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Published in: PLoS ONE

DOI: 10.1371/journal.pone.0077939

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Document Version
Publisher's PDF, also known as Version of record

Publication date: 2013

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Gonzalo-Asensio, J., Ortega, A. D., Rico-Pérez, G., Pucciarelli, M. G., & García-Del Portillo, F. (2013). A novel antisense RNA from the Salmonella virulence plasmid pSLT expressed by non-growing bacteria inside eukaryotic cells. PLoS ONE, 8(10), [e77939]. https://doi.org/10.1371/journal.pone.0077939

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A Novel Antisense RNA from the *Salmonella* Virulence Plasmid pSLT Expressed by Non-Growing Bacteria inside Eukaryotic Cells

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**Abstract**

Bacterial small RNAs (sRNAs) are regulatory molecules playing relevant roles in response to environmental changes, stressful conditions and pathogenesis. The intracellular bacterial pathogen *Salmonella enterica* serovar Typhimurium is known to regulate expression of some sRNAs during colonization of fibroblasts. Here, we characterize a previously unknown sRNA encoded in the *S*. Typhimurium pSLT virulence plasmid that is specifically up-regulated by non-growing dormant bacteria persisting inside fibroblasts. This sRNA was inferred in microarray expression analyses, which unraveled enhanced transcriptional activity in the PSLT047-PSLT046 (mig5) intergenic region. The sRNA transcript was further identified as a 597-nucleotide molecule, which we named lesR-1, for ‘intracellular-expressed-sRNA’. lesR-1 expression is low in bacteria growing in axenic cultures across a variety of experimental conditions but displays a marked increase (~200–300 fold) following bacterial entry into fibroblasts. Remarkably, induction of lesR-1 expression is not prominent in bacteria proliferating within epithelial cells. lesR-1 deletion affects the control of bacterial growth in defined fibroblast cell lines and impairs virulence in a mouse infection model. Expression analyses performed in the PSLT047-iesR-1-PSLT046 (mig5) region support a cis-acting regulatory mechanism of lesR-1 as antisense RNA over the PSLT047 transcript involving interaction at their respective 3′ ends and modulation of PSLT047 protein levels. This model is sustained by the scarce production of PSLT047 protein observed in non-growing intracellular bacteria and the high amount of PSLT047 protein produced by bacteria carrying a truncated lesR-1 version with separated 5′ and 3′ regions. Taken together, these data reveal that *S*. Typhimurium sRNAs encoded in the pSLT virulence plasmid respond to a state of persistence inside the host cell. As exemplified by lesR-1, some of these sRNAs may contribute to diminish the relative levels of proteins, such as PSLT047, which are probably dispensable for the intracellular lifestyle.

**Introduction**

Small non-coding RNAs (sRNAs) constitute a family of molecules widely distributed in prokaryotes that has emerged as important elements in regulatory circuits [1–3]. sRNAs are 50–400 nucleotides in length, most often expressed as transcriptional units from intergenic regions (IGR) and include a structured stem-loop Rho-independent terminator at the 3′ end. Computational algorithms designed for sRNA discovery in bacteria have mainly relied on the search within IGRs for conserved, relatively short sequences, flanked by orphan promoters and Rho-independent terminators [4]. Direct cloning of small RNAs and high-density microarray and RNA-seq approaches have unraveled the sRNAome of different bacterial species, including pathogens [5–9]. sRNAs can interact to either nucleic acid-binding proteins modulating their regulatory activity by titration or to target mRNAs by direct base-pairing complementarity. The latter include cis-acting sRNAs, codified in the same genetic region but in antisense orientation to their target mRNAs and trans-acting sRNAs, which are transcribed elsewhere in the chromosome and require the RNA chaperone Hfq to assist sRNA binding to multiple mRNA targets. Target sites in trans-regulated mRNAs have been most often found in the 5′ untranslated region (UTR) of the transcript. This binding results in the modulation (positive or negative) of the mRNA stability and/or translation [1].
sRNAs have been identified in intracellular bacterial pathogens such as Salmonella enterica, Legionella pneumophila, Listeria monocytogenes, Yersinia spp., Streptococcus pneumoniae and Mycobacterium tuberculosis, among others [6,10–15]. These pathogens reprogram the transcriptome/proteome to successfully survive in the harsh environment encountered within host cells. Transcriptional reprogramming involves adaptive mechanisms, including adjustment of the sRNAs levels. A recent study has shown increased expression of defined sRNAs in S. enterica serovar Typhimurium (S. Typhimurium) when this pathogen invade fibroblasts, a host cell type in which intracellular bacteria establish a non-proliferative state [16]. Induction of sRNAs by intracellular S. Typhimurium has also been reported in macrophage-like cells, as it is the case of OxyS [17], an sRNA previously shown to be up-regulated in response to oxidative stress [18]. Although most mutants lacking defined sRNAs often show mild phenotypes, a few sRNA-defective mutants display impaired virulence in vivo [11–13,15,19]. Mutants lacking IsrM are impaired for invasion of epithelial cells and exhibit reduced proliferation in macrophage-like cells. The absence of IsrM also affects the capacity of S. Typhimurium to grow in mouse organs [11]. It is also known that mutants defective in IstR or SroA exhibit reduced fitness in mice [13]. Mechanistically, the sRNA IsrM affects post-transcriptionally the expression of the virulence factor HilE and the secreted effector SopA [11]. Decreased translocation of the protein effector SptP into epithelial cells was also linked to a defect in the sRNA IstJ [17]. Whether additional S. Typhimurium sRNAs contribute to other aspects of the host-pathogen interplay, such as that occurring during non-proliferative persistent infections, is at present unknown.

Some pathogens have developed strategies to undergo a persistent infection in specific host cell types, which allow them to evade host immune responses [20]. In humans, some intracellular pathogens as S. enterica serovar Typhi, Helicobacter pylori and M. tuberculosis, are prone to cause this type of infections [21]. S. Typhimurium is a suitable model for persistent infection given the ability of this pathogen to cause chronic infections in resistant mice [22]. Recent work has shown that S. Typhimurium restrains growth of extracellular bacteria within the intestinal lamina propria of susceptible mice and that such phenotype is reproduced in primary intestinal fibroblasts [23]. Upon invasion of cultured fibroblasts, most intracellular bacteria actively attenuate growth, a response that requires a subset of pathogen regulators including the PhoP-PhoQ system [23,24]. Recently, we analyzed in S. Typhimurium the expression of chromosomally-encoded sRNAs in non-proliferating bacteria persisting within fibroblasts [16]. This study demonstrated that some sRNAs are specifically expressed along the intracellular infection process. Whether some of these sRNA play a dedicated role in modulating growth of the pathogen inside the host cell has not been yet interrogated.

The genome of the S. Typhimurium virulent strain SL1344 comprises a 4.9 Mb chromosome and a 94 Kb virulence plasmid named pSLT [6,25]. Genetic mobile elements, including plasmids, frequently comprise non-coding RNA loci [26]. An example is the hok/sok type I toxin-antitoxin system involved in the post-segregational killing mechanism employed by the R1 plasmid in E. coli [27]. Conjugal transfer of pSLT is also controlled by a cis-acting sRNA, namely FinP, which negatively regulates translation of the adjacent traJ gene [28,29]. Most studies on sRNAs have been however conducted in the chromosome, overlooking the possible presence of these molecules in the virulence plasmid. A notable exception was a comprehensive RNAseq study focused in deciphering the transcriptional map of Salmonella plasmids during the early stationary phase of growth [6]. The expression of these RNAs during infection conditions was, however, not examined.

In this work, we searched for novel sRNAs in the Salmonella virulence plasmid pSLT by microarray data obtained from intracellular bacteria that colonize fibroblasts. We selected a novel sRNA exhibiting significant expression in non-growing intracellular wild type bacteria. Further analysis of the locus displaying such transcriptional activity revealed the presence of an antisense sRNA positioned between PSLT047 and PSLT046 (mig5) genes. The expression of this sRNA, which we named IsrR-1, for ‘Intracellular-expressed-sRNA-1’, was low in most of the laboratory growth conditions tested while increased dramatically in non-growing intracellular bacteria. Manipulation of the relative levels of IsrR-1 also led to alteration of virulence in the mouse typhoid model. Collectively, the data obtained in this work support a model involving a cis-acting mechanism of IsrR-1 over PSLT047 with interaction at their respective 3’ ends, a phenomenon that could modulate PSLT047 translation. This mechanism is consistent with the marked decrease of the PSLT047 protein observed in intracellular bacteria.

Materials and Methods

Ethics Statement

Animal research adhered to the principles mandatory in the European Union, as established in the Legislative Act 86/609 CEE (November 24, 1986), and followed the specific protocols established by the Royal Decree 1201/2005 of the Government of Spain (October 10, 2005). The protocols employed in the study were reviewed by the ‘Comité Ético de Experimentación del Consejo Superior de Investigaciones Científicas (CSIC)’ and were approved on March 15, 2007.

Bacterial Strains and Growth Conditions

The S. enterica serovar Typhimurium wild-type strain SV5015 (SL1344 His") [30] and its isogenic mutant MD1120 (phoP7953::Tn10) [24], were used. The strain EG5510 (phoP-24 carrying a constitutive active allele was a gift from Eduardo A. Groisman (Yale University). The MD2218 (ΔiesR-1/5::cat) strain, derived from SV5015, was constructed using the one-step inactivation procedure [31]. Amplification products were obtained from plasmid pKD3 as a template using primers KO-995-fw and KO-995-rv (Table S1). The antibiotic resistance gene from MD2218 was eliminated by an FLP recombinase, encoded in plasmid pCP20 [31], resulting in strain MD2219 (ΔiesR-1/5). The strain MD2255 (PSLT047::3xFLAG-Km) was constructed by the method described in [32] using primers FLAG-PSLT047-fw and FLAG-PSLT047-rv (Table S1). The kanamycin resistance marker was eliminated using the pCP20-encoded FLP recombinase to obtain the strain MD2256 (PSLT047::3xFLAG).

The MD2219 (ΔiesR-1/5) strain was used as recipient in genetic procedures designed to complement the defect in IsrR-1. Two versions for complementation purposes were generated, one carrying the full-length IsrR-1molecule (‘long’ variant) and the second, 208 nt of the 5’ region (‘short’ variant). The procedure was based in the usage of the PListO promoter [33] to drive the expression of the RNA molecule of interest. The construction of the ‘long’ and ‘short’ versions of IsrR-1 and their subsequent integration in the chromosomal locus araE involved the following steps: i) insertion of a CAT-PListO promoter in the promoter region of araE by the one-step method described by Datsenko and Wanner [31]; ii) PCR-amplification of the CAT-PListO-iesR-1/5 (‘short’) or CAT-PListO-iesR (‘long’) constructs with ends containing araE sequences; and, iii) insertion of the PCR fragments in the araE...
locus by one-step method using chloramphenicol resistance as marker. The chloramphenicol-resistance cassette (CAT) and the PLtetO promoter sequences were in divergent orientations in all the constructions. The production of the “short” (iesR-1/5’) and “long” (complete iesR-1) molecules was confirmed by RT-PCR. The genotype of the resulting strains was: MD2276: ΔiesR-1/5’ CAT::PLtetO iesR-1/5’; MD2277: ΔiesR-1/5’, CAT::PLtetO iesR-1. The oligonucleotides used in these procedures are listed in Table S1.

Bacteria were grown routinely in Luria Bertani (LB) broth. When required bacteria were cultured in PCN defined medium at pH 5.8 [34] or in ISM medium [35]. Incubation was performed at 37°C in non-shaking or shaking (180 rpm) conditions. When appropriate, tetracycline (10 μg/ml), kanamycin (30 μg/ml) or chloramphenicol (10 μg/ml) were added to the growth medium.

Total RNA Isolation and cDNA Synthesis

Cultures were inoculated with overnight-grown bacteria (dilution 1:100, OD –0.02). Standing cultures were incubated overnight. Shaking cultures were incubated at 180 rpm until early-exponential phase (OD600 ~ 0.1 in PCN and ISM media, or OD600 ~ 0.2 in LB broth) or overnight to reach stationary phase (OD600 ~ 0.8, 1.0 and 2.0 in PCN, ISM and LB media, respectively). At these times, 4 ml of culture were added to 1 ml of chilled stop solution (5% phenol in ethanol) [36] and incubated at 4°C for 30 minutes. This step allows bacterial RNA stabilization prior to subsequent treatments [37]. Bacteria were harvested by centrifugation at 4,500 × g for 5 min at 4°C, and total RNA was extracted using the Trizol reagent (Life Technologies) as described [16]. RNA integrity was confirmed by agarose TAE electrophoresis, and lack of DNA contamination was assessed by lack of amplification products after 30 cycles of PCR with primers OmpA-F and OmpA-R (Table S1). Once confirmed RNA quality and purity, 1 μg RNA were reverse transcribed into cDNA libraries using the High-capacity cDNA archive kit (Life Technologies, Carlsbad, CA). For the synthesis of gene-specific cDNA, 1 μg of total RNA from standing cultures was reverse transcribed for 1 h at 42°C followed by 15 min at 85°C inactivation step with 200 U of the M-MuLV RT (New England Biolabs, Ipswich, MA), 10 pmol of reverse gene-specific primer (Table S1), 4 mM of each dNTP, 2.5 mM MgCl2, 6 μg/ml actinomycin D and 0.5 U/μl RNase inhibitor. Amplification from gene-specific and cDNA libraries was performed in 1/100 (target) or 1/400 (housekeeping) dilutions of the RT reaction by quantitative real time PCR as detailed below.

Simultaneous Mapping of 5’- and 3’-ends of RNA Molecules by RACE

Mapping of 5’- and 3’-RNA ends by RACE (Rapid Amplification of cDNA Ends) using circularized RNAs was performed as previously described [8,38]. Circular RACE involves T4-mediated RNA self-ligation followed an RT-PCR using gene-specific outward primers. RNA samples were treated or not with the Tobbaco Acid Pyrophosphatase (TAP), which specifically cleaves the 3′-terminal resulting in a 5′-monophosphorylated terminus that may be ligated to a 3′-hydroxylated terminus using T4 RNA ligase. This procedure distinguishes primary transcripts, the targets of the TAP, respect from processed monophosphorylated RNAs. Thus, primary RNA species are enriched in the TAP–treated RNA fraction as compared to the non-treated when assessing RT-PCR products in RACE assays. The method was used in this work with the following modifications. Briefly, reactions were performed on RNAs extracted from bacteria grown in LB until OD at 600 nm reached a value of 0.2. Six μg of RNA were treated with Turbo DNA free (Life Technologies) to avoid any false positive products. The RNA was divided in two aliquots. Both aliquots were incubated for 1 h at 37°C with the corresponding buffer in presence or absence of TAP (Epicentre Biotechnologies, Madison, WI) respectively. This step allows discriminating a 5′-end generated by transcription initiation from a 5′-end provided by RNA processing. After incubation, acid-phenol and chloroform extractions and ethanol precipitation were performed. Serial dilutions (from 500 ng to 0.5 ng) of the TAP+ and TAP–treated RNAs were prepared. Each dilution were ligated with 40 U of T4 RNA ligase I (New England Biolabs) in the presence or absence of TAP (Epicentre Biotechnologies, Madison, WI). This step allows discriminating a 5′-end generated by transcription initiation from a 5′-end provided by RNA processing. After incubation, acid-phenol and chloroform extractions and ethanol precipitation, the ligated RNAs were resuspended in 10 μl the RNAase-free water. RT-PCR reactions were performed using specific outward primers (Table S1) and the One-Step RT-PCR kit (Qiagen, Hilden, Germany). RT-PCR products were checked by loading on a 2% TAE agarose gel. Bands significantly enriched or only present in the TAP+ reactions were purified by PureLink Quick Gel Extraction kit (Life Technologies) and cloned into pGEMT easy (Promega, Madison, WI). Plasmids containing the expected size of insert were sent to sequencing. Sequences were compared with S. Typhimurium SL1344 genome (NC_0116810) to determine the localization of the 5′- and 3′-ends and subsequently the RNA length.

Eukaryotic Cell lines and Culture Conditions

Eukaryotic cell lines included NRK-49F normal rat kidney fibroblasts (ATCC CRL-1570), human telomerase reverse transcriptase (hTERT)-immortalized BJ-5ta fibroblasts (ATCC CRL-4001) and HeLa epithelial cells (ATCC CCL-2). NRK-49F fibroblasts were propagated in Dulbecco’s modified Eagle’s medium (DMEM) containing 5% (vol/vol) fetal bovine serum (FBS) and 4 mM L-glutamine. BJ-5ta fibroblasts were propagated in a 4:1 ratio of DMEM to medium 199 containing 10% FBS, 1 mM sodium pyruvate, and 4 mM L-glutamine. Minimum essential medium Eagle (MEM) containing 10% FBS, 1 mM sodium pyruvate, and 2 mM L-glutamine was used to grow HeLa cells.

Isolation of RNA from Intracellular Salmonella

Bacteria were grown overnight in LB broth at 37°C in non-shaking conditions. Then, bacteria were spun down and washed using Hank’s buffered saline solution (HBSS) prior to infection. An aliquot of these bacteria was used to isolate control RNA from the initial inoculum. For each RNA extraction from intracellular Salmonella, a total of 5×107 to 109 fibroblasts or epithelial cells were seeded in 500 cm2 culture square dishes (BD Biosciences, ref. 351040) and infected at a multiplicity of 10:1 (bacteria : eukaryotic cell). Infection was continued for 20 min. Infected cells were washed three times with pre-warmed HBSS and then incubated with fresh culture medium containing 100 μg/ml gentamicin for an additional hour. The media was replaced with fresh medium containing 10 μg/ml gentamicin and infection was continued for as long as indicated. At each time point, infected cells were lysed in 0.1% SDS, 1% acidic phenol, 19% ethanol in RNAse-free water [39]. This phenol-ethanol mixture acted to stabilize bacterial RNA. For each time point, intracellular bacteria were isolated from 2 to 4 culture dishes and pooled. Pellets containing intracellular bacteria were collected by centrifugation at 27,000 × g for 30 min at 4°C and washed with phosphate buffered saline (PBS). In some instances, RNA from intracellular bacteria
was extracted together with eukaryotic host cell RNA. In these experiments 1 ml of chilled Trizol reagent was added directly to a 100-mm cell culture dish and subsequently scraped at 4°C. Total RNA was isolated and then reverse transcribed into cDNA libraries as described elsewhere in this section.

**Northern Analyses**

Twenty micrograms of total RNA extracted from bacteria grown in standing conditions or from intracellular bacteria were run in 8 M urea 6% acrylamide in 1×TBE buffer for 1 h at RT. Prior to electrophoresis, gel were pre-run for 30 min. RNA was transferred to NHybond membranes (GE-healthcare) in 0.5×TBE buffer for 2 h at 50V, 4°C. Membranes were UV-cross-linked, pre-hybridized with Ultrahyb-oligo buffer (Life technologies) for 1 h at 42°C. Hybridization was carried out overnight at 42°C in the same buffer containing 1×10^6 cpms/ml of each of the following primers labelled at their 5’ termini: N-0995-1.5-CTGAAAGTAGATCGAAGACAG-CACCTGGCTCTGGGA-3’

N-0995-1.75AAATCAGAATCCCATCGAATCAGAACAG-

CAGCGCAAATT-3’

N-0995-1.5-ATTGACGAAATGGGCTCAGGATT-

TATCCGCGCCGGCTCG-3’

N-1530-1.5-CCGACTGCGACGATTCATATTTCC-

CAGTTGCACTGG-3’

Ten pmol of oligonucleotide were labelled with 30 pmol of gamma-^{32}P-ATP and using 20 U of polynucleotide-kinase (New England Biolabs) for 30 min at 37°C without shaking. Membranes were washed three times in 2×SSC, 0.5% SDS, at 45°C for 30 min per wash. Membranes were exposed at −70°C for 24 h.

**Quantitative Real-Time PCR**

Oligonucleotides were designed using the Primer Express Software (Life technologies) and are listed in Table S1. Reactions were carried out in an ABI Prism 7300 instrument (Life technologies) using the Power SYBR green PCR master mix (Life technologies) under standard reaction conditions described elsewhere[16]. The expression levels of each gene in each condition tested were normalized to the levels of 16S or OmpA transcript.

**Bacterial Infection Assays**

Fibroblasts and epithelial cells were seeded in 24-well plates to reach a density of 5×10^4 to 10^5 cells/well at the time of infection. Bacteria were incubated overnight without shaking at 37°C in LB broth. Prior to infection, bacteria were washed with pre-warmed HBSS and viable bacteria were enumerated by plating serial dilutions. Cells were infected at a MOI 10:1 and infection was left to progress 20–30 min until internalized bacteria were observed by microscope examination. Infected cells were washed three times with pre-warmed HBSS and then incubated with culture medium containing 100 μg/ml gentamicin up to 2 h. The media was then replaced with fresh medium containing 10 μg/ml gentamicin and infection was continued for 6 h (HeLa cells) or 24 h (fibroblasts). Cells were lysed at the indicated post-infection times in a solution containing PBS buffer, pH 7.4, 1% (vol/vol) Triton X-100, and 0.1% (wt/vol) SDS. The number of viable intracellular bacteria was calculated by plating appropriate dilutions of the lysate. The invasion rate was the percentage of internalized bacteria calculated by as the ratio between gentamicin-protected bacteria at 2 h after infection and the number of viable bacteria in the inoculum used. The intracellular proliferation index was calculated by the ratio of viable intracellular bacteria in each well at 24 (fibroblasts) or 6 h (HeLa) divided by the viable intracellular bacteria at 2 h post-infection.

**Competitive Index (CI) Virulence Assays**

Six-week-old female BALB/c mice (Harlan laboratories, Indianapolis, IN) were used for virulence assays. In each experiment, groups of four to eight animals were inoculated with a 1:1 ratio of the respective strains. Bacteria were grown overnight at 37°C in LB without shaking, spun down, washed with PBS and diluted. Intraperitoneal inoculation was performed with a 50 μl bacterial suspension containing c.a. 10^5 viable bacteria. The number of bacteria of each individual strain in the input inoculum was assessed by plating serial dilutions of this suspension in LB and in LB containing the appropriate antibody. To determine the output bacterial load of each strain in target organs, infected mice were sacrificed at 48 to 72 h post-infection, and spleen and liver homogenates were plated. The competitive indexes (CI) were calculated as the ratios in the output samples divided by the ratios in the input inoculum.

**Protein Extracts and Western Blot Analysis**

Total protein extracts were prepared from bacteria grown overnight at 37°C without shaking in LB broth or from intracellular bacteria. Bacteria grown overnight in 1 ml of LB broth (~10^8 viable bacteria) were collected by centrifugation and suspended in an appropriate volume of Laemmli sample buffer. Intracellular bacteria from infected fibroblasts were collected as described above for RNA isolation. Prior to the comparative protein expression analysis between extracellular and intracellular bacteria, samples were adjusted using either DnaK or OmpA as loading controls. Polyclonal rabbit anti-DnaK and anti-OmpA antibodies have been described elsewhere[23]. Proteins were resolved by Tris-glycine-PAGE using 12% gels and transferred onto polyvinylidene-difluoride (PVDF) membranes using a semidry electrophoresis transfer apparatus (Bio-Rad, Hercules, CA). FLAG-tagged proteins were detected with anti-FLAG M2 monoclonal (1:5,000 dilution; Sigma, St. Louis, MO). Goat anti-mouse- or anti-rabbit-HRP-conjugated antibodies (Bio-Rad) were used as secondary antibodies (1:5,000 dilution). Blots were developed with ECL prime reagent (GE Healthcare, Little Chalfont, United Kingdom).

**Accession Numbers**

The configuration of the ‘Salgenomics’ microarray was deposited in the MIAME database (http://www.ebi.ac.uk/ miamexpress) with accession number A-MEXP-846. Gene expression data were deposited in the Array Express database (http://www.ebi.ac.uk/arrayexpress) with accession numbers E-MEXP-1774 (intracellular phoP transcriptome), E-MEXP-1775 (intracellular wild-type transcriptome), and E-MEXP-1776 (extracellular wild-type, stationary phase).

**Statistical Analysis**

Prior to carrying out the statistical analyses, relative expression data and ratios coming from infection and virulence assays were fitted to a normal distribution by a log-based transformation. The means of the normalized data were compared by two-tailed student’s t test (two samples), one-sample t-test (one sample vs. a hypothetical value) or, by two-way ANOVA and the Bonferroni’s post-test. All data sets were analyzed using GraphPad Prism, version 5.0, software (GraphPad Inc., San Diego, CA).
Results

Identification of a Novel Antisense sRNA in the S. Typhimurium Virulence Plasmid pSLT

We have recently used the microarray ‘Salgenomics’ for genome expression analyses in intracellular S. Typhimurium persisting within fibroblasts [40] and in studies involving extracellular bacteria [16,23,30]. This oligonucleotide-based microarray contains specific probes to most known non-coding sRNAs present in intergenic regions (IGRs) [41]. To detect novel sRNAs, the microarray was also designed with a second group of probes positioned in each of the two strands of every IGR along the whole genome. Given our interest in identifying novel sRNAs functionally linked to Salmonella infections, we analyzed the expression profile associated to this set of probes in both intracellular and extracellular bacteria. Besides non-growing intracellular bacteria, a sample consisting of bacteria grown to stationary phase in LB broth was included to distinguish transcriptional profiles linked genuinely to the ‘non-proliferative intracellular lifestyle’. Additional information was also obtained by analyzing the profile of the phoP mutant overgrowing within fibroblasts. Concerning extracellular phoP bacteria, our previous transcriptomic data revealed minimal differences relative to wild-type bacteria when both strain grow actively in LB broth [23]. In all conditions tested, the profile of extracellular wild-type bacteria growing in LB broth to early-exponential phase was used as common reference sample.

The transcriptomic analyses in non-growing intracellular wild-type bacteria revealed a significant induction (>4-fold, p<0.05) in probes mapping to 73 IGRs (Figure 1A). Ten of these 73 probes were also registered with increased signal in intracellular phoP overgrowing bacteria and 32 were in common with enhanced signals obtained in bacteria grown to late-stationary phase in LB broth (Figure 1A). Interestingly, 39 transcripts encoded in IGRs were found specifically induced in non-growing intracellular dormant bacteria, which suggests the occurrence of presumptive sRNAs with a putative role in pathogen persistence within fibroblasts (Figure 1A). Among these 39 probes, two of them (CNB1344-0963 and CNB1344-0995) lie to the Salmonella virulence plasmid pSLT. The sequence of these two probes in shown as supporting information (Figure S1). At the time of designing the transcriptomic data revealed minimal differences relative to wild-type bacteria when both strain grow actively in LB broth [23]. In all conditions tested, the profile of extracellular wild-type bacteria growing in LB broth to early-exponential phase was used as common reference sample.

The transcriptomic analyses in non-growing intracellular wild-type bacteria revealed a significant induction (>4-fold, p<0.05) in probes mapping to 73 IGRs (Figure 1A). Ten of these 73 probes were also registered with increased signal in intracellular phoP overgrowing bacteria and 32 were in common with enhanced signals obtained in bacteria grown to late-stationary phase in LB broth (Figure 1A). Interestingly, 39 transcripts encoded in IGRs were found specifically induced in non-growing intracellular dormant bacteria, which suggests the occurrence of presumptive sRNAs with a putative role in pathogen persistence within fibroblasts (Figure 1A). Among these 39 probes, two of them (CNB1344-0963 and CNB1344-0995) lie to the Salmonella virulence plasmid pSLT. The sequence of these two probes in shown as supporting information (Figure S1). At the time of designing the transcriptomic analyses in non-growing intracellular wild-type bacteria revealed a significant induction (>4-fold, p<0.05) in probes mapping to 73 IGRs (Figure 1A). Ten of these 73 probes were also registered with increased signal in intracellular phoP overgrowing bacteria and 32 were in common with enhanced signals obtained in bacteria grown to late-stationary phase in LB broth (Figure 1A). Interestingly, 39 transcripts encoded in IGRs were found specifically induced in non-growing intracellular dormant bacteria, which suggests the occurrence of presumptive sRNAs with a putative role in pathogen persistence within fibroblasts (Figure 1A). Among these 39 probes, two of them (CNB1344-0963 and CNB1344-0995) lie to the Salmonella virulence plasmid pSLT. The sequence of these two probes in shown as supporting information (Figure S1). At the time of designing the transcriptomic analyses in non-growing intracellular wild-type bacteria revealed a significant induction (>4-fold, p<0.05) in probes mapping to 73 IGRs (Figure 1A). Ten of these 73 probes were also registered with increased signal in intracellular phoP overgrowing bacteria and 32 were in common with enhanced signals obtained in bacteria grown to late-stationary phase in LB broth (Figure 1A). Interestingly, 39 transcripts encoded in IGRs were found specifically induced in non-growing intracellular dormant bacteria, which suggests the occurrence of presumptive sRNAs with a putative role in pathogen persistence within fibroblasts (Figure 1A). Among these 39 probes, two of them (CNB1344-0963 and CNB1344-0995) lie to the Salmonella virulence plasmid pSLT. The sequence of these two probes in shown as supporting information (Figure S1). At the time of designing the transcriptomic analyses in non-growing intracellular wild-type bacteria revealed a significant induction (>4-fold, p<0.05) in probes mapping to 73 IGRs (Figure 1A). Ten of these 73 probes were also registered with increased signal in intracellular phoP overgrowing bacteria and 32 were in common with enhanced signals obtained in bacteria grown to late-stationary phase in LB broth (Figure 1A). Interestingly, 39 transcripts encoded in IGRs were found specifically induced in non-growing intracellular dormant bacteria, which suggests the occurrence of presumptive sRNAs with a putative role in pathogen persistence within fibroblasts (Figure 1A). Among these 39 probes, two of them (CNB1344-0963 and CNB1344-0995) lie to the Salmonella virulence plasmid pSLT. The sequence of these two probes in shown as supporting information (Figure S1). At the time of designing the
Figure 1. Identification of a novel sRNA in the virulence plasmid pSLT of S. Typhimurium. (A) Venn diagrams showing the number of oligonucleotide probes corresponding to intergenic regions (IGR) induced in three experimental conditions (intracellular wild-type, intracellular phoP, late stationary phase) in the virulence plasmid pSLT of S. Typhimurium. (B) Schematic representation of the pSLT region containing the novel sRNA. (C) Southern blot analysis showing the presence of the novel sRNA (IGR-0995) in the pSLT plasmid. (D) Distribution of the pSLT plasmid region.
in LB broth, ISM or PCN media (Figure 2A). The condition used routinely to grow bacteria for infection of eukaryotic cells, non-shaking, stationary phase in LB broth, was also included. To overcome the bias inherent to the processing at the 3’-end observed in the RACE experiments (see Figure 1D), the primers used for RT-qPCR were designed to amplify a region unique to the 5’ end of IesR-1. Expression of IesR-1 was detected, albeit at very low levels, in LB under shaking conditions (Figure 2A). Consistently with the microarray data (Figure 1A), we did not observe a significant difference in LB at stationary phase compared to exponential phase (Figure 2A). IesR-1 expression was however significantly higher (~12-fold) in bacteria grown to stationary phase in LB under non-shaking conditions (Figure 2A). Bacteria grown in ISM or PCN media did not induce IesR-1 at significant levels (Figure 2A). Taken together, these results indicate that the high expression levels of IesR-1 inferred by microarray analysis in non-growing intracellular S. Typhimurium could not be reproduced in laboratory conditions.

Based on this observation, we next quantified the relative abundance of IesR-1 at different post-infection times upon bacterial entry into eukaryotic cells. NRK-49F rat fibroblasts, the host cells in which the Salgenomics microarray was used to obtain genome expression data of non-growing dormant intracellular bacteria [23], were infected to that purpose. Intracellular bacteria were collected at 1, 8 and 24 h post-infection and the transcript levels measured by RT-qPCR. IesR-1 levels increased notoriously upon infection of NRK-49F cells compared to the bacteria of the inoculum used to infect the fibroblasts (Figure 2B). Such induction became remarkably conspicuous as the infection progressed, showing an increase of ~300-fold at 24 h post-infection (Figure 2B). Such exacerbated expression of IesR-1 was also observed in non-growing bacteria persisting within BJ5-ta human fibroblasts (Figure 2B). The pronounced expression of IesR-1 in intracellular bacteria was also corroborated by Northern blot analysis, which demonstrated the presence of two IesR-1 transcripts, of ~600 nt and ~275 nt, exclusively in samples prepared from intracellular bacteria at 24 h post-infection (Figure 2C). To differentiate whether the up-regulation of IesR-1 in non-growing dormant intracellular bacteria was linked to this particular physiological state of the pathogen or to the host cell

Figure 2. The sRNA IesR-1 is up-regulated in non-growing intracellular S. Typhimurium. Relative levels of the sRNA IesR-1 were determined by reverse transcription and RT-qPCR. Data are relative to the transcript levels of IesR-1 in bacteria cultured with shaking in LB broth to early-exponential phase (O.D. 600 nm = 0.2). 16S ribosomal RNA was used as a reference gene. Bars indicate the mean ± standard deviation of three independent experiments. (A) IesR-1 expression in axenic cultures. Bacteria were cultured in LB broth or axenic media such as ISM [35] and PCN [34] with shaking to either exponential or stationary growth phases. A third condition consisting in growth with no shaking to stationary phase was also included in the analysis. (B) Expression of IesR-1 in non-growing intracellular S. Typhimurium collected at different post-infection times from rat and human fibroblasts (NRK-49F and BJ-Sta, respectively). For comparison, IesR-1 expression was also monitored in wild-type bacteria proliferating inside HeLa epithelial cells and in phoP mutant bacteria overgrowing within NRK-49F fibroblasts. The 0 h post-infection time point corresponds to bacteria grown overnight with no shaking in LB broth that were used to infect the eukaryotic cells. Note the pronounced up-regulation of IesR-1 (~200–300 fold) in non-growing intracellular bacteria at late post-infection times, which contrasts with the moderate induction values registered in extracellular bacteria. Data are the mean and the standard deviation of three independent experiments. (C) Northern blot analysis showing the production of an sRNA of ~600 nt in intracellular bacteria compatible with the expected size of IesR-1. A second transcript of ~275 nt was also detected specifically in intracellular bacteria, which as denoted by the RACE experiments (see Figure 1D) could correspond to 5’ region of IesR-1. Note the lack of noticeable amount of these two molecules in wild-type bacteria grown extracellularly.

doi:10.1371/journal.pone.0077939.g002
type that was infected, IesR-1 expression was monitored in the phoP mutant overgrowing in fibroblasts and in wild-type bacteria proliferating within HeLa epithelial cells. In none of these two cases a pronounced up-regulation of IesR-1 as in non-growing intracellular bacteria was observed although the phoP mutant produced higher levels of the sRNA (~5-fold) at 24 h post-infection compared to bacteria present in the inoculum (Figure 2B). This finding led us to discard whether IesR-1 could be subjected to regulation by the PhoP-PhoQ system. We analyzed relative levels of transcript conforming the PSLT047-iesR-1-psLT046 (ing5) region in wild-type, phoP and a phoP strain carrying a constitutively active PhoP-PhoQ system. While PSLT046 (ing5) was clearly regulated by PhoP-PhoQ, no changes in expression were observed for IesR-1 among the strains tested (Figure S2). Collectively, these data demonstrated the existence of IesR-1 as an sRNA molecule that is selectively up-regulated by S. Typhimurium inside eukaryotic cells under conditions in which growth is restrained.

Role of IesR-1 in S. Typhimurium Virulence

Since the expression of many virulence functions of S. Typhimurium has been reported to respond to the intracellular environment, we sought to determine whether an alteration in IesR-1 levels could lead to changes in the capacity of bacteria to invade or persist inside fibroblasts or to impair infection of susceptible mice. To inactivate IesR-1 but simultaneously avoid polar effects on the overlapping 3’ end of PSLT047 or the 5’ promoter region of PSLT046 (ing5) (see Figure 1A), we replaced a region spanning the first 209 nt of the IesR 5’end with a chloramphenicol-resistance cassette (cat) flanked by two FRT sites (Figure S3A). We then checked the expression of the flanking genes as well as the 3’-remnant of IesR-1 using strand-specific primers for reverse transcription followed by RT-qPCR. Wild-type, the aforementioned mutant strain (ΔiesR-1/5’::cat) and a deletion strain in which the cat cassette was eliminated by FLPe-mediated recombination of FRT sites (ΔiesR-1/5’), were included in this RT-qPCR assay. A polar effect was observed in the ΔiesR-1/5’::cat strain, as the remaining 3’ region of iesR-1 was dramatically induced (Figure S3B). This effect was most likely due either to the presence of a cryptic driver in the cat cassette or to a read-through of the RNA polymerase as no increased expression of the 3’ region of iesR-1 was observed in the clean ΔiesR-1/5’ mutant lacking the resistance cassette (Figure S3B). This finding suggested that the natural promoter of iesR-1 still drives transcription of the remaining of this sRNA that is produced by the clean ΔiesR-1/5’ mutant. Since the genomic organization of this plasmid region made not feasible the construction of a deletion mutant lacking the entire IesR-1 molecule, we included both ΔiesR-1/5’::cat and ΔiesR-1/5’ strains in the infection assays.

Wild-type and ΔiesR-1/5’ strains were used in in vitro infection assays with rat and human fibroblasts (NRK-49F and BJ-5a cell lines, respectively) and human HeLa epithelial cells. Except for BJ-5a fibroblasts, in which the lack of IesR-1 was linked to a decrease of ~2-fold in the invasion rate, no significant differences were observed in the rest of cell lines used (Figure 3A). Concerning intracellular phenotypes, the only observed change was also observed in BJ-5a human fibroblasts, in which the lack of IesR-1 correlated to an increase of ~3-fold in the intracellular proliferation rate (Figure 3B). Since only minor alterations were observed in the in vitro infection models, we tested these strains in the mouse typhoid model. Previous studies reported a strong correlation between S. Typhimurium functions required for maintaining a persistent state within fibroblasts and their relative contribution to virulence [43–46]. So, we hypothesized a probable role in virulence of IesR-1, strongly up-regulated by non-growing intracellular bacteria (Figure 2B). We then examined the fitness of ΔiesR-1/5’::cat and ΔiesR-1/5’ strains in BALB/c mice using competitive infections upon intraperitoneal challenge. The ΔiesR-1/5’::cat mutant displayed a decreased fitness compared to wild-type bacteria (competitive index, CI ~ 0.33) (Figure 4A). This result suggested that either the overexpression of the 3’ region, the lack of the 5’ region, or a combination of both, might attenuate virulence. Similar results were obtained when infecting mice by the oral route (results not shown). Interestingly, the ΔiesR-1/5’::cat mutant also displayed decreased fitness when mixed with ΔiesR-1/5’ mutant bacteria (Figure 4A). Since both strains are devoid of an identical 5’ region of IesR-1, this finding indicates that an increased expression of the 3’ region of IesR-1 might impair virulence. Competition experiments were also performed with the ΔiesR-1/5’ strain expressing in trans two versions of IesR-1: one encompassing the entire 597 nt molecule, MD2277 (ΔiesR-1/5’, cat::PLtetO iesR), and the other carrying only the first 208 nt of the 5’ region, MD2276 (ΔiesR-1/5’, cat::PLtetO iesR-3’). Expression of these IesR-1 molecules was verified by RT-PCR (data not shown). When these two complemented strains were confronted against the ΔiesR-1/5’ mutant no changes in virulence were observed (Figure 4B). Altogether, these results indicate that deregulation of IesR-1 expression in S. Typhimurium impacts on the progression of the infection and, simultaneously, suggest that the regulation of IesR-1 exerts over the PSLT047 transcript may require interaction of both RNA molecules while they are co-expressed in a specific location of the virulence plasmid.

IesR-1 Regulates Production of the PSLT047 Protein by a cis-antisense Mechanism

sRNAs that partially overlap with transcripts originated from flanking genes usually exert a cis-regulatory mechanism by antisense pairing [26]. Since IesR-1 partially overlaps with the 3’UTR of the PSLT047 transcript, we hypothesized on a possible antisense regulatory mechanism. Our previous results indicated that IesR-1 is selectively induced in non-growing dormant intracellular bacteria (Figure 2B). An inverse correlation between IesR-1 levels and its putative target would suggest a negative post-transcriptional regulation at the level of target mRNA stability. To test this, we monitored the expression pattern of PSLT047 and iesR-1 transcripts in different growth conditions (Figure 5A). Unexpectedly, PSLT047 mRNA expression followed essentially the same induction pattern as its putative sRNA cis-acting regulator, IesR-1 (Figure 5A). These results indicated that IesR-1 could have either no influence or, alternatively, a positive effect on PSLT047 mRNA stability. In this scenario, post-transcriptional control at the level of PSLT047 translation could also occur. When analyzed in non-growing intracellular bacteria, we observed that PSLT047 protein levels dropped significantly compared to extracellular bacteria in the inoculum (Figure 5B). Experiments with two strains carrying the PSLT047-3xFLAG tagged allele but differing in the integrity of the IesR-1 molecule provided additional support to the model involving interaction between 3’ regions of IesR-1 and the PSLT047 transcript with effects in PSLT047 protein levels. Thus, the amount of PSLT047 increased significantly in extracellular bacteria having an IesR-1 molecule interrupted with a Km resistance cassette, therefore unable to interact at its 3’ end with the PSLT047 transcript (Figure 5C). Altogether, these results are consistent with a cis-acting regulatory mechanism involving interaction of the antisense IesR-1 molecule with the PSLT047 mRNA, which could ultimately modulate the translation rate of this protein.
Discussion

Computational, experimental and global RNAseq approaches have been used to search for non-coding regulatory sRNAs in S. Typhimurium [6,7,17,47]. These studies have been mostly restricted to bacteria grown in vitro conditions, providing little insights into the transcription status during ex vivo or in vivo infections. A recent work in the mouse typhoid model in which S. Typhimurium mutants lacking defined sRNAs were used, uncovered the requirement of IstR, OxyS and SroA for virulence [15]. Additional studies showed that the sRNAs IsrJ and IsrM contribute to invasion of epithelial cells and that S. Typhimurium also uses IsrM for proliferation in mouse organs [11,17]. Apart from these studies, most sRNAs identified to date in S. Typhimurium remain to be shown whether they play a role in virulence [48].

In this work, we focused on transcriptional changes in S. Typhimurium persisting within fibroblasts, a host cell type in...
Figure 4. Alteration in the integrity of the iesR-1 molecule causes a defect in virulence that is not restored by complementation in trans. (A) Strains ΔiesR-1/5':cat or ΔiesR-1/5' were used in competition experiments in susceptible BALB/c mice. While both strains are devoid of the 5' region of iesR-1, the strain ΔiesR-1/5':cat overexpresses the 3' region (lower part of the panel and Figure S3). Note that both strains are attenuated in virulence; (B) Competition experiments with strains ΔiesR-1/5' overexpressing in trans from a construct positioned in the araE locus the entire iesR-1 molecule or exclusively the 5' region (208 nt) of iesR-1 (lower part of the panel). Note that in any case the expression in trans of these molecules restored virulence. Groups of four to eight mice were inoculated with the indicated strain pairs by the intraperitoneal route. Liver and spleen were extracted 72 h post-infection and homogenated. Appropriate dilutions were plated onto LB plates with and without antibiotic and the competitive index (CI) calculated as the ratio between strains in the organs versus the ratio in the inoculum. p-values were obtained by one-sample student’s t test with log-transformed data and establishing 0 as hypothetical value.

doi:10.1371/journal.pone.0077939.g004
which intracellular bacteria establish a non-proliferative, dormant state [24]. Persistence is a strategy used by successful pathogens, including *S. enterica*, *M. tuberculosis*, or *Helicobacter pylori* among others [21]. Our previous work demonstrated that certain *S. Typhimurium* sRNAs encoded in the chromosome exhibit unique expression pattern along the fibroblast infection [16]. This observation is indicative of distinct time-dependent physiological roles for these molecules during progression of the persistence state. Here, we analyzed a novel *S. Typhimurium* sRNA encoded in the pSLT virulence plasmid that we named IesR-1. Besides FinP, IesR-1 would be the second example of a pSLT-encoded sRNA that, in this case, could have evolved to modulate the growth rate of the pathogen inside the eukaryotic cell. Interestingly, other functions encoded in the pSLT plasmid, such as the transcriptional regulator SpvR, are induced in a *Caenorhabditis elegans* persistent infection model [49]. Intriguingly, *speR* mutants are defective in their capacity to restrain growth inside fibroblasts [24]. Although pSLT is known to be required for *Salmonella* virulence, little is known about additional regulatory molecules encoded by this plasmid playing a role in pathogen persistence.

Our finding that the sRNA IesR-1 is specifically induced during persistence (Figures 1–2) highlights the importance of using physiological models to identify novel virulence factors. Indeed, we failed to detect up-regulation of IesR-1 in extracellular bacteria growing in either ISM or PCN defined media, considered to mimic the conditions of the vacuole inhabited by intracellular bacteria. This result indicates that intracellular signals resulting from host cell-microbe interaction entail a higher level of

![Figure 5. IesR-1 regulates production of the PSLT047 protein by a mechanism involving interaction at the 3' ends of the respective RNA molecules.](image-url)

(A) expression pattern of iesR-1 and PSLT047 in extracellular bacteria grown in LB broth and in intracellular bacteria at 24 h post-infection of NRK-49F fibroblasts. Transcript levels were determined by reverse transcription and RT-qPCR. Expression data were calculated relative to the levels in bacteria cultured to early-exponential phase. 5S ribosomal RNA and the ompA transcript were used as endogenous controls for iesR-1 and PSLT047 transcripts, respectively. Bars indicate the mean ± standard deviation of three independent experiments. (B) Non-growing intracellular bacteria persisting in fibroblasts down-regulate the levels of the PSLT047 protein. The relative levels of PSLT047 were determined using a PSLT047:3×FLAG-tagged variant. Samples were prepared from bacteria grown in LB broth overnight in no-shaking conditions (inoculum) and from non-growing intracellular bacteria collected from NRK-49F fibroblasts at 24 h post-infection. The results from two independent experiments are shown. (C) Increased production of the PSLT047:3×FLAG-tagged variant in bacteria in which interaction between the 3' ends of iesR-1 and PSLT047 is impeded by the presence of an antibiotic resistance cassette. The genetic configuration of the strains used is indicated. Results from two independent clones are shown. DnaK and OmpA were used as loading controls.

doi:10.1371/journal.pone.0077939.g005
complexity, and that such a particular condition could be hardly accomplished by current in vitro-culturing reagents. The persistence-dependent expression of IesR-1 was confirmed in fibroblast from different sources (human and rat origin) although it was not so evident in phoP mutant bacteria actively proliferating within host cells (Figure 2B). These results open the question of whether IesR-1 expression is regulated in response to specific signal(s) implicated in quorum sensing and/or adaptive responses linked to environmental stresses existing in the Salmonellac containing vacuole.

The identification of IesR-1 as a novel sRNA was confirmed by circular RACE experiments and further cloning of ampiclons enriched in the pyrophosphatase-treated fractions as well as Northern blot. The RACE experiments allowed to accurately map the 5’-end of the primary transcripts. Regarding the 3’ end, the transcripts showed a marked heterogeneity among the sequenced clones, which is likely to result from read-through of transcriptional terminators and/or degradation by 3’-exonucleases. This is a particularly prominent feature in the overlapping region between PSLT047 and iesR-1 transcripts, for which we got clones with gradually decreasing lengths. This result could indicate an RNase III-mediated post-transcriptional endonucleolytic processing of an hypothetical PSLT047-iesR-1 duplex. Alternatively, such heterogeneity in the 3’ ends could also be explained by the fall off of the reverse transcriptase as a result of encountering dsRNA tracks with variable size made of the PSLT047-iesR-1 hybrid RNAs. Regardless of the situation, the results are consistent with a cis-acting regulation of IesR-1 over the PSLT047 transcript by an interaction of their respective 3’-ends. This postulate is also sustained by the increased production of the PSLT047 protein following the interruption of the IesR-1 molecule (Figure 5C). Such convergent genetic configuration between an sRNA and the 3’ UTR of its target has been previously documented. Thus, the GadY sRNA of Esherichia coli overlaps with the 3’ UTR of GadX, a transcriptional regulator of acid response. This interaction leads to an RNase III-mediated processing of the GadX-GadW duplex, which in turn favors the accumulation of GadX transcript [50]. Conversely, the Bacillus subtilis RatA sRNA overlaps with the 3’ UTR of tspA, which codes for a toxic peptide that promotes cell lysis [51]. This interaction impedes tspA transcript accumulation, thus avoiding cell lysis. Nevertheless, the data obtained with IesR-1 are more consistent with a regulatory mode over PSLT047 acting on its transcript translation.

The presence of antisense transcripts in mobile genetic elements (such as plasmids) is frequently associated to type I toxin-antitoxin systems (TAS) [26,27]. These systems contribute to maintain stability of plasmids during bacterial propagation by the post-segregational killing of plasmid-free daughter bacteria. Interestingly, the 3’ end of IesR-1 shows high homology to an intergenic region (Shal223_609 to Shal223_610) from Shewanella baltica (Figure S4), in which Shal223_609 is annotated as a putative protein. This observation suggests a possible horizontal gene transfer between Shewanella baltica and S. enterica and raises the tempting idea of a possible toxin-antitoxin system conformed by PSLT047 and IesR-1. Such a scenario is reminiscent to the par stability determinant of Enterococcus faecalis encoded in the pAD1 plasmid [52,53]. RNA I and RNA II are two sRNAs transcribed in opposite orientations that constitute the toxin and antitoxin of the par post-segregational killing system, respectively. Interestingly, the interaction of RNA I and II involves the binding at the 5’ and 3’ ends in a two-step mechanism, with an initial kissing interaction between the transcriptional terminator stem-loops of both RNAs followed by the pairing of the complementary direct repeat sequences and the complete hybridization of the 5’ nucleotides to stabilize the complex [53]. The binding of RNA II antitoxin has been shown to inhibit ribosome binding to RNA I, thus impeding an efficient toxin translation initiation and cell killing [52]. In this regard, it would be of interest to investigate whether IesR-1 could control negatively PSLT047 translation by a similar mechanism. Moreover, we cannot discard the possibility that IesR-1 could regulate expression of other genes located elsewhere in the chromosome and/or the pSLT plasmid via a trans-antitoxin mechanism based on the non-overlapping 5’ region. Transcription performed in wild-type and ΔiesR-1 strains growing in the most optimal extracellular condition for IesR-1 expression, non-shaking-stationary phase in LB broth, did not result in any conclusive target (data not shown). This observation could imply that the 5’ motery of IesR-1 does not play a significant role in determining target specificity. On the other hand, it cannot be ruled out that IesR-1 also exerts its function exclusively in intracellular bacteria, therefore with no possibility of target identification in extracellular bacteria. Alternatively, IesR-1 might influence translation of pre-existing mRNAs. Similar comparative transcriptionics in intracellular bacteria might provide clues on these aspects, however such study means a real technical challenge, as purification of enough total RNA from two independent strains staying in a dormant non-growing state within the fibroblast requires a large effort. Given our interest in deciphering the biological role of IesR-1, we are currently considering this possibility.

Lastly, the data obtained in the in vitro and in vivo infection models reveal a feature repeatedly observed for many sRNAs. The lack of the regulatory molecule may result in minor alterations in the capacity of the pathogen to cause infection due to the fine-tuning that most of these sRNA molecules perform in the activity or relative level of their respective targets [54,55]. Some sRNAs ‘share’ targets, so the absence of a particular sRNA can be compensated by the action of other sRNAs [3]. In the case of IesR-1, our data allow to tentatively assign relevance to a probable coordinated production of ‘both’, the antisense IesR-1 and the PSLT047 transcript. The overexpression of the 3’ region of IesR-1 or the expression in trans of the entire IesR-1 molecule led to negative phenotypes consistent with such idea. Further studies could consider the tempting hypothesis of a coordinated expression of both RNA molecules by intracellular bacteria leading subsequently to an inverse correlation between PSLT047 protein levels and IesR-1 expression. The PSLT047-iesR-1 pair could, in this regard, behave as a type I toxin-antitoxin system resembling others implicated in bacterial growth arrest and persistence [56,57].

Supporting Information

Figure S1 Sequences (5’–3’) of the oligonucleotide probes CNB1344-0963 and CNB1344-0995 mapping in IGRs of the S. Typhimurium pSLT virulence plasmid. These two probes revealed increased transcriptional activity in their respective IGRs in non-growing intracellular bacteria located inside fibroblasts.

(DOCX)

Figure S2 The non-coding sRNA IesR-1 is not regulated by the PhoP-PhoQ system. RT quantitative PCR (RT-qPCR) assays performed on iesR-1 and its flanking genes in wild-type bacteria (14028 s), an isogenic phoP::Tn10, and constitutively active phoP-24 mutant. Expression data were calculated relative to the levels in wild type bacteria. 16S ribosomal RNA was used as endogenous control gene. Bars indicate the mean ± standard deviation of three independent experiments. * p<0.05 obtained.
by one-sample student’s t test with log-transformed data and establishing 0 as hypothetical value (TIF).

**Figure S3** ΔIesR-1/5’ mutants construction and expression analysis of flanking genes. (A) Schematic representation of the PSLT region containing the iesR-1 (ΔIesR-1/5’). In the ΔIesR-1/5’::cat mutant, this region is replaced by a claramphenicol resistance (cat) cassette flanked by two FLP recombinase sites (FRT). In the in ΔIesR-1/5’ mutant the cloramphenicol resistance is lost by FLP-mediated recombination (see also Figure 4 main text). Bended arrows indicate the predicted transcriptional start sites of RNAs expressed in this region. Dotted arrows indicate the length and orientation of transcripts identified by RACE. (B) Expression of flanking genes and of the remaining 3’-moteity of iesR-1 in the ΔIesR-1/5’ and ΔIesR-1/5’::cat mutants. Expression levels were determined by strand-specific reverse transcription using reverse gene-specific primers, followed by qPCR. Data were calculated relative to the levels in wild type bacteria, and normalized by the geometric mean of 16S, ompA and ribB endogenous control genes. Bars indicate the mean ± standard deviation of three independent experiments. ***, p<0.001 as compared to wild type by student’s t test. (TIF)

**Figure S4** Analysis of IesR-1 orthologs in other bacteria. Diagram showing regions in the pSLT plasmid of S. Typhimurium strain LT2 to the *Shewanella baltrica* OS223 chromosome with high homology to the non-coding RNA sequence. (TIF)

**Table S1** Oligonucleotide primers used in this study. (DOCX)

**Acknowledgments**

We are grateful to Alejandro Toledo-Arana and Inigo Lasa for discussions and advice in RNA methodologies; Josep Casadesus for discussions and strains; and Diana Barroso and Pablo García-Bravo for their technical assistance.

**Author Contributions**

Conceived and designed the experiments: JGA ADO MGP FGP. Performed the experiments: JGA ADO MGP GRP. Analyzed the data: JGA ADO MGP FGP. Wrote the paper: JGA ADO FGP.

**References**

1. Waters LS, Storz G (2009) Regulatory RNAs in bacteria. Cell 136: 615–628.
2. Toledo-Arana A, Repoila F, Cossart P (2007) Small noncoding RNAs controlling pathogenesis. Curr Opin Microbiol 10: 162–169.
3. Papenfort K, Vogel J (2010) Regulatory RNA in bacterial pathogens. Cell Host Microbe 8: 116–127.
4. Hershberg R, Altuvia S, Margalit H (2003) A survey of small RNA-encoding genes in Escherichia coli. Nucleic Acids Res 31: 1813–1820.
5. Ferrara S, Brugoli M, De Bonis A, Righeti F, Debl中央a F, et al. (2012) Comparative profiling of Pseudomonas aeruginosa strains reveals differential expression of novel unique and conserved small RNAs. PLoS One 7: e36535.
6. Kroger C, Dillon SC, Cameron AD, Papenfort K, Sivasankaran SK, et al. (2012) The transcriptional landscape and small RNAs of Salmonella enterica serovar Typhimurium. Proc Natl Acad Sci U S A 109: E1277–1286.
7. Sittka A, Lucchini S, Papenfort K, Sharma CM, Rolle K, et al. (2008) Deep sequencing analysis of small non-coding RNA and mRNA targets of the global post-transcriptional regulator, Hfq. PLoS Genet 4: e1000163.
8. Toledo-Arana A, Dussurget O, Nikitas G, Sesto N, Guet-Revillet H, et al. (2009) Two small ncRNAs jointly govern virulence and transmission in *Legionella pneumophila*. Mol Microbiol 72: 741–762.
9. Vogel J, Bartels V, Tang TH, Chuckarov G, Shapero-Jager JG, et al. (2003) RNomics in Escherichia coli detects new sRNA species and indicates parallel transcriptional output in bacteria. Nucleic Acids Res 31: 4635–4643.
10. Armug KB, Young DB (2009) Identification of small RNAs in *Myobacterium tuberculosis*. Mol Microbiol 73: 397–408.
11. Greng H, Ya GP, Bai Y, Chan E, Wu R, et al. (2011) A Salmonella small non-coding RNA facilitates bacterial invasion and intracellular replication by modulating the expression of virulence factors. PLoS Pathog 7: e1002120.
12. Koo JT, Alleyne TM, Schiano CA, Jafari N, Lathem WW (2011) Global discovery of small RNAs in *Yersinia pseudotuberculosis* identifies Yersinia-specific small, noncoding RNAs required for virulence. Proc Natl Acad Sci U S A 108: 7697–7701.
13. Mann B, van Opijnen T, Wang J, Obert C, Wang YD, et al. (2012) Control of virulence by small RNAs in *Streptococcus pneumoniae*. PLoS Pathog 8: e1002788.
14. Padalon-Brauch G, Hershberg R, Elgrably-Weiss M, Baruch K, Rosenshine I, et al. (2013) The intracellular sRNA transcriptome of *Listeria monocytogenes* during growth in macrophages. Nucleic Acids Res 39: 4235–4248.
15. Blaser MJ, Kirschner D (2007) The equilibria that allow bacterial persistence in human hosts. Nature 449: 843–849.
16. Monack DM, Mueller A, Falkow S (2004) Persistent bacterial infections: the interface of the pathogen and the host immune system. Nat Rev Microbiol 2: 747–766.
17. Broz P, Ohlson MB, Monack DM (2012) Innate immune response to Salmonella typhimurium, a model enteric pathogen. Gut Microbes 3: 62–70.
18. Nunez-Hernandez C, Tierrez A, Ortega AD, Pucciarelli MG, Godoy M, et al. (2013) Genome expression analysis of nonproliferating intracellular *Salmonella enterica* serovar Typhimurium unravels an acid pH-dependent PhoP-PhoQ response essential for dormancy. Infect Immun 81: 154–165.
19. Cano DA, Martinez-Moya M, Pucciarelli MG, Groisman EA, Casadesus J, et al. (2001) *Salmonella enterica* serovar Typhimurium response involved in attenuation of pathogen intracellular proliferation. Infect Immun 69: 6463–6474.
20. McClelland M, Sanderson KE, Spieth J, Clifton SW, Latreille P, et al. (2001) Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. Nature 413: 452–456.
21. Thomason MK, Storz G (2010) Bacterial antiseptic RNAs: how many are there, and what are they doing? Ann Rev Genet 44: 167–188.
22. Gersdes K, Wagner EG (2007) RNA antitoxins. Curr Opin Microbiol 10: 117–124.
23. Koraimann G, Telfel M, Markolin G, Weser U, Groisman EA (1990) The FinOP repressor system of *Salmonella typhimurium*: analysis of the antiseptic RNA control of finP expression and conjugal DNA transfer. Mol Microbiol 4: 811–821.
24. Frost I, Lee S, Yanchar N, Paranchych W (1989) *FinP* and *FinO* mutations in *FinP*-anti-sense RNA suggest a model for FinOP action in the repression of bacterial conjugation by the *F* plasmid *pJL60*. Mol Gen Genet 218: 152–160.
25. Maricasoti JF, Garcia-del Portal F (2009) Genome expression analyses revealing the modulator of the Salmonella LcrD regulator by the attenuator IgaA. J Bacteriol 191: 1655–1667.
26. Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci U S A 97: 6640–6645.
27. Uzzau S, Figueras-Bossi N, Rubino S, Bossi L (2001) Epitope tagging of chromosomal genes in *Salmonella*. Proc Natl Acad Sci U S A 98: 15264–15269.
28. Lutz R, Bujard H (1997) Independent and tight regulation of transcriptional regulatory elements in Escherichia coli via the LacR/O, the TetR/O and AraC/I1–I2 regulatory units in Escherichia coli. Nucleic Acids Res 25: 1203–1210.
29. Lober S, Jackel D, Kainer H, Hensel M (2006) Regulation of Salmonella pathogenicity island 2 genes by independent environmental signals. Int J Med Microbiol 296: 435–447.
30. Headley VL, Payne SM (1990) Differential protein expression by *Shigella flexneri* in intracellular and extracellular environments. Proc Natl Acad Sci U S A 87: 4179–4183.
31. McDowell J, Lin-Chao S, Cohen SN (1994) A+U content rather than a particular nucleotide order determines the specificity of RNAe cleavage. J Biol Chem 269: 10790–10796.
37. Tedin K, Blasi U (1996) The RNA chain elongation rate of the lambda late mRNA is unaffected by high levels of ppGpp in the absence of amino acid starvation. J Biol Chem 271: 17675–17686.
38. Fu GK, Wang JT, Yang J, Au-Young J, Stove LL (2004) Circular rapid amplification of cDNA ends for high-throughput extension cloning of partial genes. Genomics 84: 205–210.
39. Eriksson S, Lucchini S, Thompson A, Rhen M, Hinton JC (2003) Unravelling the biology of macrophage infection by gene expression profiling of intracellular Salmonella enterica. Mol Microbiol 47: 105–118.
40. Garcia-del Portillo F, Nunez-Hernandez C, Esman B, Ramos-Vivas J (2008) Growth control in the Salmonella-containing vacuole. Curr Opin Microbiol 11: 46–52.
41. Argaman L, Hebeberg R, Vogel J, Bejerano G, Wagner EG, et al. (2001) Novel RNA-encoding genes in the intergenic regions of Escherichia coli. Curr Biol 11: 941–950.
42. Valdivia RH, Falkow S (1997) Fluorescence-based isolation of bacterial genes expressed within host cells. Science 277: 2007–2011.
43. Garcia-Calderon CB, Casadesus J, Ramos-Morales F (2007) Rcs and PhoPQ regulatory overlap in the control of Salmonella enterica virulence. J Bacteriol 189: 6635–6644.
44. Matsui H, Bacot CM, Garlington WA, Doyle TJ, Roberts S, et al. (2001) Virulence plasmid-borne spvB and spvC genes can replace the 90-kilobase plasmid in conferring virulence to Salmonella enterica serovar Typhimurium in subcutaneously inoculated mice. J Bacteriol 183: 4652–4658.
45. Dominguez-Bernal G, Tierez A, Bartolome A, Martinez-Pulgarin S, Salguero EF, et al. (2008) Salmonella enterica serovar Choleraesuis derivatives harbouring deletions in rpoS and phoP regulatory genes are attenuated in pigs, and survive and multiply in porcine intestinal macrophages and fibroblasts, respectively. Vet Microbiol 130: 298–311.
46. Linehan SA, Rytkonen A, Yu XJ, Liu M, Holden DW (2005) SlyA regulates function of Salmonella pathogenicity island 2 (SPI-2) and expression of SPI-2-associated genes. Infect Immun 73: 4354–4362.
47. Chinni SV, Raabe CA, Zakaria R, Randau G, Hoe CH, et al. (2010) Experimental identification and characterization of 97 novel npcRNA candidates in Salmonella enterica serovar Typhi. Nucleic Acids Res.
48. Helbrard M, Kroger C, Srikrishn S, Colgan A, Handler K, et al. (2012) sRNAs and the virulence of Salmonella enterica serovar Typhimurium. RNA Biol 9: 437–445.
49. Alegado RA, Tan MW (2006) Resistance to antimicrobial peptides contributes to persistence of Salmonella typhimurium in the C. elegans intestine. Cell Microbiol 10: 1259–1273.
50. Opdyke JA, Feoso EM, Herrn MR, Storz G (2011) RNase III participates in GadY-dependent cleavage of the gadX-gadW mRNA. J Mol Biol 416: 29–43.
51. Silvaggi JM, Perkins JB, Losick R (2006) Small untranslated RNA antitoxin in Bacillus subtilis. J Bacteriol 187: 6641–6650.
52. Greenfield TJ, Ehl E, Kirshenmann T, Franch T, Gerdes K, et al. (2000) The antiterm RNA of the par operon of pAD1 regulates the expression of a 33-amino-acid toxic peptide by an unusual mechanism. Mol Microbiol 37: 652–660.
53. Greenfield TJ, Franch T, Gerdes K, Weaver KE (2001) Antiterm RNA regulation of the par post-segregational killing system: structural analysis and mechanism of binding of the antisense RNA, RnAI, and its target, RnAI. Mol Microbiol 42: 527–537.
54. Masse E, Majdalani N, Gottesman S (2003) Regulatory roles for small RNAs in bacteria. Curr Opin Microbiol 6: 120–124.
55. Gottesman S, Storz G (2010) Bacterial Small RNA Regulators: Versatile Roles and Rapidly Evolving Variations. Cold Spring Harb Perspect Biol.
56. Ramage HR, Connolly LE, Cox JS (2009) Comprehensive functional analysis of Mycobacterium tuberculosis toxin-antitoxin systems: implications for pathogenesis, stress responses, and evolution. PLoS Genet 5: e1000767.
57. Gerdes K, Maisonneuve E (2012) Bacterial persistence and toxin-antitoxin loci. Annu Rev Microbiol 66: 103–123.