The *Listeria* Small RNA Rli27 Regulates a Cell Wall Protein inside Eukaryotic Cells by Targeting a Long 5′-UTR Variant

Juan J. Quereda\(^1\)*a, Álvaro D. Ortega\(^1\)*b, M. Graciela Pucciarelli\(^1,2\)*, Francisco García-del Portillo\(^1\)*

1 Centro Nacional de Biotecnología-Consejo Superior de Investigaciones Científicas (CNB-CSIC), Madrid, Spain, 2 Departamento de Biología Molecular, Universidad Autónoma de Madrid, Centro de Biología Molecular ‘Severo Ochoa’ (CBMSO-CSIC), Madrid, Spain

Abstract

*Listeria monocytogenes* is a bacterial pathogen whose genome encodes many cell wall proteins that bind covalently to peptidoglycan. Some members of this protein family have a key role in virulence, and recent studies show that some of these, such as Lmo0514, are upregulated in bacteria that colonize eukaryotic cells. The regulatory mechanisms that lead to these changes in cell wall proteins remain poorly characterized. Here we studied the regulation responsible for increased Lmo0514 protein levels in intracellular bacteria. The amount of this protein increased markedly in intracellular bacteria (>-200-fold), which greatly exceeded the increase in *lmo0514* transcript levels (~6-fold). Rapid amplification of 5′-cDNA ends (RACE) assays identified two *lmo0514* transcripts with 5′-untranslated regions (5′-UTR) of 28 and 234 nucleotides. The transcript containing the long 5′-UTR is upregulated by intracellular bacteria. The 234-nucleotide 5′-UTR is also the target of a small RNA (sRNA) denoted Rli27, which we identified by bioinformatics analysis as having extensive base pairing potential with the long 5′-UTR. The interaction is predicted to increase accessibility of the Shine-Dalgarno sequence occluded in the long 5′-UTR and thus to promote Lmo0514 protein production inside the eukaryotic cell. Real-time quantitative PCR showed that Rli27 is upregulated in intracellular bacteria. In vivo experiments indicated a decrease in Lmo0514 protein levels in intracellular bacteria that lacked Rli27. Wild-type Lmo0514 levels were restored by expressing the wild-type Rli27 molecule but not a mutated version unable to interact with the *lmo0514* long 5′-UTR. These findings emphasize how 5′-UTR length affects regulation by defined sRNA. In addition, they demonstrate how alterations in the relative abundance of two transcripts with distinct 5′-UTR confine the action of an sRNA for a specific target to bacteria that occupy the intracellular eukaryotic niche.

Introduction

*Listeria monocytogenes* is a facultative intracellular food-borne bacterium responsible for serious clinical manifestations including febrile gastroenteritis, meningitis, encephalitis and maternofetal spreading to adjacent cells [4].

The bacterium responsible for serious clinical manifestations including febrile gastroenteritis, meningitis, encephalitis and maternofetal


[Image 565x695 to 602x732]
Author Summary

*Listeria monocytogenes* has evolved to adapt to numerous environments, including the intracellular niche of eukaryotic cells. Small RNAs (sRNA) play important regulatory roles in changing environments, and are thus predicted to modulate *L. monocytogenes* adaption to the intracellular lifestyle. This study shows how the regulatory activity of an sRNA on a defined target is restricted to bacteria in the intracellular infection phase. This regulation relies on a long (234-nucleotide) 5' UTR that bears the sRNA-binding site present in a transcript variant that is upregulated by intracellular *L. monocytogenes*. The concomitant increase in both the target transcript containing the long 5' UTR and the sRNA, which is postulated to facilitate opening of the Shine-Dalgarno site, culminates in markedly higher protein levels in intracellular bacteria. The limited amounts of both the target and the regulator in extracellular bacteria ensure that production of this bacterial protein is confined mainly to the host rather than the non-host environment.

Lmo0514 [12]. The mechanisms that regulate the coordinated production of such a large number of LPXTG proteins nonetheless remain largely unknown.

Bacterial small RNAs (sRNA) are a class of bacterial gene expression regulators important in many physiological processes, including virulence and cell envelope homeostasis [13,14]. sRNA coordinate target gene expression in response to environmental changes and have regulatory functions that affect protein activity and mRNA stability/translation in many microorganisms, including bacterial pathogens [15,16]. More than 100 sRNA have been identified for *L. monocytogenes* by the use of tiling arrays, global RNA sequencing (RNA-Seq) and bioinformatics methods [14,17,18]; more than 30 of these have been validated by northern blot, but their biological function and mechanisms of action are so far unknown [19]. There is little information on the regulation of sRNA expression in *L. monocytogenes*. Some reports implicate the alternative sigma factor SigB in regulating expression of the sRNA SbrA (Rli11) and SbrE (Rli47) [17,20,21]. In addition, 22 sRNA genes are preceded by putative sigma A boxes in the *L. monocytogenes* genome [14]. Recent studies also show that the sRNAs Rli31, Rli33-1, Rli38 and Rli50 modulate virulence in *L. monocytogenes* [14,17]. Despite these studies, there is no model that describes how sRNA expression in *L. monocytogenes* responds to infection of eukaryotic cells. With the exception of LhrA, which controls expression of the chitinase ChiA post-transcriptionally [22], and of the multicopy sRNA LhrC, which modulates LapB adhesin expression [23], the identity of the functions targeted by *L. monocytogenes* sRNA inside or outside eukaryotic cells, remains unknown.

Here we studied the regulatory mechanism responsible for the increase in the LPXTG protein Lmo0514 in the cell wall of intracellular bacteria [12]. Our data demonstrate an sRNA that is a key regulatory element in modulating levels of this cell wall surface protein during intracellular infection. This response to the eukaryotic niche is directed by the activity of two promoters in the target gene that generate transcripts with 5' untranslated regions (5' UTR) of distinct length. The relative abundance of these two transcripts differs in extra- and intracellular bacteria. Only the ‘long’ version, enriched in intracellular bacteria, bears the sRNA binding site. This mechanism confines the regulation of *lmo0514* by this sRNA to the intracellular eukaryotic niche.

Results

The *L. monocytogenes* gene that encodes the LPXTG surface protein Lmo0514 is expressed as two variants with distinct 5' UTR

Lmo0514, a *L. monocytogenes* LPXTG surface protein of unknown function, is encoded by a gene upregulated by bacteria located within macrophages [24]. Lmo0514 is also more abundant in the cell wall of bacteria that proliferate inside epithelial cells than in bacteria growing in laboratory media [12]. To study the basis of this regulation, we compared *lmo0514* expression in extra- and intracellular bacteria. Real-time quantitative PCR (qPCR) assays showed enhanced *lmo0514* mRNA expression (~6-fold) in intracellular bacteria after infection of JEG-3 human epithelial cells (Fig 1A). Consistent with our previous work [12], the Lmo0514 protein was detected mainly in the cell wall of intracellular bacteria, with very low levels in extracellular bacteria (Fig 1B). Changes in relative levels of Lmo0514 protein were estimated to be ~200-fold (Fig 1B), much higher than those for *lmo0514* mRNA (~6-fold). This lack of correlation between induction of *lmo0514* transcript and protein levels in intracellular bacteria led us to hypothesize that post-transcriptional regulatory mechanisms act on this gene.

To evaluate this possibility, we sought *lmo0514* gene expression control mechanisms that operate specifically in intracellular bacteria. Previous *in silico* predictions by Loh et al. [23] indicated that *lmo0514* could be expressed from three promoters at

![Figure 1. Regulation of the *L. monocytogenes* gene that encodes the LPXTG surface protein Lmo0514 in intracellular bacteria](image-url)
positions −26, −104 and −163. Two of these, −26 and −163, were assigned as tentatively regulated by sigma A (σ^A) and the third, at position −104, as controlled by sigma B (σ^B) [25] (Fig. 2A). The activity of these putative promoters and the presence of the different transcripts were analyzed by RT-PCR on RNA isolated from *L. monocytogenes* grown extracellularly and from intracellular bacteria that colonized JEG-3 epithelial cells. *lmo0514* transcripts with a long 5′-UTR were detected specifically in intracellular bacteria (Fig. 2A). To confirm these findings, rapid amplification of 5′-cDNA ends (5′-RACE) assays were used to map transcriptional start sites (TSS) of *lmo0514* in bacteria grown extracellularly and in bacteria isolated from eukaryotic cells. These 5′-RACE assays revealed two distinct TSS at positions −28 and −234 (Fig. 2B, C), and also confirmed expression of the long *lmo0514* transcript by intracellular bacteria (Fig. 2C). Putative promoters for these TSS, which we termed P1 and P2, both bear bona fide −10 TATA boxes (Fig. 2B, C). The existence of two *lmo0514* transcripts of different length was verified by northern blot (Fig. 3A), with sizes compatible with cotranscription of *lmo0514* with the downstream gene *lmo0515*, which encodes a universal stress protein [26]. *lmo0514-lmo0515* cotranscription was verified by RT-PCR (Fig. S1). qRT-PCR assays confirmed that expression of the *lmo0514* transcript variant with the long 234-nucleotide (nt) 5′-UTR was upregulated by ~12-fold in intracellular bacteria (Fig. 3B). These findings suggested that the specific induction of this mRNA variant with a longer 5′-UTR in intracellular bacteria accounts for or contributes to the 6-fold increase in total *lmo0514* mRNA (Fig. 1A). These data supported a model in which intracellular bacteria specifically upregulate expression from the P2 promoter, resulting in an *lmo0514* transcript with a long 5′-UTR. This assumption takes into account the different ratios between the two *lmo0514* transcripts when *L. monocytogenes* colonizes the eukaryotic cell.

The *lmo0514* long 5′-UTR variant has a binding site for Rli27, an sRNA induced in intracellular bacteria

The increased length of the *lmo0514* transcript variant that is upregulated in intracellular bacteria prompted us to test whether the distinctive 234-nt 5′-UTR is a target region for sRNA-mediated post-transcriptional regulation. We used in silico analysis to search for putative non-coding RNAs in the *L. monocytogenes* reference strain EGD/17 that could bind to this *lmo0514* long 5′-UTR. The targetRNA program [http://cs.wellesley.edu/~bjaden/TargetRNA2/] [28] gave a high score to a pairing between defined stretches of the *lmo0514* 234-nt 5′-UTR and a sequence in the *lmo0411-lmo0412* intergenic region. A gene in this region encodes an sRNA termed Rli27 that is upregulated by *L. monocytogenes* in the intestine of infected mice and in human blood, as shown by transcriptomics [17]; RNA-seq corroborated the expression of this sRNA [18]. Although Rli27 was identified as an sRNA induced in infection conditions [17], no further characterization of its function or targets was reported.

Genomic comparisons of pathogenic and non-pathogenic species are usually carried out to identify virulence genes,

**Figure 2.** *lmo0514* is expressed differentially from two distinct transcriptional start sites in extra- and intracellular *L. monocytogenes*. (A) Position of the three transcriptional start sites (TSS) predicted in silico for *lmo0514* by Loh et al. [25]. Primers used to amplify the *lmo0514* coding sequence are indicated (ORF, primers Lmo0514-F, Lmo0514-R, see Table S2), as well as two fragments of the 5′-UTR of different lengths, amplicon “a” (254 nt), obtained with primers UTR-B and UTR-1R (Table S2) and amplicon “b” (134 nt), obtained with primers UTR-A and UTR-1R (Table S2). Reverse transcriptase-PCR assays showing upregulation in intracellular bacteria of an *lmo0514* transcript isoform with a long 5′-UTR. 16S rRNA was monitored as loading control. RNA was obtained from extracellular bacteria grown in BHI medium to exponential logarithmic phase (log), stationary phase (stat), and from intracellular bacteria. (B) 5′-RACE assay showing a TSS at position −28 relative to the ATG site in extracellular bacteria. Colored bar indicates the position of the primer Lmo0514-PE-1rv (Table S2) used for this reaction. (C) 5′-RACE assay showing the production by intracellular bacteria of an *lmo0514* transcript with a long 5′-UTR derived from a TSS at position −234 relative to the ATG site. Colored bar indicates the position of the primer Lmo0514-PE-6rv (Table S2) used for this reaction. TAP, tobacco acid pyrophosphatase.

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Selective sRNA Targeting to a Long 5’-UTR

including sRNAs [18,29]. We analyzed the genomic region of *L. monocytogenes* containing *rli27* and those of the non-pathogenic species *L. innocua* and *L. welshimeri*. In *L. monocytogenes*, *rli27* is flanked by *lm0411* and *lm0412*, two genes that map in the opposite DNA strand (Fig. S2), whereas in the *L. welshimeri* genome, the same intergenic region has a small ORF (*lwe0373*) that codes for a predicted protein of unknown function (Fig. S2). We nonetheless found that *Rli27* is highly conserved in *L. innocua* (82% identity, Fig. 4A), in contrast with a previous report [17].

The extremely variable *rli27* genomic region might thus have been shaped by gain and/or loss of genes during *Listeria* speciation. Apart from *Listeria* species, BLAST searches did not identify *rli27* orthologs in other bacterial species. *Rli27*, identified as a 131-nt sRNA [14,18], is not predicted to encode any protein using the Small Open Reading Frame (ORF) tool in the ORF finder program (http://www.bioinformatics.org/sms2/orf_find.html).

Although the existence of *Rli27* sRNA was inferred based on its detection by genomic and transcriptomic approaches, it has not yet been formally demonstrated. The presence of *rli27* and its flanking genes in different strains ruled out the possibility that its detection by tiling arrays and RNA-seq analyses was due to transcribed regions of neighbor genes. *rli27* has its own predicted transcription start site and Rho-independent terminator sequence (Fig. 4A), and the respective promoter regions in *L. monocytogenes* and *L. innocua* showed no significant divergence (Fig. 4A).

Northern blot assays using total RNAs isolated from bacteria grown in BHI medium to exponential (log) or stationary phases (Fig. 4C). These findings indicate that *Rli27* is a bona fide sRNA that is upregulated in intracellular bacteria. Bacteria were grown in BHI medium to exponential (log) or stationary phase, or collected from epithelial cells. Data derive from a minimum of three independent experiments. ***, *P<0.001, Student’s t-test. doi:10.1371/journal.pgen.1004765.g004

**Figure 3.** Northern blot and real-time quantitative PCR (qPCR) assays confirm the predominance of *lm0514* transcripts of different lengths in extra- and intracellular *L. monocytogenes*. (A) Northern blot assays showing the short and long *lm0514* transcript isoforms in RNA isolated from extra- and intracellular bacteria, respectively. Transcript size is compatible with cotranscription of *lm0514* with downstream gene *lm0515* (Fig. S1). Relative 16S rRNA levels are shown for comparison. (B) Relative *lm0514* transcript levels detected by qPCR in extra- and intracellular bacteria, with Utr0514_qPCR_F and Utr0514_qPCR_R primers (Table S2) specific for *lmo0514* respectively. Transcript size is compatible with cotranscription of isoforms in RNA isolated from extra- and intracellular bacteria. (A) Sequence alignment of the *rli27* genomic region from *L. monocytogenes*, *L. welshimeri* and *L. innocua*. The −35 and −10 predicted sites for the *rli27* promoter and the *rli27* itself (grey background) are highlighted. Nucleotide sequence in orange corresponds to the predicted terminator shared by *rli27* and *lm0514*. Note that *rli27* is absent in *L. welshimeri*. (B) Northern blot assay performed with total RNAs isolated from bacteria grown in BHI medium to stationary phase. *L. monocytogenes* strains used included EGDe (WT) and the Δ*rli27* mutant. 5S rRNA was used as loading control. (C) Real-time qPCR showing upregulation of *Rli27* expression in intracellular bacteria. Bacteria were grown in BHI medium to exponential (log) or stationary phase, or collected from epithelial cells. Data derive from a minimum of three independent experiments. ***, *P<0.001, Student’s t-test. doi:10.1371/journal.pgen.1004765.g003

**Figure 4.** *Rli27* is a bona fide *L. monocytogenes* sRNA induced by intracellular bacteria. (A) Sequence alignment of the *rli27* genomic region from *L. monocytogenes*, *L. welshimeri* and *L. innocua*. The −35 and −10 predicted sites for the *rli27* promoter and the *rli27* itself (grey background) are highlighted. Nucleotide sequence in orange corresponds to the predicted terminator shared by *rli27* and *lm0514*. Note that *rli27* is absent in *L. welshimeri*. (B) Northern blot assay performed with total RNAs isolated from bacteria grown in BHI medium to stationary phase. *L. monocytogenes* strains used included EGDe (WT) and the Δ*rli27* mutant. 5S rRNA was used as loading control. (C) Real-time qPCR showing upregulation of *Rli27* expression in intracellular bacteria. Bacteria were grown in BHI medium to exponential (log) or stationary phase, or collected from epithelial cells. Data derive from a minimum of three independent experiments. ***, *P<0.001, Student’s t-test. doi:10.1371/journal.pgen.1004765.g003

**Rli27** interacts physically with the 5’-UTR specific to the *lm0514* long transcript

*Rli27* interaction with the *lm0514* 5’-UTR extends to several regions, although it shows a major predicted pairing region involving *Rli27* nucleotides 1 to 21 (Fig. 5A, Fig. S3). We used electrophoretic mobility shift assays (EMSA) to assess the validity of this prediction. We generated in *vivo* wild-type versions of *Rli27* and 5’-UTR-*lm0514*, together with variants of both RNA
molecules bearing mutations in 3 nt (mut-1) or 14 nt (mut-3) important for pairing (Fig. 5B). Incubation of Rli27 and 5'-UTR-lmo0514 wild-type molecules resulted in a duplex with low electrophoretic mobility (Fig. 5C). Conversely, combination of wild-type 5'-UTR-lmo0514 with mutated Rli27 (either mut-1 or mut-3 variants), reduced duplex formation (Fig. 5C). Duplex formation was partially restored by combining mutations in Rli27 with compensatory mutations in 5'-UTR-lmo0514 (Fig. 5C).

Specificity of the Rli27-5'-UTR-lmo0514 interaction was confirmed by lack of duplex formation after incubation of the 5'-UTR molecule with an unrelated sRNA, SbrA. 

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Figure 5. Rli27 interacts in vitro with the lmo0514 long 5'-UTR. (A) Scheme of the major interaction region between Rli27 and the lmo0514 5'-UTR predicted with the targetRNA program (http://cs.wellesley.edu/~btjaden/TargetRNA2/). The complete set of putative interaction sites is shown in Fig. S3. (B) Effect of Rli27-mut1 and Rli27-mut3 mutations on the predicted Rli27-5'-UTR-lmo0514 interaction. Changes are highlighted in yellow. Compensatory mutations designed in 5'-UTR molecules synthesized in vitro are also shown in Fig. S3. (C) EMSA assays showing formation of a 5'-UTR-lmo0514/Rli27 duplex with slow migration in the gel. This duplex is not formed after co-incubation of the 5'-UTR molecule with the Rli27-mut1 or Rli27-mut3 variants, and is partially restored by compensatory mutations in the lmo0514 5'-UTR. (D) Control EMSA showing no duplex formation after incubation of the lmo0514 5'-UTR with an unrelated sRNA, SbrA.

Rli27 interaction with the lmo0514 long 5'-UTR is necessary to increase Lmo0514 protein levels in intracellular bacteria

To determine the biological relevance of the 5'-UTR-lmo0514-Rli27 interaction in vivo, we analyzed the specific contribution of Rli27 binding to Lmo0514 protein upregulation in bacteria that infect eukaryotic cells. We generated a Δrli27 strain and a second isogenic mutant, Δrli27C2T, which bears an artificial strong terminator between the remaining rli27 sequences. This mutant was intended to avoid polar effects on the flanking genes lmo0411 and lmo0412 [30]. In addition, we designed a qPCR assay specific for the lmo0514 long 5'-UTR for comparison to the lmo0514 coding region. There were no notable differences among strains in the relative levels of the long 5'-UTR region or the lmo0514 ORF (Fig. 6A). In contrast, Lmo0514 protein levels were ~2.5- to 3-fold lower in the cell wall of the two Rli27-lacking mutant strains isolated from the eukaryotic cell (Fig. 6B). This phenotype was complemented by overproduction of wild-type or mut1 versions of Rli27 from a plasmid (Fig. 6C, D). In contrast, when we tested mut3, the Rli27 mutant bearing 14 nt changes in the major region predicted to interact with the lmo0514 5'-UTR (Fig. 5A), it did not restore Lmo0514 protein levels in intracellular bacteria.
bacteria (Fig. 6D). Wild-type, mut1 and mut3 Rli27 versions were all produced by the plasmid at similar levels (Fig. 6C). These data showed that Rli27 interaction with the lmo0514 long 5'-UTR was essential for induction of the protein in intracellular bacteria, and that elimination of the Rli27-lmo0514 5'-UTR interaction interfered with the Lmo0514 protein increase while levels for the long transcript isoform remained unchanged. Our findings thus supported the need for Rli27 binding for efficient Lmo0514 translation. Control qPCR experiments in extracellular bacteria showed similar lmo0514 transcript levels in this mutant series (Fig. 6E), whereas there were no marked changes in Lmo0514 protein levels (Fig. 6F).

**Discussion**

Given the unique architecture of the cell envelope in Gram-positive bacterial pathogens, cell wall-associated proteins have essential functions in the interplay of these microorganisms with the host [31]. Despite the recognized importance of these proteins in infection, relatively few studies address the spatio-temporal regulation of the production of these proteins following host colonization. Obtaining this information is particularly challenging for Gram-positive pathogens such as *L. monocytogenes* or *Staphylococcus aureus*, which produce a large arsenal of surface proteins with distinct modes of association to the cell wall [31–33]. In this study of the Gram-positive bacterium *L. monocytogenes*, we identify sRNA-mediated regulation that acts on a cell wall-associated protein, Lmo0514, during the infection process. During the review process of this work, another report showed regulation of *L. monocytogenes* adhesin LapB by the multicopy sRNA LhrC, although this regulation was not studied in the context of infection [23]. Our data for lmo0514 also distinguish two transcript isoforms with 5'-UTR of distinct length that are expressed differentially when the pathogen transits between non-host and host environments. These findings are consistent with a regulatory role for the sRNA Rli27, based on its exclusive binding to the lmo0514 long 5'-UTR variant. This long 5'-UTR is generated from a promoter, here termed P2, which must respond to environmental cues of the eukaryotic intracellular niche. The
regulator itself, Rli27, is also upregulated by L. monocytogenes following entry into host cells. Transcriptional regulators of L. monocytogenes that operate in intracellular bacteria include the alternative sigma factor SigB and the Listeria-specific virulence regulator PrfA. Transcriptomic analyses in sigB and prfA mutants grown in laboratory media did not indicate lmo0514 as a gene regulated by these factors [34]; our results in intracellular bacteria were also negative (Fig. S5A). A yet undetermined regulator might thus be involved in enhancing transcription from the lmo0514 P2 promoter. Neither SigB nor PrfA appear to upregulate Rli27 in intracellular bacteria, as determined by real-time qPCR in sigB and prfA mutants isolated from infected epithelial cells (Fig. S5B).

Comparative transcriptomic studies of L. monocytogenes and L. innocua show that ~87% of the genes are transcribed with 5’-UTR shorter than 100 nt, whereas there is a subgroup of approximately 100 genes with long 5’-UTR (>100 nt) [18]; this subgroup includes virulence-related genes and genes with riboswitches [17]. Similar distribution of 5’-UTR length was also described in the related model organism Bacillus subtilis [35]. About 80 genes shared by L. monocytogenes and L. innocua are produced with different-length 5’-UTR [18], which might indicate differences in post-transcriptional regulation of these transcripts. Our data imply a third group of genes based on distinct transcript isoforms that differ in 5’-UTR length. lmo0514 is a representative example, as it is expressed as two isoforms with 28- and 234-nt 5’-UTR in extra- and intracellular bacteria, respectively. A close parallel is found in a recent work that analyzed sRNA RydC regulation of the Salmonella enterica cfa gene, which encodes a cyclopropane fatty acid synthase [36]. RydC selectively stabilizes the longer of two cfa transcript isoforms, which is associated to the activity of a distal promoter controlled by σA and a proximal promoter modulated by σB [36]. Unlike lmo0514, both cfa isoforms are expressed by S. enterica growing extracellularly in laboratory media. These observations indicate that transcript isoforms with distinct 5’-UTR target platforms for sRNA-mediated post-transcriptional regulation could profoundly influence protein production. It is noteworthy that long 5’-UTRs are frequently associated with genes involved in pathogenesis [18,37].

An interesting feature predicted by the Mfold program is that Rli27 binding to the lmo0514 long 5’-UTR could expose the Shine-Dalgarno site, in contrast to the occluded configuration predicted when this 5’-UTR folds as single molecule (Fig. S6, S7). This led us to propose that Rli27 positively regulates Lmo0514 protein levels by altering the long 5’-UTR conformation. This mechanism resembles that of the translational regulation of the rpoS transcript in Escherichia coli [38]. The Shine-Dalgarno site is blocked by a stem-loop in the rpoS 5’-UTR, which is released by base pairing of three distinct Hfq-binding sRNA to the same
region. Other paradigmatic cases in *L. monocytogenes* include the virulence regulator *pdfA*, *actA*, and the hemolysin (*hly*) genes [25,39–41]. Our hypothesis for *lmo0514* implies that its 234-nt 5′-UTR has considerable secondary structural complexity in the absence of Rli27. This assumption is consistent with the study by Wurzel et al. [18], in which RNA-seq did not define the *lmo0514* transcriptional start site, although 2018 such sites were mapped in the *L. monocytogenes* genome, which account for 88% of all annotated transcriptional units. Our tentative model (Fig. 7) also considers the *lmo0514* transcript as ‘low-efficiency’ in terms of translation; there are marked differences in *Lmo0514* protein levels in bacteria isolated from epithelial cells (>200-fold increase) that are not reflected at the transcript level. The secondary structure prediction for the short (28-nt) 5′-UTR of the extracellular *lmo0514* isoform also suggests probable occlusion of the Shine-Dalgarno site (Fig. S8). Further work is needed to clarify the extent to which such potential structural changes in the 5′-UTR might explain Rli27-mediated regulation.

Our EMS data infer direct Rli27-5′-UTR-*lmo0514* interaction, which was also relevant in *in vivo*, based on data obtained with the Rli27-mut3 variant. This variant did not restore the *Lmo0514* protein levels produced by intracellular bacteria (Fig. 6D). We did not obtain perfect complementation with compensatory mutations in the predicted interacting regions, which allows other interpretations. For example, the targetRNA program might have predicted an incorrect pairing site, pairing between the two molecules might require additional factors with a precise stoichiometry, or the *lmo0514* transcript could undergo alternative post-transcriptional regulation; future work will address these possibilities.

We designed *in vivo* experiments to assess the *lmo0514* long 5′-UTR requirement in *Lmo0514* protein production in the cell wall of bacteria located inside eukaryotic cells. We tested strains that bear chromosomal mutations in the *lmo0514* long 5′-UTR predicted interaction site or that lack most of the 5′-UTR upstream of the P1 promoter −10 and −35 sites (Fig. S9A, S9B). *Lmo0514* protein levels dropped markedly inside the eukaryotic cells for some these mutants, especially in that lacking the *lmo0514* 5′-UTR (Fig. S9C, S9D). Nonetheless, *lmo0514* transcript levels were affected in these mutants in both extra- and intracellular conditions (Fig. S9C, S9D). Due to the clear side effect of the mutations on transcription, these findings remained inconclusive.

In summary, our results demonstrate that Rli27 is a regulatory sRNA in *L. monocytogenes*, with an essential role as a positive regulator of the *Lmo0514* surface protein during the intracellular infection cycle. We also provide evidence that the Rli27 regulatory role is directed to a transcript isoform that bears the binding site for this sRNA; in addition, we show that this isoform is specifically upregulated by intracellular bacteria. Further research will be necessary to determine how Rli27 might modify the secondary structure of the 5′-UTR after binding, and whether such a role requires additional factors also probably upregulated in intracellular bacteria. Another challenge will be to identify the host-derived signal that triggers transcription from the P2 promoter in intracellular *L. monocytogenes* and the bacterial transcriptional factor responsible.

Materials and Methods

Comparative genomics

To compare the genome bearing rli27 in *L. monocytogenes* EGD-e, *L. innocua* Clip11262 and *L. welshimeri* serovar 6b str. SLCC5334, we used the WEBACT program (http://www.webact.org/WebACT/home). Genome sequences were obtained from the Genbank repository (http://www.ncbi.nlm.nih.gov/genbank/) with entry numbers NC_003210.1, NC_003212.1 and NC_005555.1 for *L. monocytogenes* EGD-e, *L. innocua* Clip11262 and *L. welshimeri* serovar 6b str. SLCC5334, respectively.

Bacterial strains and growth conditions

The *L. monocytogenes* strains of serotype 1/2a used here are isogenic to wild-type strain EGD-e [27] (listed in Table S1). For sRNA overexpression analyses, the rli27 wild-type allele was cloned in the pP1 plasmid [42] using Lmo027-pP1-F and Lmo027-pP1-R primers (Table S2). Relative expression of cloned sRNA was monitored by semi-quantitative RT-PCR using Lmo027-F and Lmo027-R primers (Table S2). *L. monocytogenes* strains were grown at 37°C in brain heart infusion (BHI) broth. For cloning, E. coli strains were grown in Luria Bertani (LB) broth at 37°C. When appropriate, media were supplemented with erythromycin (1.5 μg/ml) or ampicillin (100 μg/ml).

Generation of Rli27 variants for overexpression in *in vivo* assays

Two Rli27 variants, Rli27-mut1 and Rli27-mut3, were constructed by amplification of the rli27 gene with degenerate primers Lmo027-pP1-F-mut1 and Lmo027-pP1-F-mut3 (Table S2) and subsequent cloning in pP1 plasmid [42]. The mut1 mutation introduces 3 nt changes and mut3, 14 nt changes in the major predicted interaction site (see Fig. 5B).

Construction of Rli27-defective *L. monocytogenes* mutants

To generate the Δ*rli27* mutant strain, fragments of ~500-bp DNA flanking rli27 were amplified by PCR using chromosomal DNA of *L. monocytogenes* strain EGD-e and cloned into the thermo-sensitive suicide integrative vector pMAD [43] with primers Lmo027-A, Lmo027B, Lmo027-C and Lmo027-D (Table S2). Genes were deleted by double recombination as described [43], and deletion was verified by PCR. To generate the Δ*lmo0514* mutant, we left 9 nt in the 3′ end and 50 nt in the 3′ end of the rli27 gene, to avoid interference with the *lmo0412* terminator (shared with rli27) and the *lmo0411* predicted promoter sequence (Fig. S4). This *Arli27* mutation affected *lmo0411* transcript levels slightly. A new deletion mutant was generated (*Arli27C2T*), which retains a 5′ extended region of the predicted *lmo0411* promoter, thus maintaining 21 nt in the 5′ end and 50 nt in the 3′ end of rli27 (Fig. S4). In addition, a strong artificial terminator sequence between the remaining rli27 sequences was introduced in the Δrli27C2T mutant (Fig. S4). All deletions were confirmed by PCR and sequencing, using primers listed in Table S2.

Construction of *L. monocytogenes* mutants with chromosomal mutations in the *lmo0514* 5′-UTR

Three types of mutants were constructed with the following chromosomal mutations: i) changes in 3 nt of the long 5′-UTR-*lmo0514* to compensate the mutation in Rli27-mut1 (see Fig. S3, S9); ii) changes in 14 nt of the long 5′-UTR-*lmo0514* to compensate the mutation in Rli27-mut3 (see Fig. S3, S9); and iii) a 174-nt deletion upstream of the −10 and −35 sites of the P1 promoter (Fig. S9). These changes were generated by double recombination as described [43] and when required, using overlapping SOEing PCR. The oligonucleotide primers for these procedures included Δ0514_P2_A, Δ0514_P2_B, Δ0514_P2_C, Δ0514_P2_D, Mut0514pXG_1-overlap, Mut0514pXG_2-over-
Isolation of intracellular bacteria for RNA expression and proteomic analyses

Intracellular bacteria were collected from the human epithelial cell line JEG-3 at 6 h post-infection, as described [12]. For total RNA isolation, epithelial cell cultures were infected (30 min) with L. monocytogenes grown in BHI medium (37°C, overnight) in static non-shaking conditions. RNA was purified using TRizol reagent method [17]. For cell wall protein analysis, intracellular bacteria were obtained from JEG-3 cells cultured on four BioDish-XL plates and infected for 6 h [12]. Subcellular fractions containing protoplasts and peptidoglycan-associated proteins were obtained by mutanolysin treatment of intact bacteria as described [10,12], except that bacterial pellets were incubated for 5 h in lysis buffer (10 mM Tris HCl pH 6.9, 10 mM MgCl₂, 0.5 M sucrose, 60 ng/ml mutanolysin, 250 μg/ml RNase-A, 0.1% protease inhibitor).

Bacterial fractionation and western blot analysis

Subcellular fractions containing protoplasts and cell wall-associated proteins of L. monocytogenes grown at 37°C in BHI media were obtained as described [10]. A volume of protoplasts and the cell wall fraction was analyzed by SDS-PAGE followed by Western blot using B. subtilis RecA-specific rabbit polyclonal antibody (a gift of Dr. JC Alonso, Centro Nacional de Biotecnología-CSIC) and rabbit polyclonal sera to the L. monocytogenes LPXTG surface proteins Lmo2653 (InlH), Lmo0435 (InlA) and Lmo0514 [12], RecA (for the protoplast fraction) and LPXTG proteins InlA and InlH (for the cell wall fraction) were used as loading controls. Goat anti-rabbit antibodies conjugated to hors-radish peroxidase (Bio-Rad) were used as secondary antibodies. Proteins were visualized by chemoluminescence using luciferin-luminol reagents.

RNA preparation and reverse transcriptase PCR assays

Total RNA from extracellular bacteria grown to exponential (OD₆₀₀ ~0.2) and non-shaking stationary phase (OD₆₀₀ ~1.0) was prepared as described [11]. Oligonucleotides for RT-PCR assays were designed using Primer Express v3.0 (Applied Biosystem-s) (listed in Table S2). RNA was treated with DNase I (Turbo DNA-free kit, Ambion/Applied Biosystems) at 37°C for 30 min. RNA integrity was assessed by agarose-TAE electrophoresis. RT-PCR was performed using the one-step RT-PCR kit (Qiagen). RNA isolation, epithelial cells cultured in BioDish-XL plates and infected for 6 h [12], were infected (30 min) with L. monocytogenes grown in BHI medium (37°C, overnight) in static non-shaking conditions. RNA was purified using TRizol reagent method [17]. For cell wall protein analysis, intracellular bacteria were obtained from JEG-3 cells cultured on four BioDish-XL plates and infected for 6 h [12]. Subcellular fractions containing protoplasts and peptidoglycan-associated proteins were obtained by mutanolysin treatment of intact bacteria as described [10,12], except that bacterial pellets were incubated for 5 h in lysis buffer (10 mM Tris HCl pH 6.9, 10 mM MgCl₂, 0.5 M sucrose, 60 ng/ml mutanolysin, 250 μg/ml RNase-A, 0.1% protease inhibitor).

To detect the sRNA Rli27 and the 5S rRNA, primers were designed using Primer3 [45] (listed in Table S2). qPCR was performed in a 10 μl final volume with 1 ng of the cDNA library as template, 500 nM of gene-specific primers and the Power SYBR Green PCR Master Mix (Applied Biosystems). Reactions and data analysis were carried out as described [46].

5’-rapid amplification of cDNA ends (5’-RACE)

5’-RACE was performed as described [47], with minor modifications. To convert 5’tiphosphates to monophosphates, 15 μg DNA-free RNA, isolated from L. monocytogenes growing extracellularly at 37°C to stationary phase or from intracellular bacteria collected at 6 h post-infection of epithelial cells, was treated with 25 U tobacco acid pyrophosphatase (TAP) (Epitcience Technologies) at 37°C for 60 min in a total reaction volume of 50 μl containing 50 mM sodium acetate (pH 6.0), 1 mM EDTA, 0.1% β-mercaptoethanol, 0.01% (v/v) Triton X-100 and 80 U RNasin (Promega). TAP-negative (TAP–) control RNA was processed in the same conditions in the absence of TAP. Following TAP treatment, RNA was phenol/chloroform-extracted and precipitated with sodium acetate and ethanol. Pellets were rinsed with 70% ethanol in DEPC-dH₂O, then resuspended in 65 μl DEPC-dH₂O; 29 μl of these TAP+ or TAP-treated RNA were combined with 5.5 μl 10× buffer, 120 U RNasin, 10% (v/v) dimethylsulfoxide, 70 U RNA ligase, 150 μM ATP and 150 ng RNA oligonucleotide adapter, in a total reaction volume of 55 μl. Samples were denatured (95°C, 5 min) and then chilled on ice. RNA adapter ligation was performed (17°C, 12 h). Following ligation, RNA was phenol/chloroform-extracted and converted to cDNA with a lmo0514-specific primer (Lmo0514-PE-3rv) and the Thermoscript RT System (Invitrogen). Reverse transcription was performed in three cycles (55°C, 60°C and 65°C; 20 min each), followed by RNaseH treatment (37°C, 20 min). lmo0514 cDNA (2 μl) was amplified by PCR with oligonucleotides RaceIN and lmo0514-PE-1rv (30 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 1 min), or with oligonucleotides RaceIN and lmo0514-PE-6rv in the same cycling conditions. PCR products were resolved on 2% agarose gels and bands of interest were excised and subcloned into pCR 2.1 TOPO-vector (Invitrogen). Plasmids containing inserts were purified using the QIAprep Spin Miniprep Kit (QIAgen) and sequenced.

Northern blot assays

To detect the sRNA Rli27 and the 5S rRNA, 15 μg total RNA was electrophoresed in a 6% polyacrylamide 8 M urea gel (1 h, 200 V in 1× TBE). RNA was transferred to a Hybond membrane (Amersham) for 2.5 h at 40 V in 0.5× TBE at 4°C and RNA was UV-crosslinked to the membrane. Membranes were pre-hybridized with UltraHyb buffer (Ambion; 65°C, 2 h) and hybridized with 10⁶ cpm 32P-labeled specific riboprobes (65°C, overnight). Membranes were washed with 2× SSC, 0.5% SDS and 1× SSC, 0.1% SDS and exposed to X-ray film. 5S rRNA was used as control [22]. A nonradioactive digoxigenin (DIG)-based RNA detection protocol was used for Northern blot analysis of lmo0514 and the 16S rRNA. Total RNA (1 μg for lmo0514 or 200 ng for 16S rRNA) was separated on a 1.5% agarose denaturing gel (2% formaldehyde, 1× MOPS), overnight capillary transferred to a Hybond membrane in 20× SSC, and UV-crosslinked. The membrane was prehybridized (68°C, 1 h) and then hybridized with DIG-labeled lmo0514 and 16S rRNA probes (68°C, overnight). Immunological detection of RNA was performed (DIG Northern starter kit; Roche) and exposed to X-ray film.

Electrophoretic mobility shift assays (EMSA)

Gel mobility shift assays were performed with 1.48 pmol in vitro-transcribed RNA corresponding to the lmo0514 5’-UTR (nucleotides −234 to −14 from the lmo0514 ATG codon) and

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increasing concentrations of in vitro-transcribed RNAs for Rli27 wild-type, Rli27-mut1 and Rli27-mut3. These in vitro-transcribed molecules included Rli27 nucleotides 1 to 131 plus an additional 60 nt at the 3’ end, as designed for optimal amplification. We produced lmo0514 5’-UTR variants with compensatory mutations in 3 nt (mut-1) or 14 nt (mut-3) for those generated in Rli27. Oligonucleotide primers used are listed in Table S2. We also generated an amplified molecule corresponding to RNA SbrA (Rli11) encompassed nucleotides 1 to 69 of the total of 71 nucleotides. The reaction was carried out in 10 µl of 1 x binding buffer (20 mM Tris-acetate pH 7.6, 100 mM sodium acetate, 5 mM magnesium acetate, 20 mM EDTA) (37°C, 1 h). The binding reactions were mixed with 2 µl loading dye (48% glycerol, 0.01% orange G) and loaded on native 4% polyacrylamide gels, followed by electrophoresis in 0.5 TBE buffer (200 V, 4°C). Gels were stained with Gel Red nucleic acid stain (Biotium) and photographed under UV transillumination with the GelDoc 2000 system (Bio-Rad).

Computational prediction of potential interactors with the lmo0514 long 5’-UTR region

The bioinformatic program TargetRNA (http://cs.wellesley.edu/~btjaden/TargetRNA2/) [28] was used to predict non-coding RNAs that could bind to the lmo0514 long 5’-UTR (234 nt from the ATG codon). Predictive folding of the lmo0514 long 5’-UTR alone or with sRNA Rli27 was done using Mfold (http://mfold.rna.albany.edu/?q=mfold),

Statistical and densitometry analyses

Statistical significance was analyzed with GraphPad Prism v5.0b software (GraphPad Inc.) using Student’s t-test. A P value ≤0.05 was considered significant. For densitometry of bands obtained in western blots, we used ImageJ software (National Institutes of Health of USA [http://imagej.nih.gov/ij]).

Supporting Information

Figure S1 lmo0514 is cotranscribed with the downstream gene lmo0515, which codes for a universal stress protein in L. monocytogenes [26]. PCR assays performed on reverse-transcribed RNA (cDNA) and genomic DNA (gDNA). C(-) refers to a control sample that lacks a template. Colors indicate relative position of promoters. Note that a strong artificial terminator was introduced to avoid expression of the remaining Rli27-specific sequences. Both Rli27 and lmo0412 genes share a Rho-independent terminator.

Figure S2 Comparison of the rli27 region of L. monocytogenes EGD-e strain (Lmo) with the respective regions of non-pathogenic species L. innocua (Lin) and L. weldshimeri (Lw). Genomes were compared using the WebACT tool (http://www.webact.org/WebACT/home). Red indicates similar genomic organization; blue indicates inversions. Orthologous genes are shown in same color. The rli27 gene of L. monocytogenes has no ortholog in L. weldshimeri and is flanked by lmo0411 and lmo0412, two genes in the opposite DNA strand predicted to encode a protein similar to phosphoenolpyruvate synthase and a protein of unknown function, respectively.

Figure S3 Scheme of all predicted interaction sites between Rli27 and the lmo0514 5’-UTR. Interactions were predicted using the targetRNA program (http://cs.wellesley.edu/~btjaden/TargetRNA2/). Scheme shows the exact positions of the predicted interaction regions between the lmo0514 5’-UTR and Rli27 as well as the mutant variants Rli27-mut1 and Rli27-mut3, with changes highlighted in yellow. Note that the hybridization energy is lower in the case of the Rli27 variants. Compensatory mutant variants of the 5’-UTR-lmo0514 molecule lmo0514-mut1, lmo0514-mut3) are also shown.

(TIF)

Figure S4 Genome region of the L. monocytogenes EGD-e strain bearing rli27 and its flanking genes, and the exact location of the deletions generated in Delt27 and Delt27C2T mutants. Blue boxes represent the predicted −10 and −35 sites of the lmo0411 promoter. Note that a strong artificial terminator was introduced in Delt27C2T to avoid expression of the remaining Rli27-specific sequences. Both rli27 and lmo0412 genes share a Rho-independent terminator.

(TIF)

Figure S5 lmo0514 and rli27 expression are not regulated by SigB or PrfA in extra- or intracellular bacteria. (A) qPCR data relative to lmo0514 obtained from total RNA isolated from extracellular bacteria grown in BHI medium to stationary phase (extracellular) or collected from epithelial cells (intracellular). Primers Utr0514_qPCR_F and Utr0514_qPCR_R were used. (B) Data relative to Rli27 expression. No significant differences were found for any of the samples. Data are derived from a minimum of three independent experiments.

(TIF)

Figure S6 Energetically favorable conformation of the lmo0514 long 5’-UTR (234 nt) as single molecule, as predicted by the M-fold program (http://mfold.rna.albany.edu/?q=mfold). Note that the Shine-Dalgarno site appears to be occluded.

(TIF)

Figure S7 Energetically favorable conformation of the lmo0514 short 5’-UTR (28 nt) as a single molecule, predicted by M-fold (http://mfold.rna.albany.edu/?q=mfold). Note the occlusion of the Shine-Dalgarno (SD) site.

(TIF)

Figure S8 Energetically favorable conformation of the lmo0514 short 5’-UTR (28 nt) as a single molecule, predicted by M-fold (http://mfold.rna.albany.edu/?q=mfold). Note the occlusion of the Shine-Dalgarno (SD) site.

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Figure S9 In vivo experiments using L. monocytogenes mutants with chromosomal mutations in the lmo0514 5’-UTR. (A) Detail of the mut1 and mut3 chromosomal mutations introduced in the lmo0514 5’-UTR. These mutations were designed to compensate the mut1 and mut3 mutations generated in the Rli27 variants. (B) Scheme of the mutations introduced in the chromosome: Δ5’-UTR-lmo0514 (174 nt deletion), 5’-UTR-mut1 (3 nt change) and 5’-UTR-mut3 (14 nt change). (C) Effect of these chromosomal mutations on Lmo0514 protein and lmo0514 ORF levels in extracellular bacteria grown in BHI medium to stationary phase. (D) Effect of these chromosomal mutations on Lmo0514 protein and lmo0514 transcript levels (differentiating production of the long 5’-UTR isoform) in intracellular bacteria collected from epithelial cells. Note the marked decrease in Lmo0514 protein by the Δ3’-UTR-lmo0514 and the 5’-UTR-mut3 mutants in the intracellular niche of the eukaryotic cell. These mutants nonetheless have low lmo0514 transcript expression, probably due to side effects linked to loss of the 5’-UTR region between the P2 and P1 promoters.

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Table S1 Listeria monocytogenes strains used in this study.

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Table S2 Oligonucleotide primers used in this study.

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Acknowledgments

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Author Contributions

Conceived and designed the experiments: JJQ ADO MGP FdGP. Performed the experiments: JJQ ADO MGP. Analyzed the data: JJQ ADO MGP FdGP. Wrote the paper: JJQ ADO MGP FdGP.

References