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Rapid, Transient, and Proportional Activation of σ^B in Response to Osmotic Stress in *Listeria monocytogenes*▽†

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The osmotic activation of sigma B (σ^B) in *Listeria monocytogenes* was studied by monitoring expression of four known σ^B-dependent genes, *opuCA*, *lmo2230*, *lmo2085*, and *sigB*. Activation was found to be rapid, transient, and proportional to the magnitude of the osmotic stress applied, features that underpin the adaptability of this pathogen.

Listeria monocytogenes is a Gram-positive bacterium that is ubiquitous in the environment and is a facultative intracellular pathogen of humans (3). Infections arise primarily in immunocompromised individuals following the ingestion of contaminated food and are associated with high (typically 25 to 30%) mortality rates (19). The remarkable adaptability of *L. monocytogenes* to different physical and chemical stresses underpins its ability to survive and grow in wide range of different environments. It can grow at temperatures as low as −0.4°C (32), it can survive over a wide pH range (31), it is extremely tolerant to bile (14, 33), and it can grow in the presence of salt concentrations as high as 2 M (10). In recent years it has become clear that many of these traits are partly under the control of the stress-inducible sigma factor SigB (σ^B) (35, 36).

Several recent studies have highlighted the role of σ^B in allowing *L. monocytogenes* to survive in the gastrointestinal tract, which is a prerequisite for establishing successful infection in the host (27, 30). Mutants of *L. monocytogenes* lacking *sigB* display decreased virulence in guinea pigs infected orally but not intravenously (12). These mutants also display reduced rates of epithelial cell invasion, a finding that is explained by the involvement of σ^B in the transcription of the *inlAB* operon (18, 21), which encodes internalin (InlA) and InlB, the surface proteins responsible for host cell invasion. Indeed, PrfA, the central regulator of virulence gene expression in *L. monocytogenes*, is itself transcribed in a manner that partly depends on the presence of σ^B (23). Further compelling evidence of the role for σ^B in the early stages of a listeriosis infection comes from a transcriptomics study that found expression of numerous genes induced in the gastrointestinal tract to be under σ^B control (30).

Although the importance of σ^B in stress adaptation and virulence is now well established, very little is known about how the activity of σ^B is regulated in this pathogen. Based on homology with the σ^B regulatory apparatus in *Bacillus subtilis*, it is likely that regulation is achieved primarily at the posttranslational level through an interaction with RsbW, an anti-sigma factor (9, 15). In order to develop an understanding of the kinetics and extent of σ^B activation in *L. monocytogenes*, we have investigated the effects of osmotic stress on the expression of four genes and loci (*opuCA*, *lmo2230*, *lmo2085*, and the *sigB* gene itself) already known to be under σ^B control in this pathogen. The *opuCA* gene encodes a component of the OpuC system that is involved in osmo- and cryotolerance (7, 11, 34) and also plays an important role in survival during the intestinal phase of infection (28), probably because it contributes to bile resistance (33). Its transcription is under σ^B control, and the promoter has been mapped to a position 58 bp upstream from the start codon of *opuCA* (11, 17). The *lmo2230* gene locus encodes a putative arsenate reductase, and its σ^B promoter has been mapped to a position 143 bp upstream from the start codon (17). This gene belongs to a category of genes that are under both σ^B and PrfA control (8, 22). The *lmo2085* gene encodes a putative peptidoglycan bound protein that has no homologue in the nonpathogenic species *L. innocua* (6). This gene has been shown in several studies to be expressed in a highly σ^B-dependent manner (1, 13, 17, 24); in recent gene microarray experiments we found it to be the gene most affected in a Δ*sigB* background (E. Starr and C. P. O'Byrne, unpublished data). The *sigB* gene itself is positively autoregulated; mutants lacking σ^B fail to induce the 4-gene *sigB* operon (consisting of *rsbV*, *rsbW*, *sigB*, and *rsbX*) in response to stress stimuli. The σ^B-dependent promoter is located upstream from the *rsbV* gene (4).

We first investigated the kinetics of σ^B activation by following σ^B-dependent transcription after the sudden imposition of an osmotic stress. Cultures of *L. monocytogenes* wild-type EGD-e and an isogenic Δ*sigB* mutant derivative (5) were grown to the mid-exponential phase (optical density at 600 nm [OD_{600}] of 0.6) in brain heart infusion (BHI) broth at 37°C, and then salt (0.5 M NaCl [in the form of solid salt crystals]) was added to the medium. RNA was prepared at several time intervals (0, 5, 10, 15, 20, and 25 min) and collected from cultures kept at 37°C and vigorously shaken. Real-time reverse transcription-PCR (RT-PCR) was then used to measure transcript levels over the indicated time period for each of the four σ^B-dependent genes studied (*opuCA*, *lmo2085*, *lmo2230*, and *sigB*) (Fig. 1; Table 1; see also Table S1 in the supplemental material). Rapid induction of each of the four genes was re-

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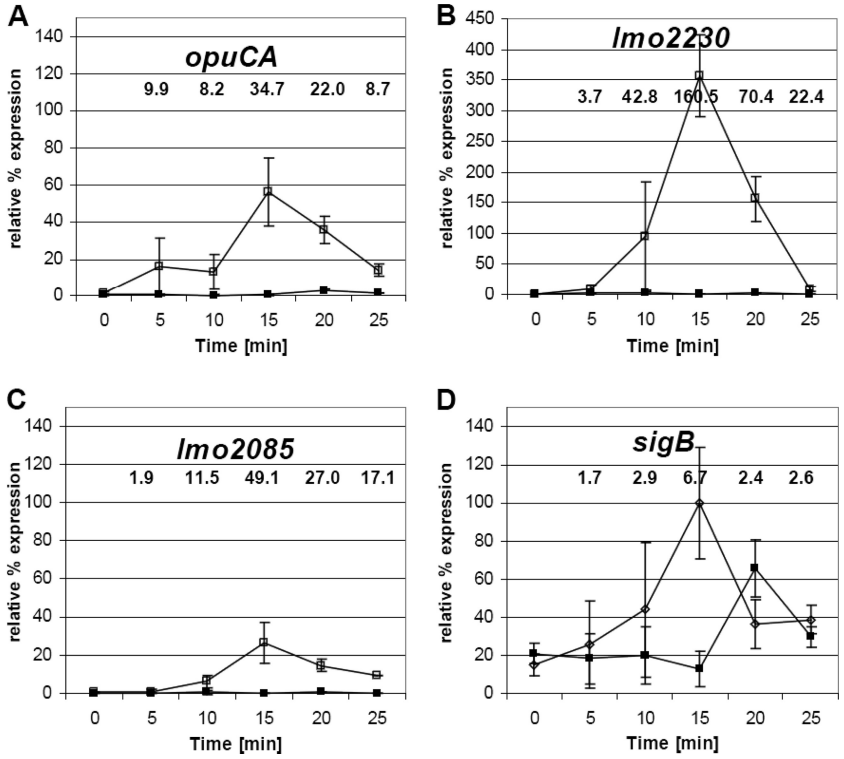

FIG. 1. Activation of σ^B by osmotic upshock occurs rapidly and transiently. Relative transcript levels of *sigB* and three other σ^B-dependent genes (*opuCA*, *lmo2230*, and *lmo2085*) were measured in exponential-phase cells in wild-type (open symbols) and Δ*sigB* (closed symbols) backgrounds grown in BHI broth at 37°C. RNA extracts were prepared either immediately before the addition of 0.5 M NaCl (0 min) or 5 min, 10 min, 15 min, 20 min, or 25 min after osmotic upshock. Real-time determination of gene transcription levels was carried out as previously described (16). All transcript levels were first normalized to the corresponding 16S RNA reference gene levels (internal control) (29), with an efficiency correction included for each primer pair as previously described (25), and then expressed as a percentage of the maximal level of *sigB* transcript detected in the experiment (thereby allowing the relative transcript levels of the 4 genes to be compared). The values presented on the graphs represent the means of the results of three independent experiments, and error bars indicate the standard deviations ($n = 3$). Numbers shown above the graphs indicate statistically significant differences in relative gene expression levels (severalfold change) for the wild type between the results seen under stressed (NaCl added at each time point) and nonstressed (time point 0 before adding NaCl) conditions ($P < 0.05$ by Student's *t* test). Note that the plot in panel B uses a different scale on the *y* axis.

corded following a sudden osmotic upshock, with *lmo2230* showing the highest level of induction (160-fold at 15 min relative to time zero). For all four genes, the maximum transcript level was observed at the 15-min time interval, with a steady decrease in transcript levels thereafter, suggesting a transient activation of transcription in response to osmotic upshock. In the Δ*sigB* mutant there was no significant increase in transcription recorded for *opuCA*, *lmo2085*, and *lmo2230* following osmotic upshock (Fig. 1A, B, and C), confirming the σ^B-dependent transcription of these genes and indicating that σ^B activation occurs transiently in response to osmotic upshock. A small delay in induction of *sigB* transcription was observed with the Δ*sigB* mutant (detected with primers directed against the undeleted part of the *sigB* gene), presumably resulting from the activation of σ^B-independent promoter (Fig. 1D). At 25 min after the addition of NaCl, transcription of each gene had returned to a level similar to that detected when cells were subjected to continuous osmotic stress during balanced growth (compare fold change values in Fig. 1 at the 25-min time point with those indicated in Fig. S1 in the sup-

TABLE 1. Primers used in the study

Target	Forward (5′–3′)	Reverse (5′–3′)	Product size (bp)	Efficiency (*E*)[c]
16S rRNA[a]	TGGGGAGCAAACAGGATTAG	TAAGGTTCTTCGCGTTGCTT	213	2.27
sigB[b]	CTATATTGGATTGCCGCTTAC	CAAACGTTGCATCATATCTTC	191	1.98
opuCA[b]	GGATGAAGCGATTAAACTG	CCGCTGTAATAGAGACTGG	206	2.19
lmo2230[b]	CATATTCGAAGTGCCATTGC	CTGAACTAGGTGAATAAGACAAAC	169	2.12
lmo2085[b]	GGTAATGGATATAATGTGCCTGC	GGGTGAACCAATAAGCAAATC	177	2.27

[a] Data are from reference 16.
[b] Designed for this study.
[c] Data were established by experiments using five serial decimal dilutions of genomic DNA and cDNA according to the equation $E = 10^{-1/\text{slope}}$ (25).

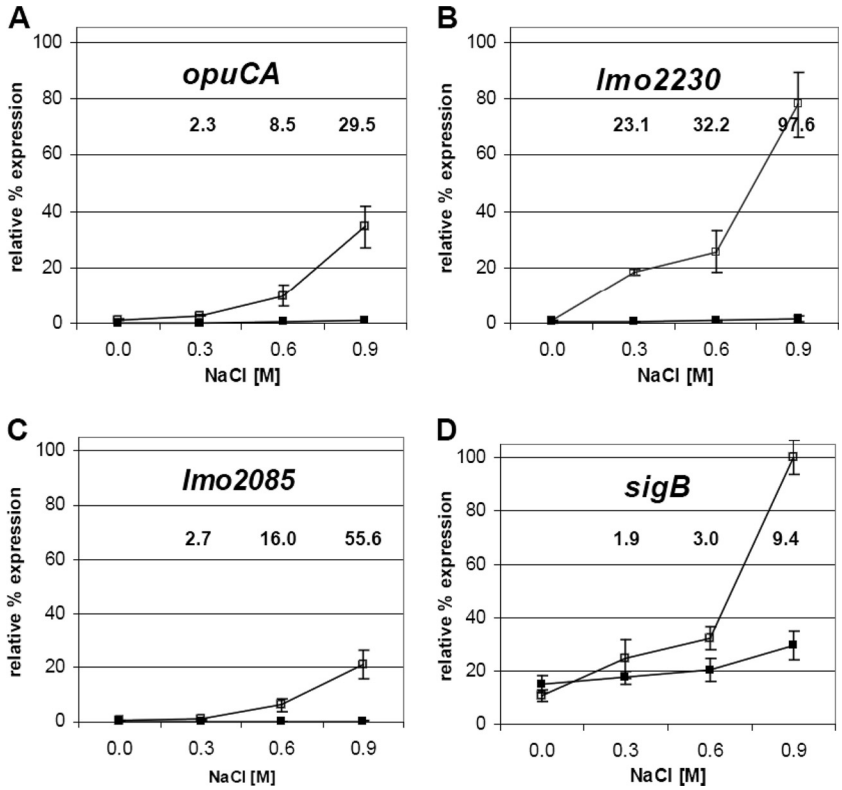

FIG. 2. Osmotic activation of σ^B is proportional to the magnitude of the stress. Relative transcript levels of three σ^B-dependent genes (*opuCA*, *lmo2230*, and *lmo2085*) and *sigB* in the wild type (open symbols) and the Δ*sigB* mutant (closed symbols) grown in BHI broth at 37°C to the exponential phase (OD_{600} of 0.6) under a range of NaCl concentrations (0 M, 0.3 M, 0.6 M, or 0.9 M) are shown. RNA extracts and cDNA were prepared as previously described (25). All transcript levels were first normalized as described in Fig. 1 and then expressed as a percentage of the maximal level of *sigB* transcript detected in the experiment (thereby allowing the relative transcript levels of the 4 genes to be compared). The values presented on the graphs represent the means of the results of three independent experiments, and error bars indicate the standard deviations ($n = 3$). Numbers shown above the graphs indicate statistically significant differences in relative gene expression levels (severalfold change) between the results seen under stressed (0.3, 0.6, or 0.9 M NaCl added at each time point) and nonstressed (0 M NaCl) conditions ($P < 0.05$ by Student's *t* test).

plemental material), a level that was still significantly higher than that detected in the absence of NaCl (Fig. 1; $P < 0.05$ [Student's *t* test]). These data suggest that σ^B is activated rapidly and transiently following sudden exposure to osmotic stress. Furthermore, the extent of σ^B activation following osmotic upshock was, for a short time, much greater than that observed during balanced growth when the same concentration of salt was present.

When the transcript levels of the four selected genes (*opuCA*, *lmo2085*, *lmo2230*, and *sigB*) were investigated during exponential growth at 37°C (RNA extraction performed when cells reached an OD_{600} of 0.6) with a range of salt concentrations (0 to 0.9 M NaCl), a dramatic and proportional increase in the transcript levels occurred in each case as the salt concentration increased (Fig. 2; see also Table S1 in the supplemental material). The NaCl-induced increase in the transcript levels was not observed for any of the four genes in a background lacking σ^B. The most substantial induction of transcription for each of the four genes was seen with 0.9 M salt. Transcription of *opuCA* at 0.9 M salt in the wild type increased dramatically (~29 times higher than that seen with the 0 M NaCl control), while *lmo2230* and *lmo2085* showed transcription ~98 and ~56 times higher at that salt concentration, respectively. No increase in transcription was detected in the Δ*sigB* mutant. Although the induction of *sigB* transcription resulting from osmotic stress was largely dependent on the presence of σ^B, a significant amount of *sigB* transcription was observed in the mutant background (Fig. 2D). This suggests that a baseline of σ^B-independent transcription of the *sigB* operon is maintained under all growth conditions, which is consistent with the requirement for a rapid transcriptional response when stress is encountered. Together, these results indicate that σ^B-dependent transcriptional activity is strongly stimulated by the presence of salt in a manner that is directly proportional to the extent of the stress, suggesting that σ^B activity can be finely tuned according to the environment encountered.

The data presented above suggest that measuring the transcription of *opuC*, *lmo2230*, or *lmo2085* is useful for measuring σ^B activity in *L. monocytogenes*. We investigated whether proportional expression of one of these genes, *opuCA*, in response to osmotic stress could also be detected at the protein level. To detect OpuCA protein levels, polyclonal antibodies against the purified OpuCA protein (ATPase subunit of the OpuC transporter) were raised in chickens as described in Fig. S2 in the supplemental material. Cultures of the wild type (EGD-e) and

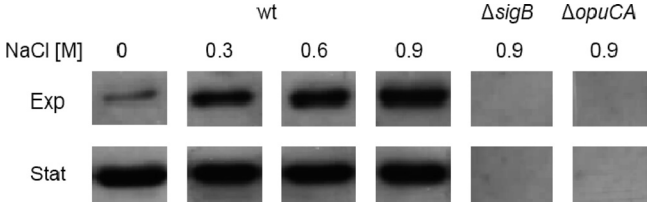

FIG. 3. OpuCA expression is induced in proportion to the osmotic stress. The OpuCA protein was detected in three biological replicates of crude cell extracts by Western blotting using anti-OpuCA-His polyclonal antibodies as described in the legend to Fig. S2 in the supplemental material. Crude cell extracts were prepared from exponential-phase cells (Exp) or stationary-phase cells (Stat) of the wild-type strain (wt) or Δ*sigB* and Δ*opuCA* mutant strains grown at 37°C in BHI broth over a range of NaCl concentrations (0, 0.3, 0.6, and 0.9). Total protein concentrations were normalized to 5 mg ml^{-1}, and 10 μl of each protein extract was loaded. Western blotting was carried out using semidry transfer with incubations and washing steps followed by chemiluminescent light detection as described in the legend to Fig. S2 in the supplemental material.

of Δ*sigB* and Δ*opuCA* mutant derivatives were grown at 37°C with vigorous shaking in BHI broth supplemented with 0, 0.3, 0.6, or 0.9 M NaCl. Crude cell extracts were prepared from each culture as described previously (2) during either the exponential phase (OD_{600} of 0.6) or the stationary phase (16 h of culture), and the levels of OpuC expression were determined by Western blotting. For all OpuCA comparisons, the total protein concentrations were normalized to 5 mg ml^{-1}, with 10 μl loaded onto gels. A clear band was detected by Western blotting for purified OpuCA; this band was absent from the Δ*opuCA* mutant blot, indicating that the antibodies produced were specific for OpuCA (see Fig. S2 in the supplemental material). OpuCA was not detected in the Δ*sigB* mutant under any of the described conditions (data are shown for the highest NaCl concentration only; Fig. 3), confirming the dependence of OpuC expression on σ^B. Strikingly, the highest level of OpuCA in the wild type occurred with the most severe osmotic challenge, specifically, 0.9 M NaCl. The levels of OpuCA were found to increase gradually and at a rate approximately in proportion to the salt concentration in the medium during exponential growth. These results correlate well with what was observed at the transcriptional level (Fig. 2) and suggest that OpuC levels reflect the extent of σ^B activation during balanced growth. Thus, the availability of antibodies against OpuCA should generally prove useful in measuring σ^B activity in *L. monocytogenes*. It is worth noting that the levels of OpuCA remained high over the whole range of salt concentrations tested in the stationary phase (Fig. 3), suggesting that expression was fully induced in the stationary phase regardless of whether or not salt was added. This result suggests that σ^B might be maximally active in the stationary phase, presumably because the entire cellular pool of σ^B is associated with RNA polymerase.

Overall, the findings of this study suggest that σ^B activation resulting from osmotic stress is proportional to magnitude of the stress, with a wide dynamic range of activities apparent. The kinetics of transcriptional activation in response to osmotic upshock are extremely fast, with a peak of activity occurring 15 min after upshock. Together, these data show that σ^B activation is carefully calibrated to meet the precise conditions encountered, a feature that is likely to contribute to the remarkable adaptability of this pathogen.

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