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σ^B Activation under Environmental and Energy Stress Conditions in *Listeria monocytogenes*

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To measure σ^B activation in *Listeria monocytogenes* under environmental or energy stress conditions, quantitative reverse transcriptase PCR (TaqMan) was used to determine the levels of transcripts for the σ^B-dependent *opuCA* and *clpC* genes in strains having null mutations in genes encoding *r*egulator of *s*igma *B* proteins (*rsbT* and *rsbV*) and sigma B (*sigB*) and in the *L. monocytogenes* wild-type 10403S strain under different stress conditions. The Δ*sigB*, Δ*rsbT*, and Δ*rsbV* strains previously exhibited increased hemolytic activities compared to the hemolytic activity of the wild-type strain; therefore, transcript levels for *hly* were also determined. RsbT, RsbV, and σ^B were all required for *opuCA* expression during growth under carbon-limiting conditions or following exposure to pH 4.5, salt, ethanol, or the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). Expression of *clpC* was RsbT, RsbV, and σ^B dependent in the presence of CCCP but not under the other conditions. *hly* expression was not RsbT, RsbV, or σ^B dependent in the presence of either CCCP or salt. *opuCA* transcript levels did not increase in the presence of rapidly lethal stresses (i.e., pH 2.5 or 13 mM cumene hydroperoxide) despite the enhanced survival of the wild type compared with the survival of the mutant strains under these conditions. These findings highlight the importance of complementing phenotypic characterizations with gene expression studies to identify direct and indirect effects of null mutations in regulatory genes, such as *sigB*. Overall, our data show that while σ^B activation occurs through a single pathway under both environmental and energy stress conditions, regulation of expression of some stress response and virulence genes in the σ^B regulon (e.g., *clpC*) appears to require networks involving multiple transcriptional regulators.

Listeria monocytogenes is a non-spore-forming, gram-positive, facultative intracellular pathogen. The emergence of this organism as a difficult-to-control food-borne pathogen is at least in part due to its ability to survive in a broad range of ecological niches (13) and in many different hosts, including both animals and humans (11, 50). Contamination of foods with *L. monocytogenes* raises both public health and economic concerns (33, 57). Although rare, listeriosis is a severe disease that results in death in 20 to 30% of reported cases (33). Infection in humans occurs predominantly among pregnant women, newborns, the elderly, and immunocompromised adults.

σ^B is a stress-responsive alternative sigma factor that has been identified in various low-G+C-content gram-positive bacteria, including the genera *Bacillus*, *Staphylococcus*, and *Listeria*. In *L. monocytogenes*, σ^B contributes to cell survival under stress conditions, including exposure to low pH, oxidative stress, carbon starvation, and growth at low temperatures (14, 15, 35, 54, 55). Loss of σ^B also reduces the ability of *L. monocytogenes* to invade human intestinal epithelial cells (20, 29, 30, 31), as well as its virulence in the murine (36, 55) and guinea pig models (20). Emerging evidence suggests that σ^B contributes to virulence in several gram-positive pathogens (27). For example, σ^B contributes to *Bacillus anthracis* virulence in the murine model (18). In *Staphylococcus aureus*, σ^B plays a major role in mouse septic arthritis (24), although it is not essential for infection in the mouse abscess model, the mouse hematogenous pyelonephritis model, or the rat osteomyelitis model (7, 38).

The σ^B activation network in *Bacillus subtilis* has been extensively studied (2, 3, 12, 26, 41, 53). While the *sigB* operons in both *B. subtilis* and *L. monocytogenes* are comprised of seven *r*egulators of *s*igma *B* (Rsb) activity (1, 16, 25, 55, 56), the initial genetic evidence that the σ^B activation pathways might be different in *B. subtilis* and *L. monocytogenes* came from the observation that while the *rsbQ-rsbP* operon products contribute to σ^B activation under energy stress conditions in *B. subtilis* (5, 51), no corresponding operon is present in *L. monocytogenes* (22). To determine the roles of *L. monocytogenes* Rsb in σ^B-mediated responses to various stresses, in-frame deletions were created in *rsbT* and *rsbV* (9), two genes predicted to encode positive regulators of σ^B activity. Phenotypic characterization of the *L. monocytogenes rsbT* and *rsbV* null mutants revealed that both mutants were similar to the Δ*sigB* strain in terms of the ability to survive under environmental or energy stress conditions, suggesting that RsbT and RsbV both convey environmental and energy stress signals to *L. monocytogenes* σ^B (9). In *B. subtilis*, RsbT contributes to regulation of σ^B activity in response to environmental stresses, while RsbV contributes to σ^B activation under both environmental and energy stress conditions (41). Taken together, these observations suggest that Rsb-dependent activation of σ^B activity in *L. monocytogenes* is different than Rsb-dependent activation of σ^B activity in *B. subtilis*.

In the present study, quantitative reverse transcriptase PCR (RT-PCR) (TaqMan) was used to compare the σ^B activity profiles in *L. monocytogenes* wild-type strain 10403S and Δ*sigB*, Δ*rsbT*, and Δ*rsbV* strains challenged with either environmental

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or energy stress conditions. As σ^B can be present in either an active or inactive state (2), measurement of transcript levels for σ^B-dependent genes was used to indirectly quantify the activity of this protein. To do this, transcript levels were determined for *opuCA*, which encodes a glycine/betaine/carnitine/choline ABC transporter (6, 19, 28, 47, 48), and for *clpC*, which encodes endopeptidase Clp ATP-binding chain C, a stress response protein that contributes to *L. monocytogenes* phagosomal escape (30, 44, 45), in the *L. monocytogenes* wild-type, Δ*sigB*, Δ*rsbT*, and Δ*rsbV* strains under different stress conditions. The previously observed higher hemolytic activities in the Δ*sigB*, Δ*rsbT*, and Δ*rsbV* strains than in the wild-type strain (9) suggested that *hly* might be expressed in a σ^B-dependent manner. Therefore, in the present study, transcript levels for the *hly* virulence gene, which encodes listeriolysin O, were also determined under σ^B-inducing conditions in all four strains.

MATERIALS AND METHODS

Bacterial strains. The *L. monocytogenes* strains used in this study were wild-type strain 10403S (4), 10403S Δ*sigB* (FSL A1-254 [55]), 10403S Δ*rsbT* (FSL C3-015 [9]), and 10403S Δ*rsbV* (FSL C3-057 [9]). Stock cultures were stored at −80°C in brain heart infusion (BHI) (Difco, Sparks, MD) broth with 15% glycerol and were streaked onto BHI agar plates prior to each experiment. The plates were incubated overnight at 37°C prior to use.

Growth conditions and stress exposure. For all experiments, overnight bacterial cultures were grown in BHI broth at 37°C with shaking (250 rpm) and then inoculated into 10 ml of BHI broth (1:100 dilution) and grown at 37°C with shaking until the optical density at 600 nm was 0.4 (representing the mid-log phase), unless indicated otherwise. Exposure to salt stress (0.3 M NaCl in BHI broth), exposure to ethanol stress (16.5% [vol/vol] ethanol in BHI broth), exposure to acid stress (BHI broth containing HCl, pH 2.5 or pH 4.5), or intracellular ATP depletion (32 μg/ml carbonyl cyanide *m*-chlorophenylhydrazone [CCCP] [22]) were each performed for 5 min at 37°C with shaking (250 rpm), as a 5-min exposure to stress was previously determined to be effective for measurement of stress-induced σ^B-dependent activity in *L. monocytogenes* (48). Specifically, cells were exposed to stress by collecting mid-log-phase cells of each strain by centrifugation (2,520 × *g*, 5 min) and then resuspending the pelleted cells in one-half the original sample volume using fresh BHI broth prewarmed to 37°C (e.g., 20-ml cultures were resuspended in 10 ml fresh BHI broth). Two-milliliter aliquots of each sample were pipetted into four 15-ml conical tubes. For each set of conditions, two tubes were exposed to stress, while two tubes were used as controls (BHI broth only). Two milliliters of each stressor was added at twice the final desired concentration to obtain the desired concentration of stress agent in a final volume of 4 ml, in which the cell density was equivalent to that of the original culture. One pair of tubes (one exposed tube and one nonexposed tube) was used for RNA collection immediately following addition of the stressor; these samples are referred to below as "no incubation." The other pair of tubes was incubated with the stressor for 5 min. In preliminary experiments, RNAprotect (QIAGEN, Valencia, CA) was observed to precipitate in the presence of BHI broth containing added NaCl; therefore, bacterial cells exposed to 0.3 M NaCl (salt stress) or CCCP (intracellular ATP depletion) were collected by centrifugation (2,520 × *g*, 5 min) either immediately or 5 min after exposure to the stressor, and then the pellets were resuspended in 8 ml of RNAprotect for 10 min and centrifuged again to obtain the cell pellets used for subsequent preparation of RNA. As a consequence, all cells, including the "no incubation" controls, were exposed to either NaCl or CCCP during the 5-min centrifugation step. In contrast, for exposure to ethanol and acid (pH 2.5 or pH 4.5), RNAprotect was added directly to cultures (both with and without the stressors) either immediately ("no incubation") or after 5 min of incubation with one of the stressors, so there was very brief (<30-s) exposure to stress in the "no incubation" treatments. After 10 min of incubation with RNAprotect, cells in these cultures were collected by centrifugation for subsequent preparation of RNA.

Stationary-phase cells were used for exposure to 13 mM cumene hydroperoxide (CHP) (14), since the number of log-phase cells dropped below the detection limit within 5 min after exposure. Stationary-phase cells were prepared by inoculating a 1:100 dilution of an overnight BHI broth culture (prepared as described above) into fresh BHI broth, followed by growth at 37°C with shaking for 12 h. Stationary-phase cells were exposed to CHP using the procedures described above for exposure to ethanol and acid stresses; i.e., RNAprotect was added either immediately following CHP addition or at 5 min after CHP addition, and then cell pellets were harvested by centrifugation following 10 min of incubation with RNAprotect.

For energy stress studies, carbon starvation was induced by growing cells in defined medium (DM) (40) containing a growth-limiting concentration of glucose (0.04%, wt/vol) (9, 14). Briefly, 0.1 ml of an overnight culture in BHI broth was first inoculated into 10 ml of DM supplemented with 0.4% (wt/vol) glucose. After 12 h of incubation with shaking (250 rpm) at 37°C, cultures were diluted 1:100 into 10 ml prewarmed DM containing 0.04% glucose and reincubated. At 6 h, 12 h, 18 h, and 24 h after dilution, 4-ml aliquots of cells grown in DM containing 0.04% glucose were removed and added to RNAprotect, and this was followed by a 10-min incubation and collection of cells by centrifugation.

RNA isolation. RNA isolation was performed essentially as described by Sue et al. (48). Briefly, pellets of RNAprotect-treated cells were lysed enzymatically using lysozyme (by addition of 200 μl of a 15-mg/ml solution and 10 min of incubation) and mechanically using sonication. Total RNA was purified using an RNeasy Midi kit (QIAGEN) and was treated with RNase-free DNase (QIAGEN). Purified RNA samples were stored in 0.3 M sodium acetate–100% ethanol at −80°C.

TaqMan RT-PCR. Quantitative reverse transcriptase PCR was performed as described by Sue et al. (49), using the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). Transcript levels were determined for *opuCA*, *clpC*, and *hly*, as well as for the housekeeping genes, *rpoB* and *gap*, using TaqMan probes and primers as previously described (31, 42, 48).The RT-PCR mixtures (25 μl) contained 25 ng total RNA, 12.5 μl TaqMan One-Step RT-PCR Master Mix, 6.25 U Multiscribe reverse transcriptase, each primer (forward and reverse) at a concentration of 900 nM, and the appropriate TaqMan probe at a concentration of 250 nM. Duplicate reaction mixtures were loaded into MicroAmp optical 96-well plates. The transcript levels for each gene (i.e., cDNA copy numbers) were determined by determining the difference between the experimental reaction mixtures and the corresponding reverse transcriptase-negative controls, which were used to quantify the amount of contaminating *L. monocytogenes* DNA in each reaction mixture. Standard curves for each gene were generated from dilutions of genomic DNA prepared as described by Flamm et al. (17). The absolute cDNA copy numbers, calculated based on DNA standard curves, reflect mRNA levels for each target gene present in each RNA sample.

Statistical analysis. All statistical analyses were performed with S-Plus 6.2 (Insightful Corp., Seattle, WA). Standard regression diagnostics were computed for all models. Statistical significance was established at a P value of <0.05.

Initial data analysis indicated that the absolute mRNA transcript level data were heteroscedastic and strongly skewed. Consequently, all mRNA transcript level data were $\log_{10}$ transformed to correct the skewness and to stabilize the variance of the counts to approximate normality. Absolute mRNA transcript levels for target genes were normalized prior to final statistical analyses as described by Vandesompele et al. (49), who demonstrated that determination of transcript levels for a single housekeeping gene can be inadequate for normalizing quantitative transcript levels obtained from target genes under much different physiological conditions. Typically, housekeeping genes that are expected to maintain stable levels of expression under the test conditions are selected as targets for control reactions in quantitative gene expression studies (34); however, housekeeping gene expression can also vary under different conditions (48, 49). Therefore, we used absolute copy numbers for two independent housekeeping genes (*rpoB* [34, 48] and *gap* [30, 31]) to generate an average value for transcript accumulation for the control genes under different physiological conditions. To ensure that the trends in expression of both housekeeping genes were similar, an analysis of variance (ANOVA) was performed for the means of the log-transformed mRNA transcript levels for *rpoB* and *gap* (i.e., [$\log_{10}$ *rpoB* mRNA transcript + $\log_{10}$ *gap* mRNA transcript]/2) with "strain," "time," and "stress" as factors. No significant difference in log-transformed mRNA transcript levels for *rpoB* and *gap* was observed among different strains and times under any stress conditions, except for growth in DM at 18 or 24 h. Thus, all statistical analyses were performed using the log-transformed ($\log_{10}$) mRNA transcript levels normalized to the geometric mean for *rpoB* and *gap* mRNA transcript levels obtained under each experimental condition, unless indicated otherwise.

ANOVA models of the normalized mRNA transcript levels were used to investigate significant effects and interactions for each of the target genes using four factors, including "strain" (wild type, Δ*sigB*, Δ*rsbT*, and Δ*rsbV*), "stress" (presence or absence of test condition), "time" (0 and 5 min or 6, 12, 18, and 24 h), and "replicate" (replicates 1, 2, and 3). For the "strain" factor, multiple comparisons were performed using Fisher's least square difference (LSD). When

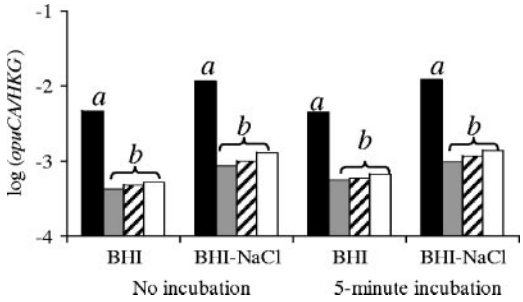

FIG. 1. Relative levels of cDNA for *opuCA*, expressed as log (*opuCA* mRNA copy number/mean housekeeping gene [*HKG*] copy number) for *L. monocytogenes* wild-type strain 10403S (solid bars), Δ*sigB* (shaded bars), Δ*rsbT* (cross-hatched bars), and Δ*rsbV* (open bars) exposed to salt stress (BHI broth containing 0.3 M NaCl). Following addition of the NaCl, cells were collected by centrifugation (5 min, 2,520 × *g*) either immediately ("no incubation") or following exposure to NaCl for 5 min. RNAprotect was added to stop transcription and stabilize the RNA following centrifugation. The values are the means from three independent experiments. Comparisons of the four strains under each condition (e.g., normalized *opuCA* transcript levels in BHI broth with no incubation for the wild-type and three mutant strains) using Fisher's LSD resulted in identification of strains whose *opuCA* transcript levels differed; the bars for strains whose *opuCA* transcript levels differed (within a given condition) are labeled with different letters.

appropriate, individual *t* tests were also performed to compare normalized mRNA transcript levels for two specific samples.

RESULTS AND DISCUSSION

σ^B activation occurs through a single pathway under both environmental and energy stress conditions. The transcript levels for *opuCA*, a σ^B-dependent gene, were determined in four *L. monocytogenes* strains (wild type, Δ*sigB*, Δ*rsbT*, and Δ*rsbV*) exposed to different environmental and energy stress conditions to measure the contributions of RsbT and RsbV to σ^B activation under conditions that have been shown to require both RsbT and RsbV (environmental stress) or only RsbV (energy stress) for induction of σ^B activity in *B. subtilis* (26, 52). Δ*sigB*, Δ*rsbT*, and Δ*rsbV* cells exposed to 0.3 M NaCl for 5 min, an environmental stress, had significantly lower *opuCA* transcript levels than the wild-type strain, and there was no difference in transcript levels among the three mutant strains (Fig. 1), showing that both RsbT and RsbV are required for induction of σ^B activity under salt stress conditions. Interestingly, compared to the *opuCA* transcript levels in the three mutant strains, the *opuCA* transcript levels in the wild-type strain were also significantly higher in cells that were not exposed to NaCl, as well as in NaCl-stressed cells collected by centrifugation immediately after exposure to the stress. Increased transcript levels in these cells likely resulted from σ^B activation from centrifugation prior to addition of RNAprotect, which is consistent with the recent observation that centrifugation induces expression of at least some σ^B-dependent genes (Y. Chan, K. J. Boor, and M. Wiedmann, unpublished results). Statistical analyses revealed that there were significant ($P < 0.05$, as determined by ANOVA) effects of "stress" (i.e., the presence of 0.3 M NaCl), "strain," and "time" on *opuCA* transcript levels, supporting the hypothesis that there was significant salt induction of σ^B activity

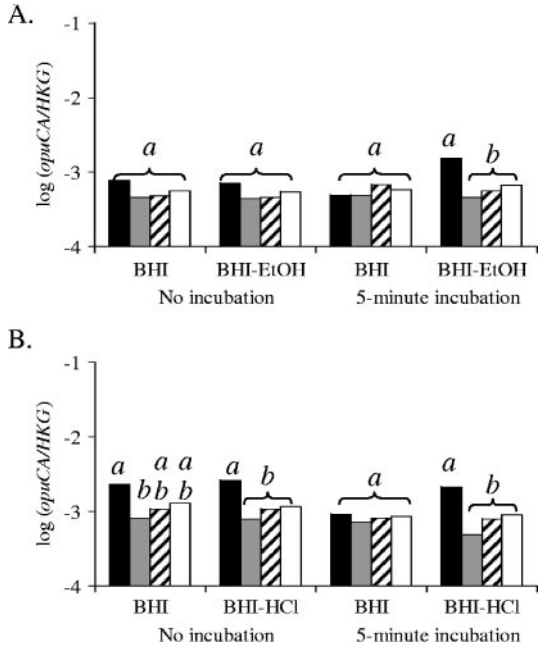

FIG. 2. Relative levels of cDNA for *opuCA*, expressed as log (*opuCA* mRNA copy number/mean housekeeping gene [*HKG*] copy number) under ethanol stress conditions (BHI broth containing 16.5% [vol/vol] ethanol) (A) or under acid stress conditions (BHI broth containing HCl, pH 4.5) (B). RNAprotect was added to stop transcription and stabilize the RNA, and then cells were collected by centrifugation (5 min, 2,520 × *g*) either immediately following addition of each stressor ("no incubation") or following exposure to stress for 5 min. The strains used were *L. monocytogenes* wild-type strain 10403S (solid bars) and the Δ*sigB* (shaded bars), Δ*rsbT* (cross-hatched bars), and Δ*rsbV* (open bars) strains. The values are means from three independent experiments. Comparisons of the four strains under each condition (e.g., normalized *opuCA* transcript levels in BHI broth with no incubation for the wild-type and three mutant strains) using Fisher's LSD resulted in identification of strains whose *opuCA* transcript levels differed; the bars for strains whose *opuCA* transcript levels differed (within a given condition) are labeled with different letters. EtOH, ethanol.

beyond the activity which may have resulted from cell handling practices under the experimental conditions used.

To further examine the contributions of RsbV and RsbT to induction of σ^B activity under environmental stress conditions, *opuCA* transcript levels were determined in bacterial cells exposed to ethanol or acid (pH 4.5). In contrast to what happened with NaCl exposure, direct addition of RNAprotect to cultures containing either ethanol or acid did not result in the formation of a precipitate; therefore, RNAprotect was added prior to collection of cells by centrifugation. The levels of the *opuCA* transcript present in the wild-type strain exposed to ethanol or acid under "no incubation" conditions (Fig. 2A and B) were lower than the levels in the wild-type strain exposed to NaCl that was centrifuged prior to addition of RNAprotect (Fig. 1), which was indicative of σ^B activation by centrifugation in the cells exposed to NaCl. Importantly, however, cells of the Δ*sigB*, Δ*rsbT*, and Δ*rsbV* strains exposed to ethanol or pH 4.5 for 5 min had significantly lower levels of the *opuCA* transcript than the wild-type strain had, and there was no difference in *opuCA* transcript levels among the three mutant strains (Fig.

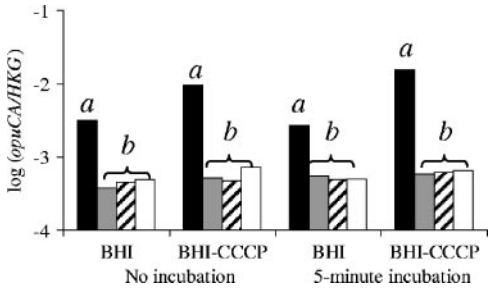

FIG. 3. Relative cDNA copy numbers for *opuCA*, expressed as log (*opuCA* mRNA copy number/mean housekeeping gene [*HKG*] copy number) under energy stress conditions (intracellular ATP depletion [BHI broth containing 32 μg/ml CCCP]). Following addition of the CCCP, cells were collected by centrifugation and treated as described in the legend to Fig. 1. The strains used were *L. monocytogenes* wild-type strain 10403S (solid bars) and the Δ*sigB* (shaded bars), Δ*rsbT* (cross-hatched bars), and Δ*rsbV* (open bars) strains. Comparisons of the four strains under each condition with Fisher's LSD resulted in identification of strains whose *opuCA* transcript levels differed; the bars for strains whose *opuCA* transcript levels differed (within a given condition) are labeled with different letters.

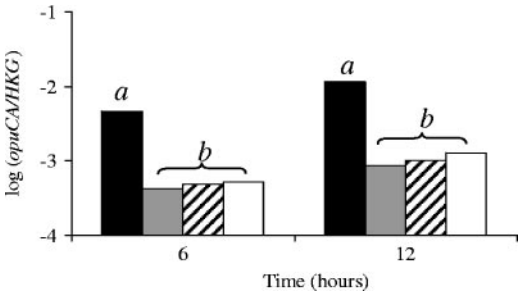

FIG. 4. Relative cDNA copy numbers for *opuCA*, expressed as log (*opuCA* mRNA copy number/mean housekeeping gene [*HKG*] copy number) under energy stress conditions (carbon starvation; growth in glucose-limiting [0.04%, wt/vol] defined media). Culture aliquots were removed at 6, 12, 18, and 24 h postinoculation. RNAprotect was added to each aliquot to stop transcription and to stabilize the RNA, and then cells were collected by centrifugation (5 min, 2,520 × *g*). As a consequence of the different patterns of housekeeping gene expression in the wild-type and mutant strains at 18 and 24 h, only data for 6 and 12 h were used to quantify the relative expression patterns of *opuCA* in the different strains. The strains used were *L. monocytogenes* wild-type strain 10403S (solid bars) and the Δ*sigB* (shaded bars), Δ*rsbT* (cross-hatched bars), and Δ*rsbV* (open bars) strains. Comparisons of the four strains under each condition (e.g., normalized *opuCA* transcript levels at 6 h for the wild-type and three mutant strains) using Fisher's LSD resulted in identification of strains whose *opuCA* transcript levels differed; the bars for strains whose *opuCA* transcript levels differed at either 6 or 12 h are labeled with different letters.

2). Overall, ANOVA showed that the factors "strain" and "time" had significant ($P < 0.01$) effects on the normalized *opuCA* transcript levels under both acid and ethanol stress conditions. Our data show that both RsbT and RsbV are required for σ^B activation under environmental stress conditions, which is consistent with previous observations for *B. subtilis* (26, 53).

Although σ^B clearly enhances *L. monocytogenes* survival following exposure to some environmental stress conditions that are rapidly lethal, such as pH 2.5 or 13 mM CHP (9, 14, 15, 55), in this study exposure to these specific conditions did not result in increased levels of the *opuCA* transcript (data not shown), probably due to rapid death of both wild-type and mutant cells under both conditions. The findings of the present study suggest that the enhanced survival of wild-type *L. monocytogenes* compared to the Δ*sigB* strain following exposure to stresses that are rapidly lethal, as observed in previous studies (9, 14, 15, 55), reflects the presence of preformed σ^B-dependent regulon products in the cell rather than de novo synthesis of these products following exposure to the lethal stresses.

To characterize the contributions of RsbV and RsbT to σ^B activation under energy stress conditions, we initially determined *opuCA* transcript levels in cells exposed to CCCP for 5 min to induce intracellular ATP depletion (21). The Δ*sigB*, Δ*rsbT*, and Δ*rsbV* strain cells exposed to CCCP for 5 min had significantly lower levels of *opuCA* transcripts than the wild-type strain cells had, and there were no differences in the *opuCA* transcript levels among the three mutant strains (Fig. 3), indicating that both RsbT and RsbV are required for induction of σ^B activity under CCCP-induced energy stress conditions. Consistent with the NaCl stress data, compared to the *opuCA* transcript levels in the three mutant strains, the *opuCA* transcript levels in the wild-type strain were also significantly higher in unexposed cells and in CCCP-exposed cells collected by centrifugation immediately after CCCP addition. Higher *opuCA* transcript levels in these cells probably reflected σ^B activation by centrifugation prior to addition of RNAprotect. Statistical analyses revealed that "stress" (exposure to CCCP) had a significant ($P < 0.001$,as determined by ANOVA) effect on *opuCA* transcript levels and that the *opuCA* transcript levels were significantly ($P = 0.0003$, as determined by a *t* test) higher in exposed wild-type cells than in unexposed wild-type cells, supporting the hypothesis that there was significant induction of σ^B activity in the presence of CCCP.

To further confirm the importance of both RsbT and RsbV for induction of σ^B activity under energy stress conditions, *opuCA* transcript levels were also determined in *L. monocytogenes* grown in glucose-limiting defined medium. Following growth for 6 or 12 h in this medium, the Δ*sigB*, Δ*rsbT*, and Δ*rsbV* strains had significantly lower *opuCA* transcript levels than the wild-type strain had, and there were no differences in the *opuCA* transcript levels among the three mutant strains (Fig. 4). Thus, we concluded that, in contrast to the σ^B activation pathway in *B. subtilis* (52), both RsbT and RsbV are necessary for σ^B activation in response to both environmental and energy stresses.

Loss of σ^B has global physiological consequences for *L. monocytogenes* during growth under carbon-limiting conditions. While we have previously shown that growth in glucose-limiting defined medium results in more rapid growth, larger maximal populations, and more rapid declines in numbers of viable cells in the Δ*sigB*, Δ*rsbT*, and Δ*rsbV* strains than in the wild-type strain (Fig. 5A) (9), determination of the numbers of *opuCA*, *gap*, and *rpoB* cDNA copies in the Δ*sigB*, Δ*rsbT*, Δ*rsbV*, and wild-type strains showed the critical role of σ^B and the regulators of σ^B (Rsbs) during growth under energy-limiting conditions. Consistent with the importance of σ^B as an activator of transcription under energy stress conditions, high absolute levels of the *opuCA* transcript were observed in the wild-type strain at both 6 and 12 h after inoculation into DM with 0.04% glucose, but the levels decreased dramatically at 18 and

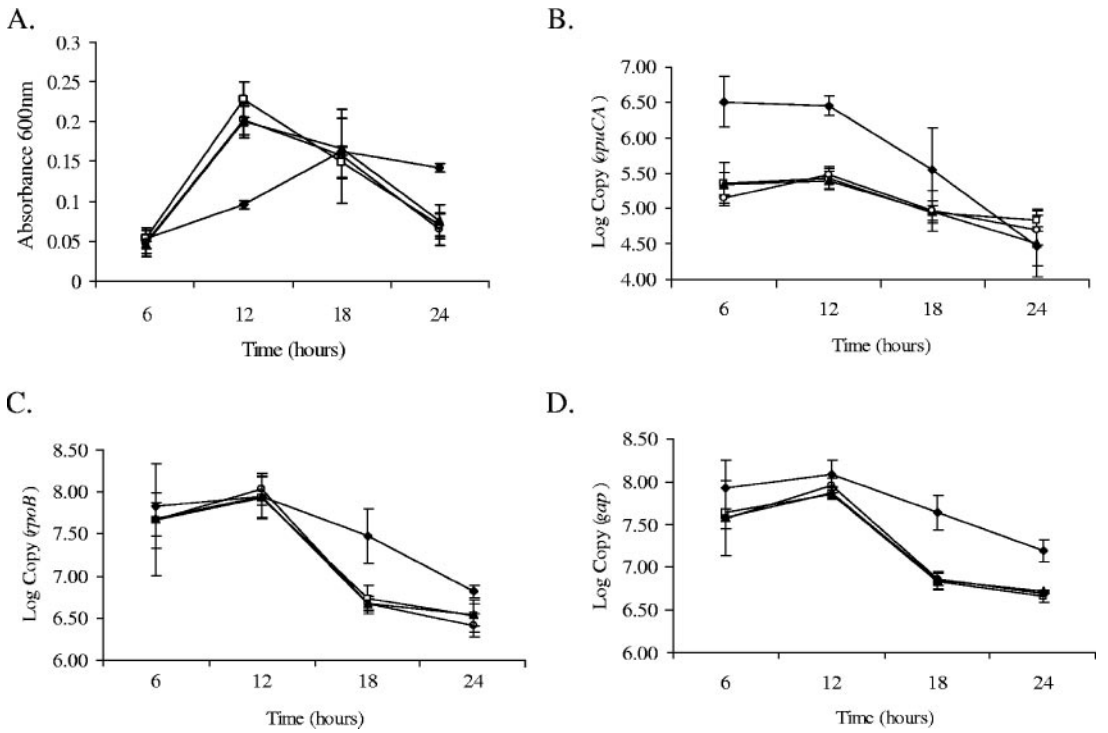

FIG. 5. Bacterial growth and expression of *opuCA*, *gap*, and *rpoB* in glucose-limiting defined medium. The strains used were *L. monocytogenes* wild-type strain 10403S (◆) and the Δ*sigB* (□), Δ*rsbT* (▲), and Δ*rsbV* (○) strains. (A) Bacterial growth, expressed in units of absorbance at 600 nm, after 6, 12, 18, and 24 h of incubation in glucose-limiting DM. (Adapted from reference 9.) (B) Log copy numbers of *opuCA* transcripts. (C) Log copy numbers of *rpoB* transcripts. (D) Log copy numbers of *gap* transcripts. The values are means from three independent experiments.

24 h after inoculation (Fig. 5B). Interestingly, the absolute transcript levels for the *rpoB* and *gap* housekeeping genes also were lower at 18 and 24 h after inoculation than at 6 and 12 h after inoculation (Fig. 5C and D). Consistent with the optical densities at 600 nm, which increased more rapidly up to 12 h and decreased more rapidly after 12 h in the Δ*sigB*, Δ*rsbT*, and Δ*rsbV* strains than in the wild-type strain, the housekeeping gene transcript levels also decreased more rapidly in the mutant strains than in the wild-type strain after 12 h. Overall, the ANOVA results supported the hypothesis that the factors "strain" and "time" had a significant effect on *rpoB* and *gap* transcript levels. As a consequence of the differences in housekeeping gene expression patterns between the wild-type and mutant strains at 18 and 24 h, only data from 6 and 12 h (Fig. 5 C and D) were used to quantify the relative expression patterns of *opuCA* in the different strains, as shown in Fig. 4. Our data provide further evidence that housekeeping gene expression can change with physiological changes in the cell (48, 49) and highlight the conclusion that expression data for housekeeping genes should be obtained under all test conditions to ensure that the data obtained for these genes can legitimately be used for normalization of target gene data. Our results also clearly demonstrate that σ^B makes important contributions to *L. monocytogenes* gene expression during exponential growth in glucose-limiting defined media, as reflected by the high levels of *opuCA* mRNA in the wild-type strain at 6 and 12 h (Fig. 4 and 5B). We hypothesize that energy expenditures necessary for production of these stress response transcripts and the resulting proteins (or other possible negative effects associated with expression of high levels of stress proteins) may contribute to the increased doubling time for the wild-type strain compared with the doubling times of the mutants, as suggested previously for *Escherichia coli* and *B. subtilis* (39, 46). However, accumulation of stress response proteins also may contribute to enhanced survival of the wild-type strain compared to the mutant strains at later times (e.g., 18 or 24 h) (Fig. 5A), consistent with the notion that the presence of preformed σ^B-dependent regulon products contributes to survival of the wild-type strain in the presence of lethal stresses.

Appropriate regulation of stress response and virulence gene expression appears to require networks involving multiple transcriptional regulators. Increasing evidence supports the hypothesis that some *L. monocytogenes* regulons (28, 34, 36, 48), including the σ^B and *p*ositive *r*egulatory *f*actor *A* (PrfA) regulons, the latter of which includes most of the well-recognized *L. monocytogenes* virulence genes, have overlapping functions. In particular, the *prfA* P2 promoter is σ^B dependent (36), and a number of PrfA-dependent genes are also regulated by σ^B (28, 34, 48). Interestingly, hemolytic activities were previously reported to be significantly higher in *L. monocytogenes* Δ*sigB*, Δ*rsbT*, and Δ*rsbV* culture supernatants than in wild-type culture supernatants (9, 36), suggesting a possible role for σ^B in hemolysin expression. Therefore, to determine if σ^B contributes to transcriptional regulation of *hly*, which encodes the *L. monocytogenes* hemolysin listeriolysin O, we determined *hly* transcript levels in the wild-type, Δ*sigB*, Δ*rsbT*, and Δ*rsbV* strains at 37°C under selected environmental (NaCl) and energy (CCCP exposure) stress conditions. Al-

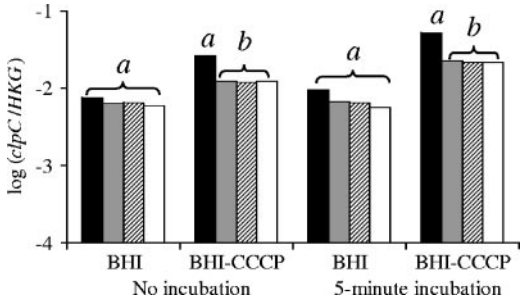

FIG. 6. Relative cDNA copy numbers for *clpC*, expressed as log (*clpC* mRNA copy number/mean housekeeping gene [*HKG*] copy number) following exposure to CCCP. Following addition of the CCCP, cells were collected by centrifugation and treated as described in the legend to Fig. 1. The strains used were *L. monocytogenes* wild-type strain 10403S (solid bars) and the Δ*sigB* (shaded bars), Δ*rsbT* (cross-hatched bars), and Δ*rsbV* (open bars) strains. Comparisons of the four strains under each condition with Fisher's LSD resulted in identification of strains whose *clpC* transcript levels differed; the bars for strains whose *clpC* transcript levels differed (within a given condition) are labeled with different letters.

though it has been suggested that σ^B influences hemolysin expression at the transcriptional level in *S. aureus* (23), we found that *L. monocytogenes hly* transcript levels were identical in the Δ*sigB*, Δ*rsbT*, Δ*rsbV*, and wild-type strains under σ^B-inducing conditions. The higher apparent hemolysin activities in the Δ*sigB*, Δ*rsbT*, and Δ*rsbV* strains observed in previous studies may have resulted from indirect effects of a loss of σ^B, possibly reflecting alterations in the translation rate of *hly* mRNA, hemolysin stability, or cellular structure (28). The findings obtained in this study highlight the importance of conducting expression analyses to identify the direct and indirect contributions of different transcriptional regulators to virulence and the stress response in bacterial pathogens under defined conditions.

In addition to σ^B and PrfA, a number of other transcriptional regulators also control expression of genes that contribute to virulence and the stress response, including *L. monocytogenes* CtsR (8, 37). Interestingly, mutations in the CtsR-regulated gene *clpC*, which encodes an ATPase that belongs to a class of heat shock proteins involved in stress tolerance, result in attenuated *L. monocytogenes* virulence and bacterial susceptibility to multiple stresses, including high temperature, high osmolarity, and iron limitation (43, 44, 45). Since expression of *clpC* is σ^B dependent in both *B. subtilis* (32) and *L. monocytogenes* (30), we hypothesized that the CtsR regulon also overlaps the *L. monocytogenes* σ^B regulon. Therefore, we determined *clpC* transcript levels under different environmental and energy stress conditions. We observed no differences in *clpC* transcript levels among the Δ*sigB*, Δ*rsbT*, Δ*rsbV*, and the wild-type strains when cells were exposed to acid (pH 4.5), ethanol, or salt, which is consistent with results reported by Conte et al. (10), who showed that *clpC* expression was not induced by exposure to acid. Interestingly, *clpC* transcript levels increased in a σ^B-dependent manner following addition of CCCP (Fig. 6); this finding was supported by an ANOVA, which showed that the factors "time," "strain," and "stress" (presence or absence of CCCP) had significant ($P < 0.05$) effects on normalized *clpC* transcript levels. However, for cells grown in DM with 0.04% glucose, the *clpC* transcript levels of the Δ*sigB*, Δ*rsbT*, Δ*rsbV*, and wild-type strains did not differ, indicating that σ^B, RsbV, and RsbT contribute to *clpC* transcription only under specific stress conditions. While existing genomic sequence data for *L. monocytogenes* EGD-e (22) do not support the presence of an apparent σ^B-dependent promoter immediately upstream of *clpC*, a putative σ^B-dependent promoter (GTTTG-27 nucleotides-GGGAT) is located 71 nucleotides upstream of the *ctsR* start codon, which is the first gene in the operon encoding *clpC*.

Overall, our data demonstrate that *L. monocytogenes* σ^B has a complex activation network which differs from that in the closely related gram-positive model organism *B. subtilis.* Furthermore, we provide evidence that *L. monocytogenes* σ^B has both direct and indirect effects on virulence and the stress response in this important food-borne pathogen.

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