σB Activation under Environmental and Energy Stress Conditions in Listeria monocytogenes

Soraya Chaturongakul and Kathryn J. Boor*
Department of Food Science, Cornell University, Ithaca, New York

Received 28 December 2005/Accepted 21 May 2006

To measure σB 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 σB-dependent opuCA and clpC genes in strains having null mutations in genes encoding regulator of sigma B proteins (rsbT and rsbV) and sigma B (sigB) and in the L. monocytogenes wild-type 10403S strain under different stress conditions. The ΔsigB, ΔrsbT, and ΔrsbV' 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 σB 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 σB dependent in the presence of CCCP but not under the other conditions. hly expression was not RsbT, RsbV, or σB 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 σB 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 σB 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.

σB 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, σB 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 σB 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 σB contributes to virulence in several gram-positive pathogens (27). For example, σB contributes to Bacillus anthracis virulence in the murine model (18). In Staphylococcus aureus, σB 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 σB activation network in Bacillus subtilis has been extensively studied (2, 3, 12, 26, 41, 53). While the sigB operon in both B. subtilis and L. monocytogenes are comprised of seven regulators of sigma B (Rsb) activity (1, 16, 25, 55, 56), the initial genetic evidence that the σB activation network might be different in B. subtilis and L. monocytogenes came from the observation that while the rsbQ-rsbP operon product contributes to σB 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 σB-mediated responses to various stresses, in-frame deletions were created in rsbT and rsbV (9), two genes predicted to encode positive regulators of σB activity. Phenotypic characterization of the L. monocytogenes rsbT and rsbV′ null mutants revealed that both mutants were similar to the ΔsigB 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 σB (9). In B. subtilis, RsbT contributes to regulation of σB activity in response to environmental stresses, while RsbV contributes to σB activation under both environmental and energy stress conditions (41). Taken together, these observations suggest that Rsb-dependent activation of σB activity in L. monocytogenes is different than Rsb-dependent activation of σB activity in B. subtilis.

In the present study, quantitative reverse transcriptase PCR (RT-PCR) (TaqMan) was used to compare the σB activity profiles in L. monocytogenes wild-type strain 10403S and ΔsigB, ΔrsbT, and ΔrsbV′ strains challenged with either environmental...
or energy stress conditions. As $\alpha^B$ can be present in either an active or inactive state (2), measurement of transcript levels for $\alpha^B$-dependent genes was used to indirectly quantify the activity of this protein. To do this, transcript levels were determined for $\alpha^P$, which encodes a glycine/betaine/carnitine/choline ABC transporter (6, 19, 28, 47, 48), and for $\Delta_2$, which encodes endopeptidase Ctp ATP-binding chain C, a stress response protein that contributes to L. monocytogenes phagosomal escape (30, 44, 45), in the L. monocytogenes wild-type, $\alpha^B_{\Delta 2}$, $\Delta_{\Delta 2}$, and $\Delta_{\Delta 2}$ strains under different stress conditions. The previously observed higher hemolytic activities in these samples are referred to below as “no incubation.” The other pair of strains was used for RNA collection immediately following addition of the stressor; $\alpha^B_{\Delta 2}$, $\Delta_{\Delta 2}$, and $\Delta_{\Delta 2}$ strains and RNA were also monitored under $\alpha^B$-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 $\alpha^B$ (FSL A1-254 [55]), 10403S $\Delta_{\Delta 2}$ (FSL C3-015 [9]), and 10403S $\Delta_{\Delta 2}$ (FSL C5-057 [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 (pH 2.5 or pH 4.5), RNAprotect was added directly to cultures (both active or inactive state (2), measurement of transcript levels for $\alpha^P$ was used for RNA collection immediately following addition of the stressor; $\alpha^B_{\Delta 2}$, $\Delta_{\Delta 2}$, and $\Delta_{\Delta 2}$ strains and RNA were also monitored under $\alpha^B$-inducing conditions in all four strains.

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 (0.01%, wt/vol) (9). Cells were grown and loaded into BHI broth to 0.1% of an overnight culture in BHI broth. After 12 h of incubation with shaking (250 rpm) at 37°C, cultures were diluted 1:100 into 10 ml prewarmed with 0.4% (wt/vol) glucose. At 24 h, 12 h, 18, and 24 h after dilution, 4-ml aliquots of cells grown in DM containing 0.4% 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 $\alpha^P$, $\alpha^B$, and $\Delta 2$, as well as for the housekeeping genes, $\alpha^B$, $\alpha^B$, and $\Delta 2$, using TaqMan probes and primers as previously described (31, 42, 48). The RT-PCR mixture (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 two separate 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 Fimm 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 level of $P < 0.05$.

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 variances. The counts to estimate the normality were 30, 44, 45), in the L. monocytogenes virulence gene, which encodes listeriolysin O, were also determined under $\alpha^B$-inducing conditions in all four strains.
opuCA strains) using Fisher’s LSD resulted in identification of strains whose 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

\( \sigma^B \) activation occurs through a single pathway under both environmental and energy stress conditions. The transcript levels for opuCA, a \( \sigma^B \)-dependent gene, were determined in four L. monocytogenes strains (wild type, \( \Delta \)sigB, \( \Delta \)rsbT, and \( \Delta \)rsbV) exposed to different environmental and energy stress conditions to measure the contributions of RsbT and RsbV to \( \sigma^B \) activation under conditions that have been shown to require both RsbT and RsbV (environmental stress) or only RsbV (energy stress) for induction of \( \sigma^B \) activity in B. subtilis (26, 52). The strains used were L. monocytogenes wild-type strain 10403S (solid bars), and \( \Delta \)sigB (shaded bars), \( \Delta \)rsbT (cross-hatched bars), and \( \Delta \)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 (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 \( \sigma^B \) 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 \( \sigma^B \) activation by centrifugation in the cells exposed to NaCl. Importantly, however, cells of the \( \Delta \)sigB, \( \Delta \)rsbT, and \( \Delta \)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.
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 $\sigma^B$ activation under environmental stress conditions, which is consistent with previous observations for $B. subtilis$ (26, 53).

Although $\sigma^B$ 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 $\Delta sigB$ strain following exposure to stresses that are rapidly lethal, as observed in previous studies (9, 14, 15, 55), reflects the presence of preformed $\sigma^B$-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 $\sigma^B$ 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 $\Delta sigB$, $\Delta rsbT$, and $\Delta rsbV$ 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 $\sigma^B$ 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 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 $\sigma^B$ 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 $\sigma^B$ activity in the presence of CCCP.

To further confirm the importance of both RsbT and RsbV for induction of $\sigma^B$ 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 $\Delta sigB$, $\Delta rsbT$, and $\Delta rsbV$ 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 $\sigma^B$ activation pathway in $B. subtilis$ (52), both RsbT and RsbV are necessary for $\sigma^B$ activation in response to both environmental and energy stresses.

Loss of $\sigma^B$ 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 $\Delta sigB$, $\Delta rsbT$, and $\Delta rsbV$ strains than in the wild-type strain (Fig. 5A) (9), determination of the numbers of opuCA, gap, and rpoB CDNA copies in the $\Delta sigB$, $\Delta rsbT$, $\Delta rsbV$, and wild-type strains showed the critical role of $\sigma^B$ and the regulators of $\sigma^B$ (RsbS) during growth under energy-limiting conditions. Consistent with the importance of $\sigma^B$ 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
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 ΔsigB, ΔrsbT, and ΔrsbV 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^H 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^H-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^H and positive regulatory factor 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^H-dependent (36), and a number of PrfA-dependent genes are also regulated by σ^H (28, 34, 48). Interestingly, hemolytic activities were previously reported to be significantly higher in L. monocytogenes ΔsigB, ΔrsbT, and ΔrsbV culture supernatants than in wild-type culture supernatants (9, 36), suggesting a possible role for σ^H in hemolysin expression. Therefore, to determine if σ^H contributes to transcriptional regulation of hly, which encodes the L. monocytogenes hemolysin listeriolysin O, we determined hly transcript levels in the wild-type, ΔsigB, ΔrsbT, and ΔrsbV strains at 37°C under selected environmental (NaCl) and energy (CCCP exposure) stress conditions. Al-
through it has been suggested that $\sigma^B$ influences hemolysin expression at the transcriptional level in S. aureus (23), we found that L. monocytogenes hly transcript levels were identical in the $\Delta$sigB, $\Delta$rsbT, $\Delta$rsbV, and wild-type strains under $\sigma^B$-inducing conditions. The higher apparent hemolysin activities in the $\Delta$sigB, $\Delta$rsbT, and $\Delta$rsbV strains observed in previous studies may have resulted from indirect effects of a loss of $\sigma^B$, 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 $\sigma^B$ 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 osmolality, and iron limitation (43, 44, 45). Since expression of clpC is $\sigma^B$ dependent in both B. subtilis (32) and L. monocytogenes (30), we hypothesized that the CtsR regulon also overlaps the L. monocytogenes $\sigma^B$ regulon. Therefore, we determined clpC transcript levels under different environmental and energy stress conditions. We observed no differences in clpC transcript levels among the $\Delta$sigB, $\Delta$rsbT, $\Delta$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 $\sigma^B$-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 $\Delta$sigB, $\Delta$rsbV, and wild-type strains did not differ, indicating that $\sigma^B$, RsbV, and RsbT contribute to clpC transcription only under specific stress conditions. While existing genomic sequence data for L. monocytogenes EGDe (22) do not support the presence of an apparent $\sigma^B$-dependent promoter immediately upstream of clpC, a putative $\sigma^B$-dependent promoter (GTTTG-27 nucleotides-GGGGAT) 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 $\sigma^B$ 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 $\sigma^B$ has both direct and indirect effects on virulence and the stress response in this important food-borne pathogen.

ACKNOWLEDGMENTS

We thank P. McGann for assistance with statistical analysis and Martin Wiedmann for helpful discussions.

This work was supported by National Institutes of Health award RO1-AI052151-01A1 (to K.J.B.). S.C. was supported by the Office of the Civil Service Commission (Thailand).

REFERENCES

Stress activation of \( \alpha^{\text{II}} \) contributes to PrfA-mediated virulence in \( L. \) monocytogenes.


