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Contributions of Two-Component Regulatory Systems, Alternative σ Factors, and Negative Regulators to *Listeria monocytogenes* Cold Adaptation and Cold Growth

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Abstract

The ability of *Listeria monocytogenes* to grow at refrigeration temperatures is critical for transmission of this foodborne pathogen. We evaluated the contributions of different transcriptional regulators and two-component regulatory systems to *L. monocytogenes* cold adaptation and cold growth. *L. monocytogenes* parent strain 10403S and selected isogenic null mutants in genes encoding four alternative σ factors (*sigB, sigH, sigC,* and *sigL*), two regulators of σ^B (*rsbT* and *rsbV*), two negative regulators (*ctsR* and *hrcA*), and 15 two-component response regulators were grown in brain heart infusion broth at 4°C with (i) a high-concentration starting inoculum (10^8 CFU/ml), (ii) a low-concentration starting inoculum (10^2 CFU/ml), and (iii) a high-concentration starting inoculum of cold-adapted cells. With a starting inoculum of 10^8 CFU/ml, null mutants in genes encoding selected alternative σ factors (Δ*sigH*, Δ*sigC,* and Δ*sigL*), a negative regulator (Δ*ctsR*), regulators of σ^B (Δ*rsbT* and Δ*rsbV*), and selected two-component response regulators (Δ*lisR,* Δ*lmo1172,* and Δ*lmo1060*) had significantly reduced growth ($P < 0.05$) compared with the parent strain after 12 days at 4°C. The growth defect for Δ*sigL* was limited and was not confirmed by optical density (OD_{600}) measurement data. With a starting inoculum of 10^2 CFU/ml and after monitoring growth at 4°C over 84 days, only the Δ*ctsR* strain had a consistent but limited growth defect; the other mutant strains had either no growth defects or limited growth defects apparent at only one or two of the nine sampling points evaluated during the 84-day growth period (Δ*sigB,* Δ*sigC,* and Δ*lmo1172*). With a 10^8 CFU/ml starting inoculum of cold-adapted cells, none of the mutant strains that had a growth defect when inoculation was performed with cells pregrown at 37°C had reduced growth as compared with the parent strain after 12 days at 4°C, suggesting a specific defect in the ability of these mutant strains to adapt to 4°C after growth at 37°C. Our data indicate (i) selected σ factors and two-component regulators may contribute to cold adaptation even though two-component regulatory systems, alternative σ factors, and the negative regulators CtsR and HrcA appear have limited contributions to *L. monocytogenes* growth at 4°C in rich media, and (ii) inoculum concentration and pregrowth conditions affect the *L. monocytogenes* cold-growth phenotype.

Listeria monocytogenes has the ability to grow at temperatures as low as −0.4°C (25, 40). Growth of *L. monocytogenes* in refrigerated ready-to-eat (RTE) foods is critical to

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transmission of this foodborne pathogen because high numbers of bacteria are required to cause human disease. Consequently, considerable efforts have been focused on design and implementation of strategies to prevent *L. monocytogenes* growth in RTE foods (4, 33). Further characterization of molecular mechanisms that facilitate *L. monocytogenes* growth at low temperature is necessary to improve our ability to reduce or prevent *L. monocytogenes* growth in refrigerated RTE foods.

The alternative sigma factor σ^B is critical for the ability of *L. monocytogenes* to respond to a number of environmental stress conditions (e.g., low pH, high salt, and carbon starvation) (17, 28, 39, 41). *L. monocytogenes* σ^B regulates transcription of a number of stress response and virulence genes, including genes with possible roles in cold adaptation (e.g., *ltrC* and *opuC*) (10, 13, 28, 30, 41). At least some *L. monocytogenes sigB* null mutant strains previously showed reduced growth in defined medium at 8°C (3) and reduced survival in raw meat stored at 4°C (34). Regulation of σ^B activity is complex and involves at least seven regulators of σ^B (12, 13). In addition to σ^B, *L. monocytogenes* has three other alternative σ factors: σ^H, σ^C, and σ^L. Although the *L. monocytogenes sigL* gene (encoding σ^L) appears to have higher transcript levels in cells grown at low temperature (10°C) compared with cells grown at 37°C (32), the specific contributions of σ^H, σ^C, and σ^L to low-temperature growth of *L. monocytogenes* have not been examined.

The negative regulators CtsR and HrcA also regulate transcription under different stress conditions (20, 27, 29, 37), particularly during heat shock (21, 22, 27). The HrcA-dependent *groEL* gene has higher transcript levels in *Bacillus subtilis* grown at 15°C compared with that grown at 37°C, suggesting a possible role of HrcA-regulated genes in cold adaptation in gram-positive bacteria (7). Although *L. monocytogenes ctsR* mutants have shown enhanced survival under different stress conditions, including heat stress (27, 35), transcriptional profiling in *B. subtilis* showed higher *ctsR* transcript levels during growth at 15°C than during growth at 37°C (7), suggesting a role for CtsR in modulating transcription at low temperatures.

In *L. monocytogenes,* 16 two-component regulatory systems (TCRSs) have been identified (19, 43), including one (encoded by *lmo0287* and *lmo0288*) that appears to be essential (26, 43). Phenotypic characterization of 15 TCRS RR in-frame deletion mutants in an *L. monocytogenes* EGD-e background indicated that none of these TCRS RR mutants contributed to growth during osmotic stress (9% NaCl) and oxidative stress (0.0025% H_2O_2); Δ*degU,* Δ*resD,* Δ*phoP,* and Δ*virR* strains had reduced growth during ethanol stress (43). In two other studies, a *lisK* null mutant had reduced osmotolerance and acid resistance (15, 38). Phenotypic characterization of five TCRS RR transposon mutant strains revealed impaired growth at 43.5°C for *lisR* and *kdpE* mutants (26). An *L. monocytogenes* strain with a deletion mutation in *kdpE* (encoding the response regulator KdpE) had growth characteristics in brain heart infusion (BHI) broth at 5°C similar to those of the EGD-e parent strain (6). No specific contributions of *L. monocytogenes* TCRSs to cold growth have been reported thus far.

In previous studies, researchers have evaluated some *L. monocytogenes* transcriptional regulators and TCRSs for contributions to cold growth through either mutant

characterization (6) or transcriptional profiling (3, 32); however, there are no comprehensive data on the contributions of alternative σ factors, TCRSs, and negative regulators to *L. monocytogenes* cold adaptation and cold growth. We used a core set of 23 in-frame deletion mutant strains to evaluate the contributions of these different regulators to *L. monocytogenes* cold growth and cold adaptation.

MATERIALS AND METHODS

Bacterial strains

The *L. monocytogenes* serotype 1/2a strain 10403S (5) and isogenic deletion mutant strains in the 10403S genetic background were used throughout this study (Suppl. Table 1; all supplemental materials are available at http://www.foodscience.cornell.edu/cals/foodsci/research/labs/wiedmann/links/chan2007.cfm). Isogenic mutant strains used carried deletions in genes encoding (i) 15 of the 16 known *L. monocytogenes* two-component response regulators (TCRRs) (our laboratory and two others (26, 43) were unable to construct a deletion mutation in *lmo0287*, suggesting that the TCRR encoded by *lmo0287* is essential); (ii) two negative regulators (CtsR and HrcA), (iii) four alternative σ factors, and (iv) RsbT and RsbV, two regulators of σ^B (12, 16). Isogenic strains with in-frame mutations in *prfA* (44), which encodes a major virulence gene regulator (9), and in *opuC* (1), which encodes an ABC transporter with a putative role in cold growth (2), also were used in selected experiments. A Δ*ctsR*Δ*hrcA* double mutant strain also was tested in selected experiments.

All isogenic in-frame deletion mutant strains have previously been constructed using splicing by overlap extension (SOE) PCR and standard allelic exchange mutagenesis (42) to generate a non-polar internal deletion within each gene of interest (see Suppl. Table 1 for SOE PCR primers used for mutant generation). For all mutant strains, allelic exchange mutagenesis has been confirmed through PCR and DNA sequencing to ensure in-frame deletions with no mutations in the sequences flanking the deletion.

For all mutant strains, their ability to grow at 37°C in BHI broth with shaking (250 rpm) was evaluated based on optical density at 600 nm (OD_{600}) measurements. None of the mutant strains, except the Δ*ctsR* and Δ*ctsR*Δ*hrcA* strains, showed evidence of growth defects at 37°C (compared with the parent strain). Reduced growth for the Δ*ctsR* and Δ*ctsR*Δ*hrcA* strains was confirmed by cell enumeration; these two strains had limited growth defects at 37°C (<1 log CFU/ml difference between mutant and parent strain after 3, 5, and 8 h of growth at 37°C) (23).

Cold growth conditions

Prior to all cold growth experiments, *L. monocytogenes* 10403S and selected mutant strains were grown overnight (16 to 18 h) in BHI broth at 37°C with shaking (250 rpm) and then diluted 1:100 into fresh BHI broth and grown with shaking to log phase (OD_{600} = 0.4), unless otherwise stated. The cold growth capabilities of both parent and mutant strains were evaluated in BHI broth (without shaking) at 4°C in borosilicate glass tubes (Fisher Scientific, Pittsburgh, Pa.). For these experiments, three different inocula were used: (i) a high-concentration (10^8 CFU/ml) and (ii) a low-concentration (10^2 CFU/ml) starting

inoculum of cells pregrown at 37°C and (iii) a high-concentration (10^8 CFU/ml) starting inoculum of cold-adapted cells. Cells were spiral plated on duplicate BHI agar plates using an Autoplate 4000 Automated Spiral Plater (Spiral Biotech, Inc., Norwood, Mass.). The plates were incubated at 37°C for 24 to 48 h, and colonies were enumerated using Q count (Spiral Biotech).

For high-inoculum experiments, log-phase cells of the *L. monocytogenes* parent strain and selected deletion mutant strains were used to inoculate BHI broth prechilled to 4°C to an OD_{600} of 0.15 ± 0.05, which is equivalent to 10^8 CFU/ml. On day 0 (immediately after inoculation) and on day 12, cells were enumerated after spiral plating, and OD_{600} values were determined; these measurements were performed in three independent experiments. In one experiment, bacterial numbers also were determined by spiral plating on days 3, 6, 9, and 12, and the OD_{600} was measured once a day for 12 days.

For low-inoculum experiments, log-phase *L. monocytogenes* cells were used to inoculate BHI broth prechilled to 4°C to achieve a starting inoculum of 10^2 CFU/ml. Cells were enumerated by spiral plating on BHI agar on day 0 (immediately after inoculation) and on days 7, 14, 21, 28, 35, 42, 56, 70, and 84. Three independent experiments were performed.

For growth experiments using a high-concentration inoculum of cold-adapted cells, bacteria that had been grown at 4°C for 43 days (as outlined for the low-inoculum experiment) were used to inoculate BHI broth prechilled to 4°C with a starting inoculum of 10^8 CFU/ml. Cells were enumerated by spiral plating on BHI agar on day 0 (immediately after inoculation) and on day 12. Three independent experiments were performed.

Statistical analyses

For all growth experiments at 4°C, bacterial numbers or growth and OD_{600} values (where available) for the *L. monocytogenes* parent strain (10403S) and deletion mutant strains were analyzed using the general linear model (GLM) in SAS v 9.1 (SAS Institute, Inc., Cary, N.C.). To allow blocking by experiments, a mixed-effects model with Tukey's studentized range test (LS means) was used when the "experiment" variable was significant in the initial GLM analysis. For time points where the initial analysis indicated a significant effect of the "strain" variable, LS means was used to determine whether there was a significant difference in bacterial numbers or OD_{600} values between a given mutant strain and the *L. monocytogenes* parent strain. An α value of <0.05 was considered significant.

RESULTS

Growth of *L. monocytogenes* strain 10403S and selected deletion mutant strains at 4°C using a high-concentration inoculum

For initial characterization of the cold growth phenotype, *L. monocytogenes* mutant strains and the parent strain were inoculated into prechilled BHI broth at ~10^8 CFU/ml. Growth at 4°C was defined as the difference in bacterial numbers (log CFU per milliliter; Fig. 1) from day 0 to day 12 or as the difference in OD_{600} (Suppl. Fig. S1) from day 0 to day 12. Overall statistical analysis showed a significant effect of strain (parent and different mutant strains) on growth over 12 days at 4°C for both the bacterial numbers data ($P < 0.0001$; mixed

effects model) and the OD_{600} data ($P < 0.0001$; mixed effects model). Based on the bacterial numbers data, three σ factor null mutant strains (Δ*sigH*, Δ*sigC*, and Δ*sigL*), the two Rsb mutant strains (Δ*rsbT* and Δ*rsbV*), three TCRR mutant strains (Δ*lisR*, Δ*lmo1172*, and Δ*lmo1060*), and the Δ*ctsR* strain had significantly reduced growth at 4°C ($P < 0.05$; LS means) compared with the parent strain (Fig. 1). The same mutants (except for Δ*sigL*) also had reduced growth after 12 days at 4°C, based on the OD_{600} measurements. These mutants (except for Δ*sigL*) had growth defects at 4°C on days 3, 6, and 9 as determined by bacterial numbers data and OD_{600} measurements (Suppl. Fig. S2). Because the Δ*sigL* strain had borderline significantly reduced growth as assessed by bacterial numbers at day 12 ($P = 0.018$) (Fig. 1) and no clear patterns of reduced growth on days 3, 6, and 9 (Suppl. Fig. S2) or as indicated by the OD*600* data (Suppl. Fig. S1), this mutant was considered to have limited evidence of reduced growth at 4°C. Although the Δ*rsbT* and Δ*rsbV* strains had significantly reduced growth over 12 days as compared with the parent strain ($P < 0.0001$), the Δ*sigB* strain did not have evidence of reduced growth (Fig. 1). Although the Δ*ctsR* strain had reduced growth as compared with the parent strain ($P < 0.001$), growth of the Δ*hrcA* and Δ*ctsR*Δ*hrcA* strains did not differ significantly from that of the parent strain (Fig. 1). The Δ*opuCA* strain had no growth defect even though an *opuC* transposon mutant previously had reduced growth at 7°C in Pine's medium containing carnitine (2).

Growth of *L. monocytogenes* 10403S and selected deletion mutant strains at 4°C using a low-concentration inoculum

To confirm the growth defects observed for selected mutant strains in the high-inoculum experiment at 4°C (and because *L. monocytogenes* typically contaminates refrigerated foods at low levels, and therefore growth is required to reach human infectious doses), we assessed the growth of selected *L. monocytogenes* mutant strains at 4°C over 84 days in experiments using a low-concentration starting inoculum (10^2 CFU/ml; Suppl. Fig. S3A). Strains tested in this experiment were (i) the *L. monocytogenes* parent strain (10403S), (ii) null mutants that had reduced growth at 4°C in the high-inoculum experiment (i.e., Δ*sigC*, Δ*sigH*, Δ*sigL*, Δ*ctsR*, Δ*rsbV*, Δ*lisR*, Δ*lmo1060*, and Δ*lmo1172*), (iii) the Δ*sigB* strain, which did not have reduced growth at 4°C in the high-inoculum experiment but had previously been reported to have reduced growth at low temperatures in at least some types of media (3), and (iii) the Δ*kdpE* and Δ*hrcA* strains, which did not have reduced growth in the high-inoculum experiment (thus serving as negative controls). A Δ*prfA* strain also was included because PrfA, which positively regulates virulence genes in L. monocytogenes (9), has temperature-dependent expression with maximum activity at 37°C (24).

The average initial (day 0) inoculum in the low-inoculum experiments was 2.13 ± 0.12 log CFU/ml (average for all strains); at day 0, the strain variable was not significant ($P > 0.2$; mixed effects model), indicating a similar starting inoculum of approximately 10^2 CFU/ml for all strains. Separate analyses of variance (ANOVAs) for bacterial numbers data for different days of growth at 4°C revealed a significant effect of the strain on bacterial numbers on most of the days later in the experiment (i.e., days 28, 35, 42, 70, and 84) but not earlier (i.e., days 7, 14, and 21) (Suppl. Fig. S3). This finding indicates that none of the mutant strains tested had reduced growth at 4°C during log phase (i.e., days 7, 14, and 21; Suppl. Fig. S3), but growth of at least some mutant strains differed from that of the parent

strain during stationary phase (i.e., after day 21; Suppl. Fig. S3). Specifically, Δ*ctsR* had clear although limited reduction in stationary-phase survival at 4°C, and Δ*ctsR* cell numbers were significantly lower than those for the parent strain at days 28, 35, 42, 70, and 84 (Suppl. Fig. S3). The Δ*sigB* strain had slightly (<1 log CFU/ml) but significantly lower numbers than the parent strain at days 35 and 42 (Suppl. Fig. S3); the Δ*lmo1172* and Δ*sigC* strains had slightly (<1 log CFU/ml) but also significantly lower numbers than the parent strain at day 42. The Δ*rsbV* and Δ*sigL* strains had slightly (<1 log CFU/ml) but significantly higher bacterial numbers than the parent strains at day 35 (9.2 log CFU/ml for both mutant strains and 9.0 log CFU/ml for the parent strain). Overall, our data indicate that none of the mutant strains tested (except for the Δ*ctsR* strain, which also showed slightly reduced growth at 37°C) had sustained and consistent reductions in growth or survival at 4°C as compared with the parent strain when a low-concentration starting inoculum (10^2 CFU/ml) was used.

Growth of selected cold-adapted *L. monocytogenes* mutant strains at 4°C using a high-concentration inoculum

To further resolve the apparent discrepancies between the high-inoculum and low-inoculum experiments (i.e., a number of *L. monocytogenes* null mutant strains had reduced growth or survival in the high-inoculum experiment but not in the low-inoculum experiment), we repeated the high-inoculum experiment with cold-adapted *L. monocytogenes* (pregrown in BHI broth for 43 days at 4°C) as an inoculum. Strains tested in this experiment were (i) the parent strain (10403S), (ii) all mutant strains that had reduced growth in the high-inoculum experiment (i.e., Δ*ctsR*, Δ*sigC*, Δ*sigH*, Δ*sigL*, Δ*rsbV*, Δ*lisR*, Δ*lmo1060*, and Δ*lmo1172*) except Δ*rsbT*, and (iii) Δ*hrcA*, which did not have a growth defect in the initial high-inoculum experiment (and thus served as a control). An ANOVA revealed no significant effect ($P > 0.1$; GLM) of the strain (parent strain or mutant strains) on growth (in log CFU per milliliter) (Suppl. Fig. S4), indicating that cold adaptation of *L. monocytogenes* results in similar growth among the parent and selected mutant strains at 4°C. These findings suggest a specific defect in the ability of these mutant strains to adapt to 4°C when they were pregrown at 37°C.

DISCUSSION

Characterization of a core set of 23 *L. monocytogenes* in-frame deletion mutant strains in genes encoding alternative σ factors, TCRSs, and negative regulators revealed that (i) selected σ factors, TCRRs, and negative regulators specifically contribute to *L. monocytogenes* cold adaptation but have limited contributions to cold growth at 4°C in rich medium and (ii) inoculum concentration and pregrowth conditions can affect *L. monocytogenes* cold growth and survival phenotypes.

Although strains with mutations in genes encoding TCRSs (i.e., strains Δ*lisR*, Δ*lmo1060*, and Δ*lmo1172*), in genes encoding alternative σ factors (i.e., strains Δ*sigC* and Δ*sigH*), and in genes encoding two regulators of σ^B activity (strains Δ*rsbV* and Δ*rsbT*) had reduced growth or survival at 4°C when BHI broth was inoculated with high bacterial numbers, these same mutant strains had very limited or no reductions in growth or survival at 4°C when

BHI broth was inoculated with a low bacterial numbers (allowing for extended growth) or when BHI broth was inoculated with high numbers of cold-adapted cells. We thus conclude that selected TCRSs (i.e., LisR, Lmo1060, and Lmo1172) and the alternative σ factors σ^C and σ^H contribute to *L. monocytogenes* cold adaptation but make limited or no contributions to *L. monocytogenes* cold growth. Previous studies indicate that LisK is involved in osmotolerance (38), which further supports a role for LisRK in cold adaptation because a number of osmotolerance genes also have been linked to cold adaptation (e.g., *opuC* and *gbu*) (2, 31).

L. monocytogenes σ^C, which appears to contribute to cold adaptation based on the data from the present study, has previously been classified as a thermal resistance regulator (45), with transcription of the *sigC* operon induced during temperature upshift. However, *sigC* is present only in *L. monocytogenes* lineage II strains (e.g., strain 10403S), and lineage II strains are generally more common in RTE foods and are usually found at considerably higher levels in naturally contaminated RTE foods than are lineage I strains (14, 36), which lack a *sigC* gene. Thus, it is tempting to speculate that the action of σ^C in cold adaptation may contribute to the apparent enhanced ability of lineage II strains to grow in refrigerated RTE foods.

Although two regulators of σ^B activity (i.e., RsbV and RsbT) appear to contribute to *L. monocytogenes* cold adaptation, an *L. monocytogenes* Δ*sigB* strain pregrown at 37°C did not have evidence of reduced ability to adapt to 4°C in BHI broth. This observation is intriguing because the same Δ*sigB*, Δ*rsbV*, and Δ*rsbT* strains consistently had identical phenotypic characteristics under a number of other stress conditions (e.g., acid stress, oxidative stress, and carbon starvation) (12). Contributions of regulators of σ^B to cold adaptation are consistent with observations that the gene strings *rsbR-rsbS-rsbT-rsbU* and *rsbV-rsbW-sigB-rsbX* have higher transcript levels in *L. monocytogenes* (11) and *B. subtilis* (7), respectively, grown at low temperatures. Overall, these findings suggest that regulators of σ^B may affect *L. monocytogenes* cold adaptation through mechanisms other than regulation of σ^B activity. Under cold stress, RsbV and RsbT may activate σ factors other than σ^B. This hypothesis is consistent with evidence in *B. subtilis* that some regulators of σ factor activity can, under specific stress conditions, act promiscuously to contribute to regulation of σ factors other than their primary targets (8). Although in previous growth studies, reduced growth of a 10403S Δ*sigB* strain (compared with its parent strain) at 8°C in defined medium (3) and reduced survival of an *L. monocytogenes* serotype 4c Δ*sigB* strain in meat stored at 4°C (33) were reported, we did not find evidence for reduced growth of the Δ*sigB* strain in BHI broth at 4°C. Cold-growth differences for Δ*sigB* strains in defined medium and rich medium may indicate that nutrients (e.g., solutes) available in the medium may affect the contributions of *L. monocytogenes* σ^B to cold growth and cold adaptation.

Although some of the mutant strains tested in this study had reduced cold growth from a starting inoculum of 10^8 CFU/ml grown at 37°C, the Δ*ctsR* strain was the only mutant that showed evidence for reduced cold growth or survival at 4°C regardless of starting inoculum (10^2 or 10^8 CFU/ml grown at 37°C). Because this mutant strain also had slightly reduced growth at 37°C, the effect of the *ctsR* null mutations on cold growth and survival may reflect a general growth defect.

Overall, our data clearly indicate that inoculum concentration and pregrowth conditions can affect cold-growth phenotypes. Among the *L. monocytogenes* mutant strains that had reduced growth at 4°C after inoculation into BHI broth at high numbers, most strains had limited or no evidence of growth defects in BHI broth at 4°C after inoculation with 10^2 CFU/ml and none had reduced growth in BHI broth at 4°C after inoculation with cold-adapted bacteria. Moorhead and Dykes (34) previously reported differences between the phenotypic characteristics of prechilled *L. monocytogenes* cells and cells grown at 30°C. Although these and other similar findings are not necessarily surprising, they reemphasize the importance of careful choice of pregrowth conditions for bacteria prior to phenotypic characterization, including the importance of using bacteria pregrown at different temperatures for experiments aimed at assessing cold adaptation and cold-growth phenotypes.

Various regulatory proteins play roles in *L. monocytogenes* cold adaptation, but null mutations in single genes encoding regulatory proteins have limited effects on cold growth and survival of cold-adapted *L. monocytogenes* in rich medium. However, the ability of *L. monocytogenes* to grow in most RTE foods at refrigeration temperatures may depend on some of the regulators tested here, because many refrigerated RTE foods impose multiple stresses (e.g., acid and osmotic stress) on *L. monocytogenes* and some of the regulatory proteins tested here (e.g., σ^B and LisRK) clearly contribute to acid and osmotolerance (15, 17, 18, 28, 38, 39, 42). Although evaluation of *L. monocytogenes* mutant strains in BHI broth can provide some initial insights into the importance of different regulatory proteins in *L. monocytogenes* cold growth and cold adaptation and may be representative for some RTE foods (e.g., milk), further characterization of the mutant strains in actual RTE foods is necessary.

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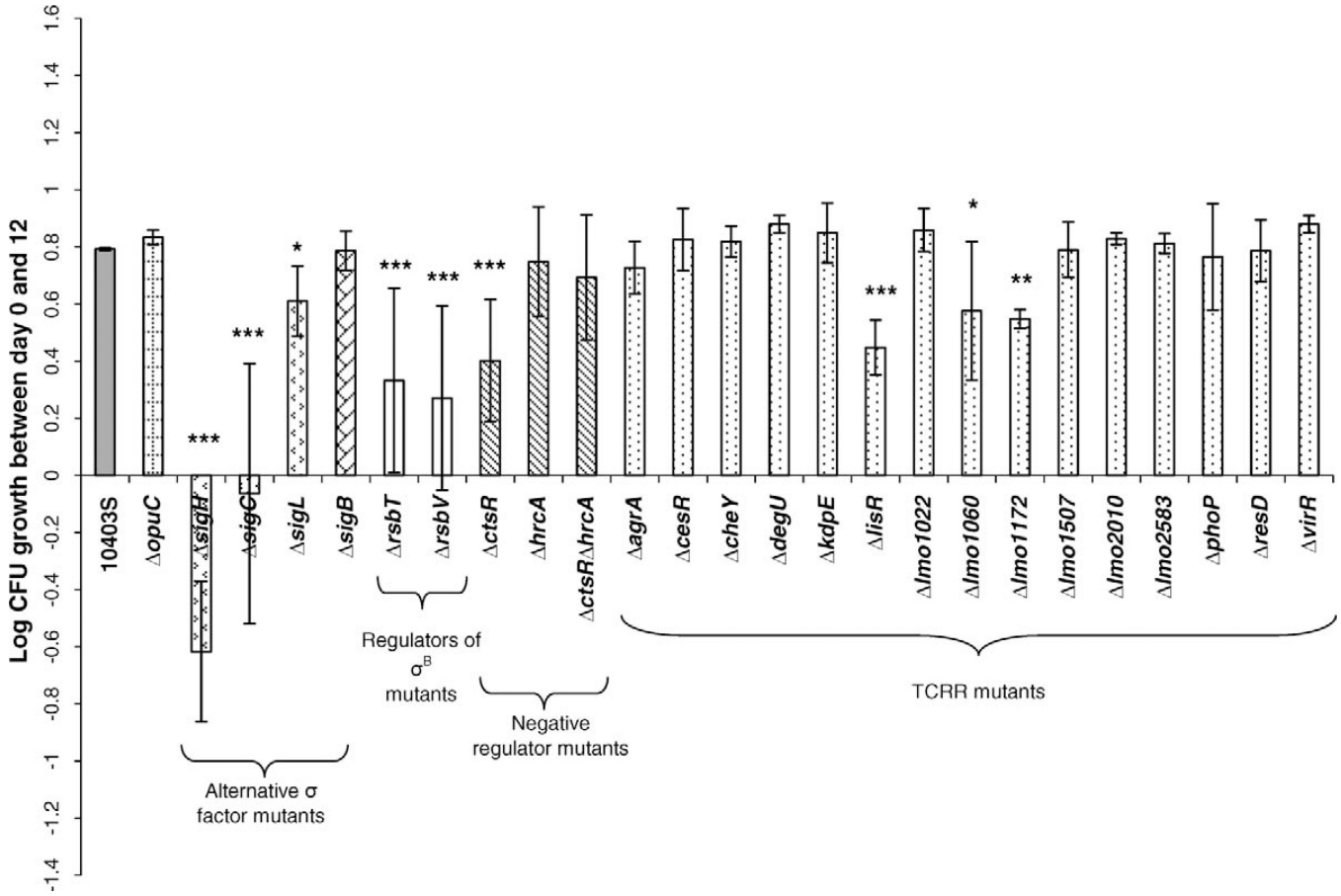

FIGURE 1.

Growth in BHI broth after 12 days at 4°C of L. monocytogenes strain 10403S and null mutant strains with a starting inoculum of 10^8 CFU/ml. Log-phase cells (OD_{600} = 0.4) grown at 37°C were inoculated into prechilled (4°C) BHI broth to an OD_{600} of 0.15 ± 0.05 (equivalent to approximately 10^8 CFU/ml). Growth between days 0 and 12 (shown on the y axis) represent the increase in CFU per milliliter from day 0 to day 12. Data represent three independent experiments; error bars indicate standard deviations. LS means in a mixed effects model with experiment as a blocking variable was used to determine whether growth for a given mutant strain differed significantly from that for the L. monocytogenes parent strain (10403S). ***, **, and *, Growth of a given mutant strain differed ($P < 0.0001$, <0.01, or <0.05, respectively) from growth of the parent strain. Corresponding OD_{600} data are shown in Supplemental Figure S1.