) may be regulated by additional temperature-dependent mechanisms (McGann *et al.*, 2007a; Chan *et al.*, 2007a). We did find though that $\sigma^B$-dependent negative regulation of *flaA* transcript levels, which was previously described by two studies (Raengpradub *et al.*, 2008; Toledo-Arana *et al.*, 2009), was only apparent here in bacteria

grown at 30°C, even though this regulation has previously been reported in bacteria exposed to salt stress at 37°C (Raengpradub *et al.*, 2008). Temperature effects on $\sigma^B$-dependent regulation of some genes may thus be dependent on other environmental conditions (e.g., osmotic stress).

Contributions of PrfA to Caco-2 cell invasion in *L. monocytogenes* grown at 37°C, but not in bacteria grown at 30°C, are consistent with previous findings of low PrfA activity in *L. monocytogenes* grown at temperatures < 37°C (Leimeister-Wachter *et al.*, 1992) as well as data showing that PrfA-dependent *inlA* expression is significantly higher at 37°C than at 25°C (Dramsi *et al.*, 1993). Interestingly, in our study here, *prfA* transcript levels and PrfA activity (as measured by *plcA* transcription levels) were not significantly different in the parent strain between 30°C and 37°C growth conditions; this observation may reflect low baseline PrfA activity in *L. monocytogenes* grown at 37°C in BHI (i.e., the conditions used here) as low levels of easily catabolized sugar and/or presence of other compounds (e.g., charcoal) seem to be required to induce PrfA activity at 37°C (Ripio *et al.*, 1996; Milenbachs *et al.*, 1997; Gilbreth *et al.*, 2004). While PrfA activity and PrfA-dependent phenotypes are thus clearly temperature dependent, with maximum PrfA activity in bacteria grown under certain conditions at 37°C, it is increasingly clear that transcriptional patterns and phenotypic characteristics of *L. monocytogenes* are governed by complex, environmental-condition-dependent interactions between multiple regulators.

Synergisms between PrfA and $\sigma^B$ were confirmed here through formal statistical analyses that showed (i) a statistically significant interaction effect between *sigB* and *prfA* deletions on Caco-2 invasion in bacteria grown at 37°C, but not in bacteria grown at 30°C and (ii) a borderline significant interaction effect between *sigB* and *prfA* deletions on *inlA* transcript levels in bacteria grown at 37°C, but not at 30°C. While this type of temperature-dependent synergism has not previously been described, contributions of both $\sigma^B$ and PrfA to Caco-2 cell invasion and co-regulation of *L. monocytogenes* virulence genes, including *inlA*, have been reported previously (Lingnau *et al.*, 1995; Kazmierczak *et al.*, 2003; Sue *et al.*, 2004; Kim *et al.*, 2005; McGann *et al.*, 2007b). The observation that *flaA* also appears to be downregulated by $\sigma^B$, possibly with an antisense RNA type mechanism (Toledo-Arana *et al.*, 2009), further supports a temperature-dependent regulatory network involving PrfA and $\sigma^B$ that affects multiple effector proteins contributing to *L. monocytogenes* invasion and virulence, even though possible (temperature-dependent) contributions of PrfA itself to transcription of *flaA* and other motility regulated genes (Michel *et al.*, 1998; Milohanic *et al.*, 2003) will require further confirmation. For example, while others (Ripio *et al.*, 1997) previously reported (based on visual examination of an RNA slot blot) negative regulation of *flaA* transcription by PrfA* (i.e., a PrfA protein that is constitutively active) in *L. monocytogenes* grown at 20°C, we did not find any effect of the *prfA* deletion on *flaA* transcript levels.

## Conclusions

Overall, our data show that *L. monocytogenes* use a number of regulatory mechanisms to modulate virulence gene expression, particularly expression of genes important for invasion, under different temperatures. Specifically, modulation of gene expression in *L. monocytogenes* grown at temperatures < 37°C (mimicking environmental conditions before host infection) appears to increase the invasiveness of this pathogen, priming it for subsequent infection of a mammalian host. Importantly, our data also suggest an initial specific model for regulation of key invasion-associated genes at transition of *L. monocytogenes* from environment to host. During growth at temperatures below mammalian body temperatures, CheY and DegU activity induce a motility phenotype, and although PrfA-dependent virulence gene expression is minimal, InlA expression is assured by $\sigma^B$-

dependent *inlA* transcription. After introduction into the host environment, passage through the gastrointestinal system can further activate $\sigma^B$ (e.g., through acid and osmotic stress), priming the cell for intestinal cell invasion through increased *inlA* transcription (Sue *et al.*, 2004). Once *L. monocytogenes* enter the intracellular environments, PrfA-dependent gene expression becomes critical for intracellular survival and spread (Freitag *et al.*, 1993) with $\sigma^B$ taking on a modulating role by downregulating expression of genes encoding cytolysins, which may cause excessive host cell damage (Ollinger *et al.*, 2008). Concurrently, motility appears to be downregulated in *L. monocytogenes* grown at 37°C, including through $\sigma^B$-dependent mechanisms (Raengpradub *et al.*, 2008; Toledo-Arana *et al.*, 2009), possibly facilitating evasion of Toll-like-receptor-mediated host responses (Hayashi *et al.*, 2001; Torres *et al.*, 2004).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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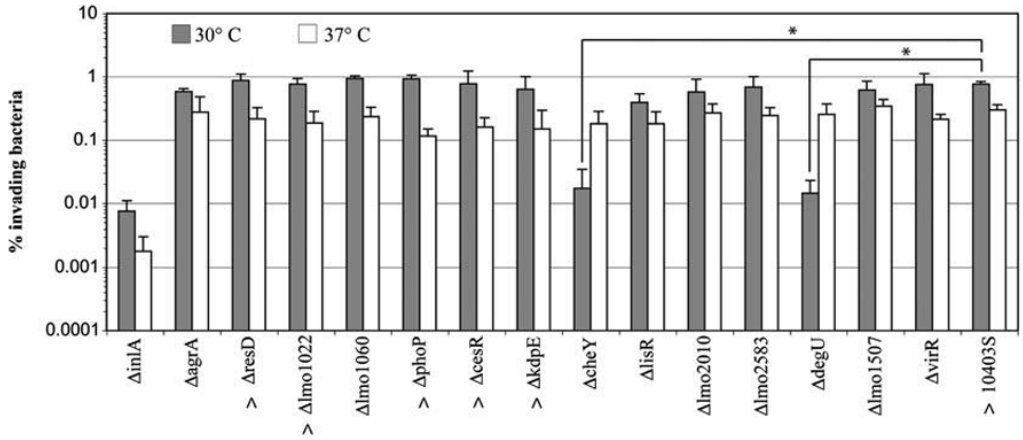


**FIG. 1.**

Caco-2 invasion efficiencies of *Listeria monocytogenes* 10403S and 14 mutant strains with deletions in genes encoding RRs. Bacteria were grown to early stationary phase (growth to $OD_{600}$ = 1.0, followed by 3 h of incubation) with aeration (i.e., shaking at 220 rpm) at 30°C or 37°C. Invasion assays were performed at 37°C. Data represent the mean of at least three biological replicates. Error bars represent SD. A Δ*inlA* strain was included as a control. Overall ANOVA analyses of the 14 RR mutants and the parent strain showed a significant effect of temperature ( $p = 0.0051$); all strains (except Δ*cheY* and Δ*degU*) showed numerically higher invasion efficiencies when grown at 30°C. Strains showing statistically higher invasion after growth at 30°C than at 37°C ( $p < 0.05$; *t*-test) are denoted by ^. For 10403S and RR mutants, ANOVA showed a significant effect of strain on invasion efficiency for bacteria grown at 30°C ( $p < 0.0001$). For bacteria grown at 37°C this effect was not significant ( $p = 0.3900$). Among RR mutants grown at 30°C, Δ*cheY* and Δ*degU* showed significantly lower invasion efficiencies than the parent strain ( $p < 0.0001$; *post hoc* Dunnett's; denoted by *). RR, response regulator; ANOVA, analysis of variance; SD, standard deviation.

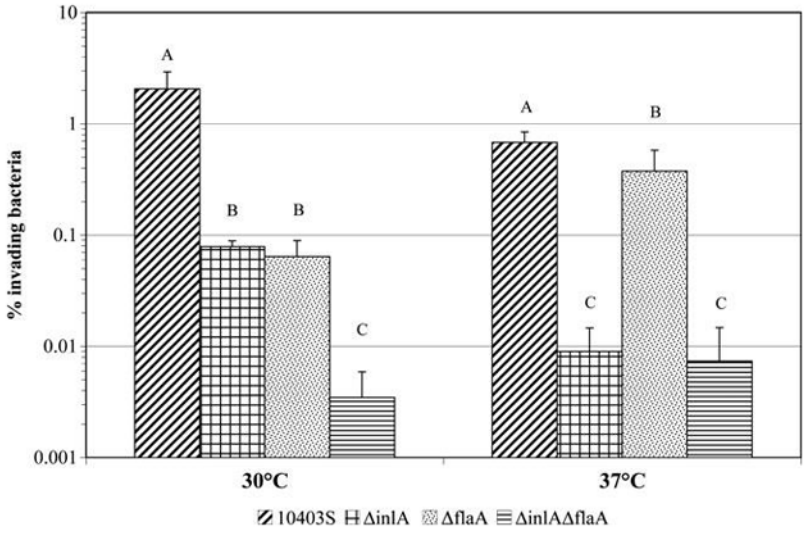


**FIG. 2.**

Caco-2 invasion efficiencies of *L. monocytogenes* 10403S, Δ*inlA*, Δ*flaA*, and Δ*inlA*Δ*flaA* strains grown to early stationary phase with aeration (i.e., shaking at 220 rpm) at either 30°C or 37°C. Data represent the mean of four biological replicates. Error bars represent SDs. Strains with different letters had significantly different invasion efficiencies at a given growth temperature ( $p < 0.05$; *post hoc* Tukey HSD); lower invasion for the Δ*flaA* strain than the parent strain (for growth at 37°C) was only borderline significant ( $p = 0.0279$; Tukey HSD). Two-way ANOVA was performed within each temperature to determine effects of single and double mutations on invasion efficiency (see Table 3 for *p*-values). HSD, honestly significantly different.

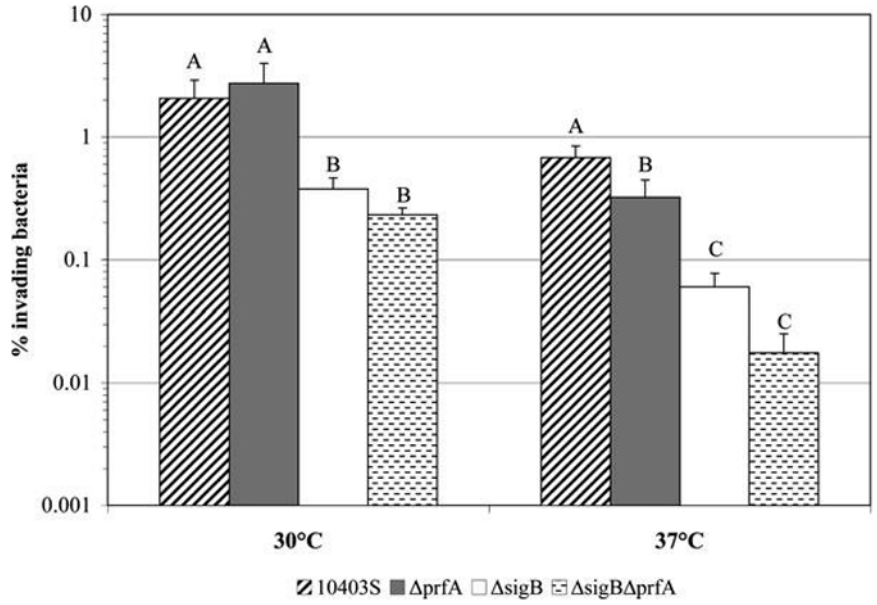


**FIG. 3.**

Caco-2 invasion efficiencies of *L. monocytogenes* 10403S, Δ*prfA*, Δ*sigB*, and Δ*sigB*Δ*prfA* strains grown to early stationary phase with aeration (i.e., shaking at 220 rpm) at either 30°C or 37°C. Data for the parent strain (10403S) are the same as those shown in Figure 2. Data represent the mean of four biological replicates. Error bars represent SD. Strains with different letters have significantly different invasion efficiencies at a given growth temperature ( $p < 0.05$; *post hoc* Tukey HSD). Two-way ANOVA was performed within each temperature to determine effects of single and double mutations on invasion efficiency (see Table 3 for *p*-values).

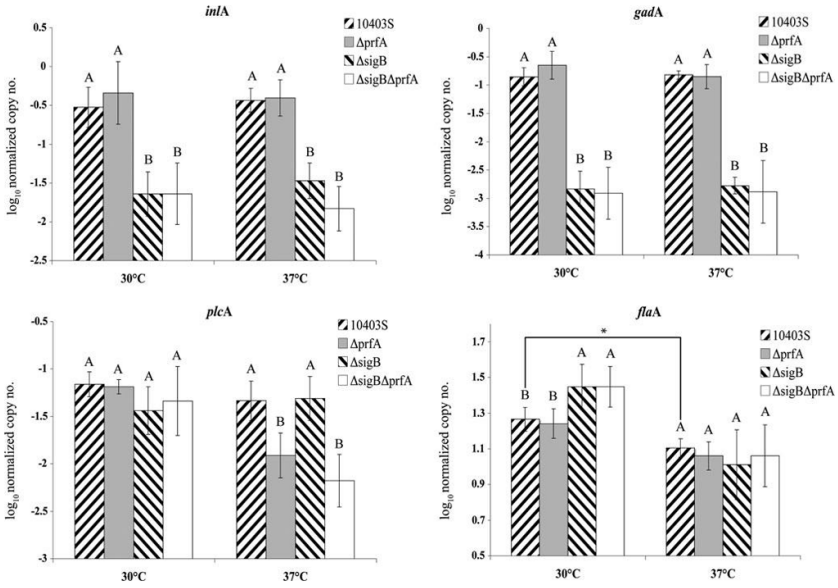


**FIG. 4.**

Normalized transcript levels of four genes in *L. monocytogenes* 10403S, Δ*sigB*, Δ*prfA*, and Δ*sigB*Δ*prfA* grown to early stationary phase with aeration (i.e., shaking at 220 rpm) at 30°C or 37°C. Data presented as $\log_{10}$ (target gene mRNA level/rpoB mRNA level). Bars represent the average of four biological replicates and error bars represent SD. Strains with different letters (e.g., A, B) had significantly different transcript levels ( $p < 0.05$; *post hoc* Tukey HSD). Two-way ANOVA was performed for each gene at each temperature to determine effects of single and double mutations on transcript levels (see Supplemental Table S2 for *p*-value summary). For each gene, a *t*-test was used to compare transcript levels in 10403S between 30°C and 37°C. Only *flaA* levels were significantly different between temperatures ( $p = 0.0014$; *t*-test; indicated by *).

## Table 1

Strains Used in This Study

| Strain designation[a] | Genotype | Description of gene product | Reference[b] |
|---|---|---|---|
| X1-001 | Parent strain | Strain 10403S; serotype 1/2a | Bishop and Hinrichs (1987) |
| K4-006 | Δ*inlA* | Internalin A, required for internalization into selected host cells | Bakardjiev *et al.* (2004) |
| H6-199 | Δ*flaA* | Flagellum structural protein | O'Neil and Marquis (2006) |
| B4-007 | Δ*lisRK*[c] | LisRK, contributes to log-phase acid resistance | This study |
| B2-078 | Δ*agrA* | Response regulator (RR), contributes to protein secretion | This study |
| B2-080 | Δ*resD* | RR, contributes to virulence gene repression in presence of select carbohydrates | This study |
| B2-086 | Δ*lmo1022* | Putative RR, no known role | This study |
| B2-096 | Δ*lmo1060* | Putative RR, no known role | This study |
| C5-017 | Δ*lmo2010* | Putative RR, no known role | This study |
| C5-019 | Δ*lmo2583* | Putative RR, no known role | This study |
| B2-100 | Δ*phoP* | RR, similar to *Bacillus subtilis* PhoP | This study |
| C5-041 | Δ*virR* | Novel RR involved in invasion of Caco-2 cells | This study |
| C5-036 | Δ*lmo1507* | Putative RR, no known role | This study |
| C5-033 | Δ*degU* | RR, regulates expression of motility genes | This study |
| B2-104 | Δ*kdpE* | RR, contributes to growth at high osmoloarity and low temperature | This study |
| B2-105 | Δ*cheY* | RR, contributes to chemotaxis | This study |
| B2-102 | Δ*cesR* | RR, contributes to ethanol and *β*-lactam tolerance | This study |
| A1-254 | Δ*sigB* | Alternative sigma factor $\sigma^B$, regulates general stress response | Wiedmann *et al.* (1998) |
| B2-046 | Δ*prfA* | PrfA, regulation of virulence genes | Cheng and Portnoy (2003) |
| B2-068 | Δ*sigB*Δ*prfA* | | McGann *et al.* (2007b) |
| I1-001 | Δ*inlA*Δ*flaA* | | Marquis Lab |

[a] All strain designations carry the prefix FSL.

[b] References where a given mutant was previously described.

[c] This mutant includes an internal deletion of the *lisR* gene, which also removed a portion of the ribosome binding site of *lisK* sensory kinase gene.

RR, response regulator.

**Table 2**

Caco-2 Invasion Efficiencies of *Listeria monocytogenes* 10403s and Select Mutants After Exposure to Varying Temperature Treatments

| Growth temperature | Temperature of PBS hold (2.5 h) | Caco-2 invasion efficiency (calculated as [CFU recovered/CFU infected] × 100) (SD) for[a] | | | | |
|---|---|---|---|---|---|---|
| | | 10403S | Δ*cheY* | Δ*degU* | Δ*flaA* | Δ*inlA* |
| 30°C | None | 0.67 (0.17)$^{A}$ | 0.018 (0.007)$^{B,*}$ | 0.015 (0.008)$^{B,*}$ | 0.014 (0.010)$^{B,*}$ | 0.024 (0.010)$^{A,*}$ |
| 30°C | 30°C | 0.64 (0.19)$^{A}$ | 0.013 (0.003)$^{B,*}$ | 0.010 (0.005)$^{B,*}$ | 0.010 (0.006)$^{B,*}$ | 0.023 (0.007)$^{A,*}$ |
| 30°C | 37°C | 0.36 (0.10)$^{A}$ | 0.006 (0.002)$^{B,*}$ | 0.007 (0.002)$^{B,*}$ | 0.007 (0.004)$^{B,*}$ | 0.014 (0.005)$^{A,*}$ |
| 37°C | None | 0.14 (0.03)$^{B}$ | 0.14 (0.06)$^{A}$ | 0.20 (0.05)$^{A}$ | 0.14 (0.06)$^{A}$ | 0.0008 (0.0003)$^{B,*}$ |
| 37°C | 37°C | 0.11 (0.04)$^{B}$ | 0.12 (0.05)$^{A}$ | 0.14 (0.10)$^{A}$ | 0.09 (0.06)$^{A}$ | 0.0007 (0.0007)$^{B,*}$ |
| 37°C | 30°C | 0.11 (0.03)$^{B}$ | 0.13 (0.05)$^{A}$ | 0.17 (0.08)$^{A}$ | 0.14 (0.07)$^{A}$ | 0.0007 (0.0005)$^{B,*}$ |

[a]Within a given column values with identical letters (A or B) are not significantly different ($p \geq 0.05$; *post hoc* Tukey HSD test). Within a given row, invasion efficiencies that are lower for a given mutant than the parent strain exposed to the same condition ($p \leq 0.05$; *post hoc* Dunnett's test) are marked with an asterisk (*). Data represent the mean and standard deviation of three biological replicates.

PBS, phosphate-buffered saline; SD, standard deviation; ANOVA, analysis of variance; HSD, honestly significantly different.

## Table 3

Effects of Various Gene Deletions on *Listeria monocytogenes* Caco-2 Invasion Efficiency

| | ***p*-Values from two-way ANOVA for *L. monocytogenes* grown at** | |
|---|---|---|
| **Deletion variable[a]** | **30°C** | **37°C** |
| sigB | 0.0001[b] | < 0.0001[b] |
| prfA | 0.4401 | 0.0014[c] |
| sigB*prfA | 0.226 | 0.0057[c] |
| inlA | 0.001[b] | < 0.0001[b] |
| flaA | 0.0009[b] | 0.0256[d] |
| inlA*flaA | 0.0015[c] | 0.0269[d] |

[a] The variables listed in this column represent either single-gene deletions (e.g., “sigB”) or interactions between two gene deletions (e.g., “sigB*prfA”). The *p*-values for the single-gene deletions measure the individual effect of deleting each respective gene. The “gene*gene” variable measures synergistic deletion effects by comparing the effect of deleting both genes to the effect of deleting either one gene or the other; significant values are marked with

[b] $p \leq 0.001$,

[c] $p \leq 0.01$, or

[d] $p \leq 0.05$. The actual data used for these analyses are presented in Figure 2 (*inlA* and *flaA* data) and Figure 3 (*sigB* and *prfA* data).