Identification of a σ^B -Dependent Small Noncoding RNA in *Listeria monocytogenes*[†]

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In *Listeria monocytogenes*, the alternative sigma factor σ^B plays important roles in stress tolerance and virulence. Here, we present the identification of SbrA, a novel small noncoding RNA that is produced in a σ^B -dependent manner. This finding adds the σ^B regulon to the growing list of stress-induced regulatory circuits that include small noncoding RNAs.

The gram-positive bacterium *Listeria monocytogenes* is an important food-borne pathogen associated with life-threatening invasive infections in humans and animals (33). In order to establish an infection, *L. monocytogenes* must be able to grow and survive under a variety of stress conditions, including those encountered during food processing, in the gastrointestinal tract of the host, and, at the later stages of infection, within the intracellular environments of host cells.

The alternative sigma factor σ^B plays an important role in general stress response and virulence in *L. monocytogenes*. Mutant strains lacking functional σ^B are more sensitive toward a variety of stress conditions, including low pH and oxidative stress (9, 36). Furthermore, σ^B contributes to the ability of *L. monocytogenes* to invade nonphagocytic human cells and plays a critical role during the gastrointestinal stage of listeriosis in guinea pigs (10, 19). Recently, the σ^B regulon in *L. monocytogenes* has been studied by genomic strategies (1, 13, 29). Whole-genome microarray and proteome analyses of the σ^B regulon in *L. monocytogenes* strain 10403S and strain EGD-e revealed more than 100 genes to be positively controlled by σ^B , including genes encoding proteins with established roles in stress resistance (e.g., *clpC*, *gadA*, and *ctc*) and virulence (e.g., *inlAB*, *inlD*, *bsh*, and *prfA*). In addition, a large number of genes were found to be negatively influenced by σ^B , in particular genes encoding flagellar and ribosomal proteins. Intriguingly, many of the σ^B -dependent effects observed in these studies appear to be indirect, i.e., through secondary gene regulatory systems, such as the stress-related transcriptional regulators HrcA and CtsR, the key virulence regulator PrfA, and the RNA chaperone Hfq (15, 16).

In recent years, genome-wide experiment- and computer-based screens have led to the discovery of hundreds of small RNA molecules (sRNAs) in various bacterial species (6, 20, 21, 22, 27, 34, 37). Intriguingly, many of these sRNAs are induced

only under specific growth conditions, such as in the stationary growth phase. In several cases, the regulatory mechanisms underlying this upregulation involve the action of alternative stress sigma factors (18, 20, 25, 31). The majority of the sRNAs studied thus far regulate gene expression by pairing with target mRNAs to change their translation and/or stability, whereas other sRNAs bind to and alter the activity of proteins (12). The RNA-binding protein Hfq is critical for the regulatory functions of multiple sRNAs. Hfq acts as an RNA chaperone to promote sRNA-mRNA duplex formation (2, 32). Furthermore, binding of Hfq increases the stability of several sRNAs, most likely by protecting them from degradation by RNases. In *L. monocytogenes*, Hfq plays a role in stress tolerance and contributes to pathogenesis in mice (7). We recently identified a number of sRNAs that interact with Hfq in *L. monocytogenes*, suggesting that Hfq-binding sRNAs are implicated in stress tolerance and virulence in this organism (6).

In several cases, it has been shown that the synthesis of sRNAs is tightly regulated at the level of transcription by known regulatory proteins, including alternative sigma factors, as exemplified by σ^E in *Escherichia coli* and *Salmonella* spp. (18, 25), and σ^{54} in *E. coli* and *Vibrio cholerae* (20, 31). These findings prompted us to search for σ^B -dependent sRNAs in *L. monocytogenes*. To identify σ^B -dependent sRNAs in *L. monocytogenes* EGD-e, we used a straightforward bioinformatics approach. Since bacterial sRNAs are often encoded by intergenic regions (IGRs), we searched for putative σ^B binding sites (GTTT-N_{14 to 17}-GGKWWWW) and rho-independent transcriptional terminator structures within the IGRs of the *L. monocytogenes* EGD-e genome using the ListiList web page (<http://genolist.pasteur.fr/ListiList/>). Here, we defined an intrinsic terminator as an inverted repeat with a ΔG no higher than -10 kcal/mol, followed by a stretch of at least five U's. Putative σ^B binding sites located within 150 bases upstream from and in the same orientation as an open reading frame were discarded. Our initial screen resulted in the identification of four putative sRNA loci, named *sbrA*, *sbrB*, *sbrC*, and *sbrD* for sigmaB-dependent small RNA A to D (Table 1). These four loci all contain sequences with a high level of homology to the σ^B consensus sequence followed by putative rho-independent transcription terminator sequences. To examine whether σ^B -

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TABLE 1. Genetic organization of candidate σ^B -regulated sRNAs in *L. monocytogenes* EGD-e

Gene	Flanking genes ^a	Length	σ^B promoter ^b
<i>sbrA</i>	lmo1374 > > > lmo1375	70	TAAAAATGTTTTAATCTAGGTTTAGCGGGTATTGTTTAGT
<i>sbrB</i>	lmo1251 > < < lmo1252	168	TTATTTGGTTTTACTTGTTGGTGGCTTGGTTTTGTTTTTC
<i>sbrC</i>	lmo1515 > < > lmo1516	73	ATATAGCGTTTTCAATTGTTTGCTTTTGGTATTTATTTTCAG
<i>sbrD</i>	<i>atpI</i> < < < lmo1537	68	TCCGGCTGTTTTTCCGTTCTTTTCTTGGGAAAAGAACTTT

^a The orientation of the proposed sRNA gene as well as upstream and downstream genes is indicated by arrows.
^b Proposed σ^B consensus -10 and -35 regions are shown in bold.

dependent sRNAs were expressed from these four loci, we performed Northern blotting using RNA sampled from wild-type (wt) and $\Delta sigB$ mutant strains subjected to osmotic stress (Fig. 1A). In these experiments, we observed that SbrA was induced in the wt strain in a transient fashion, reaching a maximum level around 20 min after the addition of NaCl. Importantly, SbrA was not observed in the $\Delta sigB$ strain. No σ^B -dependent expression of SbrB, SbrC, and SbrD could be observed under these experimental conditions (data not shown).

In addition to osmotic stress conditions, the activity of σ^B in *L. monocytogenes* is known to increase when cells enter the stationary growth phase. By Northern blotting, we tested the expression of the four putative sRNAs in wt and $\Delta sigB$ strains during growth in rich medium. We found that SbrA was present in wt cells in a growth-phase-dependent manner, reaching a maximum level when the cells enter the stationary growth phase (Fig. 1B). Again, sRNAs corresponding to SbrB, SbrC, and SbrD were not observed under these growth conditions (data not shown). With respect to SbrB and SbrC, the lack of induction may be due to the presence of the T nucleotide instead of the G consensus nucleotide within the putative -10 region of the σ^B binding site (Table 1). The lack of σ^B -dependent expression of SbrD may be due to the rather long spacing (17 nucleotides) separating the consensus -10 and -35 sequence elements of the putative σ^B binding site (Table 1). To map the 5' end of SbrA, we performed primer extension analysis with RNA samples from wt and $\Delta sigB$ strains subjected to osmotic stress (Fig. 1C). We found that the 5' end of SbrA maps to the region located immediately downstream from the putative σ^B promoter element.

To verify the presence of a σ^B -dependent promoter upstream from *sbrA*, we constructed a transcriptional fusion of the *sbrA* promoter region to a promoter-less *lacZ* gene in the vector pTCV-*lac* (28). The resulting plasmid, named *psbrA*(-T)-*lacZ* (see Fig. 2A), was introduced into the wt and $\Delta sigB$ strains, and the level of β -galactosidase activity was determined under various growth conditions. During growth in rich medium, we observed an increase in *sbrA*(-T)-*lacZ* expression in the wt cells, reaching a maximum level in the stationary growth phase (Fig. 2B). In contrast, only negligible levels of β -galactosidase activity were recorded for the $\Delta sigB$ cells. Next, we examined the expression of *sbrA*(-T)-*lacZ* under various stress conditions (Fig. 2C). We observed that the level of β -galactosidase activity increased dramatically in the wt strain subjected to 4% NaCl or 2% ethyl alcohol, whereas the activity in $\Delta sigB$ cells remained constant. These results clearly indicate the presence of a σ^B -dependent promoter in the *sbrA* promoter region.

FIG. 1. Identification of novel σ^B -regulated sRNAs. (A) Northern blot showing the expression of SbrA after NaCl stress. Overnight cultures of wild-type (Wt) and $\Delta sigB$ cells were diluted 100 times in brain heart infusion (BHI) medium and grown to early exponential phase, at which point 4% NaCl was added. The samples were drawn for RNA extraction at the indicated time points after the addition of NaCl. RNA purification and Northern blotting were performed as previously described (6) using a specific ^{32}P -labeled RNA probe prepared by the in vitro transcription of a PCR product generated by using the primers *sbrA*-Fwd T7 and *sbrA*-Rev (the primers are listed in Table S1 in the supplemental material). (B) Northern blot showing the expression of SbrA during growth in BHI. Samples were drawn at the indicated time points. The time points of 2, 3, and 6 h correspond to the early to late exponential growth phases, whereas the 8-, 14-, and 24-h time points correspond to the early to late stationary growth phases. Lane C is a positive control corresponding to NaCl-induced SbrA. Wt, wild type. (C) Mapping of the 5' transcriptional start site of SbrA by primer extension. Wild-type (Wt) and $\Delta sigB$ cells were grown in BHI to early exponential phase, at which point 4% NaCl was added. The samples were drawn at the indicated time points. Following RNA extraction, primer extension was performed as previously described (6) using the primer *sbrA*-I (see Table S1 in the supplemental material).

To investigate the function of the putative rho-independent transcriptional terminator located 42 to 70 nucleotides downstream from the *sbrA* transcription start site, we fused the entire *sbrA* gene, including the *sbrA* promoter region and the putative transcription terminator, to the *lacZ* gene in pTCV-*lac*, resulting in the plasmid construct *psbrA*(+T)-*lacZ* (Fig. 2A). The level of β -galactosidase activity in the wt cells and the Δ *sigB* cells harboring the *sbrA*(+T)-*lacZ* fusion plasmid remained negligible under all conditions tested, suggesting that the majority of the σ^B -dependent transcripts originating from the *sbrA* promoter region indeed ends at this transcription terminator structure (Fig. 2B).

Based on the findings described above, we infer that *sbrA* is a 70-nucleotide σ^B -dependent sRNA encoded by the IGR between lmo1374 and lmo1375. Furthermore, we note that no potential open reading frames are likely to be encoded by *sbrA*. We conducted a thorough BLAST and FASTA search for homologues of SbrA. We found that while SbrA is highly conserved in sequenced *Listeria* species (see Fig. S1 in the supplemental material), it was not identified in closely related bacterial species such as *Bacillus subtilis* and *Staphylococcus aureus*. The lmo1374 gene encodes a lipamide acyltransferase and is part of the *bkd* operon which plays a crucial role in the ability of *L. monocytogenes* to modulate its membrane lipid composition in response to temperature and pH (11, 39). The -35 box of the σ^B -dependent promoter is located immediately downstream from the stop codon of lmo1374, suggesting that the SbrA transcript and the transcripts originating from the *bkd* operon all terminate at the same transcription terminator structure. The lmo1375 gene is located 265 bp downstream from the *sbrA*-*bkd* transcription terminator and encodes a putative aminotripeptidase.

To analyze the functional importance of SbrA, we constructed a Δ *sbrA* mutant strain using splicing by overlap extension PCR (14). The mutant strain lacks the -10 box of the σ^B -dependent promoter and 37 nucleotides at the 5' end of the SbrA-encoding gene, thus conserving the *sbrA*-*bkd* transcription terminator. The growth or survival of wt and Δ *sbrA* strains was followed under a variety of conditions, including low-temperature, osmotic, acid, and alcohol stress. Under all conditions tested, no significant differences in growth or survival could be recorded for the wt and Δ *sbrA* strains (data not shown).

In order to predict a putative function of a sRNA, it may be helpful to know its secondary structure at the molecular level. The MFOLD program predicts a rather simple structure composed of a 5' stem-loop and a 3' transcription terminator stem-loop (Fig. 3B). We confirmed the structure predicted by

MFOLD by probing in vitro-transcribed and 5'-end-labeled SbrA with lead(II) acetate, RNase A, and RNase III (Fig. 3A and B). Many of the sRNAs studied thus far act as antisense RNAs by base pairing with target mRNAs near the ribosome binding site and translation start site, resulting in the inhibition of translation and an increase in mRNA degradation (12). The nucleotides that participate in this antisense base pairing often lie in single-stranded regions of the sRNAs. We note that the loop of the 5' stem-loop of SbrA (termed L1) contains 19 single-stranded nucleotides rich in CUs which could potentially base-pair with a variety of GA-rich ribosomal binding sites (Fig. 3B), a regulatory mechanism employed by several sRNAs, including *Salmonella* CyaR (26), *S. aureus* RNAIII (5), and *E. coli* OxyS (3).

In an attempt to identify putative mRNAs targeted by SbrA, we used the program TargetRNA (<http://snowwhite.wellesley.edu/targetRNA/>), which identifies and scores target mRNAs based on the minimum free energy of the target interaction with an sRNA gene (30). We used a hybridization seed of at least seven consecutive base-pairing nucleotides and focused our search on genomic regions surrounding start codons. A number of the top candidates predicted by TargetRNA (*kdpA*, lmo2421, lmo0397, lmo0698, lmo1602, and lmo1292) were selected for further analysis. In order to test whether the translation of these mRNAs was affected by SbrA, in-frame translational fusions of the target genes to *lacZ* were produced in pCK-*lac* (see Fig. S2 and S3 in the supplemental material). Furthermore, we tested the mRNA levels of lmo0397, lmo0698, and lmo1602 by Northern blotting, semiquantitative reverse transcriptase PCR, and primer extension experiments, respectively, before and after the induction of SbrA production from an IPTG (isopropyl- β -D-thiogalactopyranoside)-inducible promoter (see Fig. S4 in the supplemental material). Under all experimental conditions, none of the putative target genes predicted by TargetRNA appeared to be under the control of SbrA.

A large number of sRNAs depend on Hfq for function and/or stability. To test whether the stability of SbrA was affected by Hfq, we carried out stability assays of SbrA in wt and Δ *hfq* strains (Fig. 4A). SbrA expression was first induced by an osmotic upshift. After 20 min, transcription was blocked by the addition of rifampin. In both strains, the half-life of SbrA was approximately 5 to 6 min, suggesting that SbrA does not depend on Hfq for stability (Fig. 4B). Even though SbrA does not depend on Hfq for stability, it still may interact with Hfq. To investigate whether SbrA interacts with Hfq in vivo, we conducted a coimmunoprecipitation experiment using Hfq antibodies (Fig. 4C). As a positive control, we included a

FIG. 2. β -Galactosidase assay of transcriptional *sbrA*-*lacZ* fusions. (A) Description of transcriptional fusions of the *sbrA* promoter to *lacZ*. For the construction of *psbrA*(-T)-*lac* (containing the *sbrA* promoter-*lacZ* fusion without the intrinsic terminator [i.e., from -253 to $+53$ relative to the SbrA transcription start site]), primers *sbrA*-2 and *sbrA*-4 were used (see Table S1 in the supplemental material). For the construction of *psbrA*(+T)-*lac* (containing the *sbrA* promoter fusion, including the intrinsic terminator [i.e., from -253 to $+157$ relative to the SbrA transcription start site]), primers *sbrA*-2 and *sbrA*-3 were used (see Table S1 in the supplemental material). The PCR products were inserted into the vector pTCV-*lac* (26). (B) *sbrA*(+T)-*lacZ* and *sbrA*(-T)-*lacZ* expression in wild-type (Wt) and Δ *sigB* strains during growth in BHI medium. The samples were drawn at the indicated time points, and the amount of β -galactosidase activity was measured as previously described (7). (C) *sbrA*(-T)-*lacZ* expression in wild-type (Wt) and Δ *sigB* strains in response to various stress conditions. Cells were grown to an optical density at 600 nm of 0.3 and subjected to the indicated stress condition for 30 or 60 min. At these time points, 1-ml samples were drawn, and the amount of β -galactosidase activity was measured.

FIG. 3. Secondary structure of SbrA RNA. (A) The secondary structure of SbrA was mapped by RNase and lead(II) acetate cleavage. SbrA was prepared by the in vitro transcription of a PCR product generated by using the primers T7-*sbrA*-Fwd and *sbrA*-Rev-2 (see Table S1 in the supplemental material) using the MaxiScript in vitro transcription kit (Ambion). For structural probing, 50 fmol SbrA and 3 μ g tRNA were incubated in structure buffer with or without (–) the specific ribonuclease RNase A (which cleaves the 3' end of unpaired C and U residues) or RNase III (which is specific for double-stranded RNA) or with 5 mM lead(II) acetate (which cleaves single-stranded RNA) for 5 or 10 min. For a sequencing reaction of SbrA (lane T₁), 50 fmol SbrA and 3 μ g tRNA were incubated in sequencing buffer (Ambion) with RNase T₁ (cleaves single-stranded RNA at G residues) for 5 min. An RNA ladder (lane OH) was prepared by boiling 100 fmol SbrA in hydroxide buffer (Ambion) for 5 min. After the indicated time, all enzyme reactions were stopped by the addition of 40 μ l stop solution (Ambion). Lead(II) acetate cleavage reactions were stopped by the addition of EDTA to a final concentration of 10 mM. (B) Secondary structure of SbrA proposed by MFOLD (<http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/rna-form1.cgi>). The arrows indicate cleavage by ribonucleases and lead(II) acetate.

known Hfq-binding sRNA, LhrA (6). In these experiments, only LhrA was retained by the Hfq-specific antibodies, suggesting that Hfq does not bind SbrA in vivo. Thus, we conclude that SbrA is an Hfq-independent sRNA.

Conclusion. The sRNA-encoding gene *sbrA* is part of the σ^B regulon, and we find it likely that SbrA plays a role in the σ^B -dependent regulation of stress response, metabolism, and virulence. The lack of phenotypes for the Δ *sbrA* mutant strain indicates that SbrA, like many other sRNAs, may be involved in the fine-tuning of gene expression. Alternatively, a role for SbrA may be observed under very specific growth conditions only, which still remain to be defined. Regarding a mechanism of action for SbrA, our initial characterization showed that it does not interact with Hfq and most likely functions indepen-

dently of this RNA chaperone. In *E. coli*, all sRNAs that control gene expression by base pairing with target mRNA interact with Hfq (12). In contrast to this, *S. aureus* RNAIII acts as an antisense RNA independently of Hfq (5, 17). Thus, although SbrA does not interact with Hfq, the possibility that SbrA could function by an antisense mechanism should not be excluded. However, an antisense-based mechanism is clearly not supported by our initial test of mRNAs predicted to be targeted by SbrA. As an alternative, SbrA may exert its function by interacting with target proteins, as exemplified by the sRNAs CsrB and CsrC (4) and 6S RNA (35). A key to the functional role and mechanism of action of SbrA may be found in the genomic studies of the σ^B regulon in *L. monocytogenes* (1, 13, 29). These studies revealed that more than 100 genes in

FIG. 4. SbrA stability and interaction with Hfq. (A) Northern blot showing the stability of SbrA after the addition of rifampin. Wild-type (Wt) and Δhfq cells were grown to early exponential phase, and SbrA expression was induced by the addition of 4% NaCl. After 20 min, transcription was blocked by the addition of 15 μ g/ml rifampin. (B) Quantification of the Northern blot in panel A. The approximate half-lives ($T_{1/2}$) are indicated. (C) The in vivo interaction between SbrA and Hfq was tested by coimmunoprecipitation using α -Hfq antiserum. Coimmunoprecipitations and Northern blots of immunoprecipitated RNA was performed as described previously (6). Coimmunoprecipitations were performed using α -Hfq antiserum (αHfq). As negative controls, we used no serum (-) and pre-immune serum (Pre) in the coimmunoprecipitation experiments. In the left panels (Unbound), control RNA that did not bind to the Protein A-Sepharose-coupled antibodies was used for Northern blotting. In the right panels (Bound), the coimmunoprecipitated RNA was tested by Northern blotting. For the Northern blotting experiments, we used the SbrA-specific probe described in the legend to Fig. 1A. As a positive control, we used a probe directed against the Hfq-binding sRNA LhrA (6).

L. monocytogenes are negatively influenced by σ^B . Furthermore, a large portion of the σ^B -activated genes are not preceded by a σ^B -dependent promoter. Thus, σ^B affects the expression of multiple genes in an indirect manner that could involve the action of regulatory sRNAs, such as SbrA.

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