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Phosphotransferase System-Dependent Extracellular Growth of *Listeria monocytogenes* Is Regulated by Alternative Sigma Factors σ^L and σ^H

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Alternative sigma (σ) factors and phosphotransferase systems (PTSs) play pivotal roles in the environmental adaptation and virulence of *Listeria monocytogenes*. The growth of the *L. monocytogenes* parent strain 10403S and 15 isogenic alternative σ factor mutants was assessed in defined minimal medium (DM) with PTS-dependent or non-PTS-dependent carbon sources at 25°C or 37°C. Overall, our results suggested that the regulatory effect of alternative σ factors on the growth of *L. monocytogenes* is dependent on the temperature and the carbon source. One-way analysis of variance (one-way ANOVA) showed that the factor "strain" had a significant effect on the maximum growth rate (μ_{max}), lag phase duration (λ), and maximum optical density (OD_{max}) in PTS-dependent carbon sources ($P < 0.05$) but not in a non-PTS-dependent carbon source. Also, the OD_{max} was not affected by strain for any of the three PTS-dependent carbon sources at 25°C but was affected by strain at 37°C. Monitoring by quantitative real-time PCR (qRT-PCR) showed that transcript levels for *lmo0027*, a glucose-glucoside PTS permease (PTS^{Glc}-1)-encoding gene, were higher in the absence of σ^L, and lower in the absence of σ^H, than in the parent strain. Our data thus indicate that σ^L negatively regulates *lmo0027* and that the increased μ_{max} observed for the Δ*sigL* strain in DM with glucose may be associated with increased expression of PTS^{Glc}-1 encoded by *lmo0027*. Our findings suggest that σ^H and σ^L mediate the PTS-dependent growth of *L. monocytogenes* through complex transcriptional regulations and fine-tuning of the expression of specific *pts* genes, including *lmo0027*. Our findings also reveal a more important and complex role of alternative σ factors in the regulation of growth in different sugar sources than previously assumed.

The facultatively intracellular pathogen *Listeria monocytogenes* has a "Jekyll and Hyde" lifestyle (1). *L. monocytogenes* can effectively adapt to environmental conditions outside the eukaryotic host cells and can multiply using various carbon sources. The transition of *L. monocytogenes* from an extracellular saprophyte to an intracellular parasite is modulated mainly by the activation of the master virulence regulatory protein PrfA, triggered by a variety of environmental cues, such as carbon source and temperature (2, 3). PrfA is activated in host cells but remains inactive in broth cultures. While the mechanisms of regulation of PrfA activity are not fully understood (4, 5), carbon sources may serve as an environmental signal for *L. monocytogenes* to switch between the life cycle of an extracellular saprophyte and that of an intracellular pathogen (6). Specifically, when the bacterium is outside the host cell, extracellular carbon sources, such as glucose and cellobiose, are transported by phosphoenol pyruvate (PEP)-dependent phosphotransferase systems (PTSs) (7). In the presence of active PTSs, the activity of PrfA appears to be downregulated through complex regulatory interactions (2, 8) that are not yet fully elucidated. In contrast, *L. monocytogenes* can utilize non-PTS-dependent carbon sources available in the cytoplasm of eukaryotic cells, such as phosphorylated glucose and glycerol, for intracellular growth (9). PrfA is thus activated in the absence of active PTSs (10). By *in silico* analysis, Stoll and Goebel have identified 86 genes encoding 29 complete PTSs and 10 single PTS components (11). Among these, two mannose-fructose-sorbose PTS permeases, PTS^{Man}-2 (encoded by *lmo0096* to *lmo0098*) and PTS^{Man}-3 (encoded by *lmo0781* to *lmo0784*), as well as a glucose-glucoside PTS permease (PTS^{Glc}-1, encoded by *lmo0027*), have been found to play key roles in glucose transportation in *L. monocytogenes* EGD-e (11).

Alternative σ factors play key roles in the adaptation of *L. monocytogenes* to changing environmental conditions (12). Under certain environmental conditions, alternative σ factors reprogram the RNA polymerase holoenzyme to recognize specific promoters and hence allow for rapid induction of the transcription of stress response and virulence genes (12). Four alternative σ factors (σ^B, σ^C, σ^H, and σ^L) have been identified in *L. monocytogenes*. A number of studies on σ^B have demonstrated that this alternative σ factor controls a large regulon and contributes both to the stress response and to the virulence of *L. monocytogenes* (12–17). σ^B has been shown to positively regulate at least one PTS-encoding operon, *lmo0781* to *lmo0784* (PTS^{Man}-3), which has been suggested to play an important role in the regulation of PrfA activity (18). However, σ^H, σ^L, and σ^C have not been as extensively characterized. A transcriptomic analysis of the *L. monocytogenes* parent strain EGD-e and an isogenic *sigL* deletion mutant indicated that σ^L controls the expression of genes encoding four PTSs and thus controls carbohydrate metabolism via direct regulation of PTS activity (19). Proteomics using strain 10403S and isogenic mutants have also shown that σ^L positively regulates the expression of PTS^{Man}-2, another PTS suggested to play a key role in the activation of PrfA (18), and negatively regulates the expression of other PTSs, such as those encoded by *lmo0027* (PTS^{Glc}-1) and *lmo2097* to *lmo2098* (PTS^{Gat}-2) (20). Chaturongakul et al. identified 51 and

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TABLE 1 Bacterial strains and plasmid used in this study

Strain or plasmid	Genotype	Source or reference
Strains		
FSL X1-001	Parent strain 10403S	38
FSL A1-254	Δ*sigB*	39
FSL B2-124	Δ*sigL*	28
FSL C3-126	Δ*sigH*	28
FSL C3-113	Δ*sigC*	28
FSL C3-119	Δ*sigB* Δ*sigC*	This study
FSL C3-123	Δ*sigB* Δ*sigH*	This study
FSL B2-127	Δ*sigB* Δ*sigL*	This study
FSL C3-124	Δ*sigC* Δ*sigH*	This study
FSL B2-129	Δ*sigC* Δ*sigL*	This study
FSL B2-130	Δ*sigH* Δ*sigL*	This study
FSL C3-139	Δ*sigC* Δ*sigH* Δ*sigL*	This study
FSL C3-128	Δ*sigB* Δ*sigC* Δ*sigH*	20
FSL C3-137	Δ*sigB* Δ*sigC* Δ*sigL*	20
FSL C3-138	Δ*sigB* Δ*sigH* Δ*sigL*	20
FSL C3-135	Δ*sigB* Δ*sigC* Δ*sigH* Δ*sigL*	20
FSL B2-392	Δ*sigH* Δ*sigL* Δ*lmo0027*	This study
FSL B2-393	Δ*sigL* Δ*lmo0027*	This study
FSL B2-394	Δ*lmo0027*	This study
FSL B2-395	Δ*sigH* Δ*lmo0027*	This study
Plasmid pSW1	Δ*lmo0027*	This study

TABLE 2 SOE PCR primers for mutant construction

Primer	Sequence (5′ to 3′)
SW01 soeA	TGGAAGCTTAATGCTTGCAACCGTTTTCTTTAT
SW02 soeB	TTGACCACCACTTTTAATGACAGA
SW03 soeC	TCTGTCATTAAAAGTGGTGGTCAAACAACTAT CTTCCCAACTGGT
SW04 soeD	TGGTCTAGACATAAAAACAGCTTGAGCAAGT TACT
SW05 *lmo0027* XF	TGGTTAGTTGAGCAAGGCGTTA
SW06 *lmo0027* XR	AGTGCAATTCTGTTCTCATCTTCTTT

169 genes as differentially regulated by σ^L and σ^H, respectively, including 8 and 3 genes encoding components of PTSs (14). An *L. monocytogenes* EGD-e Δ*sigH* strain showed significantly impaired growth in minimal medium as well as slightly reduced virulence potential in a murine model (21). Through proteomics, Mujahid et al. identified three PTS components positively regulated by σ^H: (i) PTS^{Glc}-1, encoded by *lmo0027*; (ii) one component of PTS^{Man}-2, encoded by *lmo0096*; and (iii) PTS^{Glc} (EIIBC), encoded by *lmo1255* (20). Moreover, a σ^H-dependent promoter was identified 54 nucleotides upstream of the start codon of *lmo0027*, suggesting that σ^H may directly regulate the expression of this gene (20). σ^C has been described only for *L. monocytogenes* strains belonging to lineage II, and studies conducted to date on the σ^C regulon identified a few *pts* genes as σ^C dependent (14, 20, 22). Furthermore, considerable overlap has been found between different *L. monocytogenes* alternative σ factor regulons (14, 20).

The previous studies collectively suggest that alternative σ factors other than σ^B may play an important role in the environmental adaptation and/or virulence of *L. monocytogenes* through the regulation of specific *pts* genes. To more precisely characterize the significance of alternative σ factors and PTSs for listerial carbon utilization and pathogenesis, we assessed the growth of *L. monocytogenes* 10403S and 19 isogenic alternative σ factor and/or *pts* gene mutants in PTS-dependent and non-PTS-dependent carbon sources at either 25°C or 37°C.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *L. monocytogenes* 10403S and the 19 isogenic mutant strains used in this study (Table 1) were stored at −80°C in brain heart infusion broth (BHIB) (Becton Dickinson, Sparks, MD) with 15% glycerol (IBI Scientific, Peosta, IA). In preparation for the experiments, isolates were streaked onto brain heart infusion agar (BHIA) from frozen stocks and were incubated at 37°C for 24 h. A single colony was transferred to 5 ml BHIB and was incubated at 37°C with shaking (230 rpm) for 18 h, followed by transfer of a 1% inoculum to 5 ml BHIB. After growth to early-exponential phase (defined as an optical density at 600 nm [OD_{600}] of 0.4), 2 μl of a 0.01% inoculum was transferred to a 100-well honeycomb plate (Oy Growth Curves AB, Raisio, Finland) containing 198 μl prewarmed (37°C) chemically defined *L. monocytogenes* minimal medium (DM) (23) in each well. DM was supplemented with one of the following carbon sources at a final concentration of 10 mM: glucose (Sigma, St. Louis, MO), mannose (Sigma), cellobiose (Sigma), or glycerol. The carbon sources were added to the medium at the required final concentrations, followed by filter sterilization.

The 100-well honeycomb plates were incubated in the Bioscreen C automated turbidimetric system (Growth Curves USA, Piscataway, NJ) at 25°C or 37°C. OD measurements were taken every 10 min using the wide-band filter (420 to 580 nm) of the instrument for a total of 72 h. Growth parameters were determined in duplicate wells in two independent replications. Additionally, spread plating was used to determine the growth parameters of the parent strain 10403S and the Δ*sigH*, Δ*sigL*, and Δ*sigH* Δ*sigL* mutants in DM supplemented with glucose, cellobiose, or glycerol at a final concentration of 10 mM. Briefly, early-exponential-phase cell cultures were diluted in phosphate-buffered saline (PBS, pH 7.4) and transferred to 5 ml DM to reach a final level of approximately 1×10^3 CFU per milliliter. Cultures were sampled every 12 h for 96 h. Samples were diluted in PBS and were plated onto BHIA using an Autoplate 4000 system (Spiral Biotech, Bethesda, MD). Plates were incubated at 37°C for 24 h before enumeration of colonies with a Q-Count system (Spiral Biotech). Growth was monitored for three independent replicates of each strain with each carbon source.

Growth parameters and statistical analyses. Optical-density-based growth curves were fitted by the Gompertz model using the grofit package, version 1.0, in R, version 2.13.1 (24), to estimate the lag phase duration (λ), maximum growth rate (μ_{max}), and maximum optical density (OD_{max}). One-way analysis of variance (one-way ANOVA) was used to examine the effect of strain on the growth parameters for a given temperature and carbon source. Linear regression models were subsequently applied to the data by using the presence of the genes encoding alternative σ factors as predictors of the response variables (i.e., λ, μ_{max}, and OD_{max}) for each of the four carbon sources (cellobiose, mannose, glucose, and glycerol). The models were created using the lm function, while the stepAIC function from the MASS package in R, version 2.13.1, was used with both the forward and backward algorithms to identify the best model. Confidence intervals at an α value of 0.05 (95% CI) were estimated using the "predict" function.

For plate count data, the growth parameters of each strain in each carbon source were estimated using the Baranyi model without λ (25) implemented in the NLStools package, version 0.0-11, in R, version 2.13.1. Plate counts (in CFU per milliliter) for each strain at every time point were log transformed and were used to estimate the μ_{max} and maximum cell density (N_{max}) values. Differences among the strains and carbon sources were analyzed with a separate fixed-effect ANOVA for each growth parameter. The linear model used for ANOVA included the strain and carbon source as fixed effects. ANOVA and a *post hoc* Tukey test were performed with JMP Pro, version 9.0.2 (SAS Institute, Inc., Cary, NC). Adjusted *P* values of <0.05 were considered significant.

TABLE 3 Results of one-way ANOVA on the effects of strain on optical density-based growth parameters

Temp (°C)	Carbon source	PTS dependent[a]	Prob > F^b for:		
			μ_{max}	λ	OD_{max}
25	Glucose	Y	<0.0001	<0.0001	0.134
	Mannose	Y	<0.0001	<0.0001	0.170
	Cellobiose	Y	<0.001	0.390	0.199
	Glycerol	N	0.302	0.560	0.806
37	Glucose	Y	<0.0001	<0.0001	<0.0001
	Mannose	Y	<0.0001	<0.0001	0.017
	Cellobiose	Y	<0.0001	<0.0001	<0.0001
	Glycerol	N	0.780	0.082	0.280

[a] Y, yes; N, no.
[b] Prob > *F*, probability of obtaining an *F* value greater than the one calculated if, in reality, there is no difference in the population group means. Observed significance probabilities of <0.05 are considered as indicating that there are differences in the group means.

RNA isolation and quantitative PCR (qPCR). RNA was extracted from cultures of strain 10403S and its isogenic Δ*sigL*, Δ*sigH*, and Δ*sigH* Δ*sigL* mutants after growth in DM with 10 mM glucose at 37°C to an OD_{600} of 0.4. Total RNA was extracted using the PowerSoil total-RNA isolation kit (Mo Bio, Carlsbad, CA) by following the manufacturer's protocol. After extraction, RNA was treated with DNase I (Invitrogen, Foster City, CA), followed by purification with RNeasy minicolumns (Qiagen, Valencia, CA). RNA quality was assessed on a Bioanalyzer system (Agilent, Santa Clara, CA), and samples with an RNA integrity number (RIN) of >8.0 were used for subsequent analyses.

cDNA was synthesized from 500 ng total RNA using 4 μl qScript cDNA SuperMix (5×) (Quanta Biosciences, Gaithersburg, MD) in a reaction mixture with a total volume of 20 μl. Reverse transcription reactions were carried out under the following conditions: 5 min at 25°C, 30 min at 42°C, 5 min at 85°C, and a hold at 4°C. Tenfold serial dilutions of cDNA were used as the input for qPCR assays. RNA samples without 10-fold dilution and reverse transcription were used to determine background levels of DNA. Primer sequences for *lmo0027* and *rpoB* were taken from previous studies (26, 27). The qPCR mixtures contained 10 μl PerfeCTa SYBR green FastMix (2×) (Quanta Biosciences), 500 nmol (each) primer, and 5 μl of the template and were run on the CFX96 system (Bio-Rad Laboratories, Hercules, CA) under the following conditions: 40 cycles at 95°C for 15 s and 60°C for 1 min. The threshold cycle (C_T) and reaction efficiencies were determined using CFX Manager software (Bio-Rad). Quantitative PCRs were carried out in duplicate for each cDNA sample tested. Target gene copy numbers were determined using genomic DNA standard curves and were normalized to copy numbers of *rpoB*. The ratios of normalized *lmo0027* transcript levels for the Δ*sigH*, Δ*sigL*, and

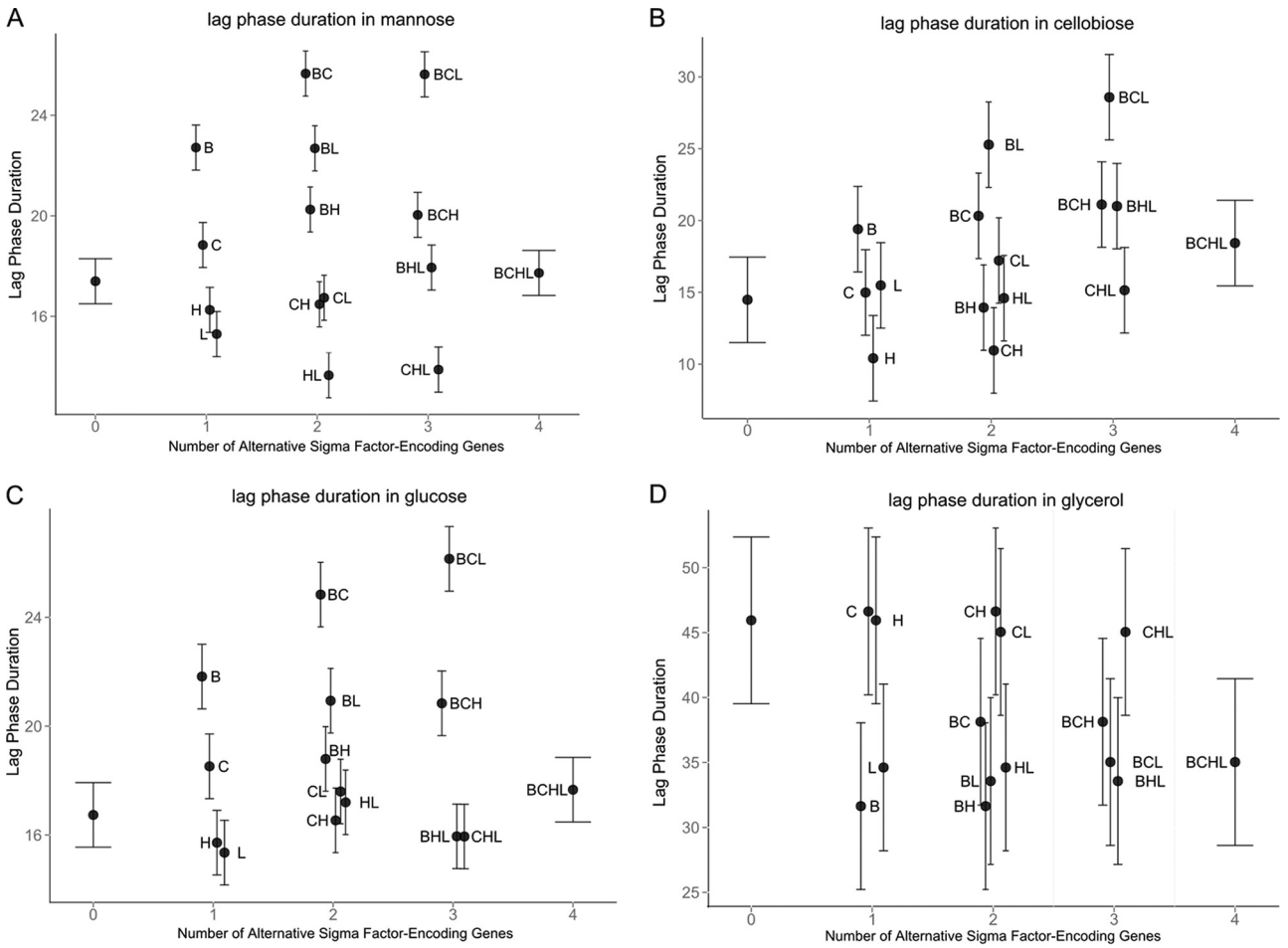

FIG 1 Estimates for OD-based lag phase duration (λ) in DM with mannose (A), cellobiose (B), glucose (C), or glycerol (D) for 10403S and 15 isogenic mutants at 37°C. Error bars indicate 95% confidence intervals. The lag phase duration (in hours) is shown along the *y* axis. The number of alternative σ factor-encoding genes present, ranging from zero (Δ*sigB* Δ*sigC* Δ*sigH* Δ*sigL* quadruple mutant) to 4 (parent strain 10403S), is shown along the *x* axis. B, σ^B; C, σ^C; H, σ^H; L, σ^L.

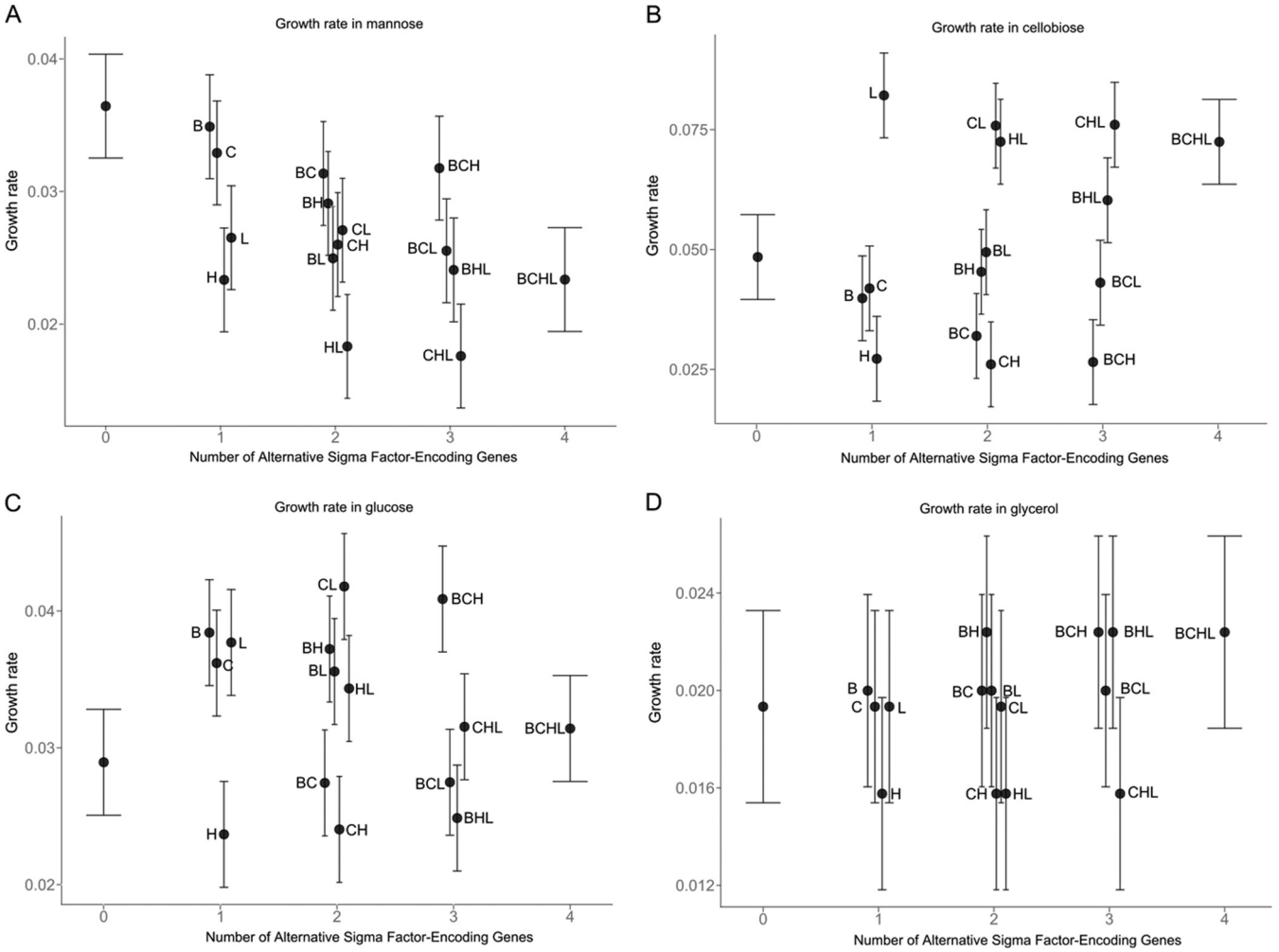

FIG 2 Estimates for OD-based maximum growth rate (μ_{max}) in DM with mannose (A), cellobiose (B), glucose (C), or glycerol (D) for 10403S and 15 isogenic mutants at 37°C. Error bars indicate 95% confidence intervals. The value of μ_{max} (OD increase per hour). The number of alternative σ factor-encoding genes present, ranging from zero (Δ*sigB* Δ*sigC* Δ*sigH* Δ*sigL* quadruple mutant) to 4 (parent strain 10403S), is shown along the *x* axis.

Δ*sigH* Δ*sigL* strains to transcript levels for the parent strain 10403S were calculated.

Mutant construction. In-frame deletion mutations of *lmo0027* were constructed in the 10403S, 10403S Δ*sigH*, 10403S Δ*sigL*, and 10403S Δ*sigH* Δ*sigL* backgrounds, using the splicing by overlap extension (SOE) method and the pKSV-7 vector as described previously (28). Table 2 lists the primer sequences used in mutant construction. All mutations were confirmed by PCR and by sequencing of the chromosomal copy of the deletion allele (data not shown).

RESULTS AND DISCUSSION

The effects of deletion of alternative σ factors on the growth of *L. monocytogenes* are dependent on the growth temperature and carbon source. One-way ANOVA was used for initial assessment of the effect of the factor "strain" on each OD-derived growth parameter in each of the four carbon sources at 25°C or 37°C (Table 3; detailed data are provided in Tables S1 and S2, and in Fig. S1 and S2, in the supplemental material). Overall, this factor affected more growth parameters when *L. monocytogenes* was grown at 37°C than when it was grown at 25°C; only 5 of the 12 growth parameters were significantly affected by strain at 25°C, while 9 of the 12 growth parameters were significantly affected by strain at 37°C (Table 3). At 25°C, this factor contributed significantly to the observed differences in the maximum growth rate (μ_{max}) and lag phase duration (λ) ($P < 0.05$), but not in the maximum optical density (OD_{max}), in DM with glucose and mannose, while in DM with cellobiose, only μ_{max} was significantly affected by strain (Table 3). At 37°C, this factor contributed significantly to the observed differences in μ_{max}, λ, and OD_{max} in all three PTS-dependent carbon sources (glucose, cellobiose, and mannose) ($P < 0.05$). Interestingly, no significant effect of strain on growth parameters was observed when *L. monocytogenes* was grown in glycerol, a non-PTS-dependent carbon source, at either 25°C or 37°C ($P > 0.05$) (Table 3).

These results suggested that the effects of deletions of genes encoding alternative σ factors on the growth of *L. monocytogenes* in DM with different sugars are more pronounced at 37°C than at 25°C. This may be explained by the temperature-dependent expression of alternative σ factors and/or the alternative σ factor regulon, which has been reported previously (29–32). For example, *sigL* transcript levels have been shown in one study to be

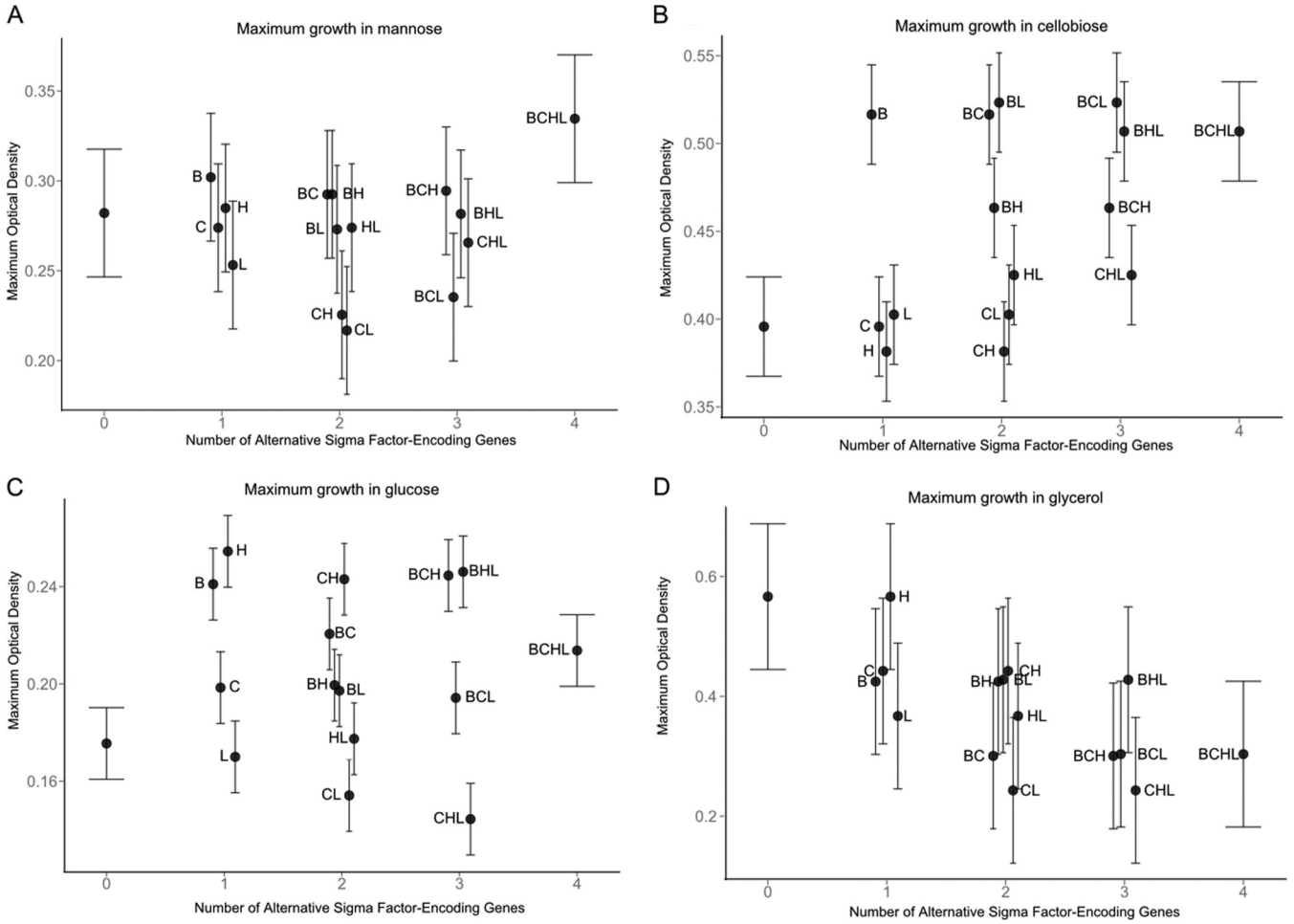

FIG 3 Estimates for maximum OD (OD_{max}) in DM with mannose (A), cellobiose (B), glucose (C), or glycerol (D) for 10403S and 15 isogenic mutants at 37°C. Error bars indicate 95% confidence intervals. The OD_{max} value is shown along the *y* axis. The number of alternative σ factor-encoding genes present, ranging from zero (Δ*sigB* Δ*sigC* Δ*sigH* Δ*sigL* quadruple mutant) to 4 (parent strain 10403S), is shown along the *x* axis.

higher at 10°C than at 37°C in BHIB (29). Chan et al., on the other hand, reported that *sigL* transcript levels were lower in stationary-phase cells at 4°C than at 37°C in BHIB (30). Moreover, previous studies have shown that the transcript levels of the σ^B regulon are temperature dependent (31, 32); Toledo-Arana et al. specifically found that σ^B-mediated transcription of virulence genes, including *prfA*, *inlA*, *inlB*, and *ctsR*, was upregulated in stationary phase relative to exponential phase at 37°C but not at 30°C (31). McGann et al. found that the transcript levels of four σ^B-dependent internalin genes (*inlC2*, *inlD*, *lmo0331*, and *lmo0610*) were highest at 16°C and generally lowest at 37°C (32). While these findings do not provide direct evidence that alternative σ factor expression and activity are higher at 37°C than at 25°C, they do demonstrate the effect of temperature on alternative σ factor expression and activity, consistent with our findings suggesting an influence of temperature on the growth parameters observed for different alternative σ factor-null mutants in different carbon sources.

The growth of *L. monocytogenes* is dependent on σ^B, σ^H, and/or σ^L in PTS-dependent carbon sources at 37°C according to an OD-based growth study. Since ANOVA results showed a significant effect of strain on the three growth parameters at 37°C, we used linear regression to specifically assess the effects of deletions of the genes encoding the four alternative σ factors on growth parameters in different carbon sources at 37°C. The best model for each combination of carbon source and growth parameter was selected by the Akaike information criterion (AIC) using stepwise algorithms (Fig. 1, 2, and 3 present results for λ, μ_{max}, and OD_{max}, respectively; results are discussed by carbon source below). Predictor variables and their respective estimates are presented in Tables S3 to S14 in the supplemental material.

In DM with mannose, the presence of *sigB* had a significant positive effect on λ (i.e., longer λ in the presence of *sigB*) while the presence of *sigL* had a significant negative effect on λ (Fig. 1A; see also Table S3 in the supplemental material). Under the same conditions, the interaction of *sigB* and *sigL* resulted in a significant positive effect on λ (Fig. 1A; see also Table S3), suggesting a regulatory interaction between the two alternative σ factors encoded by these genes. The presence of *sigC* also showed a significant positive effect on λ, and the interaction of *sigB* and *sigC* resulted in a significant further increase in this parameter (Fig. 1A; see also Table S3). μ_{max} was negatively affected by the presence of *sigH* and *sigL*, but these effects were not statistically significant ($P = 0.17$), while the interaction between *sigH* and *sigB* resulted in a significant increase in this parameter in DM with mannose (Fig. 2A; see

TABLE 4 Colony enumeration-derived growth parameters of *L. monocytogenes* 10403S and mutants at 37°C[a]

Carbon source	PTS dependent[b]	Strain or genotype	μ_{max} [log(CFU/ml)/h]	N_{max} [log(CFU/ml)]
Glucose	Y	10403S	0.23 ± 0.01 C	8.36 ± 0.34 A
		Δ*sigH*	0.18 ± 0.02 DEF	8.27 ± 0.17 A
		Δ*sigL*	0.32 ± 0.03 A	8.63 ± 0.14 A
		Δ*sigH* Δ*sigL*	0.22 ± 0.02 CDE	8.72 ± 0.08 A
		Δ*lmo0027*	0.24 ± 0.01 BC	8.62 ± 0.07 A
		Δ*sigH* Δ*lmo0027*	0.17 ± 0.01 DEF	8.88 ± 0.04 A
		Δ*sigL* Δ*lmo0027*	0.15 ± 0.01 F	8.96 ± 0.06 A
		Δ*sigH* Δ*sigL* Δ*lmo0027*	0.15 ± 0.01 F	8.83 ± 0.15 A
Cellobiose	Y	10403S	0.22 ± 0.01 CD	8.43 ± 0.41 A
		Δ*sigH*	0.17 ± 0.01 EF	8.41 ± 0.21 A
		Δ*sigL*	0.28 ± 0.01 AB	8.71 ± 0.11 A
		Δ*sigH* Δ*sigL*	0.22 ± 0.03 CD	8.63 ± 0.26 A
Glycerol	N	10403S	0.20 ± 0.01 CD	7.99 ± 0.20 A
		Δ*sigH*	0.22 ± 0.02 CDE	8.09 ± 0.37 A
		Δ*sigL*	0.21 ± 0.02 CDE	8.09 ± 0.20 A
		Δ*sigH* Δ*sigL*	0.21 ± 0.02 CDE	8.21 ± 0.35 A
		Δ*lmo0027*	0.20 ± 0.01 CDE	8.07 ± 0.23 A
		Δ*sigH* Δ*lmo0027*	0.19 ± 0.01 CDEF	8.13 ± 0.22 A
		Δ*sigL* Δ*lmo0027*	0.18 ± 0.01 DEF	8.25 ± 0.23 A
		Δ*sigH* Δ*sigL* Δ*lmo0027*	0.21 ± 0.01 CDE	8.16 ± 0.20 A

[a] Results are means ± standard deviations for each bacterial strain tested in triplicate. Means followed by the same letter within a given column are not statistically different from each other (overall α, 0.05 by Tukey's honestly significant difference test).
[b] Y, yes; N, no.

also Table S4 in the supplemental material). OD_{max} was not significantly affected by any alternative σ factor-encoding gene alone or by any interaction among alternative σ factor-encoding genes in DM with mannose (see Table S5 in the supplemental material).

In DM with cellobiose, the presence of *sigB* significantly increased λ (Fig. 1B; see also Table S6 in the supplemental material). The presence of *sigL* alone led to a significant increase in μ_{max} in cellobiose (Fig. 2B; see also Table S7 in the supplemental material). The presence of *sigH* significantly reduced μ_{max}, but the interaction between *sigB* and *sigH* led to an increase in this parameter, in DM with cellobiose (see Table S7). Conversely, the interaction of *sigB* and *sigL* resulted in a significant decrease in μ_{max} (see Table S7), suggesting differential regulatory interactions between σ^B and σ^H, as well as between σ^B and σ^L, in DM with cellobiose. OD_{max} was significantly affected only by the presence of *sigB*, which led to an increase in this parameter, in DM with cellobiose (Fig. 3B; see also Table S8 in the supplemental material).

Growth in DM with glucose was the most affected by the presence of alternative σ factor-encoding genes. λ was significantly increased by (i) the presence of *sigB*, (ii) the presence of *sigC*, and (iii) the interaction between *sigH* and *sigL* (Fig. 1C; see also Table S9 in the supplemental material). Conversely, λ was significantly reduced by the interactions between (i) *sigB* and *sigH*, (ii) *sigB*, *sigH*, and *sigL*, and (iii) *sigC*, *sigH*, and *sigL* (see Table S9), suggesting a complex network of regulatory interactions among σ factors in DM with glucose. In DM with glucose, μ_{max} was also significantly affected by several σ factor-encoding genes and their interactions; the presence of *sigB*, *sigC*, and *sigL* and the interaction between *sigB*, *sigC*, and *sigH* positively affected μ_{max} (Fig. 2C; see also Table S10 in the supplemental material). The presence of *sigH* and the interactions between (i) *sigB* and *sigC*, (ii) *sigB* and *sigL*, (iii) *sigC* and *sigH*, and (iv) *sigB*, *sigH*, and *sigL* had negative effects on μ_{max} (Fig. 2C; see also Table S10). OD_{max} was also highly affected by the presence of alternative σ factor-encoding genes. Interestingly, *sigB*, *sigC*, and *sigH* all had positive effects on OD_{max}, while the presence of *sigL* had a negative though nonsignificant effect on this parameter (Fig. 3C; see also Table S11 in the supplemental material). However, all possible combinations of interactions between two σ factor-encoding genes resulted in significantly negative effects on OD_{max} (see Table S11). Yet interactions among three of the four alternative σ factor-encoding genes, excluding the interaction among *sigC*, *sigH*, and *sigL*, resulted in significantly positive effects on OD_{max}, and the interaction among all four alternative σ factor-encoding genes resulted in a significantly negative effect on OD_{max} (see Table S11). These data suggested that in DM with glucose, alternative σ factors form a regulatory network that can drastically affect OD_{max} during the growth of *L. monocytogenes*.

The initial ANOVA did not identify a significant effect of strain on growth parameters in DM with glycerol, the only non-PTS-dependent carbon source analyzed. However, linear regression indicates that the presence of *sigB* or *sigL* significantly reduced λ, while the interaction between these two σ factor-encoding genes significantly increased λ (Fig. 1D; see also Table S12 in the supplemental material). μ_{max} was not affected by the alternative σ factor-encoding genes (Fig. 2D; see also Table S13 in the supplemental material), while OD_{max} was negatively affected by the presence of *sigC* and *sigL* (Fig. 3D; see also Table S14 in the supplemental material). These results suggested that the effects of the presence or absence of alternative σ factors on growth in DM with glycerol may not necessarily be identified in strain level analysis but can be identified with linear regression, since only significant variables and their interactions are retained in the final model (e.g., only 4 of the 15 predictor variables and their interactions are included in the final model for OD_{max} in DM with glycerol at 37°C).

Overall, our data showed that in DM with PTS-dependent carbon sources, the presence of *sigB* significantly increases λ, while in DM with a non-PTS-dependent carbon source (i.e., glycerol), the presence of *sigB* significantly reduces λ. Moreover, with the exception of mannose, OD_{max} in a PTS-dependent carbon source was positively affected by the presence of *sigB*. The presence of *sigH* significantly reduced μ_{max} in DM with any of the three PTS-dependent carbon sources. The presence of *sigL*, on the other hand, showed a PTS-dependent carbon-specific effect on μ_{max}; while μ_{max} was positively affected by *sigL* in DM with glucose or cellobiose, this parameter was negatively affected by *sigL* in DM with mannose. Interactions between alternative σ factor-encoding genes were also dependent on carbon sources. These results collectively suggested a complex regulatory network among σ^B, σ^H, and σ^L for the growth of *L. monocytogenes* in PTS-dependent carbon sources. This is not surprising, because there are considerable regulon overlaps among the alternative σ factors (14). For example, the PTS^{Man}-2 encoded by *lmo0096* to *lmo0098* is the major transporter for glucose, followed by the PTS^{Man}-3 encoded by the *mpoABCD* (*lmo0781*-to-*lmo0784*) operon (11, 18). It has been reported that *lmo0096* to *lmo0098* are positively regulated by σ^L (19, 33), while *mpoABCD* showed evidence of positive regulation by σ^B (13, 14). σ^B also has been reported to be involved in the regulation of other *pts* genes, including *lmo0021*, *lmo0027*, *lmo0398* to *lmo0400*, *lmo2733*, *lmo0631*, and *lmo2665* (13, 14).

The growth of *L. monocytogenes* is positively affected by the presence of σ^H and negatively affected by the presence of σ^L in PTS-dependent carbon sources, but not in a non-PTS-dependent carbon source, at 37°C. Unlike that of σ^B, which is well known to play a role in the stress response and virulence of *L. monocytogenes*, the functional roles of σ^L and σ^H have not been clearly defined to date (12). We recognize that OD cannot be used as the sole indicator for the evaluation of growth rates for *L. monocytogenes* (34). Therefore, in order to confirm the roles of σ^L and σ^H in the growth of *L. monocytogenes*, we used spread plating to compare the growth of the Δ*sigH*, Δ*sigL*, and Δ*sigH* Δ*sigL* mutants to that of the parent strain 10403S in PTS-dependent carbon sources (glucose and cellobiose) and a non-PTS-dependent carbon source (glycerol) at 37°C (Table 4; Fig. 4). In DM with glucose or cellobiose, the μ_{max} of the Δ*sigH* strain decreased significantly ($P < 0.01$) from that of the parent strain, while the growth rate of the Δ*sigL* strain increased significantly ($P < 0.01$). In contrast, the μ_{max} of the Δ*sigH* Δ*sigL* mutant was similar to that of the parent strain ($P > 0.05$) (Table 4; Fig. 4A and B). While the plate-counting results were generally consistent with the OD-based growth results, OD- and plate-counting-based parameters yielded different conclusions about μ_{max} for the Δ*sigL* strain grown in DM with cellobiose. Specifically, the OD-based data indicated a significantly higher μ_{max} for the Δ*sigL* strain than for the parent strain, while the plate count-based data indicated a significantly lower μ_{max} than for the parent strain ($P < 0.05$). Since OD-based results are highly dependent on bacterial culture conditions (e.g., cell geometry, presence and concentrations of secreted compounds) (35), these data may suggest that the Δ*sigL* strain shows distinct cell geometry or physiology when exposed to cellobiose. This is consistent with a previous study that reported that the surfaces of certain rumen bacterial strains became smoother and contained fewer protuberant structures when grown in cellobiose (36). Further morphological studies on *L. monocytogenes* grown in DM with cellobiose will be needed to elucidate this phenomenon. In

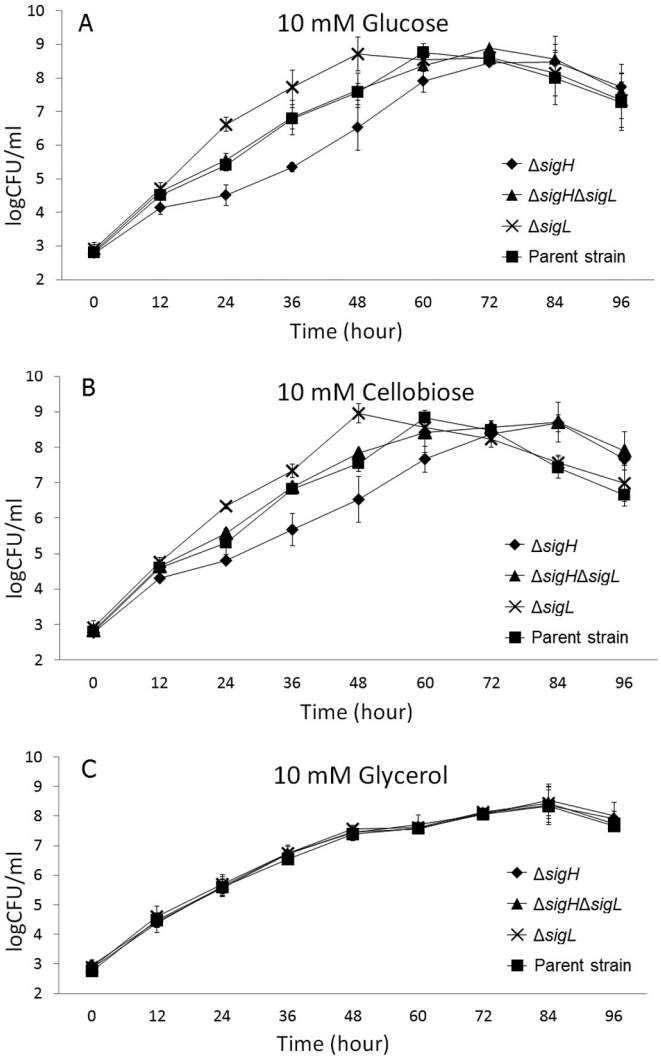

FIG 4 Growth of the parent strain 10403S and the Δ*sigH*, Δ*sigL*, and Δ*sigH* Δ*sigL* mutants at 37°C in DM with 10 mM glucose (A), DM with 10 mM cellobiose (B), or DM with 10 mM glycerol (C).

DM with glycerol, the plate-counting-based μ_{max} values of the Δ*sigH*, Δ*sigL*, and Δ*sigH* Δ*sigL* mutants were not significantly different from that of the parent strain ($P > 0.05$) (Table 4; Fig. 4C). Also, deletion of *sigH* and/or *sigL* did not affect N_{max} in any carbon source at 37°C (Table 4; Fig. 4).

These findings indicated that σ^H and σ^L play positive and negative roles, respectively, in the utilization of PTS-dependent carbon sources for the growth of *L. monocytogenes*. However, previous studies have suggested that σ^L plays multiple roles in the regulation of PTSs. Among the genes encoding three major PTSs for glucose transport (i.e., PTS^{Man}-2, PTS^{Man}-3, and PTS^{Glc}-1), *lmo0096* to *lmo0098*, which encode PTS^{Man}-2, have been reported to be positively regulated by σ^L (19), and *lmo0781* to *lmo0784*, which encode PTS^{Man}-3, have been reported to be constitutively transcribed with additional positive regulation by σ^B (11). On the other hand, *lmo0027*, which encodes PTS^{Glc}-1, has not only been reported to be negatively regulated by σ^L (14, 19) but also is preceded by two putative σ^A-dependent and one putative σ^H-dependent promoter (20). Rea et al. also found that deletion of *sigH*

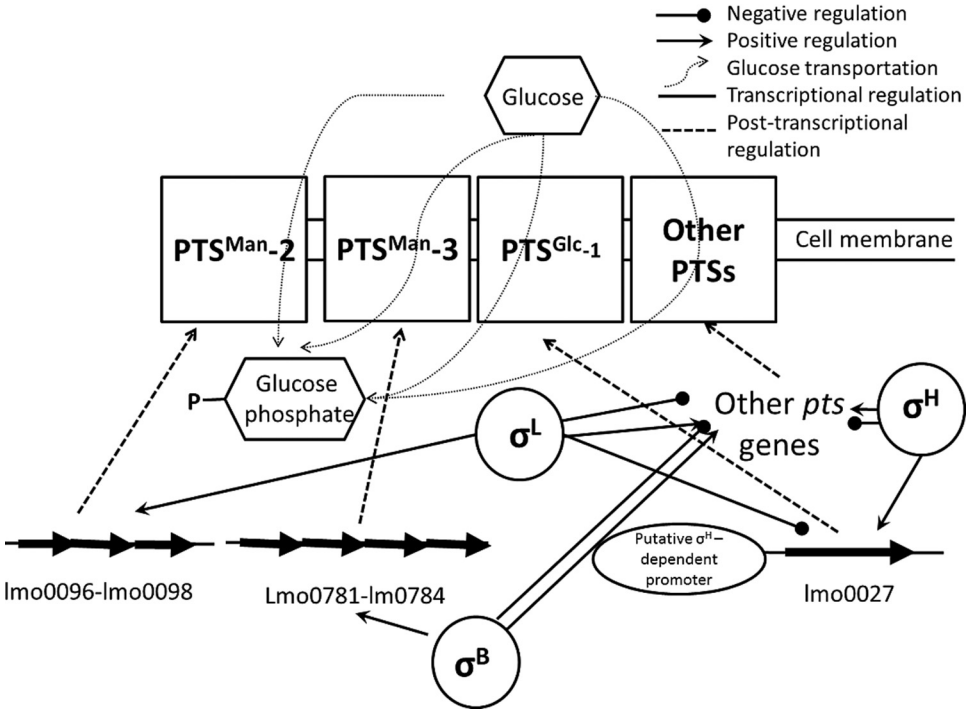

FIG 5 Proposed mode of regulation of PTS-dependent *L. monocytogenes* growth in glucose by σ^B, σ^H, and σ^L. Solid lines indicate transcriptional regulation (i.e., regulation at the DNA level); dotted lines indicate posttranscriptional regulation (i.e., regulation at the RNA level).

resulted in reduced growth in minimal medium but not in BHIB (21), suggesting a role for σ^H in the acquisition or utilization of nutrients in minimal medium. However, the role of σ^H in PTS-dependent carbon source acquisition or utilization was not elucidated in previous studies.

No significant regulatory effect of σ^H or σ^L on the growth in DM with glycerol was observed at 37°C, even though the transcription of genes involved in glycerol catabolism has been shown to be σ^B dependent in *L. monocytogenes* (37). Joseph et al. specifically reported that genes known to be involved in glycerol uptake and metabolism (*glpFK* and *glpD*) showed significantly higher transcript levels with σ^B in the presence of glycerol than in the presence of glucose or cellobiose (2). Chaturongakul et al. reported that the transcription of *glpFK* and *glpD* was not dependent on σ^H or σ^L at 37°C (14). Arous et al. found that *glpD* and *glpF* were significantly upregulated with the loss of σ^L in BHIB at 42°C (19), suggesting negative regulation of these genes by σ^L at 42°C but not at 37°C. Collectively, these findings suggest a temperature-dependent regulation by σ^L of *L. monocytogenes* growth in glycerol.

The growth of *L. monocytogenes* in glucose is dependent on the regulation of *lmo0027* by σ^L. Since *lmo0027* encodes an important glucose transporter (i.e., PTS^{Glc}-1), and prior evidence for σ^H- and σ^L-dependent transcription has been shown, qPCR was used to quantify the change in *lmo0027* transcript levels in the absence of *sigH* and *sigL*. The transcript levels of *lmo0027* were 14.3- ± 3.1-fold ($P < 0.01$) and 10.1- ± 5.5-fold ($P < 0.01$) higher in the Δ*sigL* and Δ*sigH* Δ*sigL* strains, respectively, than in the parent strain. In the Δ*sigH* mutant, the *lmo0027* transcript level was 2.2- ± 1.5-fold lower ($P < 0.05$) than that in the parent strain. These results are consistent with previous findings (19, 20), suggesting a weak positive regulatory effect of σ^H, and a strong negative regulatory effect of σ^L, on *lmo0027* (Fig. 5). While σ^L has also been found to positively regulate other *pts* genes (14, 19), we hypothesized that the negative regulation of *lmo0027* by σ^L plays a role in the reduced μ_{max} in glucose associated with the *sigL* deletion.

To further test the effect of *lmo0027* on the growth of *L. monocytogenes* in DM with glucose and glycerol at 37°C, Δ*lmo0027*, Δ*sigH* Δ*lmo0027*, Δ*sigL* Δ*lmo0027*, and Δ*sigH* Δ*sigL* Δ*lmo0027* deletion mutants of the parent strain 10403S were generated and evaluated. In agreement with the findings by Stoll and Goebel (11), the μ_{max} of the Δ*lmo0027* mutant was similar to that of the parent strain in glucose ($P > 0.05$). However, the Δ*sigL* Δ*lmo0027* and Δ*sigH* Δ*sigL* Δ*lmo0027* mutants showed significantly lower μ_{max} values at 37°C ($P < 0.05$) than did the parent strain, although the deletion of *sigL* alone resulted in significantly increased μ_{max} at 37°C (Table 4). A previous study suggested that the loss of *lmo0027* alone did not result in a reduced growth rate but that the loss of *lmo0027* along with deletion of the PTS^{Man}-2 operon and the PTS^{Man}-3 operon led to a drastically reduced growth rate in DM with glucose (11). Taken together, these observations suggested that the negative regulation of *lmo0027* by σ^L indeed plays a role in the growth of *L. monocytogenes* in glucose, even though the absence of *lmo0027* alone did not impact the growth of *L. monocytogenes* in DM with glucose (Table 4; Fig. 5), likely because *lmo0027* transcription was repressed by σ^L under these conditions in the parent strain.

The Δ*sigH* Δ*lmo0027* strain demonstrated a μ_{max} similar to that of the Δ*sigH* strain, which was significantly lower than that of the parent strain ($P < 0.05$) (Table 4). This suggests that σ^H does not regulate *lmo0027* to an extent that affects the growth of *L.*

monocytogenes in DM with glucose. The reduced μ_{max} of the Δ*sigH* mutant relative to that of the parent strain in DM with glucose is possibly associated with the positive regulation by σ^H of other *pts* genes, such as *lmo0738*, encoding PTS^{Glc}-2, and *lmo2355*, encoding PTS^{Fru}-6 (14). The expression of *lmo0738* has been reported previously to be upregulated in an *hprK* mutant, which was defective in carbon catabolite repression (CCR) control (10). The expression of *lmo2355* was downregulated in DM with glycerol relative to DM with glucose (2). Yet the biological significance of these genes in the growth of *L. monocytogenes* using glucose requires further elucidation.

Conclusions. Our present study suggests that the regulation of the growth of *L. monocytogenes* by alternative σ factors is both temperature and carbon source dependent. Besides σ^B, σ^L and σ^H coregulate PTS-dependent carbon source uptake for *L. monocytogenes* through complex, temperature-dependent regulatory networks that allow *L. monocytogenes* to fine-tune its response to changing growth conditions. σ^H positively regulates the growth of *L. monocytogenes*, and σ^L negatively regulates the growth of *L. monocytogenes*, in PTS-dependent carbon sources at 37°C. The negative regulatory effect of σ^L on the growth of *L. monocytogenes* is associated with the negative regulation of the PTS^{Glc}-1-encoding gene, *lmo0027*. While *lmo0027* transcription is positively regulated by σ^H, this regulatory effect does not seem to affect the growth of *L. monocytogenes* in DM with glucose at 37°C, possibly due to transcription from two identified putative σ^A-dependent promoters, in addition to a putative σ^H-dependent promoter, upstream of *lmo0027*. Due to the complexities of transcriptional regulation and the considerable gaps in knowledge about the function of *pts* genes in *L. monocytogenes*, further work on elucidating the regulatory effects of alternative σ factors on these *pts* genes will be necessary for better understanding of the carbon metabolism regulation of this important bacterial pathogen.

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