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Submitted on 2 Dec 2019

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Highlights

- *L. monocytogenes* secretes an RNA-binding protein, Zea
- Zea binds and protects *L. monocytogenes* RNA, resulting in extracellular RNA accumulation
- During infection, Zea binds RIG-I and modulates RIG-I-dependent IFN response
- Zea plays a role in *L. monocytogenes* virulence in mice

In Brief
Extracellular RBPs have only been described in eukaryotes. Pagliuso et al. report that *L. monocytogenes* secretes an RNA-binding protein, Zea, which associates extracellularly with *L. monocytogenes* RNA. During infection, Zea binds RIG-I and modulates the type I IFN response. These results reveal that RNA can traffic between organisms via RBPs.
An RNA-Binding Protein Secreted by a Bacterial Pathogen Modulates RIG-I Signaling

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https://doi.org/10.1016/j.chom.2019.10.004

SUMMARY

RNA-binding proteins (RBPs) perform key cellular activities by controlling the function of bound RNAs. The widely held assumption that RBPs are strictly intracellular has been challenged by the discovery of secreted RBPs. However, extracellular RBPs have been described in eukaryotes, while secreted bacterial RBPs have not been reported. Here, we show that the bacterial pathogen Listeria monocytogenes secretes a small RBP that we named Zea. We show that Zea binds a subset of L. monocytogenes RNAs, causing their accumulation in the extracellular medium. Furthermore, during L. monocytogenes infection, Zea binds RIG-I, the non-self-RNA innate immunity sensor, potentiating interferon-β production. Mouse infection studies reveal that Zea affects L. monocytogenes virulence. Together, our results unveil that bacterial RNAs can be present extracellularly in association with RBPs, acting as “social RNAs” to trigger a host response during infection.

INTRODUCTION

RNA-binding proteins (RBPs) are found in all living organisms. By binding RNAs, RBPs assemble in ribonucleoprotein complexes that dictate the fate and the function of virtually every cellular RNA molecule. In bacteria, RBPs interact with their cognate RNAs via classical RNA-binding domains (RBDs), structurally well-defined signatures that recognize specific RNA sequences and/or motifs (reviewed in Holmqvist and Vogel, 2018). Previously thought to be mainly involved in transcriptional regulation, bacterial RBPs have now been implicated in a wide variety of cellular processes such as translation, RNA turnover, decay, processing, and stabilization (Holmqvist and Vogel, 2018). Although bacterial RBPs regulate vital functions in bacterial physiology, their number remains limited. It is therefore conceivable that many bacterial RBPs remain to be discovered.

One feature of all bacterial RBPs described so far is their exquisite intracellular localization. Few extracellular RBPs have been described only in eukaryotes and shown to stabilize RNA in the extracellular milieu and participate in cell-to-cell communication (Arroyo et al., 2011; Vickers et al., 2011; Wang et al., 2010; Shurtleff et al., 2016; Maori et al., 2019). At present, no secreted RBPs have been identified in bacteria. A recent study screened
Figure 1. Zea Is a Secreted Oligomeric Protein of *L. monocytogenes*

(A) Synthony analysis of the lmo2686/zea-containing genomic locus between *L. monocytogenes* and *L. innocua*. Arrows and stem and circle represent the transcriptional start sites (TSSs) and the transcriptional terminators, respectively.

(B) Schematic representation and primary sequence of the Zea protein. The N-terminal signal peptide is highlighted in red.

(C) Bacterial cytosol and culture medium from wild-type (*wt*) and Azea strains. IB: Zea, IB: InIC, IB: EF-Tu.

(D) Bacterial cytosol and culture medium from wild-type (*wt*) and Azea strains. IB: FLAG (Zea), IB: InIC, IB: EF-Tu.

(E) Monomeric Zea and Zea-FLAG in 90° samples. IB: HA, IB: FLAG.

(F) Monomeric Zea and Zea-FLAG in 90° samples. IB: HA, IB: FLAG.

(G) Western blot analysis of Zea-HA and Zea-FLAG in bacterial cytosol and culture medium. IB: HA, IB: FLAG.

(H) Western blot analysis of Zea-HA and Zea-FLAG in bacterial cytosol and culture medium. IB: HA, IB: FLAG.


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thousands of secreted effectors of Gram-negative symbionts and bacterial pathogens for the presence of known RBPs and failed to unambiguously identify any RBPs (Tawk et al., 2017). It is therefore likely that secreted bacterial RBPs harbor unconventional RBDs, which render them undetectable by using conservation-based searches.

In this study, we report the identification of a secreted bacterial RBP, the *Listeria monocytogenes* protein Lmo2686. We provide evidence that Lmo2686 is secreted in the culture supernatant, where it is associated with a subset of *L. monocytogenes* RNAs. Protein sequence analysis of Lmo2686 revealed the absence of any canonical RBD, suggesting a non-canonical mode of RNA binding. We show that Lmo2686 induces the extracellular accumulation of its RNA targets, possibly by protecting them from degradation. Furthermore, during infection of mammalian cells, Lmo2686 interacts with RIG-I and modulates RIG-I-dependent type I interferon (IFN) response. We further show that Lmo2686 affects *L. monocytogenes* virulence in vivo. Based on these findings, we propose to rename this protein Zea—as Zea, also known as Hecate, is an ancient Greek goddess who protected and guided the travelers. The presence of Zea orthologs in other bacterial species revealed that secretion of RBPs is a conserved phenomenon in prokaryotes.

**RESULTS**

**Zea Is a Secreted Protein of *L. monocytogenes***

The lmo2686/zea open-reading frame is 534 bp long (Figure 1A). Zea is found in half of the *L. monocytogenes* strains sequenced to date as well as in the animal pathogen *Listeria ivanovii* (Bécavin et al., 2017). Orthologs of zea are also found in other species, mainly bacteria of the genus Bacillus (Figure S1). Zea is absent from the genome of the nonpathogenic species *L. innocua* (Glaser et al., 2001) and *Listeria marthi* (Graves et al., 2010), which suggests that it may contribute to *L. monocytogenes* virulence (Figure 1A).

RNA sequencing (RNA-seq) data have revealed a transcriptional start upstream of the start codon of zea (Figure 1A) (Wurtzel et al., 2012). Zea appears constitutively expressed at 37°C, albeit at low levels, and is slightly upregulated under microaerophilic conditions and at 4°C (Bécavin et al., 2017; Wurtzel et al., 2012).

The zea gene encodes a protein of 177 amino acids (aa) (Figure 1B). Analysis of the Zea protein sequence predicted the presence of an N-terminal signal peptide of 25 aa for Sec-mediated secretion, resulting in a putative 152 aa-mature protein with a basic isoelectric point (pI = 8.4) (Figure 1B). Of note, the signal peptide is conserved in almost all the Zea orthologs, suggesting that the major function of the protein is outside bacteria (Figure S1). We could not identify any other domain of known function.

The presence of a signal peptide prompted us to test whether Zea could be secreted. We generated three antibodies against three peptides of the C terminus of the protein and used them to assess the presence of Zea in the *L. monocytogenes* cytosol and in the culture medium. Immunoblot analysis revealed that Zea could be recovered from the culture medium, indicating secretion of the protein (Figure 1C). Culture medium collected from the zea-deleted strain (Δzea) did not show any immunoreactive band, thus confirming the specificity of our antibodies. The secretion of Zea was also confirmed by engineering a *L. monocytogenes* strain carrying a chromosomally integrated copy of the C-terminally FLAG-tagged zea gene under the control of a constitutive promoter (zeaFLAG) (Figure 1D). Quantitative analysis of the distribution of Zea between bacterial cytosol and culture medium in stationary phase revealed a strong accumulation in the extracellular medium, indicating that the protein was efficiently secreted (Figure 1D).

**Zea Is an Oligomeric Protein that Interacts with RNA**

The structure of Zea was previously solved by X-ray crystallography at a resolution of 2.75 Å and deposited in the Protein Data Bank (PDB) by Minasov and colleagues (PDB: 4K15). Zea is a toroid-shaped homohexamer in which every monomer essentially contacts the neighboring molecule via a beta sheet-hairpin-beta sheet unit (Figure 1E). As this structure is not shared by any other polypeptide of known function, the role of Zea and its orthologs is currently unknown.

Nevertheless, we noticed several other proteins that assemble as a torus (e.g., in Hfq) have the intrinsic capability to bind RNA (Gabitzke et al., 1995; Lee et al., 2007; Antson et al., 1995; Vogel and Luisi, 2011; Thomsen and Berger, 2009). Interestingly, Zea shows a positively charged surface on one side of the torus due to the presence of several lysine residues, which might accommodate the negatively charged RNA (Figure 1F). These features led us to hypothesize that Zea might bind RNA.

Before addressing this hypothesis, we sought to verify whether the oligomeric state of Zea observed by X-ray crystallography also existed under physiological conditions, ruling out possible crystallization artifacts. We used three different approaches: (1) co-immunoprecipitation of hemagglutinin (HA)- and FLAG-tagged versions of Zea (Figure 1G), (2) size-exclusion chromatography of *L. monocytogenes* cytosol and culture medium (Figure 1H), and (3) size-exclusion gel chromatography of recombinant His-tagged Zea expressed and purified from *E. coli* (Figure 1I). Collectively, our data show that Zea has a high tendency to oligomerize, in line with the hexameric structure.
Figure 2. Zea Associates with RNA

(A) Enrichment of Zea-bound RNAs (n) from bacterial cytosol and culture medium. Blue squares and red circles depict individual RNAs. The y axis shows the enrichment of the Zea-interacting RNAs relative to immunoprecipitation with IgG.

(B) Expression of *L. monocytogenes* RNAs grown in BHI at stationary phase measured by tiling array compared with the enrichment of the Zea-bound RNAs.

(C) Circular genome map of *L. monocytogenes* showing the position of the Zea-interacting RNAs. The first two circles from the inside show the genes encoded on the + (inner track) and - (outer track) strands, respectively. The positions of Zea-interacting small RNAs (rlis) are pointed at outside of the circular map. Dotted lines highlight the phage A118 locus.

(D) Examples of normalized read coverage (reads per million) visualized by IGV from Zea and control (IgG) IP for a selection of phage A118 genes (blue arrows). Gene names marked in red show no significant enrichment in the Zea IP.

(legend continued on next page)
shown by X-ray crystallography. We noticed, however, that the molecular mass of the recombinant His-tagged Zea (HisZea) exceeded that of the hexameric Zea, indicating that high molecular weight assemblies composed of several hexameric units or, potentially, other components are formed.

We then examined whether Zea could bind RNA. We performed RNA immunoprecipitation (IP) of cytosolic extract and culture supernatant followed by high-throughput sequencing (RIP-seq) (Figure S2A). Given the low amount of Zea protein produced in vitro, we made use of a Zea-overexpressing strain (zea*). Zea was immunoprecipitated from *L. monocytogenes* cytosol and culture medium, and the Zea-bound RNAs were subsequently extracted and sequenced. As a control, we performed a mock IP using an unrelated antibody of the same isotype. Remarkably, RIP-seq analysis revealed the presence of *L. monocytogenes* RNAs almost exclusively in the culture medium compared with the control samples (Figure 2A), indicating that Zea can form complexes with RNA extracellularly. An enrichment threshold of log2 fold change (log2FC) >1.5, corresponding to almost 3-fold increase, was used for the identification of Zea-associated RNAs. Importantly, the enrichment of specific RNAs in the Zea IP was uncorrelated to their expression levels (Figure 2B) (Bécavin et al., 2017). Zea preferentially bound a subset of protein-coding mRNAs and small regulatory RNAs, to a lesser extent (Table S1).

We then analyzed the genomic distribution of Zea-bound RNAs on the *L. monocytogenes* chromosome (Figure 2C). It was striking that there was one region particularly overrepresented. This locus contains the prophage A118 (Figures 2C–2E). The phage A118 is a temperate phage belonging to the *Syphoviridae* family of double-stranded DNA bacterial viruses (Dorscht et al., 2009). Cluster of Orthologous Genes (COG) classification highlighted the phage A118 RNA as the most enriched class of Zea-bound RNAs in the culture medium (Figure S2B).

To validate the interaction of phage A118 RNA with Zea found by RIP-seq (Figures 2C–2E), we next performed RNA IP coupled with quantitative PCR (RIP-qPCR) analysis. RIP-qPCR confirmed a strong association of Zea to phage RNA, but we could not find any binding to control transcripts that were not enriched in our RIP-seq dataset (Figure 2F). In agreement with the RIP-seq data, the enrichment of phage RNA in the culture medium fraction was particularly strong, indicating that the phage RNA accumulates extracellularly together with Zea (Figure 2F). Taken together, our data revealed that Zea is an oligomeric protein that is found associated in the extracellular compartment with a subset of *L. monocytogenes* RNAs enriched in phage RNA.

**Zea Directly Binds *L. monocytogenes* RNA**

We next investigated whether Zea could directly bind RNA. We first performed electrophoretic mobility gel shift assay (EMSA) by using recombinant HisZea and in vitro-transcribed radiolabeled RNA. We selected *rli143* and *rli92*, two small RNAs that showed a significant enrichment in the RIP-seq dataset (8- and almost 3-fold enrichment compared with control immunoglobulin G [IgG] IP, respectively; see Figure 3A) and have a small size, which is appropriate for in vitro transcription. Incubation of *rli143* or *rli92* with HisZea produced several shifts, most likely due to the binding of different Zea oligomers to RNA (Figures 3A and 3B). Importantly, the binding of Zea with both *rli143* and *rli92* was specific, as it was displaced by the addition of increasing amounts of each unlabeled small RNA (Figures 3C and 3D).

To further prove direct binding of Zea with its target RNAs, we performed an RNA pull-down assay using in vitro-transcribed biotinylated RNA and recombinant HisZeea. Here, in addition to *rli143* and *rli92*, we tested two other small RNAs (*rli18* and *rli1*), which also displayed specific binding to Zea in the RIP-seq data set (Figure 2C). As a control, we employed a small RNA (*rli80*) that was not specifically bound by Zea. Using this alternative approach, we confirmed binding above background level for *rli143* (Figure 3E), which also showed the highest enrichment in the RIP-seq dataset. Collectively, these data clearly indicate that Zea directly binds RNA.

**Extracellular Zea-Bound RNAs Do Not Derive from Bacterial Lysis**

It was important to verify that the extracellular RNAs in complex with Zea were not due to bacterial lysis. Thus, we first analyzed the presence of one abundant cytosolic protein of *L. monocytogenes* (EF-Tu) in the culture medium. EF-Tu was undetectable in the culture medium, indicating that bacterial lysis under our experimental conditions was negligible (Figure 1H). As bacteria lyse after death, we also quantified both live and dead bacteria. Confocal microscopy analysis revealed less than 2% of dead bacteria, further confirming minimal bacterial lysis (Figure S3A). Finally, we designed an experiment in which we used the strict intracellular localization of the RBP Hfq and its RNA targets as a readout of bacterial lysis. In *L. monocytogenes*, Hfq has been shown to bind three small RNAs, LhrA, LhrB, and LhrC (Christiansen et al., 2006). We reasoned that if bacterial lysis occurred, we should find Hfq complexed to its RNA targets in the medium. We thus grew *L. monocytogenes* under the conditions used for the Zea RIP-seq experiment and then immunoprecipitated Hfq from the bacterial cytosol and culture medium by using an anti-Hfq antibody (Christiansen et al., 2006). Hfq was recovered from the bacterial cytosol but was undetectable in the culture medium, indicating minimal bacterial lysis (Figure S3B). RNA was extracted from the immunopurified Hfq ribonucleoprotein complexes and used to assess the abundance of the Hfq targets by qPCR. Given the low expression of LhrB and LhrC in stationary phase (Christiansen et al., 2006), we focused on LhrA. LhrA was detected in association with intracellular Hfq but remained undetectable in the culture medium (Figure S3C). Collectively, these results strongly indicate that extracellular RNAs complexed with Zea are not originating from lysed bacteria.
the three cellular abundance of the phage transcripts was comparable in *L. innocua* (Lee et al., 2016; Göhmann et al., 1990). Overexpression of Zea is considered to be a cryptic prophage whose function remains elusive (Lee et al., 2016; Göhmann et al., 1990). The lma-monocin RNAs (Figure S2 B). The lma-monocin WT and Δzea RNAs and not their expression level. However, when comparing cating that Zea specifically affects the quantity of extracellular amount of extracellular phage RNA. This is probably due to the low expression level of Zea by WT bacteria in MM, as revealed by qPCR (Figure S4 B). We next evaluated the abundance of by promoting its export from bacteria and/or by promoting the abundance in the culture medium. We found that Zea promotes the abundance of in the culture medium when Zea was co-overexpressed (Figure 4 C) but not when Lmo2595 was co-overexpressed. The intracellular expression level of rli143 was comparable in all of the strains (Figure S4 D).

To further establish a role for Zea in the regulation of the extracellular amount of RNA, we generated a *L. innocua* strain overexpressing either rli143 alone or rli143 with Zea and then measured the abundance of rli143 in the culture medium. As a control, rli143 was co-expressed with Lmo2595. We found that co-expression of Zea and rli143 induced an even greater accumulation of rli143 in the culture medium compared with *L. monocytogenes* (Figure 4 D). The intrabacterial abundance of rli143 was comparable in the three *L. innocua* strains (Figure S4 E). Altogether, these data show that the overexpression of Zea induces accumulation in the culture medium of phage-derived and small RNAs that are Zea-binding RNA species.

Zea overexpression could increase the amount of extracellular RNA by promoting its export from bacteria and/or by promoting its stabilization in the culture medium. We found that Zea protected rli143 from RNase-mediated degradation *in vitro* (Figure S5 A), indicating that RNA protection may partially account for the increased amount of extracellular RNA.

As a last approach to definitively establish the impact of Zea on secreted RNA, we performed RNA-seq analysis on extracellular RNAs prepared from the WT and Δzea+ strains. We reasoned that if Zea increases the secretion and/or protection of a specific
subset of extracellular *L. monocytogenes* RNAs, then its overexpression should increase the overall amount of those RNAs in the medium. We thus purified extracellular RNA from three independent samples (3 from WT and 3 from *zea*+) and performed sequencing. Differential gene expression analysis revealed that, besides the overexpressed Zea RNA, 36 endogenous transcripts were significantly more abundant in the medium of the *zea*+ strain (Figure S5B). The vast majority of these transcripts were mRNAs (78%), while a small percentage represented small non-coding RNAs (sRNAs) and antisense RNAs (10% and 8%, respectively) (Figure S5B). We then examined the correlation between Zea overexpression and the higher amount of extracellular RNA; we intersected the differential extracellular abundance dataset with the dataset of the Zea RIP-seq experiment performed in the culture medium (i.e., the RNAs in complex with Zea). Strikingly, we found that one-third (12 out of 37 RNAs) of the transcripts enriched in the culture medium when Zea was overexpressed were also associated with Zea in the RIP-seq dataset (Figure S5C). This indicates that a subset of transcripts found in complex with Zea becomes more abundant in the medium following Zea overexpression (exact right rank Fisher’s test: *p* = 7.18 \(10^{-3}\)). Of note, among these 12 enriched secreted RNAs, 8 RNAs proceeded from the A118 phage. qPCR analysis of intrabacterial phage RNA from the WT and *zea*+ strains revealed similar amounts of the majority of the phage genes tested, indicating that Zea does not affect the expression of phage genes (Figure S5D). Altogether, our results show that Zea binds a subset of *L. monocytogenes* RNAs and that its overexpression increases their abundance in the extracellular medium.

**Zea Affects *L. monocytogenes* Virulence**

The absence of a Zea ortholog in *L. innocua* (Figure 1A) prompted us to assess whether Zea could impact *L. monocytogenes* virulence. We thus examined the properties of the WT and *Δzea* strains in a mouse infection model. After intravenous inoculation, the *Δzea* strain showed a significant increase in bacterial load after 72 h, both in the liver (Figure 5A) and in the spleen (Figure 5B). These results indicate that Zea is an effector that affects *L. monocytogenes* virulence.

**Zea Modulates the Type I IFN Response in a RIG-I-Dependent Fashion**

Three RBPs of the RIG-I-like receptor (RLR) family (RIG-I, MDA5, and LGP2) can sense non-self RNA in the cytoplasm, but only RIG-I and MDA5 can trigger the type I IFN signaling cascade
A recent approach has successfully helped to identify viral RNA sequences bound to RIG-I, MDA5, or LGP2 during viral infections (Sanchez David et al., 2016; Chazal et al., 2018). This method is based on the affinity purification of stably expressed Strep-tagged RLRs followed by the sequencing of their specific viral RNA partners. We thus applied this approach to obtain L. monocytogenes-specific RNAs bound to each of the RLRs upon infection with L. monocytogenes WT. We infected HEK293 cells stably expressing Strep-tagged RLRs (or Strep-tagged mCherry as a negative control) with L. monocytogenes WT and pulled down the Strep-tagged proteins. Co-purified RNA molecules from three independent replicates were sequenced and mapped to the L. monocytogenes genome. We found 15 RNAs specifically enriched in the RIG-I pull-down, and 9 of them (60%) belonged to the phage A118 locus (Figure S6). We did not identify specific RNAs bound to MDA5 and LGP2 (Figure S6), in agreement with a previously suggested major role of RIG-I and minor role of MDA5, in L. monocytogenes-induced IFN response infection (Abdullah et al., 2012; Hagmann et al., 2013). These data indicate that during infection, L. monocytogenes phage RNAs gain access to the host cytoplasm, where they specifically bind to RIG-I.

Since Zea is a secreted protein and binds phage RNA, we investigated whether it could participate in RIG-I-dependent signaling. We first compared the expression of IFN-β in cells infected with L. monocytogenes WT versus cells infected with zeα (Figure 6A). qPCR analysis revealed that overexpression of Zea increased the amount of IFN-β while IFN-γ was undetectable (Figure 6A). Zea overexpression did not increase the expression of the proinflammatory cytokine interleukin 8 (IL-8). Notably, a L. monocytogenes strain overexpressing another secreted protein had no effect on INF-β expression (Figure 6A). Thus, Zea can modulate a type I IFN-β response. Next, to address whether the increased IFN response was mediated by RIG-I, we repeated the same experiment in RIG-I knockout cells. The Zea-induced IFN-β upregulation was strongly impaired after RIG-I silencing (Figure 6B). These data indicate that Zea plays a role in the RIG-I-dependent type I IFN response.

These findings led us to examine whether Zea and RIG-I might share the same compartment in cells. Attempts to detect endogenous Zea in infected cells by immunoblotting or immunofluorescence were unsuccessful, as our antibodies cross-reacted with some mammalian proteins. We thus infected cells with zeα and L. monocytogenes and used an anti-FLAG antibody for detection. Immunoblotting analysis of cytosolic and nuclear fractions prepared from infected cells revealed that Zea was present in both host cell compartments (Figure S7A), whereas RIG-I is mostly cytosolic (Sánchez-Aparicio et al., 2017; Liu et al., 2018). Transfected FLAG-tagged Zea also localized both to the cytoplasm and the nucleus. (Figure S7B). Thus, the fraction of Zea present in the cytosol might be compatible with the RIG-I-dependent signaling. Next, we tested whether Zea and RIG-I co-localized in cells. FLAG-tagged Zea partially co-localized with endogenous RIG-I, indicating a spatial vicinity of the two proteins (Figure 6C). Of note, a negative control FLAG-tagged mCherry protein did not co-localize with RIG-I (Figure 6C). These results prompted us to test whether Zea could interact with RIG-I. Immunopurified Zea pulled down Strep-tagged RIG-I from cell lysates, indicating interaction between the two proteins (Figure 6D). This interaction did not absolutely require the presence of L. monocytogenes RNA, as pre-treatment of immunopurified Zea with RNaseA reduced Zea-RIG-I binding without abolishing it (Figure 6D). In agreement with a minor role of RNA in the Zea-RIG-I interaction, transfection of mammalian cells with FLAG-tagged Zea, which is therefore not bound to L. monocytogenes RNA, was able to interact with co-expressed Strep-tagged RIG-I independently of RNA presence (Figure 6E). Altogether, our results show that Zea interacts with RIG-I and modulates RIG-I-dependent type I IFN response.

Since RIG-I activation implies RNA binding, we sought to determine whether Zea-interacting RNAs could trigger an IFN response. We used a reporter-cell line stably transfected with
Figure 6. Zea Interacts with RIG-I and Modulates a RIG-I Dependent IFN Response

(A) qPCR analysis of IFN-β, IFN-γ, and interleukin 8 (IL8) (n = 3) expression in response to infection with WT and zea+ L. monocytogenes in LoVo cells (left); qPCR analysis of interleukin 8 (IL8) expression in response to infection with WT and Lmo1656+ L. monocytogenes in LoVo cells infected as above (right). The relative expression was calculated after normalization to (1) the GAPDH as a housekeeping gene and (2) to the WT sample. †, not detected. Statistical significance determined by two-tailed t test.

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During infection, Zea interacts with RIG-I and modulates RIG-I-dependent signaling. This modulation likely depends on Zea-bound RNA (Figure 7).

**DISCUSSION**

The major finding of this study is the identification of a secreted RBP from bacteria. We show that *L. monocytogenes* secretes Zea, a small RBP, which associates extracellularly with a subset of *L. monocytogenes* RNAs. We found that the overexpression of Zea correlates with an increased amount of its RNA ligands in the culture medium. This might be due to two non-mutually exclusive phenomena: (1) increased secretion of Zea-RNA complexes and (2) increased stabilization of Zea RNA targets following Zea accumulation in the extracellular medium. In the latter case, the pathway regulating RNA secretion would remain to be identified. The presence of Zea orthologs in other bacterial species indicates that the secretion of RBPs is a conserved phenomenon in prokaryotes. Our results suggest that additional RBPs remain to be identified in bacteria; however, their identification might be difficult, as bacterial RBPs are likely devoid of classical RBDs (Tawk et al., 2017). In agreement with this hypothesis, the analysis of the Zea protein sequence showed that Zea does not possess any recognizable RNA-binding region. Future work will shed light on which amino acids are important for RNA binding as well as whether specific RNA sequences or structures are recognized by Zea.

Our work reveals that the secretion of an RBP during bacterial infection regulates the induction of the INF response. We provide evidence that during *L. monocytogenes* infection, a Zea-containing ribonucleoprotein complex binds to RIG-I and modulates RIG-I-dependent signaling. In agreement with this hypothesis, Zea devoid of its RNA targets has no effect on RIG-I, while Zea-bound RNAs are able to induce an INF response. In addition, Zea and RIG-I bind a similar subset of *L. monocytogenes* RNAs that is enriched in phage RNAs. We do not exclude that additional factors or signaling events might contribute to the Zea-mediated modulation of RIG-I signaling during *L. monocytogenes* infection. This study provides the identification of the bacterial RNA species recognized by RIG-I during bacterial infection and uncovers that RIG-I binds viral (phage) RNAs from an invading bacterium. These data reinforce the concept that RIG-I has primarily evolved to sense viral RNAs.
and highlight that phage RNAs from a bacterial pathogen contribute to RIG-I activation.

Our data show that Zea dampens L. monocytogenes virulence in vivo as its deletion results in an increased bacteria burden in the organs of infected mice. Strikingly, and in agreement with our results, Zea is absent in the hypervirulent strains of L. monocytogenes (lineage I), but it is well conserved in the strains from lineage II, which include a smaller number of clinical isolates. Given the complexity of the innate immune response to L. monocytogenes infection (Stockinger et al., 2009; Dussurget et al., 2014; O’Connell et al., 2004; Carrero et al., 2004; Auerbuch et al., 2004), it is difficult to establish the precise role of Zea in vivo. We show that Zea participates in the modulation of the IFN response, but we do not exclude that Zea might have additional roles during infection. We found that Zea also localizes to the nucleus of infected cells (Figures 6C and S7), possibly to affect host nuclear functions. It is conceivable that the phenotype observed in vivo is a consequence of these additional features of Zea. Notably, Zea orthologs are also present in other bacteria that normally reside in the environment and are rarely associated with disease. In addition to its role in L. monocytogenes virulence, Zea might also play a role during the saprophytic life of L. monocytogenes. In conclusion, this study revealed that RNA can traffic between different organisms and that an RBP mediates the transfer of this “social RNA,” thereby triggering a host response. In line with these findings, a recent paper showed that honeybees secrete an RBP that stabilizes RNA in the environment and facilitates RNA sharing thereby triggering a host response. In line with these findings, a recent paper showed that honeybees secrete an RBP that stabilizes RNA in the environment and facilitates RNA sharing.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.chom.2019.10.004.

**ACKNOWLEDGMENTS**

We thank all members of the Cossart laboratory for helpful discussions. We thank Dr. B. Kalilpolitis for providing the anti-Listeria Hfq antibody and Dr. E. Hajnsdorf for the anti-E.coli Hfq. We acknowledge the platforms of the Grenoble Instruct-ERIC Center (ISBG); UMS 3518 CNRS-CEA-UJAG-EMBL within the Grenoble Partnership for Structural Biology (PSB), Platform access was supported by FRISBI (ANR-10-INBS-05-02) and GRAL, a project of the University Grenoble Alpes graduate school (Écoles Universitaires de Recherche) CBH-EUR-GS (ANR-17-EURE-0003). The IBS acknowledges integration into the Interdisciplinary Research Institute of Grenoble (IRIG, CEA).

We are grateful to R.Y. Sanchez David and R. Viber for technical support. Work in A.L.’s team was supported by Inserm ATIP-Avenir and Mairie de Paris (Programme Émergences—Recherche médicale). This work was supported by grants from the European Research Council (ERC) advanced grant BacCellEpi (670823) and the Fondation Le Roch to P.C.

**AUTHOR CONTRIBUTIONS**

A.P. designed and conducted most experiments, analyzed data, and wrote the manuscript with inputs from F.S., A.L., and A.V.K.; T.N.T. performed cloning, immunoblotting, and generated several bacterial strains; E.A. performed gel filtration and RIP-PCR; S.R. performed qPCRs and pull-downs; B.D. performed EMSA experiments; M.K. performed RLR pull-downs; Q.B. and A.D. purified HisZea and performed gel filtrations; C.B. performed bioinformatics analysis; V.N. performed RLR pull-downs and RNA purifications; M.-A.N. performed animal experiments; F.T. supervised A.V.K.; F.S. performed immunofluorescence experiments; S.B. performed initial experiments; C.M. supervised E.A.; A.L. performed qPCRs and provided expertise on data analysis; A.V.K. provided expertise and tools for the RLR part; and P.C. proposed the project to A.P., supervised the project, and edited the manuscript.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

Received: May 24, 2019
Accepted: October 7, 2019
Published: November 21, 2019

**REFERENCES**


**STAR METHODS**

**KEY RESOURCES TABLE**

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Deposited Data

| RIP-Seq | This paper | EMBL-EBI [https://www.ebi.ac.uk/ arrayexpress]; ArrayExpress: E-MTAB-7665 |
|RNA Seq | This paper | EMBL-EBI [https://www.ebi.ac.uk/ arrayexpress]; ArrayExpress: E-MTAB-7665 |
|RLRs purification and sequencing | This paper | EMBL-EBI [https://www.ebi.ac.uk/ arrayexpress]; ArrayExpress: E-MTAB-7665 |
|RIP-Seq, RNA Seq, RLRs purification and sequencing analysis | This paper | [https://github.com/becavin-lab/RIPSeq-Listeria](https://github.com/becavin-lab/RIPSeq-Listeria) |

Experimental Models: Cell Lines

| LoVo cells | ATCC | CCL-229 |
|HEK293 Strep-mCherry | Sanchez David et al., 2016 | N/A |
|HEK293 Strep-RIG-I | Sanchez David et al., 2016 | N/A |
|HEK293 Strep-MDA5 | Sanchez David et al., 2016 | N/A |
|HEK293 Strep-LGP2 | Sanchez David et al., 2016 | N/A |
|STING-37 | Lucas-Hourani et al., 2013 | N/A |

Experimental Models: Organisms/Strains

| Mouse: BALB/c | Charles River | 028 |

Oligonucleotides

For more oligonucleotides, see Table S2

| Zea fw (for pAD cloning) | GAGTCAGGGCGGATAAA GCAAGCATATAATA | N/A |
|Zeaflag rv (for pAD cloning) | ACGTGTCGACTTTTGAAGCATATAATA TTTTTGTGAGTTTAAC TTTTTCCGCT | N/A |
|Zea no tag rv (for pAD cloning) | ACGTGTCGACTTTTGTGAGTTTAAC TTTTTCCGCT | N/A |
|Zea2595 fw (for pAD cloning) | GAGTCAGGGCGGATAAA GCAAGCATATAATA | N/A |
|Lmo2595 rv (for pAD cloning) | ACGTGTCGACTTTTGTGAGTTTAAC TTTTTCCGCT | N/A |
|ZeaHA fw (for pP18 cloning) | GCAGGGATCCATGAAGGAATTTTTATTTTTTGC TGTATTTTACT | N/A |
|ZeaHA rv (for pP1 cloning) | ACGTGTCGACTTTTGTGAGTTTAAC TTTTTCCGCT | N/A |
|Zea fw (for pet28a cloning) | CCGGCGGATCCATGAAGGAATTTTTATTTTTTGCC | N/A |
|ZeaHis rv (for pet28a cloning) | CGCGGATCCATGAAGGAATTTTTATTTTTTGCC | N/A |
|Lmo2282 qPCR fw | AAAATATTGAACCTTCAATAATCG AAAACGCGC | N/A |

(Continued on next page)
LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Pascale Cossart (pcossart@pasteur.fr).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Bacterial Strains and Cell Lines

*L. monocytogenes* EGDe strain was used as the parental strain (detailed informations on the strains used in this study are provided in the Key Resources Table). *L. monocytogenes* strains were grown in brain heart infusion (BHI) medium (GIBCO) with shaking at 200 rpm at 37°C. *E. coli* cells were grown in LB broth. When required, antibiotics were added (chloramphenicol at 35 μg/mL for *E. coli* or 7 μg/mL for *L. monocytogenes*, erythromycin 5 μg/mL for *L. monocytogenes*). LoVo cells were maintained in Ham’s F-12K medium (GIBCO) supplemented with 20% fetal calf serum and Glutamax (GIBCO). Strep-tagged RIG-I, MDA5, LGP2 and mCherry cell lines (Sánchez-Aparicio et al., 2017) were maintained in Dulbecco’s modified Eagle medium (GIBCO) supplemented with 10% heat-inactivated fetal calf serum (GE Healthcare) and 10,000 U/mL of Penicillin-Streptomycin (Life Technologies) and G418 (Sigma) at 500 μg/mL. The ISRE reporter cell line (STING-37) corresponding to HEK293 cells stably transfected with an ISRE-luciferase reporter-gene was previously described (Lucas-Hourani et al., 2013). All cell lines were maintained and propagated at 37°C with 10% CO₂.

Bacterial Mutant Generation

For the deletion of *zea*, PCR products comprising ~500 bp upstream and downstream of the *zea* open reading frame (ORF) were fused via splicing by overlap extension PCR and cloned with appropriate restriction sites into the integrative suicide vector pMAD as previously described (Arnaud et al., 2004).

Mice

BALB/c mice (8-week-old female) were purchased by Charles River, Inc. All animal experiments were carried out in strict accordance with the French national and European laws and conformed to the Council Directive on the approximation of laws, regulations, and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes (86/609/Eec). Experiments that relied on laboratory animals were performed in strict accordance with the Institut Pasteur’s regulations for animal care and use protocol, which was approved by the Animal Experiment Committee of the Institut Pasteur (approval no. 03-49).
**METHOD DETAILS**

**Plasmid Vectors and Antibodies**

Information about the oligonucleotides used for cloning are provided in the Key Resources Table. To create the plasmids for the overexpression in *L. monocytogenes* of both full-length ZeaFlag, full-length untagged Zea and Lmo2595, the entire ORFs (with or without a Flag tag at the C terminus for Zea) were synthesized as a gBlocks (Integrated DNA Technologies) and subcloned into the integrative plasmid pAD, downstream of the Phyper promoter (Balestrino et al., 2010). The same strategy was used to generate a plasmid overexpressing ZeaHA (C-terminal HA-tag), but the cloning was subsequently performed in the pPlasmid [pAT18 derivative (Trieu-Cuot et al., 1991)]. To create the plasmid for the overexpression of rl143, a fusion fragment corresponding to the entire rl143 gene downstream the *phyper* promoter was synthesized as a gBlock (Integrated DNA Technologies) and cloned in the pPlasmid plasmid with the appropriate restriction enzymes. To create a plasmid for the overexpression of HisZea in *E. coli* (N-terminal His-tag), the *zea* ORF was amplified by PCR from *L. monocytogenes* genomic DNA and cloned with the appropriate restriction enzymes into the pET28a plasmid, downstream of the polyhistidine tag. To create a plasmid for the overexpression of ZeaFlag in mammalian cells, the cDNA encoding the predicted mature form of Zea was codon-optimized for human expression and synthesized (GeneCust) downstream of the polyhistidine tag. The resulting construct was then subcloned into pcDNA3.1(+) using the appropriate restriction sites. Modified pCineo plasmid carrying GW cassette (pCineoGW) and the Cherry coding sequence was provided by Dr. Yves Jacob (Institut Pasteur). pEXP-IBA105-RIG-I and pEXP-IBA105-mCherry plasmids for the overexpression of Strep-RIG-I and Strep-mCherry, respectively, were previously described (Sanchez David et al., 2016).

Anti-Zea polyclonal antibodies were raised against three synthetic peptides spanning the C terminus of the protein (CSFNAKINVSKGKKTIS; FYSPLGDVKSKKLSKTIS; TLKASVSGKKLTTSFK). Two rabbits were injected with each antigen supplemented with Freund’s adjuvant (Covalabs, Villeurbanne, France). The total IgG fractions were affinity-purified via a resin column containing the antigenic peptide. The affinity-purified antibodies were dialyzed against PBS and 50% glycerol and stored at −20 °C. A mix of the three antibodies (1 μg/mL in total) was used for immunoblotting. The specificity of the anti-Zea antibodies in immunoblotting and immunoprecipitation was verified by comparing bacterial extract and culture medium prepared from the zea^+ and the zea^− strains.

**Bacterial Fractionation**

For detection of endogenous Zea in culture medium, *L. monocytogenes* was grown to exponential phase (OD_{600} = 1). Bacteria were harvested by centrifugation (4000 × g, 30 min, 4 °C) and proteins in the culture medium fraction were precipitated by addition of 40% ammonium sulfate and incubated at 4 °C (overnight, gentle shaking). Protein were recovered by centrifugation (30 min, 16,000 × g, 4 °C) and resuspended in water. Samples were dialyzed against water (overnight, 4 °C), concentrated using an Amicon centrifugal filter units (3K cut-off, Millipore) and resuspended in LDS-PAGE sample loading buffer (NuPage, Life Technologies). The bacterial pellet was washed twice in PBS and resuspended in lysis buffer [20mM Tris pH 8.0, 1 mM MgCl2, 150 mM KCl supplemented with protease inhibitors mixture (Complete, EDTA-free, Roche)]. Bacteria were transferred to 2 mL lysing matrix tubes (MP Biomedicals) and mechanically lysed by bead beating in a FastPrep apparatus (45 s, speed 6.5 three cycles). Subsequently, tubes were centrifuged (10 min at 16,000 × g, 4 °C) to remove cellular debris. To quantify the partition of Zea between bacterial cytosol and culture medium, equal volumes of culture supernatant and bacterial cytosol were analyzed by gradient SDS-PAGE and subjected to immunoblotting via western transfer onto 0.45 μm nitrocellulose membrane (Millipore). EF-Tu and InlC proteins were used as markers of intracellular and extracellular fractions (Prokop et al., 2017). Detection of overexpressed ZeaFlag in *L. monocytogenes* was performed as described above, except for the protein precipitation from culture medium which was performed as previously described (Archambaud et al., 2005). Briefly, 16% of trichloroacetic acid (TCA) (Sigma) was added to the filtered culture medium and samples were left on ice for 2 h. Precipitated proteins were recovered by centrifugation (20 min, 16,000 × g, 4 °C). The protein pellets were washed twice with ice-cold acetone and dried at 95 °C for 5 min. Proteins were resuspended in NuPage LDS sample buffer and an equal percentage of bacterial cytosol and culture medium were subjected to immunoblotting as above.

**Expression and Purification of HisZea**

pET28a-HisZea (described above) was used to transform *E. coli* C43 bacteria which were grown at 37 °C in Terrific broth (TB) (Thermo Fisher Scientific) supplemented with 50 μg/mL kanamycin. Expression was induced by the addition of IPTG to a final concentration of 1 mM at OD_{600nm} = 0.7 AU. Cultures were incubated overnight, and cells were harvested by centrifugation (5,500 × g, 20 min, 4 °C). The bacterial pellet was resuspended in Buffer A (50 mM potassium phosphate pH 7.0, 300 mM NaCl, 10% glycerol, 20 mM imidazole, 2 mM beta-mercaptoethanol). All subsequent steps were performed at 4 °C. Cell lysis was carried out by passing the samples three times through a pre-cooled microfluidizer operating at 17,000 psi. The soluble fraction was then obtained by centrifugation at 39,000 × g for 45 min at 4 °C. Subsequently, the supernatant was loaded onto a pre-equilibrated Ni-NTA column (Qiagen) at 0.5 mL/min with a peristaltic pump at 4 °C. The washing and elution steps were performed on an AKTA system using steps of 35% and 100% Buffer B (50 mM potassium phosphate pH 7.0, 300 mM NaCl, 10% glycerol, 300 mM imidazole, 2 mM beta-mercaptoethanol). The fractions containing Zea were pooled, concentrated with an Amicon centrifugal filter unit (10K cut-off, Millipore), and further purified by size-exclusion chromatography on a Hi Load S200 10/300 column (GE Healthcare) pre-equilibrated in Buffer C (50 mM potassium phosphate pH 7.0, 300 mM NaCl, 10% glycerol, 2 mM beta-mercaptoethanol). Peak fractions were pooled, concentrated to 10 mg/mL, and subsequently dialyzed against Buffer D (50 mM potassium phosphate pH 7.0, 100 mM NaCl, 2 mM beta-mercaptoethanol).
10% glycerol, 2 mM beta-mercaptoethanol). After dialysis, protein concentration was assessed again and the sample was flash-frozen in liquid nitrogen. During purification, the purity and homogeneity of the sample were monitored by SDS-PAGE.

**RNA Extraction**

Total RNA from *L. monocytogenes* was extracted as previously described (Mellin et al., 2013). Briefly, bacteria grown either to exponential phase (OD_{600nm} = 0.8-1.0 for growth cultures in BHI or OD_{600nm} = 0.4 for growth cultures in MM) or stationary phase (overnight culture: OD_{600nm} = 3.0-3.5 for growth cultures in BHI, or OD_{600nm} = 1.0 for growth cultures in MM) were pelleted by centrifugation (2862 × g, 20 min, 4°C). Pellets were resuspended in 1 mL TRIzol Reagent (Ambion), transferred to 2 mL Lysing Matrix tubes and mechanically lysed by bead beating in a FastPrep apparatus (45 s, speed 6.5 followed by an additional 30 s, speed setting 6.5). Subsequently tubes were centrifuged (5 min at 8,000 × g, 4°C) in a tabletop centrifuge and lysates were transferred to a 2 mL Eppendorf tube. RNA isolation proceeded according to the manufacturer’s instructions. Briefly, 200 μL of chloroform (Sigma) were added to the lysate, shaken and incubated for 10 min at room temperature, followed by centrifugation (15 min at 13,000 × g, 4°C). The upper aqueous phase was removed and transferred to a new 1.5 mL Eppendorf tube and RNA was precipitated by the addition of 500 μL isopropanol and incubation at room temperature for 5–10 min. RNA pellets (10 min at 13,000 × g, 4°C) were washed twice with 75% ethanol and resuspended in 50 μL of nuclease-free water (Ambion).

To extract total secreted RNA from MM, *L. monocytogenes* was grown until exponential phase (OD_{600nm} = 0.4). Medium was recovered by centrifugation (2862 × g, 20 min, 4°C), filtered (0.22 μm) and concentrated 10 times using an Amicon centrifugal filter unit (3K cut-off). Medium was then brought back to the initial volume by adding nuclease-free water and concentrated again. This desalting process was repeated three times to avoid co-precipitation of salts during the subsequent RNA isolation. RNA was then extracted twice with acid phenol/chloroform, precipitated with ethanol/0.3 M sodium acetate and resuspended in nuclease-free water.

RNA extraction from LoVo cell monolayers in 6-well plates was performed by using TRIzol Reagent. Briefly, cells were washed once with ice-cold PBS and directly lysed in the well by adding 1 mL of TRIzol and gentle pipetting. Samples were vortexed thoroughly for 30 s before the addition of 200 μL chloroform and then incubated 3 min at room temperature. After centrifugation (15 min, 12,000 × g, at 4°C), the upper aqueous phase was transferred to a new Eppendorf tube and RNA was precipitated by the addition of an equal volume of isopropanol and incubation at room temperature for 10 min. RNA pellet was washed twice with 70% ethanol and resuspended in 50 μL of nuclease-free water.

**In Vitro RNA Transcription**

cDNA templates of the *L. monocytogenes* small RNAs fused with a T7 promoter were obtained by PCR amplification from genomic DNA with the appropriate primers. The cDNA quality was verified on a 1% agarose gel and visualized by ethidium bromide staining. cDNA was purified from agarose gel with a Gel extraction kit (QIAGEN) and resuspended in nuclease-free water. Purified cDNA DNA with the appropriate primers. The cDNA quality was verified on a 1% agarose gel and visualized by ethidium bromide staining.

**Electrophoretic Mobility Gel Shift Assay**

In vitro formation of HisZea - RNA complexes was assessed by electrophoretic mobility gel shift assay (EMSA). For in vitro RNA synthesis, 1 μg of cDNA template carrying a T7 promoter was amplified by PCR and in vitro-transcribed using the MAXiSscript T7 in vitro transcription kit according to the manufacturer’s recommendation. The quality of the in vitro-transcribed RNA was verified by SYBR Gold (Life Technologies) staining after running a 1% agarose gel. In vitro-transcribed RNA was purified and concentrated using “RNA clean & concentrator” (Zymo Research) before dephosphorylation and 5% end labeling as previously described (Chevalier et al., 2009). Labeled RNA was denatured for 1 min at 95°C, chilled on ice (5 min) and renatured by slowly cooling down to 25°C. Upon addition of HisZea (concentrations as indicated in the figure legends) the complex was formed in 20 μL of binding buffer [50 mM Tris pH 8.0, 300 mM NaCl, 10% glycerol, 50 μg/mL fatty acid-free BSA (Roche), supplemented with 1 μg of yeast tRNA (Invitrogen)] for 20 min at room temperature. Unlabeled competitor was added and samples were incubated for an additional 20 min. Samples were mixed with loading buffer (50% glycerol, 0.5% tris-borate EDTA and 0.1% xylene cyanol) before running on native 8% Novex TBE gels (Thermo Fisher Scientific). Signals were detected by autoradiography (at least one-h exposure at −80°C in presence of an intensifying screen).

**Biotin Pull-Down Assay**

HisZea (2.5 μg) was incubated with 50 μL of equilibrated streptavidin magnetic beads (BioLabs) in 250 μL of binding buffer (150 mM KCl, 25 mM Tris pH 8.0, 5 mM EDTA, 0.5 mM DTT, 0.5% NP40) for 45 min at 4°C with shaking. Beads were recovered by centrifugation (500 × g, 5 min, 4°C) and discarded. The HisZeap-containing supernatant was used in the subsequent steps. This precleaving step was performed in order to remove the Zea fraction which aggregated non-specifically onto the beads. Biotinylated RNA (500 nM) was added to the HisZea-containing supernatant and incubated for 30 min, 4°C with shaking. Then, 50 μL of equilibrated streptavidin magnetic beads were added for a further incubation (30 min, 4°C with shaking). The beads were then washed four times in binding buffer and bound HisZea was recovered by addition of NuPage LDS sample buffer.
RNase Protection Assay

Equimolar concentrations of HisZea or GST (1 μM) were mixed with 32P radiolabelled rli143 in 20 μL of binding buffer (50 mM Tris pH 8.0, 300 mM NaCl, 10% glycerol and 50 μg/mL fatty acid-free BSA) and incubated at 25 °C for 30 min. Then, 0.0033U of RNaseI (Ambion) were added before incubation for either 1 or 3 min at 37 °C. Reactions were stopped by addition of NuPage LDS sample buffer and samples were loaded on 8% Novex TBE-Urea gels (Thermo Fisher Scientific). Signals were detected by autoradiography (at least one-h exposure at ~80 °C in presence of an intensifying screen).

Immunoprecipitations

To assess the interaction between ZeaFlag and ZeaHA, 25 mL of L. monocytogenes overnight cultures expressing either ZeaFlag alone, or both ZeaFlag and ZeaHA were centrifuged (2862 × g, 20 min, 4 °C) to collect bacteria and culture medium. The recovered medium was filtered by using Millex-GP 0.22 μm filters (Millipore), supplemented with 0.2% of Triton X-100, before adding 20 μL of M2 Flag magnetic beads (Sigma). Samples were shaken for 2 h at 4 °C and then washed four times with lysis buffer (20mM Tris pH 8.0, 1 mM MgCl2, 150 mM KCl, supplemented with protease inhibitors mixture). The immunoprecipitated material was finally eluted using 100 μg/mL of 3xFlag peptide (Sigma) according to the manufacturer’s instructions. Bacterial pellet was washed twice in ice-cold PBS, resuspended in lysis buffer and lysed in a FastPrep apparatus (45 s, speed 6.5, thrice). The samples were then clarified by centrifugation (14000 × g, 10 min, 4 °C, twice) and protein concentration determined by Bradford assay. The same percentage of bacterial cytosol compared to the culture medium was used to immunoprecipitate ZeaFlag, under the same condition used for the culture medium. Equal amounts of eluted proteins were subjected to immunoblotting via wet transfer onto a 0.45 μm nitrocellulose membrane.

For the interaction between ZeaFlag and Strep-RIG-I, 25 mL of culture medium from L. monocytogenes wt and zeafag overnight cultures were recovered and washed four times with washing buffer (20 mM MOPS-KOH pH 7.4, 120 mM KCl, 0.2% Igepal, 2 mM beta-mercaptoethanol) and left on ice while preparing the cell lysates. HEK293 cells stably transfected with Strep-RIG-I were lysed in lysis buffer (20mM MOPS-KOH pH 7.4, 120 mM KCl, 0.2% Igepal, 2 mM beta-mercaptoethanol, supplemented with protease inhibitors mixture and 12.5 U/μl Rnasin (Promega), sonicated twice for 15 s at 20% amplitude and incubated on ice for 30 min. The cell lysate was cleared by centrifugation (14000 × g, 10 min, 4 °C) with the supernatant assayed for protein concentration with Bradford assay and used fresh. At least 1 mg of cell lysate was added to the Zea-containing washed Flag magnetic beads (prepared above) and incubated overnight at 4 °C with shaking. Beads were washed four times in washing buffer and twice in washing buffer without Igepal. For RNaseA treatment, 100 μg/mL of RNaseA (Roche) in lysis buffer without Igepal were added to the beads (30 min, ice) followed by two further washes in the same buffer. Zea was eluted from the magnetic beads with 3xFlag peptide at 100 μg/mL, according to the manufacturer’s recommendations, in a total volume of 50 μL. Samples were then subjected to immunoblotting via wet transfer onto a 0.45-μm nitrocellulose membrane.

For ZeaFlag immunoprecipitation from mammalian cells, LoVo cells in 10-cm2 dishes were transiently co-transfected with 7 μg of each DNA (ZeaFlag and Strep-RIG-I) using 24 μL of Lipofectectamine LTX (Thermo Fisher Scientific). 24 h after transfection, the cells were washed twice with PBS and lysed using 1 mL lysis buffer per dish (20 mM MOPS-KOH pH 7.4, 120 mM KCl, 0.2% Igepal, 2 mM beta-mercaptoethanol, supplemented with protease inhibitors mixture). The lysate was sonicated for 15 s, at 20% amplitude and incubated on ice for 30 min with shaking. The lysate was then clarified (13,000 × g, 10 min, 4 °C) and assayed for protein concentration (Bradford). 0.5 mg of total lysate was incubated with 15 μL of M2 Flag magnetic beads (overnight, 4 °C, shaking). Beads were recovered and washed three times in lysis buffer before treatment with RNase A and elution with the 3xFlag peptide (both performed as above). Samples were then subjected to immunoblotting via wet transfer onto a 0.45-μm nitrocellulose membrane.

For Hfq immunoprecipitation, bacteria were grown in 20 mL of BHI until stationary phase and pelleted (2862 g, 30 min, 4 °C). The bacterial pellet was washed twice with ice-cold PBS and mechanically lysed in 1 mL of lysis buffer (20 mM Tris pH 8.0, 1 mM MgCl2, 150 mM KCl, 1 mM DTT, supplemented with protease inhibitors) in 2 mL Lysing Matrix tubes by bead beating in a FastPrep apparatus (45 s, speed 6.5, thrice). Bacterial lysate was clarified by centrifugation (18,407 × g, 30 min, 4 °C) and protein concentration was determined by Bradford assay and used fresh. At least 50 μL of protein A Sepharose beads (GE Healthcare) were added for a further h of incubation (4 °C, shaking). The immune complexes were collected by centrifugation (500 × g, 5 min, 4 °C). After three washes with lysis buffer, the bound protein was eluted from the protein A Sepharose beads by boiling (10 min) in 50 μL LDS sample buffer.

For immunoprecipitation of ZeaFlag from nuclear and cytosolic fractions of infected LoVo cells (6 h, MOI 50), 20 μL of pre-equilibrated M2 Flag magnetic beads were added to equal percentage of cytosolic and nuclear fractions. Samples were incubated overnight at 4 °C with shaking. After three washes in lysis buffer (20 mM Tris pH 8.0, 150 mM NaCl, 1 mM DTT, 1% Igepal), immune complexes were retrieved by adding 100 mg/mL of 3xFlag peptide. Samples were then subjected to immunoblotting via wet transfer onto a 0.45-μm nitrocellulose membrane.

Immunofluorescence

Immunofluorescence was performed as previously described (David et al., 2018). Briefly, cells were fixed for 10 min in 4% paraformaldehyde/PBS at room temperature, permeabilized for 5 min in 0.1% Triton X-100/PBS and blocked for 10 min in 1% BSA, 10%
goat serum/PBS. Cells were then incubated for 1 h with primary antibody, washed in PBS, incubated for 45 min with secondary antibody/DAPI, washed again as above and mounted in Vectashield.

**Cell Fractionations**

Fractionation of cultured cell lines was performed as previously described (Pereira et al., 2018). Cell were resuspended in buffer A (20 mM HEPES pH 7, 0.15 mM EDTA, 0.15 mM EGTA, 10 mM KCl). 1% NP40 was added, followed by SR buffer (50 mM HEPES pH 7, 0.25 mM EDTA, 10 mM KCl, 70% (v/v) saccharose). Samples were centrifuged for 500 x g, 4°C. The supernatant was isolated as the cytosolic fraction and recentrifuged as before to eliminate cell nuclear debris. The pellet was washed in buffer B (10 mM HEPES pH 8, 0.1 mM EDTA, 100 mM NaCl, 25% (v/v) glycerol) and centrifuged 5 min at 500 x g, 4°C. Buffer A, B and SR were supplemented with 0.15 mM spermidine, 0.15 mM spermine, 1 mM DTT and protease inhibitor. The washed pellet was resuspended in sucrose buffer (20 mM Tris pH 7.65, 60 mM NaCl, 0.34 M sucrose, 0.15 mM MgCl2) to obtain a final salt concentration of 250 mM. Samples were incubated for 25 min and centrifuged for 10 min at 13,000 x g, 4°C. The supernatant was isolated as the nuclear soluble fraction from the pellet which represents chromatin and nuclear insoluble material. The pellet was resuspended in sucrose buffer supplemented with 0.0025 U/µL and 1 mM CaCl2 and was incubated at 37°C for 10 min. 4 mM EDTA was added and samples were sonicated using the Bioruptor (Diagenode) for 7.5 min (15 s on and 1 min off) and centrifuged for 15 min at 13,000 x g, 4°C. The supernatants represent a soluble chromatin fraction.

**Live/Dead Bacterial Staining**

*L. monocytogenes* was grown in 20 mL of BHI until stationary phase. Bacteria were pelleted (2862 x g, 20 min, room temperature) and then stained with LIVE/DEAD BacLight (Molecular Probes), following the manufacturer’s recommendation. Bacterial suspension (20 µL) was then deposited on a glass coverslip and immediately imaged by using a Zeiss AxioObserver.Z1 inverted fluorescence microscope equipped with an Evolve EM-CCD camera (Photometrics). Images were acquired with a 100 x N.A. 1.4 oil objective using MetaMorph.

**RIP-Seq**

50 mL of *L. monocytogenes* stationary phase (OD_{600nm} = 3.5) culture (a zea-deletion strain in which one copy of the zea gene was integrated in the *L. monocytogenes* genome under the control of a constitutive promoter) was centrifuged (2862 x g, 20 min, 4°C) to recover the culture medium and the bacterial pellet. 10 mL culture medium were filtered (0.22-µm) and concentrated to 1 mL by using Amicon centrifugal filter unit (3K cut-off). Concentrated medium was then supplemented with 0.05% of Triton X-100, centrifuged again (18407 x g, 20 min, 4°C) and left on ice while preparing bacterial cytosolic extracts. The bacterial pellet was washed thrice in ice-cold PBS and lysed by mechanical shaking in a FastPrep apparatus (described above) in 1 mL of lysis buffer (25mM Tris pH 7.4, 150mM KCl, 1mM DTT, 0.05% Triton X-100) supplemented with protease inhibitors mixture. Bacterial cytosol was recovered by centrifugation (two serial centrifugations at 18407 x g, 20 min, 4°C) and protein concentration determine by Bradford assay. One-milliliter of bacterial cytosol and concentrated culture medium (corresponding to 50 mL and 10 mL of the bacterial cytosol and culture medium, respectively) was individually loaded on a Superose 6 10/300 GL column pre-equilibrated with lysis buffer without Triton X-100. About 161 fractions of 220 µL were collected, and one out of every seven fractions was concentrated by acetone precipitation and the presence of Zea and EF-Tu was then determined by immunoblotting to eliminate nuclear debris. The aqueous upper phase was recovered and fractions containing the complexes (A or B) were then pooled and processed for immunoprecipitation assays. Briefly, each sample was incubated overnight at 4°C with shaking, with a mix of 30 µg of anti-Zea antibodies (10 µg of each antibody) or 30 µg of normal rabbit IgG (CellSignaling). Then, 50 µL of Protein A Sepharose were added for further 2 h to recover immunocomplexes. The beads were washed four times with lysis buffer and treated with Turbo DNase (Ambion) for 10 min at 37°C in 200 µL 1X Turbo DNase buffer. Samples were vortexed for 30 s after the addition of 200 µL of acid phenol (Ambion). Then, 50 µL chloroform (Sigma) were added and samples were centrifuged for 5 min at 10 000 x g at 4°C. The aqueous upper phase was recovered and transferred to a new Eppendorf tube. RNA was precipitated by adding the same volume of isopropanol and 0.3 M sodium acetate (Ambion) and 1 µL of glycogen (Invitrogen). Samples were centrifuged (30 min, 10 000 x g, 4°C), and RNA was washed once with 70% ethanol before being resuspended in 25 µL of nuclease-free water. RNA was analyzed with the Bioanalyser RNA pico kit (Agilent). Purified RNA was fragmented with the “RNA fragmentation reagents” (Thermofisher), purified by ethanol precipitation and quality-controlled with the Bioanalyzer, as described above. Directional RNA-seq libraries were prepared with 30 ng of purified RNA for each sample by using NEBNext Multiplex Small RNA Library Prep Set for Illumina (New England Biolabs) according to the manufacturer’s instructions. When required, the RNA samples were spiked-in with a synthetic *in vitro*-transcribed AdML splicing reporter (Allemand et al., 2016) in order to have 30 ng of total RNA. Libraries were sequenced on an Illumina HiSeq 2500 platform (SR100).

**RIP-Seq Data Analysis**

The *L. monocytogenes* EGD-e genome (NC_003210) and a list of 3160 transcripts (genes, small-RNAs, tRNAs, and rRNAs) were downloaded from the Listeriomics database (Bécavin et al., 2017). After the sequencing of all RIP-Seq samples, the resulting reads were trimmed (AllenTrimmer 0.4.0, default parameters) (Crisculo and Brisse, 2013). They were mapped on the EGD-e genome using Bowtie2 2.1.0 (very-sensitive parameter) (Langmead and Salzberg, 2012). Mapping files were filtered to keep uniquely mapped reads using SAMtools 0.1.19 (samtools view -b -q 1 parameters) (Li et al., 2009), and saved to BAM files after indexation. Read Per Million
coverage files were saved in BigWig format using bamCoverage package from deepTools 3.1.3 (Ramirez et al., 2016). The quality of the sequencing and mapping was assessed using FastQC 0.10.1 and MultiQC 0.7 (Ewels et al., 2016). The number of reads per transcript (mRNA, sRNA, tRNA, rRNA) was counted using FeatureCount (1.4.6-p3 default parameters) (Liao et al., 2014). Statistical analysis was performed using SARTools package (Varet et al., 2016) and in-house R scripts (https://github.com/becavin-lab/ RIPSeq-Listeria). Data were normalized with the TMM (Robinson et al., 2010) (edgeR package) normalization method. Finally, the log2(Fold changes) were calculated by subtraction of log2(TMM) normalized expression values.

**Sequencing of Total Secreted L. monocytogenes RNA**

To extract total secreted RNA from the culture medium, L. monocytogenes strains (wt and Zea+) were grown to exponential phase (OD600nm = 0.4) in 14 mL of MM under microaerophilic conditions using Oxoid AnaeroGen 2.5L gas packs (Thermo Fisher) at 25°C. Under this condition, Zea appeared slightly upregulated compared to standard growth conditions (i.e. 37°C, BHI medium) (Bécavin et al., 2017). A parallel culture (same conditions) was set-up to check the OD and arrest the bacterial growth when the strains reached the same OD. The culture medium was then recovered by centrifugation (2862 g (Be´cavin et al., 2017). A parallel culture (same conditions) was set-up to check the OD and arrest the bacterial growth when the strains reached the same OD. The culture medium was then recovered by centrifugation (2862 g, 20 min, 4°C) and filtered (0.22 µm). The bacterial pellet was stored at −80°C for subsequent RNA extraction. 10 mL of the filtered culture medium were desalted and the RNA was extracted as described above. The quality of the RNA was checked by the Bioanalyser RNA nano kit. The amount of recovered RNA was similar in all the samples. Total secreted RNA (5 µg) was ribodepleted by using the Rib Zero rRNA removal kit (Illumina) following the manufacturer’s instructions. Ribodepletion was controlled by the Bioanalyser RNA pico kit. Directional RNA-seq libraries were prepared with 100 ng of purified RNA for each sample by using NEBNext Multiplex Small RNA Library Prep Set for Illumina according to the manufacturer’s instructions. Libraries were sequenced on an Illumina NextSeq500 platform (SR75).

**Sequencing of Secreted L. monocytogenes RNA**

The RNA-seq datasets were first trimmed to keep only reads longer than 45bp (AlienTrimmer 0.4.0, -l45) (Crisculo and Brisse, 2013). They were mapped on the EGD-e genome using Bowtie2 2.1.0 (very-sensitive parameter) (Langmead and Salzberg, 2012). Mapping files were filtered to keep uniquely mapped reads using SAMtools 0.1.19 (samtools view -b -q 1 parameters) (Li et al., 2009), and saved to BAM files after indexation. Read Per Million coverage files were saved in BigWig format using bamCoverage package from deepTools 3.1.3 (Ramirez et al., 2016). The quality of the sequencing and mapping was assessed using FastQC 0.10.1 and MultiQC 0.7 (Ewels et al., 2016). The number of reads per transcript (mRNA, sRNA) was counted using HTSeq 0.9.1 (–s no –m union–nonunique all parameters) (Anders et al., 2015). Differential analysis was performed using SARTools (Varet et al., 2016) and DESeq2 R (Love et al., 2014) packages.

**RIP-qPCR**

L. monocytogenes bacterial cultures (Zeaz+ Zea+) were processed essentially as described for the RIP-seq experiment unless otherwise stated. In summary, L. monocytogenes was grown until the stationary phase (OD600nm = 3.5) and, for each sample, 50 mL of bacterial culture were processed as follows. Bacteria were pelleted at 2862 g, 20 min, 4°C and culture supernatant was filtered and processed (5 mL) for total RNA purification (input, 10%), by performing two sequential phenol/chloroform extractions followed by ethanol/sodium acetate precipitation. The RNA pellet was washed once with ethanol 70% and resuspended in 20 µL of nuclease-free water. Purified RNA was then treated with Turbo DNase and purified again, as described above. The remaining medium (45 mL, 90% of the initial sample) was processed for Zea immunoprecipitation. Briefly, 20 µg of a mix of Zea antibodies (6.6 µg of each antibody) were coupled to 100 µL of Protein A Dynabeads (Invitrogen) for 2 h in 500 µL of PBS (room temperature, shaking). Beads were then washed twice with PBS and once with lysis buffer (25mM Tris pH 7.4, 150mM KCl, 1mM DTT, 0.05% Triton X-100). Culture medium was supplemented with 0.05% Triton X-100 before the addition of the anti-Zea antibody-coupled beads. Samples were then incubated overnight (4°C, shaking). Beads were washed four times with lysis buffer, treated with Turbo DNase and processed for RNA extraction. Purified RNA was stored at −80°C until use. The bacterial pellet was washed thrice in ice-cold PBS and then mechanically lysed by using FastPrep apparatus in 1 mL of lysis buffer supplemented with protease inhibitors mixture and RNasin (Promega) at 12.5 U/µL. Bacterial lysate was clarified by two sequential centrifugations and the final volume was carefully measured. A volume corresponding to 10% of the total sample (input) was treated with DNase and processed for RNA isolation by phenol/chloroform extraction and ethanol/sodium acetate precipitation. Purified RNA was resuspended in nuclease-free water and stored at −80°C until use. The remaining bacterial cytosol was incubated with an anti-Zea antibody coupled to Protein A as described above (overnight, 4°C, shaking). Beads were washed four times with lysis buffer, treated with Turbo DNase and processed for RNA extraction. For qPCR analysis, 100 ng of purified RNA were subjected to reverse transcription in 20 µL final volume using the Reverse Transcription Kit (Qiagen) according to the manufacturer’s instructions. Reactions were then diluted by adding 180 µL of nuclease-free water. qPCR was assayed in 10 µL reactions with Brilliant III Ultra Fast SYBR-Green qPCR Master Mix (Agilent). Reactions were carried out in a Stratagene MX3005p system with the following thermal profile: 5 min at 95°C, 37 cycles of 10 s at 95°C and 12 s at 60°C. Results were analyzed with an MxPro software, as described earlier (Batsché et al., 2006).

**Quantitative Real-Time PCRs**

For qPCR of L. monocytogenes secreted RNA (phage, lma-monocin and rli143 RNAs), bacterial strains were grown in MM until exponential phase (OD600nm = 0.4). L. monocytogenes wt, Zeaz+ Zea+ strains were used for the phage and lma-monocin...
quantification; *L. monocytogenes* wt, zea, zeα and *imo2595*. *L. monocytogenes* strains were used for the quantification of rli143; *L. innocua* wt, zeα-pAD and *imo2595-pAD* strains were employed for the quantification of rli143. The bacterial OD was measured, and the cultures were recovered when OD was equal for all the strains. MM was collected by centrifugation, filtered and processed for RNA extraction (as described above). Purified RNA (5-10 μg) was subjected to DNase treatment using the DNase treatment and removal kit (Ambion). Treated RNA (500 ng) was mixed with an equal amount of CleanCap™ OVA mRNA (TriLink) which serves as an internal control for normalization, and processed for reverse transcription and qPCR, as above. Gene expression levels were normalized to the OVA mRNA, and the fold change was calculated using the ΔΔCT method.

For qPCR of *Listeria* genes from total (intracellular) *L. monocytogenes* RNA, the RNA was extracted, as described in the RNA extraction section and treated, as described above, except that the OVA mRNA was not included in the reverse transcription reaction. Gene expression levels were normalized to the *rpoB* gene, and the fold change was calculated using the ΔΔCT method.

For qPCR of Hfq-associated RNAs, RNA was extracted from immunoprecipitated Hfq, by using the protocol described for the RIP-seq of Zea. DNase-treated RNA (120 ng) was subjected to reverse transcription. Gene expression levels were normalized to the input fractions, and the fold change was calculated using the ΔΔCT method.

For qPCR of IFNβ, IFNγ and IL-8, mammalian RNA was extracted, as described in the RNA extraction section. Purified RNA (5-10 μg) was subjected to DNase treatment, and 1 μg processed for reverse transcription, as described above. Gene expression levels were normalized to the actin mRNA and to the uninfected samples, and the fold change was calculated using the ΔΔCT method.

**Purification of RLRs and RNA Extraction**

Four 15-cm² tissue culture dishes per cell line were pretreated with 0.1 mg/mL poly-L-Lysine-hydrobromide (Sigma), rinsed with distilled water and dried for 1 h before plating the cells. Cells (30-40x10⁶) were plated per dish in 20 mL of DMEM medium for 24 h before infection. Overnight *L. monocytogenes* EGDe cultures in BHI were diluted 1/20 in fresh BHI the day of infection and grown up to OD₅₀₀nm = 1. Each plate was infected with an MOI of 50 for 1 h before replacing the media with complete DMEM containing 10 μg/mL of gentamicin to kill extracellular bacteria. After an additional 3 h (in total 4 h of infection), plates were rinsed twice with ice-cold PBS, crosslinked at 400 mM/cm² in 10 mL of ice-cold PBS/plate and cells were then scraped, pelletized and resuspended in 8 mL of MOPS lysis buffer (20 mM MOPS-KOH pH 7.4, 120 mM KCl, 0.5% Igepal, 2 mM beta-mercaptoethanol, supplemented with protease inhibitors mixture and RNasin at 0.2 U/μL and protease inhibitors mixture (Roche). Cell lysates were incubated on ice for 20 min with gentle mixing every 5 min and then clarified by centrifugation at 16000 x g for 15 min at 4°C. Streptactin Sepharose beads (GE Healthcare, 100 μL/dish) were washed in MOPS washing buffer (20 mM MOPS-KOH pH 7.4, 120 mM KCl, 2 mM beta-mercaptoethanol, supplemented with RNasin 0.2 U/μL and protease inhibitors mixture and finally resuspended in 1 mL of MOPS-lysis buffer per initial culture dish. Clarified cell lysate was incubated with Streptactin beads for 2 h at 4°C. The beads were washed three times with MOPS washing buffer and centrifuged at 1600 x g, 5 min at 4°C. Strep-tagged proteins were then eluted twice for 15 min at 4°C in 250 μL/dish of 1X elution buffer (IBA, Biotin Elution Buffer 10X). Each sample was treated with proteinase K (Roche) in v/v of 2X proteinase K buffer (200 mM Tris pH 8, 100 mM NaCl, 20 mM EDTA, 4M urea) for 20 min at 4°C. Proteins were then subjected to DNase treatment and RNA purification was performed using TRI Reagent LS (Sigma). RNA was dissolved in 50 μL of DNase-free and RNase-free ultrapure water. Extracted RNAs were analyzed using Nanovue (GE Healthcare) and Bioanalyzer RNA nano kit (Agilent) before being processed for next-generatio sequencing (HiSeq 2500, SR50).

**Data Analysis of RLR-Associated RNAs**

Due to the high number of eukaryotic RNAs in the datasets and presence of insertions and deletions, the reads were trimmed (AlienTrimmer 0.4.0) (Criscuolo and Brisse, 2013), and mapped using GSNAP (v2018-07-04) (Wu et al., 2016), a special mapping software allowing variability in reads sequence. Mapping files were filtered to keep uniquely mapped reads using SAMtools 0.1.19 (samtools view -b -q 1 parameters) (Li et al., 2009), and saved to BAM files after indexation. Read Per Million coverage files were saved in BigWig format using bamCoverage package from deepTools 3.1.3 (Ramirez et al., 2016). The quality of the sequencing and mapping was assessed using FastQC 0.10.1 and MultiQC 0.7 (Ewels et al., 2016). The number of reads per transcript (mRNA, sRNA, tRNA, rRNA) was counted using HTSeq 0.9.1 (–s no -m union–nonunique all parameters) (Anders et al., 2015). Differential analysis was performed using SARTools (Varet et al., 2016) and EdgeR packages (Robinson et al., 2010) (https://github.com/becavin-lab/RIPSeq-Listeria).

**Transfection of Zea-Interacting RNAs**

The ISRE reporter cells (STING-37 cell line) (Lucas-Hourani et al., 2013) were seeded in 24-well plates and 2 h later transfected with 100 ng of *in vitro*-transcribed rli143, rli18, rli92 and 250 nucleotides-long fragments of mCherry RNAs (Chazal et al., 2018) using Lipofectamine 2000 (Thermofisher Scientific). 100 ng of high molecular weight (HMW, ttrl-pic, Invivogen) and low molecular weight Poly(I:C) (LMW, ttrl-picw, Invivogen), and 100 ng of short 5’3P RNA (produced as previously described (Lucas-Hourani et al., 2013)) were used as positive controls. Cells were lysed 24 h post-transfection with 200 μL Passive Lysis buffer (Promega), The Firefly luciferase activity was measured using the Bright-Glo Luciferase Assay System (Promega) following the manufacturer’s recommendation.
Mice Infections

L. monocytogenes was thawed from glycerol stocks stored at –80°C and diluted in phosphate-buffered saline (PBS) before injection. A sublethal dose (10⁴ L. monocytogenes) was injected into the lateral vein of the tail of each mouse. The number of bacteria in the inoculum was confirmed by plating serial dilutions of the bacterial suspension onto BHI agar plates. For determination of bacterial loads, livers and spleens were recovered and disrupted in PBS at the indicated time points post-infection. Serial dilutions of organ homogenates were plated onto BHI agar plates, and colony forming units (CFUs) were counted after growth at 37°C for 48 h.

QUANTIFICATION AND STATISTICAL ANALYSIS

All data are expressed as mean and standard error of the mean. Student’s t test or ANOVA were used for statistical analysis. Differences in means were considered statistically significant at p < 0.05. Sample number (n) indicates the number of independent biological samples in each experiment, for each set of experiments this information is provided in the figure legends.

DATA AND CODE AVAILABILITY

The accession number for RIP-Seq, RNA-seq and RLRs purification and sequencing data reported in this paper is [Array Express at EMBL-EBI]: [E-MTAB-7665]. All scripts used for the analysis have been deposited on the Institut Pasteur GitLab: https://github.com/becavin-lab/RIPSeq-Listeria.