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An mRNA-mRNA Interaction Couples Expression of a Virulence Factor and Its Chaperone in *Listeria monocytogenes*

**Highlights**
- FUSE can identify and structurally characterize regulatory events in 5' UTRs
- An mRNA encoding listeriolysin O stabilizes the mRNA encoding its chaperone PrsA2
- An RNA thermoswitch controls expression of the CspA protein in *L. monocytogenes*
- Simultaneous binding of ribosomes and small RNAs on an mRNA can be defined by FUSE

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**In Brief**
By using a comparative structural analysis method targeting 5' untranslated regions, Ignatov et al. identify different RNA-based regulatory mechanisms such as an RNA thermoswitch, a small RNA (sRNA)-mRNA interaction, and strikingly, an mRNA-mRNA interaction.
An mRNA-mRNA Interaction Couples Expression of a Virulence Factor and Its Chaperone in *Listeria monocytogenes*

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SUMMARY

Bacterial pathogens often employ RNA regulatory elements located in the 5′ untranslated regions (UTRs) to control gene expression. Using a comparative structural analysis, we examine the structure of 5′ UTRs at a global scale in the pathogenic bacterium *Listeria monocytogenes* under different conditions. In addition to discovering an RNA thermoswitch and detecting simultaneous interaction of ribosomes and small RNAs with mRNA, we identify structural changes in the 5′ UTR of an mRNA encoding the post-translocation chaperone PrsA2 during infection conditions. We demonstrate that the 5′ UTR of the prsA2 mRNA base pairs with the 3′ UTR of the full-length hly mRNA encoding listeriolysin O, thus preventing RNase J1-mediated degradation of the prsA2 transcript. Mutants lacking the hly-prsA2 interaction exhibit reduced virulence properties. This work highlights an additional level of RNA regulation, where the mRNA encoding a chaperone is stabilized by the mRNA encoding its substrate.

INTRODUCTION

Within the cytosol of living cells, RNA molecules fold into complex structures that are important for their functions (Miao and Westhof, 2017). In bacteria, regulatory RNAs control gene expression in response to changes in physical and chemical parameters, and binding of proteins, metabolites, or other RNA molecules, and their mechanism of action is often based on alteration of RNA structure (Meyer, 2017).

Small RNAs (sRNAs) represent a widespread class of regulators in bacteria and have long served as a paradigm for trans-encoded RNA regulators. However, recent data suggest that protein-coding transcripts can also base pair in trans and perform regulation. Genomes of different bacterial species encode dual-function sRNAs, which not only encode peptides or short proteins but also base pair with other RNAs (Raina et al., 2018). Moreover, even canonical mRNAs with lengths exceeding 500 nt can engage in regulatory base-pairing interactions. As an example, intramolecular base-pairing between the 5′ and 3′ untranslated regions (UTRs) regulates expression of the icaR mRNA in *Staphylococcus aureus* (Ruiz de los Mozos et al., 2013). A similar mechanism regulates expression of toxin mRNAs in some type I toxin-antitoxin systems (Masachis and Darfeuille, 2018). Moreover, a canonical mRNA invA in *Streptococcus mutans* has been shown to base pair with another mRNA, gbpC, and protect it from degradation (Liu et al., 2015). These discoveries suggest that the network of regulatory base-pairing interactions in bacteria can be more complex than previously anticipated.

Pathogenic bacteria employ regulatory RNAs to adapt to living conditions inside the host and orchestrate the development of their virulence programs (Chakravarty and Massé, 2019). The facultative pathogen *Listeria monocytogenes* has served as a model for extensive research on RNA regulation (Lebreton and Cossart, 2017). Studies of several *L. monocytogenes* sRNAs facilitated the identification of genes that they regulate in the context of pathogenesis (Dos Santos et al., 2018; Nielsen et al., 2011, 2011; Quereda et al., 2014; Ross et al., 2019; Sievers et al., 2014, 2015). Another example of RNA regulation is an RNA thermometer controlling translation of the prfA mRNA, encoding the master regulator of *L. monocytogenes* virulence. During infection, PrfA induces expression of the major virulence genes (de las Heras et al., 2011). The RNA thermometer is located in the 5′ UTR of prfA mRNA and inhibits translation initiation at 30°C, but not 37°C, the latter temperature encountered when the pathogen enters the host (Johansson et al., 2002). The prfA 5′ UTR is also able to form base-pairing interactions with processed SAM riboswitches, which adds further complexity to RNA regulatory networks in *L. monocytogenes* (Loh et al., 2009).
Remarkably, our data also provided evidence for an mRNA-sRNA and the ribosome competing for the same transcript. CspA. We also identified a case of simultaneous binding of a moswitch regulating expression of the cold shock protein (McGinnis et al., 2015; Mizrahi et al., 2018; Righetti et al., 2016).

Discover novel regulatory elements in bacteria and eukaryotes RNAs or the whole transcriptome has previously been used to analyses of their structures. Comparison of structures of selected RNA molecules inside the cell on a transcriptome-wide scale (Mitchell et al., 2019; Strobel et al., 2018). These approaches were employed in bacteria to study how RNA structure affects translation (Burkhardt et al., 2017; Mustoe et al., 2018), discover G-quadruplexes (Guo and Bartel, 2016), and monitor RNA folding states during the response to the cold shock (Zhang et al., 2018). The action of regulatory RNA elements can be traced by the alterations of their structures. Comparison of structures of selected RNAs or the whole transcriptome has previously been used to discover novel regulatory elements in bacteria and eukaryotes (McGinnis et al., 2015; Mizrahi et al., 2018; Righetti et al., 2016).

The development of high-throughput RNA structure probing approaches has made it possible to profile the secondary structure of RNA molecules inside the cell on a transcriptome-wide scale (Mitchell et al., 2019; Strobel et al., 2018). These approaches were employed in bacteria to study how RNA structure affects translation (Burkhardt et al., 2017; Mustoe et al., 2018), discover G-quadruplexes (Guo and Bartel, 2016), and monitor RNA folding states during the response to the cold shock (Zhang et al., 2018). The action of regulatory RNA elements can be traced by the alterations of their structures. Comparison of structures of selected RNAs or the whole transcriptome has previously been used to discover novel regulatory elements in bacteria and eukaryotes (McGinnis et al., 2015; Mizrahi et al., 2018; Righetti et al., 2016).

The 5’ UTRs of bacterial mRNAs often serve as an important hub for RNA regulation, affecting the level of translation initiation or stability of transcripts (Oliva et al., 2015; Waters and Storz, 2009). To identify new RNA-based regulatory mechanisms in L. monocytogenes, we performed comparative analysis of 5’ UTR structures using an approach based on dimethyl sulfate (DMS) mutational profiling with sequencing (DMS-MaPseq) method (Zubradt et al., 2017). Our work revealed an RNA ther-moswitch regulating expression of the cold shock protein CspA. We also identified a case of simultaneous binding of a sRNA and the ribosome competing for the same transcript. Remarkably, our data also provided evidence for an mRNA-mRNA interaction in virulence-inducing conditions. The 5’ UTR of the prsA2 mRNA interacts with the 3’ UTR of hly, encoding listeriolysin O (LLO), an important virulence factor in L. monocytogenes. The PrsA2 chaperone is necessary for the folding of LLO and other virulence factors during infection. The hly-prsA2 interaction protected the prsA2 transcript from degradation by RNase J1, thus allowing increased PrsA2 expression. An absence of the mRNA-mRNA interaction decreased cell-to-cell spread and reduced bacterial virulence in infected mice. Our results suggest an additional level of L. monocytogenes virulence regulation beyond the master virulence regulator PrfA, where an mRNA encoding a chaperone can directly bind and stabilize the mRNA encoding its substrate.

RESULTS

Profiling the RNA Structure of 5’ UTRs in Listeria monocytogenes

We adapted a DMS-MaPseq protocol (Zubradt et al., 2017) to focus on the dynamics of 5’ UTR structures in the bacterial pathogen L. monocytogenes (Figure 1). Most bacterial regulatory RNA domains so far identified are located in the 5’ UTRs of mRNA molecules or in sRNAs. Our protocol (FUSE for 5’ UTR structure elucidation) selectively enriches for 5’ UTRs and sRNAs. In brief, DMS selectively methylates the Watson-Crick surfaces of unpaired adenine or cytosine nucleotides, but not when these bases are paired (intramolecularly or intermolecularly) or bound to proteins. After ligation of 5’ adapters, the RNA is fragmented, and the 3’ adapters are ligated. Using a reverse transcriptase (TGIRTIII) that incorporates a random base upon encountering a methylated adenine or cytosine, cDNA is produced. Base substitutions in the sequences indicate unpaired adenines or cytosines and using dedicated algorithms, the nucleotides changing their base-pairing interactions can be identified. Bacterial cells with different genetic backgrounds (Table S1) were grown at different conditions (Table S2) and treated with DMS during growth (in vivo samples). Alternatively, RNA isolated from cells was refolded, and DMS modification was performed (in vitro samples). After DMS treatment, DMS values were calculated for each adenine and cytosine in the 5’ UTRs and sRNAs. The DMS value represents a normalized measure of DMS modification and was used to predict RNA secondary structures, and the FUSE protocol increases the sequencing coverage of 5’ UTRs (Figure S1; Table S3).

Because no crystal structures have been solved for L. monocytogenes regulatory RNAs, we predicted the secondary structure of evolutionarily conserved 4.5S RNA using its structure in B. subtilis and E. coli as a blueprint (Nakamura et al., 1992). To verify the reliability of the method, we studied how well the calculated DMS values fit the predicted structure. In general, all base-paired nucleotides in 4.5S RNA had DMS values lower than 1, whereas most of the unpaired nucleotides had DMS values higher than 1 (Figures S2A and S2B). Some of the nucleotides not predicted to base pair also showed low DMS values, suggesting that these nucleotides can participate in tertiary interactions or be protected from DMS modification by other mechanisms. Indeed, during the analysis of 4.5S RNA structure, we noticed that several nucleotides in the evolutionarily conserved domain IV had significantly lower DMS values in vivo compared with...
in vitro (Figures 2A and S2C). The most dramatic changes were observed for nucleotides A151 and A159. In Escherichia coli, nucleotides in these positions directly interact with the M domain of the Ffh protein (Figure 2B; Batey et al., 2000, 2001).

Second, we used the prfA thermosensor as a reference to compare our FUSE data with the previously identified secondary structure of the prfA RNA thermoswitch (Johansson et al., 2002). In most samples, the expression of the monocistronic prfA mRNA was too low to probe its DMS reactivity. Only in FUSE libraries where DMS treatment was performed in vitro and in the prfA bacterial strain with PrfA regulator constantly activated did we obtain enough coverage to profile the structure of prfA 5’ UTR. The structure of the thermoswitch in the in vitro and in vivo samples agree well with each other and with the published structure (Figure S2D; Johansson et al., 2002). To detect changes associated with prfA thermoswitch melting, we employed targeted DMS-MaPseq of the prfA 5’ UTR region in bacteria growing at 26°C and 37°C. The structural differences identified in the thermoswitch between two temperatures were limited. However, we identified a higher DMS reactivity of some critical bases, such as C98, which needs to be base paired to prevent PrfA expression and virulence gene expression at 30°C (Figure S2E; Johansson et al., 2002). In conclusion, we consider FUSE to be a reliable approach to determine RNA secondary structures.

Shine-Dalgarno Sequences Are Occupied by Ribosomes In Vivo
We compared the average DMS values of 5’ UTRs in vitro and inside the cell. Globally, the nucleotides in positions 10–15 upstream of the start codon had considerably lower DMS values in vivo compared with in vitro (Figure 2C). This region overlaps with the distribution of Shine-Dalgarno (SD) sequences in L. monocytogenes mRNAs. A previous study performed in vitro on a selected mRNA demonstrated that binding of the 30S ribosome subunit confers DMS protection for the nucleotides of the SD sequence (Hüttenhofer and Noller, 1994). Interestingly, the DMS values of SD sequences in samples prepared in vitro (without ribosomes) showed the opposite trend and were slightly higher compared with flanking regions (Figure 2C). This suggests that SD sequences generally do not base pair, thereby facilitating the interaction with 30S ribosome subunits, as has been suggested previously (Righetti et al., 2016). We suggest that the low DMS reactivity of the SD sequences we observed in vivo on a global level can be explained by the interaction with ribosomes, through base-pairing interactions between the SD sequences of mRNAs and the anti-SD sequences of 16S rRNA. Hence FUSE can predict structural changes that might indicate the binding of other factors.

Comparison of 5’ UTR Structures at Different Temperatures Uncovers a Thermoswitch Controlling Expression of a Cold Shock Protein
A comparison of DMS values in L. monocytogenes cells grown at different temperatures detected significant structural changes in the 5’ UTR of the cspA (lmo1364) mRNA (Table S4). This gene encodes a cold shock protein that plays an important role in the adaptation of L. monocytogenes to low temperatures (Schmid et al., 2009). Using DMS values as guides for structure modeling, we reconstructed two alternative conformations for this 5’ UTR: at 26°C, the structure adopted an “open” conformation, where the SD sequence was available for ribosome binding, whereas at 37°C, the 5’ UTR rearranged to a “closed” conformation.
Figure 3. FUSE Identifies an RNA Thermosensor and Determines Simultaneous Binding of an sRNA and the Ribosome to an mRNA

(A) The 5' UTR of cspA (lmo1364) mRNA assumes alternative conformations at different temperatures. The secondary structures of the “open” and “closed” conformations are shown with DMS values of the 26°C sample for the open conformation and the 37°C sample for the closed conformation. A red color denotes high DMS values of adenine (A) and cytosine (C) nucleotides indicating their unpaired status. On the contrary, a yellow color indicates low DMS values suggesting the nucleotides to be base paired or interacting with a protein. The locations of mutations M1–M4 are indicated.

(B) The cspA 5' UTR inhibits translation at 37°C, and this effect is mediated by the rearrangement of its structure. The coding sequence of the β-galactosidase gene was fused with the cspA 5' UTR (wild type [WT]), the cspA 5' UTR carrying mutations M1–M4 (see A for their location) and the 5' UTRs of control mRNAs (lmo0277, lmo0354, and lmo2110, respectively). The constructs were expressed in E. coli at different temperatures, and β-galactosidase activity was measured (n = 3). The error bars represent standard deviations. The statistically significant differences between β-galactosidase expression levels at 26°C and 37°C are shown by asterisks (Student’s t test, *p < 0.05; **p < 0.01; ***p < 0.001).

(C) Electrophoretic mobility of in-vitro-synthesized WT and mutant cspA 5' UTRs at 26°C and 37°C. The location of the mutations M1–M4 are shown in (A). The in-vitro-transcribed cspA 5' UTR and the mutants were denatured, refolded at the indicated temperatures, and resolved on non-denaturing polyacrylamide gel. The experiments were repeated twice with similar results. Pictures were 1.5 times extended on the vertical axis to facilitate visualization.

(legend continued on next page)
with the SD sequence hidden in an RNA hairpin (Figures 3A and S3A). This was especially evident after a transient shift from 26°C to 37°C. To study the putative effect of the rearrangement on translation, we introduced mutations in the cspA 5’ UTR structure that destabilized either the open or closed conformation (Figures 3A and S3B) and created translational fusions of the wild-type (WT) and mutant 5’ UTRs to the β-galactosidase gene. As controls, translational fusions of three short 5’ UTRs that do not form stable secondary structures were constructed. The expression of the translational fusions was measured in *Escherichia coli* at different temperatures. Expression of the control fusions was higher at 37°C than at 26°C, whereas the WT cspA 5’ UTR reduced β-galactosidase expression at 37°C (Figure 3B). Mutation M1 destabilized the open conformation and inhibited expression at both 26°C and 37°C, whereas introduction of mutation M2 complementary to M1 restored the WT expression pattern. Mutation M3 destabilized the closed conformation and abolished inhibition at 37°C, causing high expression at both temperatures, whereas its complementary mutation M4 restored the expression pattern observed in the WT. To examine whether other cellular factors were required for the structural rearrangement of the cspA 5’ UTR, we synthesized the WT thermoswitch and its mutated isoforms *in vitro* and denatured and refolded them at different temperatures. The folding into open or closed conformation was identifiable by their different electrophoretic mobilities in non-denaturing polyacrylamide gels. The WT thermoswitch migrated more rapidly when refolded at 26°C than at 37°C, suggesting a higher electrophoretic mobility of the open conformation, whereas the M1 mutant RNA, which is locked in the closed conformation, demonstrated low mobility at both temperatures (Figure 3C). In contrast, the electrophoretic mobility of the M3 mutant RNA, which is locked in the open conformation, was high at both temperatures, whereas the compensatory mutations (M1+M2 and M3+M4) restored the migration to the pattern observed for the WT cspA 5’ UTR (Figure 3C). Refolding of the WT cspA 5’ UTR at different temperatures ranging from 26°C to 34°C demonstrated that the transition from the open to closed conformation occurs at approximately 30°C (Figure 3D). These data were further verified by performing DMS-MaPseq of *in vitro*-synthesized cspA 5’ UTR that was denatured and refolded at 26°C or 37°C. The structure of the cspA 5’ UTR refolded at 26°C showed a more open conformation, whereas the cspA 5’ UTR refolded at 37°C adopted a closed conformation (Figure S3C). Together, our data show that the cspA 5’ UTR in *L. monocytogenes* represents a bona fide RNA thermostwitch. The structure corresponding to the open conformation of the thermostwitch is conserved in all *Listeriaceae* and in some other species in the orders *Bacillales* and *Lactobacillales* (Figure S3D). However, it is structurally different from the cspA thermostwitch in *Escherichia coli* (Figure S3E; Giuliani et al., 2010; Zhang et al., 2018).

**Simultaneous Binding of Ribosomes and sRNAs Can Be Defined by FUSE**

Many bacterial species require Hfq to establish a functional interaction between sRNAs and their target mRNAs (Updegrove et al., 2016). However, in Firmicutes and *L. monocytogenes* in particular, the role of Hfq seems to be limited (Nielsen et al., 2010). We therefore studied how deletion of the RNA chaperone Hfq affects the structure of 5’ UTRs globally. Indeed, the Δhfq strain showed very modest changes in the 5’ UTR structurome in comparison with the WT (Table S4). The strongest changes were observed in the sRNA LhrA (Figure S4) and the 5’ UTR of the *lmo0850* mRNA (Figure 3E). It has previously been shown that LhrA interacts with the *lmo0850* mRNA in a Hfq-dependent manner both *in vivo* and *in vitro* (Nielsen et al., 2010). In agreement with this, we observed that the nucleotides of the *lmo0850* mRNA known to form base-pairing interactions with LhrA had significantly higher DMS values in the Δhfq strain, supporting the fact that the interaction is abolished in the absence of the chaperone (Figures 3E and 3F). On the contrary, the DMS values of nucleotides in the SD sequence of *lmo0850* were decreased in the Δhfq strain, correlating with stronger ribosome binding when LhrA is not bound. These data agree fully with the previous study showing that the LhrA-*lmo0850* interaction inhibits formation of a translation initiation complex and represses translation (Nielsen et al., 2010). A similar profile was observed in the Δhfq mutant and in the LhrA-mut strain, bearing substitutions in the LhrA sequence at its interaction site with *lmo0850* (i.e., increased reactivity at the LhrA interaction site and decreased reactivity at the SD region). Hence, at the single-nucleotide resolution, we were able to simultaneously follow the binding of the ribosome and LhrA sRNA to the *lmo0850* transcript *in vivo* (Figure 3F).

**The Activation of the *L. monocytogenes* Virulence Program Induces DMS Protection of the 5’ UTR of the *prsA2* mRNA**

*L. monocytogenes* is a facultative pathogen that can switch between the lifestyles of an environmental bacterium and a dangerous intracellular pathogen. The major player in this transition is the transcriptional regulator PrfA (de las Heras et al., 2011). Upon entry of the bacterium into the mammalian cell, PrfA recognizes glutathione as a host signal and directly activates expression of the most important virulence genes (Reniere et al., 2015). We hypothesized that an induction of the virulence program (D) The rearrangement of the cspA 5’ UTR structure in *vitro* upon the temperature increase. The *in vitro*-transcribed cspA 5’ UTR was denatured and refolded at 26°C. Gradual increase in temperature induced structural rearrangement at approximately 30°C. M1 and M3 mutants locked in the “closed” and “open” conformations, respectively, are shown as controls.

(E) DMS values of the *lmo0850* 5’ UTR in the WT, Δhfq, and lhrA-mut strains, respectively. Red bars indicate statistically significant increase of DMS values, and blue bars indicate statistically significant decrease of DMS values in the Δhfq and the lhrA-mut strains relative to the WT strain, respectively. The error bars for the WT strain represent standard deviation (n = 2).

(F) Model of the interaction between *lmo0850* mRNA, LhrA sRNA, Hfq protein, and the ribosome. Binding of LhrA inhibits ribosome binding to the SD sequence of *lmo0850*. Absence of Hfq or a mutation in LhrA (at its interaction site with *lmo0850*) decreases LhrA binding to *lmo0850* and thus increases ribosome binding to the SD region.

See also Figures S3 and S4 and Table S4.
upon PrfA activation might have an influence on the 5' UTR structures of the virulence-associated mRNAs, especially because *L. monocytogenes* appear to harbor many such transcripts with long 5' UTRs (Loh et al., 2006). Also, several genes are positively regulated by PrfA but do not have an obvious PrfA binding site, suggesting they are indirectly regulated by PrfA (Milohanic et al., 2003). To examine this, we compared the 5' UTR structures in the WT and *prfA* strains of *L. monocytogenes*. The *prfA* strain carries a G145S substitution in the PrfA protein, which makes the regulator constitutively active and permits high expression of virulence genes even during growth in broth culture (Ripio et al., 1997). Our comparison identified a group of nucleotides in the 5' UTR of *prsA2* (*lmo2219*) mRNA that were protected from DMS in the *prfA* strain compared with WT (Figure 4A; Table S4). *PrsA2* is predicted to function as a peptidyl-prolyl isomerase chaperone that assists in the folding of secreted proteins at the interface between the bacterial membrane and cell wall. In particular, *PrsA2* promotes secretion and stability of the most significant virulence factor, LLO (Alonzo et al., 2009; Zemansky et al., 2009). Although expression of the *prsA2* gene was shown to be dependent on PrfA activation (Figure S5A; Milohanic et al., 2003), the deletion of the PrfA binding site from the *prsA2* promoter did not eliminate *prsA2* expression (Zemansky et al., 2009).

The 3' UTR of *hly* Directly Interacts with the 5' End of *prsA2*

One possible reason why the nucleotides in the *prsA2* 5' UTR showed an altered DMS protection may be due to RNA-RNA or RNA-protein interactions. Previously, it has been shown

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**Figure 4. The 3' UTR of Full-Length hly mRNA Interacts with the 5' UTR of prsA2 mRNA, thereby Increasing the Level of prsA2 mRNA and PrsA2 Protein**

(A) Several nucleotides in the 5' UTR of *prsA2* mRNA show reduced DMS values in a *prfA* strain relative to the WT strain. The stretch of DMS-protected nucleotides corresponds to the predicted base-pairing interaction with the 3' UTR of *hly* mRNA. Red and blue boxes indicate the locations of the M1 and M2 mutations, respectively. Inset shows sequence of the M1 and M2 mutations, respectively. The start codon of *prsA2* and the stop codon of *hly* are indicated by purple boxes. The error bars for the WT strain represent standard deviation (*n* = 2).

(B) Expression of *prsA2* and *hly* genes in the WT strain and strains carrying mutations M1, M2, and M1+M2, respectively. Expression of the genes was measured by qRT-PCR, and the data were normalized to the expression level of 16S rRNA (*n* = 3). The error bars represent standard deviation, and the statistically significant differences in the levels of mRNAs are shown by asterisks (Student’s t test, *p* < 0.05, **p** < 0.01).

(C) Expression of *PrsA2*, LLO, and P60 (control) in the WT strain and strains carrying mutations M1, M2, and M1+M2, respectively. Expression of the proteins was measured by western blot, and the data were normalized to the expression level in the WT strain. Representative western blots showing *PrsA2*, LLO, and P60 levels are shown (*n* = 2). The error bars represent standard deviation, and the statistically significant differences in the levels of proteins are shown by asterisks (Student’s t test, *p* < 0.05). The products of partial proteolysis or truncated LLO proteins in M1 and M2 strains are indicated by an arrow.

(D) The 3' UTR of *hly* mRNA binding to *prsA2* is part of the full-length *hly* transcript. Northern blot was performed with RNA isolated from the *prfA* and the WT strains. The membrane was probed with radioactively labeled oligonucleotides complementary to the 3' UTR (3' UTR probe) and the coding sequence (CDS probe) of *hly* mRNA. To demonstrate the ability to detect short transcripts, the membrane was probed with oligonucleotides complementary to the PrfA-regulated sRNA Rli51, originating from the 5' UTR of *mpl*. The probe complementary to the transfer-messenger RNA (tmRNA) was used as a loading control. See also Figure S5 and Table S5.
that the trans-acting sRNA RoxS is able to bind and prevent degradation of the yflS mRNA, by binding at its extreme 5’ end (Durand et al., 2017). With this in mind, we performed a whole-genome search for sequences complementary to the identified DMS footprint using the CopraRNA software (Table S5; Wright et al., 2014). Unexpectedly, the best match was in the 3’ UTR of the hly (limo0202) mRNA, encoding LLO. Expression of the hly gene is directly regulated by PrfA and is much higher in the prfA* strain than in the WT strain (Figures S5A and S5B; Ripio et al., 1997). Our data thus suggested a base-pairing interaction between the 5’ and S5B; Ripio et al., 1997). Our data thus suggested a prfA* expression of the prsA2 gene is directly regulated by PrfA and is much higher in the prfA* strain than in the WT strain (Figures S5A and S5B; Ripio et al., 1997). Our data thus suggested a base-pairing interaction between the 5’ UTR of prsA2, encoding the chaperone, and the 3’ UTR of hly mRNA, encoding its substrate. To validate this interaction, we introduced complementary base substitutions in the chromosome corresponding to the proposed site of interaction between the prsA2 5’ UTR (M1) and the hly 3’ UTR (M2) (Figure 4A). These mutations disrupt the GC-rich motif located in the center of the putative hly-prsA2 interaction site and when introduced alone should significantly diminish the mRNA-mRNA interaction. However, the simultaneous introduction of the complementary M1 and M2 mutations (strain M1+M2) should restore the base-pairing interaction between hly and prsA2. Despite several attempts, the strain simultaneously carrying both M1 and M2 mutations could not be created in the prfA* strain background. Instead, we introduced chromosomal mutations in WT L. monocytogenes and induced virulence factor expression by growing bacteria in a special medium containing glucose-1-phosphate and a polymeric non-polar adsorbent (Figure S5B; Ermolaeva et al., 2004). The M1 and M2 mutant strains had significantly lower prsA2 mRNA levels and protein levels compared with the WT strain (Figures 4B and 4C). In contrast, the M1+M2 mutant had prsA2 and PrsA2 levels almost as high as the WT. Our results thus suggest that the 3’ UTR of the hly transcript is able to directly bind 5’ UTR of the prsA2 transcript. Importantly, none of these mutations significantly affected the levels of the hly mRNA or LLO protein expression. An increased appearance of truncated LLO was observed in the M1 and M2 mutant strains, when the level of PrsA2 was reduced, as has been shown previously (Figure 4C, arrow; Alonzo et al., 2009). Whether this is due to an increased proteolysis and/or synthesis of shorter peptide due to partial degradation of the prsA2 transcript is unknown.

The DMS profile of the prsA2 (limo2219) 5’ UTR in the Δhfq strain did not show any difference with the WT L. monocytogenes (Table S4), suggesting that the interaction between hly and prsA2 mRNAs does not require the Hfq chaperone.

**The Regulatory Region in the 3’ UTR Is Part of the Full-Length hly Transcript**

The 3’ UTR regions of bacterial mRNAs can serve as reservoirs for sRNAs and are produced either by transcription from internal promoters or by mRNA processing (Miyakoshi et al., 2015). We therefore examined whether the regulatory region in the hly 3’ UTR was present as a short form or as a part of the full-length hly transcript. The northern blot results showed that the hly 3’ UTR was exclusively detected as part of the full-length hly transcript (Figures 4D and S5C). This is in line with previous studies where analysis of L. monocytogenes transcriptomes did not detect any short transcripts generated from the 3’ end portion of the hly mRNA (Mraheil et al., 2011; Toledo-Arana et al., 2009). In contrast, we readily detected the short RNA RII51, originating from the 5’ UTR of the mpl mRNA (Toledo-Arana et al., 2009). Hence our data strongly indicate that the full-length hly mRNA acts in trans by a direct interaction with the prsA2 mRNA.

hly Protects prsA2 mRNA from RNaseJ1-Mediated Degradation

In contrast with the LhrA-imo0850 interaction (Figure 3E), we did not observe increased DMS protection of the prsA2 SD sequence upon interaction with hly, indicating that the hly-prsA2 interaction does not affect ribosome binding (Figure 4A). Furthermore, abolition of the interaction between the prsA2 and hly mRNAs decreased the quantities of both prsA2 mRNA and PrsA2 protein equally, suggesting that the hly-prsA2 interaction might instead affect the stability of the prsA2 mRNA (Figures 4B and 4C). To examine this, we measured prsA2 mRNA stability following addition of rifampicin to prevent new transcription initiation. The half-life of prsA2 mRNA was reduced in the M1 and M2 mutant strains lacking a functional hly-prsA2 interaction (Figures 5A and S5D). In agreement with the expression data, the half-life of the prsA2 transcript was increased in the M1+M2 double-mutant strain, in which the hly-prsA2 interaction was restored. Unexpectedly, we observed a similar pattern of prsA2 transcript stability in the strains also at non-inducing conditions (Figure 5A), suggesting that hly also stabilizes prsA2 under non-inducing conditions, despite being expressed at much lower levels.

Our data suggest that the hly mRNA interacts with the extreme 5’ end of the prsA2 mRNA (Figure 4A). In Bacillus subtilis, RoxS binding to the extreme 5’ end of the yflS mRNA protects it from the 5’–3’ exoribonuclease activity of RNase J1 (Durand et al., 2017). L. monocytogenes is very similar to B. subtilis with respect to the set of RNases that these organisms use for RNA degradation (Durand et al., 2015). We therefore examined whether hly binding could protect the prsA2 mRNA from degradation by RNase J1. RNA fragments representing the 5’ UTR of the WT prsA2 and the M1 mutant derivative were transcribed in vitro. The RNAs contained a 32P-labeled 5’-monophosphate group to mimic removal of the 5’-triphasate group by RNA pyrophosphohydrolase activity in vivo, allowing access to RNase J1. RNase J1-mediated 5’–3’ exoribonuclease activity caused the liberation of the first nucleotide, 32P-labeled GMP, and this was used as a measure of RNase J1 activity (Durand et al., 2017). The WT prsA2 and prsA2-M1 RNAs were subjected to degradation by RNase J1 alone, or in the presence of the hly mRNA with a WT 3’ UTR or hly-M2 mutant mRNA (124 nt) (Figures 5B and 5C). The accumulation of free GMP indicated rapid degradation of both prsA2 and prsA2-M1 transcripts by RNase J1 in the absence of the hly mRNA (Figures 5B and 5D). The degradation of the WT prsA2 was significantly inhibited by the addition of hly, whereas the effect of adding hly-M2 was negligible (Figures 5B and 5D). For the prsA2-M1 mutant, we observed the reverse situation: addition of hly-M2 (but not hly) caused significantly stronger inhibition of RNase J1 activity compared with the WT hly transcript (Figures 5C and 5D).
Collectively, these data suggest that hly binding increases prsA2 stability by protecting it from degradation by RNase J1, and that the interaction sites in the hly 3′ UTR and prsA2 5′ UTR are required for this protection. Despite undertaking several strategies, we have been unable to create a L. monocytogenes strain lacking RNase J1. Although RNase J1 is not essential in B. subtilis, it has been shown to be essential in some Gram-positive bacteria (Bugrysheva and Scott, 2010).

Figure 5. Interaction with hly Protects prsA2 mRNA from Degradation by RNase J1
(A) The half-life of prsA2 mRNA in L. monocytogenes EGDe (WT) strain and prsA2 and hly UTR mutants under inducing and non-inducing conditions. RNA was isolated from indicated strains grown at virulence-inducing or non-inducing conditions, respectively, at indicated time points after addition of rifampicin. The prsA2 transcript was detected by northern blot (representative figure is shown in Figure S5D) and quantified before being normalized to the quantity of tmRNA. The expression levels were normalized to the expression level before rifampicin addition (0 min) for each strain, respectively. For each time point, the mean and standard deviation of two measurements are shown. For each sample, the fitted log-linear curve that was used for half-life calculations is shown. In the WT and M1+M2 samples, the 0-, 2-, 5-, and 10-min time points after rifampicin addition were used for half-life calculations. For the M1 and M2 samples, the 0-, 2-, and 5-min time points after rifampicin addition were used for half-life calculations. Because prsA2 had very low quantities at 10 min in these strains, this time point was excluded from half-life calculations. The data in the table (right) represent the mean and 95% confidence interval calculated on the basis of two measurements.

(B) The 3′ UTR of hly mRNA protects the 5′ UTR of prsA2 mRNA from degradation by RNase J1 in vitro. The RNA fragments corresponding to the first 53 nt of the prsA2 mRNA carrying a 32P-labeled 5′-monophosphate group. The fragment was incubated with RNase J1 for the indicated amount of time in the absence or in the presence of WT hly transcript (hly) or a hly transcript carrying the M2 mutations (hly-M2). In parallel, the control was incubated in the absence of the enzyme (10-). The bands correspond to the full-length prsA2 5′ UTR and its fragments (upper panels) and the released GMP (lower panels).

(C) The 3′ UTR of hly-M2 mRNA protects the 5′ UTR of prsA2-M1 mRNA from degradation by RNase J1 in vitro. The RNA fragments corresponding to the first 53 nt of prsA2-M1 mRNA carrying a 32P-labeled 5′-monophosphate group. The fragment was incubated with RNase J1 for the indicated amount of time in the absence or in the presence of WT hly transcript (hly) or a hly transcript carrying the M2 mutations (hly-M2). In parallel, the control was incubated in the absence of the enzyme (10-). The bands correspond to the full-length prsA2-M1 and fragments (upper panels) and the released GMP (lower panels).

(D) The dynamics of GMP release from prsA2 (left panel) and prsA2-M1 (right panel) substrate RNAs by RNase J1 ± hly or hly-M2 mRNAs. The data shown correspond to the quantification of two independent experiments from Figures 5B and 5C (technical replicates). The error bars represent standard deviations, and the asterisks indicate statistically significant differences in the amounts of released GMP after 10-min incubation for prsA2 versus prsA2+hly and prsA2-M1 versus prsA2-M1+hly-M2 (Student’s t test, *p < 0.05).
The hly-prsA2 Interaction Is Important for L. monocytogenes Pathogenicity

PrsA2 is an important virulence factor, and a L. monocytogenes strain lacking PrsA2 is severely attenuated during infection (Alonzo and Freitag, 2010; Alonzo et al., 2009, 2011; Cahoon and Freitag, 2015; Cahoon et al., 2016; Port and Freitag, 2007; Zemansky et al., 2009). To examine whether the hly-prsA2 interaction is required for infectivity, we first monitored our set of strains (WT, M1, M2, and M1+M2) in a plaque assay, where the ability of the bacteria to spread from cell to cell is monitored (Sun et al., 1990). The M1 and M2 mutant strains lacking a functional hly-prsA2 interaction showed a significantly reduced capacity to spread between cells as compared with the WT and M1+M2 strains (Figures 6A and 6B). In line with the results from the plaque assay, the M1 and M2 strains were attenuated (2–3 orders of magnitude) in their ability to colonize the liver and spleen in mice compared with the WT strain (Figure 6C). It should be noted that strains completely lacking PrsA2 exhibit more severe infection attenuation in comparison with the levels observed for the M1 and M2 mutants (Alonzo and Freitag, 2010; Alonzo et al., 2009, 2011; Cahoon and Freitag, 2015; Cahoon et al., 2016; Port and Freitag, 2007; Zemansky et al., 2009). This suggests that the residual PrsA2 expressed in the M1 and M2 mutants (~30% of WT) provides some measure of chaperone activity that contributes to infection. The double-mutant M1+M2 strain showed a significantly enhanced ability to colonize the liver and spleen compared with the M1 and M2 single-mutant strains, although in contrast with the full complementation observed for the plaque assay, full virulence was not restored. This could indicate that either the 5' UTR of prsA2 or...
The action of regulatory RNAs is often based on dynamic transcripts. However, the functional changes of RNA structure are possible to simultaneously compare RNA structures for multiple transcripts from cell to cell and to colonize the liver and spleen of mice was reduced significantly compared with the PrfA* strain alone (Figures S6A–S6C). Overall, our data suggest that a direct hly-prsA2 interaction is required for L. monocytogenes infectivity.

The genus Listeria contains 20 species. Of these, only three are hemolytic and encode LLO (L. monocytogenes, L. ivanovii, and L. seeligeri). To examine whether the hly-prsA2 interaction was conserved in these strains, we compared the 3′ UTRs of hly (Figures 6D and S6). The region of the hly 3′ UTR that interacts with prsA2 in L. monocytogenes is conserved in L. ivanovii and L. seeligeri. This is especially evident for the region interacting with the extreme 5′ end of prsA2, strongly suggesting that the hly-prsA2 interaction is also functionally important in other hemolytic Listeria species (Figure S6D).

## DISCUSSION

The action of regulatory RNAs is often based on dynamic changes in base-pairing interactions. We employed this principle to search for novel regulatory RNAs in the human pathogenic bacterium L. monocytogenes. The recent development of high-throughput approaches for RNA structure probing made it possible to simultaneously compare RNA structures for multiple transcripts. However, the functional changes of RNA structure can be small (Meyer et al., 2017), and their detection can require very high sequencing coverage. Because the 5′ UTRs have been shown to be an important regulatory region, we focused on the structures of the 5′ ends of bacterial mRNAs and used an enrichment protocol that increases their sequencing coverage. A conceptually similar approach has previously been used to selectively profile the structure of eukaryotic 3′ UTRs (Wu and Bartel, 2017).

Comparison of DMS reactivities at different temperatures uncovered a structural rearrangement in the 3′ UTR of an mRNA encoding the major cold shock protein CspA. When the temperature increases, the cspA 5′ UTR rearranges from the “open” to “closed” conformation, and its ribosome-binding site becomes occluded in a hairpin. Our study suggests that this new element acts as an RNA thermoswitch that inhibits cspA translation when the temperature increases to above ~30°C. A functionally similar RNA thermoswitch has previously been discovered in E. coli, where it also regulates translation of the cspA mRNA (Figure S3E; Giuliodori et al., 2010; Zhang et al., 2018). In E. coli and S. aureus, the cspA 5′ UTR also plays a role in autoregulation of CspA expression. In E. coli, the CspA protein binds to the 5′ UTR of its own mRNA and shifts the equilibrium to a conformation that inhibits translation (Zhang et al., 2018). In S. aureus, RNase III increases translation of the cspA mRNA by cleaving a hairpin in its 5′ UTR. CspA binds and unwinds a U-rich motif in the hairpin, thus interfering with RNase III cleavage and leading to inhibition of cspA translation (Caballero et al., 2018). A similar autoinhibitory loop might exist in L. monocytogenes: the U-rich motif that participates in formation of the hairpin in the open conformation might represent an attractive target for CspA binding (Figure 3A). The unwinding of this hairpin would shift the structure equilibrium to the closed conformation.

The other regulatory events we detected in our study involved the base-pairing of two independent transcripts to each other. When studying how the deletion of the RNA chaperone Hfq affects RNA structure, we detected a “footprint” in the Imo0850 mRNA. The LhrA-Imo0850 interaction has been thoroughly studied before, showing that LhrA blocks ribosome binding to the SD in a Hfq-dependent manner (Nielsen et al., 2010). Previously, the footprints of sRNAs on their targets have almost exclusively been demonstrated in vitro (Mollerup et al., 2016; Sharma et al., 2007). The detection of an interaction between two RNAs in vivo suggested that RNA structure profiling can help to discover novel trans-acting regulatory RNAs. The structure probing method provides orthogonal information about interactions between RNA molecules and can be used to complement other methods to search for novel trans-acting (small) RNA targets. The traditional approaches to search for the targets of sRNAs in bacteria include genetic screens (Vogel and Sharma, 2005), mutation analysis or overproduction of sRNAs, and studying how this affects the transcriptome or proteome (Borgmann et al., 2018; Nielsen et al., 2011). They also include bioinformatics approaches (Pain et al., 2015). More recently developed approaches are based on pull-down of proteins mediating RNA-RNA interactions followed by proximity ligation and high-throughput sequencing of the hybrid fragments (Melamed et al., 2016; Waters et al., 2017). Proximity ligation methods have proved useful to detect novel (and unexpected) RNA-RNA interactions.

In this work, we also discovered an interaction between two mRNAs, through the detection of a “footprint” that appeared on the prsA2 5′ UTR upon activation of the L. monocytogenes virulence program (Figures 4, 5, and S5). This was followed by bioinformatics searches for transcripts predicted to form base pair interactions with this footprint, with the 3′ UTR of the full-length hly transcript showing the best match. Most trans-acting RNAs discovered in bacteria represent short transcripts. Although some of them code for polypeptides, typically their length does not exceed 80 amino acids (Raina et al., 2018). Currently, there is only one example of a “classical” mRNA acting in trans to regulate expression of another mRNA: the 5′ UTR of the relatively short invA mRNA (~520 nt) from S. mutans base pairs with the coding region of gbpC mRNA and protects it from RNase J2 degradation (Liu et al., 2015). In this study, we expand the repertoire of described mRNA-mRNA interactions by showing that in L. monocytogenes the 3′ UTR of the full-length hly transcript directly binds the 5′ UTR of prsA2 mRNA, protecting it from degradation by RNase J1 (and perhaps other RNases), thereby increasing PrsA2 chaperone production (Figure 7). Surprisingly, even the low amount of hly mRNA expressed in non-virulence conditions is sufficient to stabilize the prsA2 transcript, suggesting a role for the hly transcript also outside the host.

It is intriguing to speculate that the hly-prsA2 mRNA interaction may serve to couple translation and secretion of the two proteins, thereby potentially providing readily available PrsA2 for...
LLO folding. So far, most 3’ UTRs with a regulatory role have been shown to act as individual sRNAs, through processing from the full-length mRNA or by having an ORF-internal promoter (Miyakoshi et al., 2015). Here, we have identified a regulatory 3’ UTR that remains attached to its coding sequence. Because the LLO and PrsA2 proteins interact, in this case it would make sense that the 3’ UTR remains attached to the hly transcript so that the hly and prsA2 transcripts stay associated with each other to facilitate the interaction of their products.

It was unexpected that only a 3-fold reduction in PrsA2 levels would have such a strong impact on L. monocytogenes virulence. However, although the results from plaque assays indicate full restoration of cell-to-cell spread in the M1+M2 double mutant, it was apparent that the strain bearing complementary mutations in both the 3’ UTR of hly and the 5’ UTR of prsA2 did not fully restore virulence in mice to the WT levels (although virulence was significantly increased in comparison with the single mutants). We therefore considered the possibility that hly might have other (PrfA-regulated) targets than prsA2 or vice versa, especially because we were unable to create a M1-M2 double mutant in the PrfA+ background. The only obvious binding candidate for the prsA2 mRNA was hly (Table S5). However, when examining for possible targets of the hly 3’ UTR that could explain the phenotype, one significant candidate appeared that had a stronger binding score than prsA2: the transcriptional termination region of lmo2494 (Table S5). This region also involves an antisense RNA, Rli142, which overlaps the terminator of lmo2494 (Wurtzel et al., 2012). lmo2494 encodes the phosphate uptake regulator PhoU and is part of an operon encoding proteins important for phosphate uptake (pstSCAB-phoU). Phosphate uptake has been shown to be important for Salmonella pathogenicity, and its regulation also involves an antisense RNA, ArgR, that targets the virulence-associated protein MgtC (Lee and Groisman, 2010). Expression of the genes in the pstSCAB-phoU operon is upregulated in the intestine in a SigB- and PrfA-dependent manner (Toledo-Arana et al., 2009), but do not have an obvious PrfA-box. Interestingly, the suggested interaction site in hly overlaps with the prsA2 binding site by 11 bases. Further work is required to define whether the expression of the lmo2494 operon is controlled by hly and/or whether phosphate uptake might be important for virulence.

Although we did not comprehensively assess the performance of our 5’ UTR enrichment strategy, it allowed us to discover functional elements by sequencing DMS-MaPseq libraries of moderate size (=10 million sequencing reads). Because lmo0850 and prsA2 are expressed only at low levels, the 5’ UTR enrichment was necessary to observe differences in DMS reactivity between different conditions. Larger libraries would increase the depth and might aid in the identification of more complex regulatory events. Also, the datasets obtained by FUSE in different conditions stems from few replicates. Despite this, we were able to confirm the discoveries made by FUSE using other methods.

In summary, we have identified RNA regulatory interactions through the targeted structural analyses of L. monocytogenes 5’ UTRs in vitro and in vivo. As exemplified in this and other studies, different MaP-seq methods can be used to identify both intragenic and intergenic RNA-RNA interactions, and we consider FUSE to be an excellent screening approach that requires further analysis to reveal details of mechanisms. Hence such approaches will be very fruitful to reveal new hidden RNA-based mechanisms.

**STAR METHODS**

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Supplemental Information can be found online at https://doi.org/10.1016/j.cellrep.2020.03.006.

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The authors declare no competing interests.

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REFERENCES
Alonzo, F., 3rd, and Freitag, N.E. (2010). Listeria monocytogenes PrsA2 is required for virulence factor secretion and bacterial viability within the host cell cytosol. Infect. Immun. 78, 4944–4957.
Alonzo, F., 3rd, Port, G.C., Cao, M., and Freitag, N.E. (2009). The posttranslational chaperone PrsA2 contributes to multiple facets of Listeria monocytogenes pathogenesis. Infect. Immun. 77, 2612–2623.
Alonzo, F., 3rd, Xayarath, B., Whisstock, J.C., and Freitag, N.E. (2011). Functional analysis of the Listeria monocytogenes secretion chaperone PrsA2 and its multiple contributions to bacterial virulence. Mol. Microbiol. 80, 1530–1548.
Anders, S., Pyl, P.T., and Huber, W. (2015). HTSeq—a Python framework to work with high-throughput sequencing data. Bioinformatics 31, 166–169.
Arnaud, M., Chastanet, A., and Débarbouillé, M. (2004). New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, gram-positive bacteria. Appl. Environ. Microbiol. 70, 6887–6891.
Barrett, T., Wiillite, S.E., Ledoux, P., Evangelista, C., Kim, I.F., Tomashevsky, M., Marshall, K.A., Phillippy, K.H., Sherman, P.M., Holko, M., et al. (2013). NCBI GEO: archive for functional genomics data sets–update. Nucleic Acids Res. 41, D991–D995.

Batey, R.T., Rambo, R.P., Lucat, L., Rha, B., and Doudna, J.A. (2000). Crystal structure of the ribonucleoprotein core of the signal recognition particle. Science 287, 1232–1239.
Batey, R.T., Sagar, M.B., and Doudna, J.A. (2001). Structural and energetic analysis of RNA recognition by a universally conserved protein from the signal recognition particle. J. Mol. Biol. 307, 229–246.
Benjamini, Y., and Hochberg, Y. (1995). Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. J. R. Stat. Soc. Ser. B Stat. Methodol. 57, 289–300.
Borgmann, J., Schäkermann, S., Bandow, J.E., and Narberhaus, F. (2018). A Small Regulatory RNA Controls Cell Wall Biosynthesis and Antibiotic Resistance. mBio 9, e02100–e2118.
Bugay, J.V., and Scott, J.R. (2010). The ribonucleases J1 and J2 are essential for growth and have independent roles in mRNA decay in Streptococcus pyogenes. Mol. Microbiol. 75, 731–743.
Burkhardt, D.H., Rouskin, S., Zhang, Y., Li G.W., Weissman, J.S., and Gross, C.A. (2017). Operon mRNAs are organized into ORF-centric structures that predict translation efficiency. eLife 6, e22037.
Caballero, C.J., Menendez-Gil, P., Catalan-Moreno, A., Vergara-Ingary, M., Garcia, B., Segura, V., Iruzun, N., Villanueva, M., Ruiz de Los Mozos, I., Solano, C., et al. (2018). The regulon of the RNA chaperone CspA and its auto-regulation in Staphylococcus aureus. Nucleic Acids Res. 46, 1345–1361.
Cahoon, L.A., and Freitag, N.E. (2015). Identification of Conserved and Species-Specific Functions of the Listeria monocytogenes PrsA2 Secretion Chaperone. Infect. Immun. 83, 4028–4041.
Cahoon, L.A., Freitag, N.E., and Prehna, G. (2016). A structural comparison of Listeria monocytogenes prsA2 protein chaperones PrsA1 and PrsA2 reveals molecular features required for virulence. Mol. Microbiol. 101, 42–61.
Chakravarty, S., and Massé, E. (2019). RNA-Dependent Regulation of Virulence in Pathogenic Bacteria. Front. Cell. Infect. Microbiol. 9, 337.
Christiansen, J.K., Larsen, M.H., Ingmer, H., Segaard-Andersen, L., and Kallipolitis, B.H. (2004). The RNA-binding protein Hfq of Listeria monocytogenes: role in stress tolerance and virulence. J. Bacteriol. 186, 3355–3362.
Darby, K., Denis, A., and Ponty, Y. (2009). VARNA: Interactive drawing and editing of the RNA secondary structure. Bioinformatics 25, 1974–1975.
de las Heras, A., Cain, R.J., Bielecka, M.K., and Vázquez-Boland, J.A. (2011). Regulation of Listeria virulence: Pfrα master and commander. Curr. Opin. Microbiol. 14, 118–127.
Di Tommaso, P., Moretti, S., Xenarios, I., Orobitg, M., Montanyola, A., Chang, J.M., Taly, J.F., and Notredame, C. (2011). T-Coffee: a web server for the multiple sequence alignment of protein and RNA sequences using structural information and homology extension. Nucleic Acids Res. 39, W13–W17.
Dos Santos, P.T., Menendez-Gil, P., Sabharwal, D., Christensen, J.H., Brunhede, M.Z., Lillebaek, E.M.S., and Kallipolitis, B.H. (2018). The Small Regulatory RNA LrHCl1-5 Contribute to the Response of Listeria monocytogenes to Heme Toxicity. Front. Microbiol. 9, 599.
Durand, S., Tomasini, A., Braun, F., Condon, C., and Romby, P. (2015). sRNA and miRNA turnover in Gram-positive bacteria. FEMS Microbiol. Rev. 39, 316–330.
Durand, S., Braun, F., Hefter, A.C., Romby, P., and Condon, C. (2017). sRNA-mediated activation of gene expression by inhibition of 5′-3′ exonucleolytic mRNA degradation. eLife 6, e23602.
Ermolaeva, S., Novella, S., Vega, Y., Ripio, M.T., Scortti, M., and Vázquez-Boland, J.A. (2004). Negative control of Listeria monocytogenes virulence genes by a diffusable autorepressor. Mol. Microbiol. 52, 601–611.
Gaubig, L.C., Waldminghaus, T., and Narberhaus, F. (2011). Multiple layers of control govern expression of the Escherichia coli ibpAB heat-shock operon. Microbiology 157, 66–76.
Giuiliodori, A.M., Di Pietro, F., Marzi, S., Masquida, B., Wagner, R., Romby, P., Qualeri, C.O., and Pon, C.L. (2010). The cspA mRNA is a thermosensor that modulates translation of the cold-shock protein CspA. Mol. Cell 37, 21–33.
Good, J.A., Andersson, C., Hansen, S., Wall, J., Krishnan, K.S., Begum, A., Grundström, C., Niemiec, M.S., Vaitekvičius, K., Chorrell, E., et al. (2016). Attenuating Listeria monocytogenes Virulence by Targeting the Regulatory Protein PrfA. Cell Chem. Biol. 23, 404–414.

Guo, J.U., and Bartel, D.P. (2016). RNA G-quadruplexes are globally unfolded in eukaryotic cells and depleted in bacteria. Science 353, 8a5537i.

Huttenhoffer, A., and Noller, H.F. (1994). Footprinting mRNA-ribosome complexes with chemical probes. EMBO J. 13, 3892–3901.

Johansson, J., Mandin, P., Renzoni, A., Chiaruttini, C., Springer, M., and Cossart, P. (2002). An RNA thermosensor controls expression of virulence genes in Listeria monocytogenes. Cell 110, 551–561.

Klinkert, B., Cimdins, A., Gaubig, L.C., Roßmanith, J., Aschke-Sonnenborn, U., and Narberhaus, F. (2012). Thermogenetic tools to monitor temperature-dependent gene expression in bacteria. J. Biotechnol. 160, 55–63.

Langmead, B., and Salzberg, S.L. (2012). Fast gapped-read alignment with Bowtie 2. Nat. Methods 9, 357–359.

Lebreton, A., and Cossart, P. (2017). RNA- and protein-mediated control of Listeria monocytogenes virulence gene expression. RNA Biol. 14, 460–470.

Lee, E.J., and Groisman, E.A. (2010). An antisense RNA that governs the expression kinetics of a multifunctional virulence gene. Mol. Microbiol. 76, 1020–1033.

Liu, N., Niú, G., Xie, Z., Chen, Z., Itzek, A., Kreth, J., Gillaspy, A., Zeng, L., Byrne, R., Qi, F., and Merritt, J. (2015). The Streptococcus mutans inA gene encodes a trans-acting riboregulatory mRNA. Mol. Cell 57, 179–190.

Loh, E., Gripenland, J., and Johansson, J. (2006). Control of Listeria monocytogenes virulence by 5′-untranslated RNA. Trends Microbiol. 14, 294–298.

Loh, E., Dussurget, O., Gripenland, J., Vaitekvičius, K., Tiensuu, T., Mandin, P., Repolla, F., Buchrieser, C., Cossart, P., and Johansson, J. (2009). A trans-acting riboswitch controls expression of the virulence regulator PrfA in Listeria monocytogenes. Cell 139, 779–789.

Lorenz, R., Bernhart, S.H., Höner Zu Siederdissen, C., Tafer, H., Flammar, C., Stadler, P.F., and Hofacker, I.L. (2011). ViennaRNA Package 2.0. Algorithms Mol. Biol. 6, 26.

MacKenzie, G.B. (1964). The Immunological Basis of Acquired Cellular Resistance. J. Exp. Med. 120, 105–120.

Masachis, S., and Darfeuille, F. (2018). Type I toxin-antitoxin Systems: Regulating Toxin Expression via Shine-Dalgarno Sequence Sequestration and Small RNA Binding. Microbiol. Spectr. 6, WRR-0030-2018.

Mathy, N., Bénard, L., Pellegrini, O., Daou, R., Wen, T., and Condon, C. (2007). Small RNA Binding. Microbiol. Spectr. 0, 4039–4048.

Guo, J.U., and Bartel, D.P. (2016). Virus-Induced Changes in mRNA Secondary Structure Uncover cis-Regulatory Elements that Directly Control Gene Expression. Mol. Cell 72, 862–874.e5.

Cell Reports 30, 4027–4040, March 24, 2020 4039
et al. (2013). Base pairing interaction between 5′- and 3′-UTRs controls icar mRNA translation in Staphylococcus aureus. PLoS Genet. 9, e1004001.

Schmid, B., Klumpp, J., Raimann, E., Loessner, M.J., Stephan, R., and Tasara, T. (2009). Role of cold shock proteins in growth of Listeria monocytogenes under cold and osmotic stress conditions. Appl. Environ. Microbiol. 75, 1621–1627.

Sharma, C.M., Darfeuille, F., Plantinga, T.H., and Vogel, J. (2007). A small RNA regulates multiple ABC transporter mRNAs by targeting C/A-rich elements inside and upstream of ribosome-binding sites. Genes Dev. 21, 2804–2817.

Sievers, S., Sternkopf Lillebæk, E.M., Jacobsen, K., Lund, A., Mollerup, M.S., Nielsen, P.K., and Kallipolitis, B.H. (2014). A multicopy sRNA of Listeria monocytogenes regulates expression of the virulence adhesin LapB. Nucleic Acids Res. 42, 9383–9398.

Sievers, S., Lund, A., Menendez-Gil, P., Nielsen, A., Storm Mollerup, M., Lambert Nielsen, S., Buch Larsson, P., Borch-Jensen, J., Johansson, J., and Kallipolitis, B.H. (2015). The multicopy sRNA LhrC controls expression of the oligopeptide-binding protein OppA in Listeria monocytogenes. RNA Biol. 12, 985–997.

Strobel, E.J., Yu, A.M., and Lucks, J.B. (2018). High-throughput determination of RNA structures. Nat. Rev. Genet. 19, 615–634.

Sun, A.N., Camilli, A., and Portnoy, D.A. (1990). Isolation of Listeria monocytogenes small-plaque mutants defective for intracellular growth and cell-to-cell spread. Infect. Immun. 58, 3770–3778.

Thorvaldsdóttir, H., Robinson, J.T., and Mesirov, J.P. (2013). Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. Brief. Bioinform. 14, 178–192.

Toledo-Arana, A., Dussurget, O., Nikitas, G., Sesto, N., Guet-Revillet, H., Bailestrino, D., Loh, E., Grieppenjohann, J., Tiensuu, T., Valtkevicius, K., et al. (2009). The Listeria transcriptional landscape from saprophytism to virulence. Nature 459, 950–956.

Updegrove, T.B., Zhang, A., and Storz, G. (2016). Hfq: the flexible RNA matchmaker. Curr. Opin. Microbiol. 30, 133–138.

Vogel, J., and Sharma, C.M. (2005). How to find small non-coding RNAs in bacteria. Biol. Chem. 386, 1219–1238.

Waters, L.S., and Storz, G. (2009). Regulatory RNAs in bacteria. Cell 136, 615–628.

Waters, S.A., McAteer, S.P., Kudla, G., Pang, I., Deshpande, N.P., Amos, T.G., Leong, K.W., Wilkins, M.R., Strugnell, R., Gally, D.L., et al. (2017). Small RNA interactome of pathogenic E. coli revealed through crosslinking of RNase E. EMBO J. 36, 374–387.

Wright, P.R., Georg, J., Mann, M., Sorescu, D.A., Richter, A.S., Lott, S., Klein-kauf, R., Hess, W.R., and Backofen, R. (2014). CopraRNA and IntaRNA: predicting small RNA targets, networks and interaction domains. Nucleic Acids Res. 42, W119–W123.

Wu, X., and Bartel, D.P. (2017). Widespread influence of 3′end structures on mammalian mRNA processing and stability. Cell 169, 905–917.e11.

Wurtzel, O., Sesto, N., Mellin, J.R., Karunker, I., Edelheit, S., Bécavin, C., Archambaud, C., Cossart, P., and Sorek, R. (2012). Comparative transcriptomics of pathogenic and non-pathogenic Listeria species. Mol. Syst. Biol. 8, 583.

Zhang, Y., Burkhardt, D.H., Rouskin, S., Li, G.W., Weissman, J.S., and Gross, C.A. (2018). A Stress Response that Monitors and Regulates mRNA Structure Is Central to Cold Shock Adaptation. Mol. Cell 70, 274–286.e7.

Zubradt, M., Gupta, P., Persad, S., Lambowitz, A.M., Weissman, J.S., and Rouskin, S. (2017). DMS-MaPseq for genome-wide or targeted RNA structure probing in vivo. Nat. Methods 14, 75–82.
STAR★METHODS

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Anti-Listeriolysin (LLO) antibody | Abcam | Cat# ab200538 |
| Anti-P60 antibody | Biosite | Cat# P6017 |
| Rabbit polyclonal anti-PrsA2 antibody | (Alonzo et al., 2009) | N/A |
| Polyclonal anti-ActA antibody | (Netterling et al., 2015) | N/A |
| Anti-rabbit-horseradish peroxidase secondary antibody | Agrisera | Cat# AS09 602 |
| Bacterial and Virus Strains |        |            |
| Listeria monocytogenes EGD-e | (MacKaness, 1964) | N/A |
| L. monocytogenes EGD-e Δhfq | (Christiansen et al., 2004) | N/A |
| L. monocytogenes EGD-e LhrA_mut | (Nielsen et al., 2010) | N/A |
| L. monocytogenes EGD-e prfA* | (Good et al., 2016) | N/A |
| L. monocytogenes EGD-e 5UTR2219-M1 | This study | N/A |
| L. monocytogenes EGD-e 3UTR0202-M2 | This study | N/A |
| L. monocytogenes EGD-e 5UTR2219-M1 3UTR0202-M2 | This study | N/A |
| L. monocytogenes EGD-e prfA* 5UTR2219-M1 | This study | N/A |
| L. monocytogenes EGD-e prfA* 3UTR0202-M2 | This study | N/A |
| Chemicals, Peptides, and Recombinant Proteins |        |            |
| Purified C-terminal His tagged RNase J1 | (Mathy et al., 2007) | N/A |
| Deposited Data |        |            |
| Raw and analyzed NGS data | This study | GEO: GSE118387 |
| Scripts for data analysis | This study | https://github.com/dimaignatov/5-UTR-structure-elucidation |
| Experimental Models: Cell Lines |        |            |
| L2 mouse fibroblasts | (Sun et al., 1990) | N/A |
| Experimental Models: Organisms/Strains |        |            |
| Swiss Webster (CFW) Mouse, female | Charles River Laboratories | Cat# 024, https://www.criver.com/products-services/find-model/swiss-webster-cfw-mouse?region=3616 |
| Oligonucleotides |        |            |
| See Table S6 |        | N/A |
| Recombinant DNA |        |            |
| See Table S8 |        | N/A |
| Software and Algorithms |        |            |
| Bowtie2 | Langmead and Salzberg, 2012 | http://bowtie-bio.sourceforge.net/bowtie2/index.shtml |
| HTSeq framework | Anders et al., 2015 | https://htseq.readthedocs.io/en/release_0.11.1/index.html |
| Integrative Genomics Viewer | Thorvaldsdóttir et al., 2013 | https://software.broadinstitute.org/software/igv/ |
| VARNA | Darty et al., 2009 | http://varna.lri.fr/ |
| RNAfold WebServer | Lorenz et al., 2011 | http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi |
| T-Coffee Multiple Sequence Alignment Server | Di Tommaso et al., 2011 | http://tcoffee.crg.cat/ |

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and resource requests should be directed to and will be fulfilled by the Lead Contact, Jörgen Johansson (jorgen.johansson@umu.se). This study did not generate new unique reagents.
**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Bacterial strains and growth conditions**
For cloning purposes and reporter gene assay we used E. coli strain DH5α, which was grown in LB medium at constant shaking. For RNA structure profiling and other experiments L. monocytogenes EGDe strain and its mutants (Table S8) were used. Before each experiment L. monocytogenes cells were grown overnight in BHI medium (BD Biosciences) at 37 °C and constant shaking. For structure probing, the bacteria were diluted 1:100 in 25 mL fresh BHI and grown to OD600 = 1.0. Growth was followed using a spectrophotometer (Amersham Biosciences).

For the experiments requiring induction of PrfA virulence regulator, L. monocytogenes cells were diluted 1:100 in 25 mL of the Activation medium: 1x LB broth buffered with 50 mM MOPS pH = 7.3 and supplemented with 25mM glucose-1-phosphate and 1% Amberlite XAD4 resin (Sigma-Aldrich) (Ermolaeva et al., 2004; Ripio et al., 1997). As a negative control of the Activation medium, the bacteria were grown in 1x LB broth. The cultures were grown at constant shaking at 37°C for 5 hours until early stationary growth phase.

**Cell lines**
Mouse L2 fibroblasts were cultivated in DMEM + 5% Fetal bovine serum at 37°C in the 5% CO₂ incubator. For performing the plaque assay, 2 × 10⁸ L2 cells per well were seeded into 6-well plates and grown for 16 h before infection with L. monocytogenes.

**Animal studies**
Animal procedures were approved by the University of Illinois at Chicago Animal Care Committee and were conducted in the Biological Resources Laboratory. For infection studies female 7-9-week-old Swiss Webster mice (Charles River Laboratories) were administered 200μL containing 2×10⁴ CFU of bacteria by tail vein injection. The experimental groups consisted of randomly assigned littersmates.

**METHOD DETAILS**

**RNA isolation**
10 mL of bacterial culture was mixed with 2 mL of 1:20 phenol:ethanol solution and centrifuged at 7000 g for 10 minutes. The pellet was resuspended in Disruption solution (10% glucose, 12.5 mM Tris-HCl pH 7.6, and 5 mM EDTA) and immediately transferred to 2 mL screw-capped tubes with roughly 0.4 g glass beads and 500 μL of acid phenol (pH 4.5). The bacteria were disrupted using a mini bead beater (Biospec products) for 30 s. After centrifugation (5 min, 12000 g) RNA was recovered by addition of 1 mL of TRI Reagent Solution (Thermo Fisher) and 100 μL of chloroform, followed by centrifugation. Samples were thereafter subjected to two additional chloroform extractions. The aqueous phase was precipitated by adding isopropanol (0.7 ×) and incubation at −20°C for 20 min. For collection of the pellet, the RNA samples were centrifuged for 25 min. The pellet was washed with 80% ethanol and dissolved in 50 μL of RNase-free water.

**Treatment of L. monocytogenes cells and RNA with dimethyl sulfate**
L. monocytogenes cells (Table S1) were grown overnight in BHI media at 37 °C and constant shaking. The stationary phase cultures were diluted 1:100 in a fresh BHI buffered with 50 mM MOPS pH = 7.3 and grown with constant shaking at 26 °C or 37 °C until OD600 = 1 (mid-logarithmic growth phase). Three milliliters of cells were put at 37°C for 3 minutes. DMS was inactivated by adding 300 μL of Quenching solution (50% isoamyl alcohol and 30% β-mercaptoethanol). The cells were centrifuged and washed with 10 μL of 30% β-mercaptoethanol. The pellet was resuspended in Disruption solution and RNA was isolated. For in vitro treatment with DMS, RNA was isolated from L. monocytogenes cells grown at the same conditions. 5 μg of RNA was denatured in mQ water and folded at 37°C for 30 min in 300 μL Folding buffer (50 mM HEPES, 100 mM KCl, 6 mM MgCl₂). RNA was treated at 37°C with 3% DMS solution for 3 minutes. DMS was inactivated by adding 300 μL of 30% β-mercaptoethanol and RNA was ethanol precipitated.

**FUSE libraries**
After modification with DMS, RNA was treated with DNase I (Roche) and purified on RNeasy MinElute columns (QIAGEN). RNA was treated with RNA 5’ Polyphosphatase (Epitect) and once again purified on the columns. RNA was depleted of ribosomal fraction with Ribo-Zero tRNA Removal Kit (Illumina), and purified on RNeasy columns. The RNA solution was concentrated on a SpeedVac Concentrator (ThermoFisher Scientific) to 4.5 μL, mixed with 1 μL of 15 μM PAGE-purified 5’ RA (RNA adaptor; Table S6), heated for 3 min at 65°C and cooled on ice. The ligation reaction was assembled with T4 RNA Ligase 1 (NEB) and 10% DMSO, and incubated for 2 hours at room temperature. RNA was ethanol precipitated, dissolved in 9 μL of mQ water and fragmented with RNA Fragmentation Reagents (Thermo Fisher Scientific) for 3.5 min at 70°C. Following the fragmentation, RNA was run on 6% denaturing polyacrylamide gel, and fragments ranging in size from 125 to 400 nucleotides were isolated from the gel. RNA fragments were dephosphorylated with T4 Polynucleotide Kinase (Thermo Fisher Scientific) in the dephosphorylation buffer (100 mM MES pH6.0, 10 mM MgCl₂) and purified on RNeasy columns according to a modified protocol (100 μL of RNA solution was mixed with 350 μL RLT buffer and 550 μL 96% ethanol). The modified protocol preserves fragments shorter than 200 nucleotides and is used at the steps following...
RNA fragmentation. 3’ DA (DNA adaptor) was adenylated with a 5’ DNA Adenylation Kit (NEB). The RNA solution was concentrated to 4.5 μL and mixed with 1 μL of 15 μM adenyl-3’ DA. The ligation reaction with truncated T4 RNA Ligase 2 (NEB) and 25% PEG-8000 was allowed to proceed for 2 hours at room temperature. RNA was ethanol precipitated, dissolved in 20 μL of mQ water and treated with 1.6x vol:vol AMPure XP beads (Beckman Coulter) to remove non-ligated adaptors. cDNA was synthesized with TGIRTIII enzyme (InGex); the RNA solution was concentrated to 4.5 μL, mixed with 1 μL of 1 μM RT primer and heated for 2 min at 80°C. The primer was annealed for 5 minutes at room temperature, and the reverse transcription reaction was assembled in RT buffer (50 mM Tris-HCl pH8.3, 75 mM KCl, 3 mM MgCl₂) with 1 mM dNTPs mix, 5 mM DTT and 100U of TGIRTIII enzyme. The reverse transcription was incubated for 2 hours at 57°C. After that 1 μL of 5N NaOH was added to the reaction and the reverse transcriptase was inactivated by heating at 95°C for 3 min. The first strands of cDNA were ethanol precipitated, dissolved in mQ water and cDNA libraries were amplified by 20 rounds of PCR. The first 12 cycles of PCR were performed with primers LibAmp_F and LibAmp_RPIXX_R, which introduced sequences identical to Illumina TruSeq adapters (Oligonucleotide sequences © 2018 Illumina, Inc. All rights reserved) to cDNA. The product of the first PCR reaction was purified with 1x vol:vol AMPure XP beads and used as the matrix for the second 6-8 cycles PCR reaction with primers Enrich_F and Enrich_R. The product of the second PCR reaction was sequentially purified with RNeasy PCR Purification Kit (QIAGEN) and 1x vol:vol AMPure XP beads. The resulting libraries were sequenced on Illumina sequencing platform in 2 × 76 bp paired-end mode.

The whole-transcriptome DMS-MaPseq libraries

To prepare DMS-MaPseq libraries without 5’ UTR enrichment, RNA was depleted of ribosomal fraction with Ribo-Zero rRNA Removal Kit (Illumina), and purified on RNeasy columns. RNA was fragmented with RNA Fragmentation Reagents (Thermo Fisher Scientific) for 3.5 min at 70°C, and purified on RNeasy columns according to the modified protocol (100 μL of RNA solution was mixed with 350 μL RLT buffer (QIAGEN) and 550 μL 96% ethanol). RNA fragments were dephosphorylated with Shrimp Alkaline Phosphatase (NEB), purified on RNeasy columns according to the modified protocol, 5’ phosphorylated with T4 Polynucleotide Kinase (NEB), and once again purified on the columns according to the modified protocol. RNA solution was concentrated to 4.5 μL, mixed with 1 μL of 15 μM PAGE purified 5’RA, heated for 3 min at 65°C and cooled on ice. The ligation reaction was assembled with T4 RNA Ligase (NEB) and 10% DMSO, and incubated for 2 hours at room temperature. RNA was ethanol precipitated, run on a 6% denaturing polyacrylamide gel, and fragments ranging in size from 100 to 400 nucleotides were isolated from the gel. Adenylated 3’ DA adaptor was ligated to the RNA fragments with truncated T4 RNA Ligase 2 (NEB) in the presence of 25% PEG-8000 for 2 hours at room temperature. RNA was treated with 1.6x vol:vol AMPure XP beads (Beckman Coulter) to remove unligated adaptors. cDNA was synthesized with TGIRTIII enzyme, amplified and purified exactly the same as for the 5’ UTR-enriched libraries. The resulting libraries were sequenced on Illumina sequencing platform in 2 × 76 bp paired-end mode.

Targeted DMS-MaPseq of prfA 5’ UTR

L. monocytogenes EGD-e cells grown at 26°C or 37°C were treated with DMS and used for RNA isolation as described in the respective section of Methods. Total RNA was depleted of rRNA using Ribo-Zero rRNA Removal Reagent for Gram-positive bacteria (Illumina). Specific cDNA was synthesized with TGIRTIII reverse transcriptase (InGex) using 10 μM primer prfA_RT (Table S6). The region of prfA 5’ UTR was amplified with primers prfA_DMS_F and prfA_DMS_R. Indexes for both amplicons were added by PCR amplification using primers LibAmp_F and LibAmp_RPIXX_R (with a unique index). The libraries were further enriched by PCR using Enrich_F and Enrich_R primers as described for FUSE or the whole-transcriptome DMS-MaPseq in the respective section of STAR Methods.

Reporter gene assay

The region comprising lmo1364 (cspA) 5’ UTR and the first 10 codons was amplified from L. monocytogenes EGD-e genome with primers cspA_Nhel_F and cspA_EcoRI_R (Table S6) and cloned into pBAD2-bgaB vector (Klinkert et al., 2012). The 5’ UTRs and the first codons of the genes lmo0277, lmo0354 and lmo2110 were cloned into pBAD2-bgaB in the same manner. Mutations were introduced to the cloned cspA 5’ UTR sequence by inverse PCR with back-to-back primers cspA_M1/2/3/4_F and cspA_M1/2/3/4_R. The pBAD2-bgaB-5’ UTR plasmids (Table S8) were introduced to E. coli DH5α. Cells were grown at 26°C in LB medium supplemented with ampicillin (150 mg/L) to OD₆₀₀ = 0.5. Transcription was induced by adding 0.01% arabinose, and half of each culture was transferred to 37°C. After 30 minutes of induction, 1 mL of cultures were harvested and β-galactosidase activity was measured as described (Gaubig et al., 2011).

Native PAGE of in vitro folded cspA 5’ UTR

Matrices for in vitro transcription were amplified from the plasmids pBAD2-bgaB containing cspA 5’ UTR and its mutated forms with primers cspA_T7_F and cspA_T7_R (Table S6). In vitro transcription was performed with MAXIscript T7 Transcription Kit (Thermo Fisher Scientific) and the synthesized RNA was purified on 6% denaturing polyacrylamide gel (19:1 acrylamide/bis-acrylamide ratio). To study the effect of temperature on unfolding, 50 ng of cspA 5’ UTR was dissolved in 9 μL of Loading buffer (10 mM Tris-HCl pH7.0, 1 mM EDTA, 10% glycerol, 0.01% xylene cyanol), denatured at 95°C for 3 minutes and quickly cooled on ice. To initiate refolding, 1 μL of 500 mM NaCl solution was added, and RNA was incubated at temperatures ranging from 26°C to 37°C for 5 minutes. The folded RNA was applied to the 10% acrylamide gel containing 100 mM Tris-HEPES (pH = 7.5), 0.1 mM EDTA, 3 mM MgCl₂, and run at 4°C with a running buffer of the same composition. The RNA fragments in the gel were visualized by staining with SYBR Gold stain (Thermo Fisher Scientific).
**DMS-MaPseq of in vitro folded cspA 5’ UTR**

Template for in vitro transcription of cspA 5’ UTR was generated by PCR-amplification using primers cspA_T7_F and cspA_T7_R (Table S6). cspA 5’ UTR was produced using MEGAscript T7 Transcription kit and gel purified from 6% denaturing polyacrylamide gel (19:1 acrylamide/bis-acrylamide ratio). RNA was concentrated by ethanol precipitation and equilibrated to a desired buffering solution using gel filtration on PD SpinTrap G-25 columns (GE).

cspA 5’ UTR was dissolved to a final concentration of 5.6 ng/µl in 10 mM MOPS-Na pH 7.0, 1 mM EDTA and 10% (w/vol) glycerol (90 µl). RNA was denatured for 3 minutes at 95°C and cooled on ice. Folding was initiated by adding NaCl to a final concentration of 50 mM (100 µl final volume) and equilibrating temperature to either 26 or 37°C for 10 minutes. DMS was added at 5% and 3% (vol/vol) for 26°C and 37°C samples respectively, and incubated for 3 minutes. DMS was quenched by adding 50 µL 2-mercaptoethanol, RNA was ethanol precipitated and further processed for sequencing library preparation as described in FUSE protocol.

**Creation of L. monocytogenes mutants**

The genome fragment encompassing the 5’ UTR of prsA2 gene as well as 606 and 811 nucleotides upstream and downstream of the 5’ UTR of cspA gene was amplified with primers UTR2219_loc_NcoI and UTR2219_loc_Sall (Table S6). Similarly, the genome locus of the 3’ UTR of hly gene was amplified with primers UTR0202_loc_NcoI and UTR2219_loc_Sall. The fragments were digested with NcoI and Sall restriction enzymes and cloned to pMAD vector digested with the same enzymes (Arnaud et al., 2004). Mutations were introduced to the cloned fragments by inverse PCR with primers UTR0202_mut_F and UTR0202_mut_R, and UTR2219_mut_F and UTR2219_mut_R. The resulting vectors (Table S8) were introduced to L. monocytogenes EGD-e cells by electroporation. The mutants (Table S1) were generated according to the procedure described in Arnaud et al. (2004) and the loci later sequenced to assure that the mutations had been introduced on the chromosome.

**Quantitative RT-PCR**

L. monocytogenes EGD-e cells were grown overnight in BHI media at 37°C and constant shaking. The stationary phase cultures were diluted 1:100 in the Activation media: 1x LB broth buffered with 10 mM MOPS-Na pH 7.3 (non-inducing conditions), or same medium with addition of 25 mM Glucose-1-Phosphate and 50 mM MOPS-Na pH 7.3 (1x LB broth buffered with 50 mM MOPS-Na pH 7.3 (inducing conditions)). RNA was isolated from cells. 1 µg of RNA was treated with DNase I (Roche) and purified on RNeasy MinElute columns (QIAGEN). cDNA synthesis was performed with RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). The levels of hly and prsA2 mRNAs were measured by quantitative RT-PCR with respective primers (Table S6) and normalized to the level of 16S rRNA. Quantitative RT-PCR was performed with Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific).

**Western blotting**

L. monocytogenes EGD-e strain and its derivatives with M1, M2 and M1+M2 mutations (Table S1) were grown in LB medium supplemented with 50 mM MOPS-Na pH 7.3 (non-inducing conditions), or same medium with addition of 25 mM glucose-1-phosphate and 1% (w/vol) Amberlite XAD4 resin (Sigma-Aldrich) (Ernolaeva et al., 2004; Ripio et al., 1997). The culture was grown at constant shaking at 37°C for 5 hours until early stationary growth phase. RNA was isolated from cells. 1 µg of RNA was treated with DNase I (Roche) and purified on RNeasy MinElute columns (QIAGEN). cDNA synthesis was performed with RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). The levels of hly and prsA2 mRNAs were measured by quantitative RT-PCR with respective primers (Table S6) and normalized to the level of 16S rRNA. Quantitative RT-PCR was performed with Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific).

Preparation of the cellular protein fraction was performed according to the following protocol: 600 µL of culture was mixed with 600 µL of 1:1 acetone:ethanol solution, incubated on ice for 10 min and centrifuged at 20,800 × g for 5 min. The pellet was washed two times with 500 µL of wash buffer (30 mM Tris-HCl pH 8.0, 50 mM NaCl, 5 mM EDTA), resuspended in 45 µL of Mutanolysin mix: 50 mM Tris-HCl (pH 6.8), Mutanolysin (0.1 U/µl) and DNase (0.2 U/µl), and incubated at 37°C for 1 h. After centrifugation at 20000 × g at 4°C for 30 min, the supernatant was removed, and the pellet was resuspended in 580 µL of 80% ice-cold acetone. The suspension was centrifuged at 20800 × g at 4°C for 30 min before the pellet was dried and resuspended in 15 µL of 1x Laemmli sample buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.001% bromophenol blue).

Preparation of the cellular protein fraction was performed according to the following protocol: 600 µL of culture was mixed with 600 µL of 1:1 acetone:ethanol solution, incubated on ice for 10 min and centrifuged at 20,800 × g for 5 min. The pellet was washed two times with 500 µL of wash buffer (30 mM Tris-HCl pH 8.0, 50 mM NaCl, 5 mM EDTA), resuspended in 45 µL of Mutanolysin mix: 50 mM Tris-HCl (pH 6.8), Mutanolysin (0.1 U/µl) and DNase (0.2 U/µl), and incubated at 37°C for 1 h. Afterward, 15 µL of 4x Laemmli buffer (250 mM Tris-HCl pH 6.8, 8% SDS, 40% glycerol, 20% β-mercaptoethanol, 0.004% bromophenol blue) was added, the sample was heated for 30 min at 95°C and centrifuged at 20,800 × g for 5 min.

After isolation, the protein fractions were separated on 12% SDS-polyacrylamide gels and transferred to the BioTrace NT Nitrocellulose Transfer Membrane (Pall) using Trans-Blot Turbo Transfer System (Bio-Rad). Membranes were blocked in 5% dry milk. To detect LLO levels, the membrane with the secreted protein fraction was incubated with primary polyclonal anti-LLO antibody (Abcam) diluted 1:2500. The level of p60 protein in the secreted fraction was detected with the polyclonal antibodies diluted 1:3000 (Netterling et al., 2015). To detect PrsA2 levels, the membrane with the intracellular proteins fraction was incubated with primary polyclonal anti-PrsA2 antibody diluted 1:2500 (Alonzo et al., 2009). The level of ActA protein in the intracellular fraction was detected with the polyclonal antibodies diluted 1:4000 (Netterling et al., 2015). Membranes were washed and incubated with anti-rabbit-horseradish peroxidase secondary antibody (Agrisera), diluted 1:10000. The expression levels of each protein (the intensity
of chemiluminescence) were detected and measured using a LAS4000 image analyzer (Fuji). The Coomassie stained gel served as a loading control for PrsA2 expression levels, and the expression of LLO was normalized to the area under the growth curves of the bacterial cultures.

**Northern blotting**

10 μg of RNA was separated on a formaldehyde agarose gel as described in Durand et al. (2015) and transferred to the Hybond-N membrane (GE Life Sciences) by the capillary transfer according to the manufacturer’s protocol. The RNA was UV cross-linked to the membrane with Spectrolinker XL-1000 machine (Spectroline). The oligonucleotides complementary to hly mRNA CDS and 3’ UTR, RliS1 and tmRNA (Table S6) were labeled with γ-32P ATP using T4 polynucleotide kinase (Thermo Fisher Scientific) and hybridized with the membrane in the Rapid-hyb buffer (GE Life Sciences). The radioactive signal was detected with a storage phosphor screen and Typhoon FLA 9500 laser scanner (GE Life Sciences).

**Measurement of prsA2 mRNA stability**

*L. monocytogenes* EGDe strain and its derivatives with M1, M2 and M1+M2 mutations (Table S1) were grown in LB medium supplemented with 50 mM MOPS-Na pH 7.3 (non-inducing conditions), or same medium with addition of 25 mM Glucose-1-Phosphate and 1% (w/vol) Amberlite XAD4 (virulence inducing conditions). At culture density of OD600 = 0.5 a 1 mL sample was mixed with 200 pmol) were pre-heated 5 min in a 95

**RNase J1 assays**

The 53-nt prsA2 and prsA2-M1 RNA fragments were transcribed in vitro from PCR fragments containing the 5’ UTR and the first 6 nucleotides of the prsA2 coding sequence. The prsA2 and prsA2-M1 templates were amplified using *L. monocytogenes* chromosomal DNA and oligo pairs 5UTR2219_rnj_F / 5UTR2219_rnj_R and 5UTR2219_M1_rnj_F / 5UTR2219_rnj_R, respectively. The hly and hly-M2 templates were amplified by PCR using oligos 3UTR0202_rnj_F / 3UTR0202_rnj_R and 3UTR0202_M2_rnj_F / 3UTR0202_rnj_R, respectively. The prsA2 and prsA2-M1 RNAs were dephosphorylated with Calf Intestinal Phosphatase (CIP) (10 U/ml; Biolabs) and 5’ end-labeled with T4 polynucleotide kinase (PNK) and [γ-32P]-ATP. The prsA2 and prsA2-M1 RNAs (10 pmol) were pre-heated 5 min in a 95°C water bath with or without hly or hly-M2 (20 pmol), and cooled to 4°C. Then, the appropriate volume of 5x J1 buffer (100 mM Tris pH 6.8, 40 mM MgCl2, 500 mM NH4Cl, 0.5 mM DTT) was added for n+5 μL reactions. The reaction mixture was incubated at 37°C. 5 μL of reaction mix was stopped with SuL RNA loading dye (Ambion) before (time 0) and 2.5, 5 and 10 min after addition of RNase J1 (0.8 μg per 5 μL reaction) at 37°C (Mathy et al., 2007). The prsA2 and prsA2-M1 RNAs were also incubated without RNase J1 for 10 min at 37°C.

**L. monocytogenes infection of mice**

Animal procedures were approved by the University of Illinois at Chicago Animal Care Committee and were conducted in the Biological Resources Laboratory. Saturated overnight cultures of *L. monocytogenes* EGDe were diluted 1:20 in BHI broth, grown to mid-log phase and normalized based on OD600 values. Bacteria were washed twice and re-suspended in PBS pH 7. Female 7-9-week-old Swiss Webster mice (Charles River Laboratories) were administered 200μL containing 2×10^6 CFU of bacteria by tail vein injection. At 72 hours post infection, organs of infected animals were collected, homogenized, and 10-fold serial dilutions were plated for total CFUs.

**Plaque assay**

Plaque assays were conducted as previously described (Sun et al., 1990). Briefly, monolayers of L2 fibroblasts in 6-well culture dishes were infected at an MOI of 30:1 for 1 hour. Then infected monolayers were washed three times with PBS pH 7 and overlaid with DMEM/agarose containing 10 μg/ml gentamicin to kill extracellular bacteria. Plaques were measured at 72 hours with a micrometer.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

**Analysis of DMS-MaPseq and FUSE data**

The sequencing reads were mapped to *Listeria monocytogenes* EGD-e genome (NC_003210) with Bowtie 2 aligner using the –end-to-end–very-sensitive mode (Langmead and Salzberg, 2012). The coordinates of mismatches were extracted from the CIGAR string
of the SAM files. The coordinates of transcriptional start sites were obtained from Wurtzel et al. (2012) and verified using our data. Genome regions corresponding to non-coding RNAs, 5’ UTRs and the first 30 nucleotides of coding sequences were selected for further analysis. For each adenine and cytosine nucleotide position of these regions, the coverage was calculated as the number of reads mapped at that position. The mismatch rate \( MR \) of position \( i \) in sample \( j \) was calculated as the ratio between the number of mismatches \( mis \) and coverage \( cvg \) at that position:

\[
MR_{ij} = \frac{mis_{ij}}{cvg_{ij}}
\]

Our DMS treatment conditions resulted in the average mismatch rates for adenine and cytosine nucleotides ranging from 3 to 6% in different samples. In the untreated control, the average mismatch rates for these nucleotides were close to 0.3% (Table S7). The nucleotides with mismatch rate higher than 1% in the untreated control were mostly located in the regions with low sequencing coverage and were excluded from further analysis. To account for variability in DMS treatment conditions of different samples, the mismatch rates of adenines and cytosines were separately divided by the average mismatch rates in the respective sample. The resulting values got the name ‘DMS values’ (Table S3):

\[
DMS_{ij} = \frac{MR_{ij}}{MR_j}
\]

DMS values of each nucleotide were compared between different samples in a pairwise manner. The significance of the differences was estimated by a two-step strategy. At first, we performed Fisher’s exact test. To account for uneven DMS treatment conditions, the number of mismatches at the position \( i \) and samples 1 and 2 were normalized according to the average mismatch rates in the compared samples:

\[
mis_{1i} = \text{round}\left(\frac{mis_{1i} \times MR_1 + MR_2}{2MR_1}\right)
\]

\[
mis_{2i} = \text{round}\left(\frac{mis_{2i} \times MR_1 + MR_2}{2MR_2}\right)
\]

where \( MR_j = MR_A \) if \( i \) corresponds to an adenine positions and \( MR_j = MR_C \) otherwise, and the values were rounded to the nearest integers. The normalized number of mismatches and matches were used for the calculation of Fisher’s exact test statistics using the following normalized contingency table:

|                | Sample 1 | Sample 2 |
|----------------|----------|----------|
| Number of mismatches at position \( i \) | \( mis_{1i} \) | \( mis_{2i} \) |
| Number of matches at position \( i \) | \( cvg_1 - mis_{1i} \) | \( cvg_2 - mis_{2i} \) |

The calculated \( p\)-values were adjusted for multiple comparisons (Benjamini and Hochberg, 1995), and positions with the adjusted \( p\)-values < 0.05 were selected for further analysis. Among the statistically significant differences only the differences with a high effect size were regarded as significant. The effect size criterion demanded that the relative difference between the samples’ DMS values should be larger than 20% and that the absolute difference between the values should be larger than 0.1. The results of the performed comparisons are represented in Table S4. To discover structural rearrangements, we manually searched for the regions where nucleotides with pronounced changes of DMS reactivity were located close to each other.

Data manipulation was performed with custom Python scripts using HTSeq framework (Anders et al., 2015). Statistical analysis was performed with R scripts, and data visualization with Integrative Genomics Viewer and VARNA software (Darty et al., 2009; Thorvaldsdóttir et al., 2013).

**Analysis of quantitative data**

We have included the group sizes, number of replicates, statistical tests and significance criteria in figure legends. In most plots the mean and the standard deviation are indicated, and the Student’s t test was used for estimation of statistical significance. For the mouse infection data at Figures 6C and S6A the CFU values are presented as boxplots and the statistical significance of differences was estimated with the Wilcoxon Rank-Sum test. The differences were considered significant if \( p\)-value was less than 0.05.
DATA AND CODE AVAILABILITY

All sequencing data are deposited in NCBI's Gene Expression Omnibus (Barrett et al., 2013). The accession number for the sequencing data reported in this paper is GEO: GSE118387 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE118387). The scripts are deposited at GitHub under the project name 5'-UTR-structure-elucidation' (https://github.com/dimaignatov/5-UTR-structure-elucidation).

ADDITIONAL RESOURCES

There are no additional resources associated with this study.