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An RpoS-dependent sRNA regulates the expression of a chaperone involved in protein folding

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ABSTRACT

Small noncoding RNAs (sRNAs) are usually expressed in the cell to face a variety of stresses. In this report we disclose the first target for SraL (also known as RyjA), a sRNA present in many bacteria, which is highly induced in stationary phase. We also demonstrate that this sRNA is directly transcribed by the major stress σ factor σS (RpoS) in Salmonella enterica serovar Typhimurium. We show that SraL sRNA down-regulates the expression of the chaperone Trigger Factor (TF), encoded by the tig gene. TF is one of the three major chaperones that cooperate in the folding of the newly synthesized cytosolic proteins and is the only ribosome-associated chaperone known in bacteria. By use of bioinformatic tools and mutagenesis experiments, SraL was shown to directly interact with the 5′ UTR of the tig mRNA a few nucleotides upstream of the Shine-Dalgarno region. Namely, point mutations in the sRNA (SraL∗) abolished the repression of tig mRNA and could only down-regulate a tig transcript target with the respective compensatory mutations. We have also validated in vitro that SraL forms a stable duplex with the tig mRNA. This work constitutes the first report of a small RNA affecting protein folding. Taking into account that both SraL and TF are very well conserved in enterobacteria, this work will have important repercussions in the field.

Keywords: S. Typhimurium; RyjA; σS; PPIase; SraL

INTRODUCTION

Small noncoding RNAs (sRNAs) perform a wide diversity of regulatory functions in both prokaryotic and eukaryotic cells. The majority of the sRNAs act by base-pairing with mRNA targets (antisense sRNAs) or by binding to proteins to modify their activity (for a review, see Storz et al. 2011). Most of the antisense sRNAs are trans-encoded since they are encoded in a separate locus in relation with the mRNA target. Consequently, these sRNAs exhibit only partial complementarity with the target and usually regulate the RNA chaperone Hfq for base-pairing. Typically, trans-encoded sRNAs are induced under environmental stress conditions and also upon entry into stationary phase of growth in order to up- or down-regulate their target(s) (Gottesman and Storz 2011).

A plethora of sRNAs have been identified in the last years; for instance, in Salmonella enterica serovar Typhimurium (S. Typhimurium), 140 sRNAs were reported in early stationary phase of growth by using a combination of RNA-seq and dRNA-seq analyses and Hfq-coIP-seq approach (Kröger et al. 2012).

sRNAs are generally highly controlled at the transcriptional level. Nearly one-third of the functional characterized sRNAs contribute to the control of the outer membrane protein (OMP) production. Some of these sRNAs are under the control of the σ factor RpoE (also known as σE or σ24) (Johansen et al. 2006; Papenfort et al. 2006; Udekwu and Wagner 2007; Johansen et al. 2008), which regulates gene expression upon the accumulation of misfolded OMPs in the periplasmic space (Mecsas et al. 1993; Missiakas et al. 1996; Raivio and Silhavy 1999). However, only a few sRNAs have been reported to be transcribed by the σ factor RpoS (also known as σS or σ38) (Opdyke et al. 2004; Padalon-Brauch et al. 2008; Fröhlich et al. 2012). This major stress σ factor regulates 10% of the Escherichia coli genes (Weber et al. 2005) and is induced under several stress conditions, namely, the entry into the stationary phase of growth (Battesti et al. 2011). RpoS is known to play important roles in the virulence of many bacterial pathogens, including S. Typhimurium (Dong and Schellhorn 2010).

SraL (also known as RyjA) is a 140-nucleotide (nt) antisense sRNA first described in 2001 in two exhaustive genetic studies (Argaman et al. 2001; Wassarman et al. 2001), in which a combination of different approaches was used in
order to identify novel sRNAs in *E. coli*. Subsequently, this sRNA was also detected in *S. Typhimurium* (Viegas et al. 2007; Ortega et al. 2012). SraL sRNA is localized between *soxR* (Amábile-Cuevas and Demple 1991) and a gene encoding a putative glutathione S-transferase (*STM4267*), but it is transcribed in the opposite strand. SraL sRNA was only detected in cells during the entry into stationary phase (Argaman et al. 2001; Wassarman et al. 2001; Viegas et al. 2007), and its expression is particularly high in stationary phase (OD$_{600}$ 2 + 6 h). Moreover, its expression was also highly detected under *Salmonella* pathogenicity island (SPI)-2-inducing conditions (Viegas et al. 2007), which indicates a possible role for SraL in *Salmonella* virulence since SPI-2 genes are important for intramacrophage survival and systemic disease. SraL is also expressed in intracellular *S. Typhimurium* persisting inside eukaryotic cells (Ortega et al. 2012). The study of the post-transcriptional regulation of SraL through the use of several *Salmonella* ribonucleases mutants showed that this sRNA is controlled by RNases such as PNPase and the degradosome complex (Viegas et al. 2007). Moreover, it was shown that Poly(A) Polymerase I (PAP I) has a major impact in the control of the stability of this sRNA (Viegas et al. 2007). This fact was in agreement with previous 3’ RACE experiments that revealed the existence of 3’ A-tails of different lengths in the *E. coli* SraL transcript (Argaman et al. 2001).

In this work, we have determined that RpoS (the major stationary phase regulator) is a transcriptional regulator of the highly conserved sRNA SraL in *S. Typhimurium*. SraL transcription is dependent on the presence of RpoS in the cell, and we have proved that this regulation is direct since RpoS directly binds to the promoter of the *sraL* gene. We have also investigated the biological role of SraL since no targets were yet discovered for this sRNA. A proteomic analysis using a *S. Typhimurium* SraL null mutant and a SraL overexpressing strain detected Trigger Factor (TF) as a possible target. TF is one of the three major cytosolic chaperone proteins found in all eubacteria and assists in protein folding (Hesterkamp et al. 1996). This chaperone is the only ribosome-associated chaperone known in bacteria (Hoffmann et al. 2010). By using mutational analysis both in vivo and in vitro, we have determined that SraL represses *tig* mRNA through a short stretch of complementarity in the *tig* 5' UTR near the Shine-Dalgarno region. The results obtained in this study constitute the first link between sRNAs and protein folding.

**RESULTS**

**SraL sRNA is conserved among several enteric bacteria**

SraL sRNA (also known as RyjA) was first discovered in two independent studies in *E. coli*, in which the use of comparative genomics and microarrays allowed the identification of novel sRNAs (Argaman et al. 2001; Wassarman et al. 2001). More recently, it was confirmed that this sRNA is also expressed in *S. Typhimurium* (Viegas et al. 2007; Ortega et al. 2012).

Since SraL expression was detected in both *E. coli* and *S. Typhimurium*, we performed an extensive search over the genomes of other enteric bacteria using BlastN. This sRNA was shown to be highly conserved, being identified in bacteria such as *Shigella*, *Citrobacter*, and *Klebsiella* (Fig. 1A).

By use of the MFold program (http://mfold.rna.albany.edu/?q=mfold) (Zuker 2003), we predicted related secondary structures for SraL sRNA of several enteric bacteria. The most stable predicted structure of the sRNA in *S. Typhimurium* is shown in Figure 1B. Despite some small differences, the majority of SraL structures represented show a high resemblance, and all contain the same Rho-independent terminator (Fig. 1B; Supplemental Fig. S1).

We compared the expression of *S. Typhimurium* SraL under different growth conditions. When grown in LB at 37°C, SraL is mostly detected in the cells upon entry in stationary phase of growth (Fig. 1C). Moreover, this sRNA is also expressed in conditions that induce SPI-1 and SPI-2, indicating a possible role of SraL in *Salmonella* virulence (Fig. 1C). More specifically, SraL seems to be necessary after internalization of this bacterium into host cells since its expression is much higher under SPI-2-inducing conditions. In fact, it was recently shown that SraL is expressed in intracellular bacteria located in fibroblasts at 24 h post-infection (Ortega et al. 2012). SraL is also expressed in cells subjected to heat shock, as well as 4 h after growth in cold-shock conditions and under SPI-1-inducing conditions, albeit at much lower levels (Fig. 1C).

**SraL sRNA is directly regulated by σ$^5$**

Since SraL sRNA is conserved among *Enterobacteriaceae* and its expression is induced preferentially in stationary phase, we hypothesized that it could be part of the general stress response orchestrated by the σ$^5$ factor of the RNA polymerase that operates in this growth phase. To this aim, we first examined the *sraL* promoter in search of conserved sequence elements that show specific features of promoters of bona fide RpoS-regulated genes. From the alignment of the immediately 75 nt upstream sequence of *sraL* in several enteric bacteria, we noticed some traits that are characteristic of an RpoS-regulated promoter (Fig. 1A; Tycas et al. 2007). In this regard, we observed a conserved −10 box that fits well with the consensus sequence retrieved from experimentally determined RpoS-regulated genes, including the A/T-rich motif downstream from the −10 box (Fig. 1A; Weber et al. 2005; Tycas et al. 2007). Moreover, the −35 box is also characteristic of an RpoS-regulated promoter. These observations suggested a plausible selectivity of RpoS for the *sraL* promoter.

To test experimentally the putative RpoS dependence on SraL expression, we first constructed an rpoS null mutant by P22 transduction from SV4210 strain (Tierrez and Garcia-del Portillo 2004) and also a complemented strain in which rpoS was cloned into a constitutive expression plasmid.
Then, we compared SraL levels among the wild-type, the isogenic rpoS null mutant, and the complemented rpoS mutant strain throughout stationary phase, the growth condition where SraL is highly expressed (see Fig. 1C). Results presented in Figure 2A (upper panel) show that SraL sRNA is practically absent in the rpoS null mutant in this growth condition. In fact, reverse transcription and real-time quantitative PCR revealed that in stationary phase SraL is about 500 times less abundant in the rpoS mutant than in the wild-type strain (data not shown). Consistently, SraL expression is partially restored in the rpoS mutant upon ectopic expression of a wild-type rpoS allele from a constitutive

**FIGURE 1.** Analysis of SraL sequence, structure, and expression. (A) Alignment of the sraL gene including the upstream promoter region in several enterobacteria. All nucleotides are colored regarding their degree of conservation (red indicates high conservation; blue, partial conservation; black, little or no conservation). The asterisks (*) below the sequences indicate the nucleotides conserved between all the species analyzed. The putative −10 and −35 boxes of the sraL promoter are indicated (Argaman et al. 2001). The consensus sequences of −10 and −35 boxes of RpoS and the RpoS-specific promoter sequence features located around the −10 core promoter element (A/T-rich discriminator) are indicated (Typas et al. 2007). Parts of the −35 and −10 elements that are often degenerate in RpoS-dependent promoters are shown in italics (the least conserved nucleotides in lowercase letters) (Typas et al. 2007). +1 marks the transcriptional start site. The Rho (ρ)-independent terminator is indicated by arrows. The SraL sRNA interaction region with trg mRNA is indicated in yellow. Y represents a C or a T. STM, Salmonella enterica serovar Typhimurium; STY, *Salmonella enterica* serovar Typhi; SBO, *Salmonella bongori*; SBD, *Shigella boydii*; SFL, *Shigella flexneri*; SDR, *Shigella dysenteriae*; ECO, *Escherichia coli*; CRT, *Citrobacter rodentium*; CKO, *Citrobacter koseri*; ENT, *Enterobacter*; and KPN, *Klebsiella pneumoniae*. (B) S. Typhimurium SraL sRNA structure predicted by the Mfold program (Zuker 2003). (C) SraL sRNA expression under different growth conditions. Cells were grown in LB at 37°C until an OD600 of 0.5 (OD0.5), 1 (OD1), and 2 (OD2); 3 h after OD2 (OD2 + 3 h); and 6 h after OD2 (OD2 + 6 h). Cells were also grown under conditions of induction of SPI-1 and SPI-2 genes. For heat and cold shock, cells were grown in LB until an OD600 of 0.5 and then subjected to cold shock (10°C) for 30 min and 4 h (CS 30′ and CS 4 h, respectively) and heat shock (42°C) for 15 min (HS 15′). Fifteen micrograms of total RNA were run on a 6% PAA/8.3 M urea gel. SraL was detected using a riboprobe; probing for 5S rRNA was used as a loading control.
promoter (Fig. 2A, upper panel). This might be due to the fact that ectopic RpoS is expressed at a much lower level in the complemented strain than in the wild-type strain (Fig. 2A, lower panel). Even so, these low levels of RpoS protein suffice to restore SraL expression (Fig. 2A). These results indicate that SraL expression in stationary phase is highly dependent on RpoS activity.

To further examine the SraL regulation by RpoS, we analyzed sraL promoter response in a transcriptional fusion to lacZ reporter gene in both wild-type and rpoS mutant genetic backgrounds. RpoS is known to be induced during entry into stationary phase and/or many other stress conditions. Thus, we first analyzed the transcriptional activity of sraL promoter in stationary phase, and observed a significantly lower sraL promoter-driven β-galactosidase activity when RpoS is not available (Fig. 2B). To rule out any possible bias derived from the growth phase in which these analyses were performed, we investigated the RpoS dependence of sraL expression under high osmolarity, a stress condition that triggers an RpoS-mediated response (Hengge-Aronis et al. 1993). Bacteria were grown to early exponential phase and then 0.5 M NaCl was added, maintaining the bacteria in these stress conditions for 1 h. As a result of the increase in osmolarity, sraL transcriptional activity underwent an almost threefold induction in the wild-type strain, while in the rpoS mutant strain, the sraL promoter expression remained unchanged (Fig. 2C). Consistent with our previous observations on the SraL expression pattern during bacterial growth, the transcriptional activity of sraL promoter was much higher in stationary phase (Fig. 2B) than in exponential growth phase (Fig. 2C). These data suggest that the increase in SraL expression in stationary phase is the result of transcriptional regulation mediated by RpoS.

Up to now, our analysis supports that SraL expression is regulated by RpoS, but it does not differentiate between a direct or indirect regulation. To address this question, we analyzed in vivo the existence of binding of RpoS to the sraL promoter by chromatin immunoprecipitation (ChIP) assays (Raffaele et al. 2005). The extent of sraL promoter enrichment in the immunoprecipitates (IPs), which is indicative of the binding in vivo of the σ factor to the promoter, was determined by real-time quantitative PCR. We first confirmed the suitability and the specificity of the monoclonal antibody for the immunoprecipitation of RpoS. Immunoprecipitation of uncrosslinked wild-type and rpoS mutant bacterial samples revealed that the antibody has a high affinity and specificity for RpoS, since no immunoreactive bands were visualized in rpoS mutant IPs, while a strong signal around the expected molecular weight for RpoS was obtained with the wild-type strain (Fig. 3A). Two additional bands with a lower mobility were also immunoprecipitated (Fig. 3A, asterisks). Nevertheless, as they are not detected in the rpoS mutant input or IP samples, we reasoned that these immunoreactive bands might correspond to RpoS aggregates rather than an unspecific contaminating protein. These results confirm that the antibody displays a high affinity for RpoS and that it can be used to precipitate specifically DNA–RpoS complexes in vivo in ChIP assays. To assess the specificity of the ChIP assay, we first used osmY promoter as a target DNA sequence (Fig. 3B). OsmY is a periplasmic protein of unknown function previously shown to be regulated by RpoS, and we have used it here as a positive control (Hengge-Aronis et al. 1993; Yim et al. 1994). Consistently, we found a 10-fold enrichment of osmY sequence in RpoS IPs, which indicates a relative high occupancy of osmY promoter by RpoS (Fig. 3B). Interestingly, sraL target sequence was more than 100 times enriched in RpoS IPs compared with the input, which strongly supports the binding of RpoS to sraL promoter in vivo (Fig. 3B). The higher enrichment of sraL promoter in RpoS IPs compared with that of osmY suggests that the transcriptional activity of sraL promoter is larger at the stationary phase, which points out the relevance of the induction of this sRNA at this specific growth phase. No enrichment in rnpB sequence, used here as a negative control, was observed in
RpoS IPs. Collectively, these results strongly support that the increased expression levels of SraL sRNA observed during stationary phase result from a transcriptional induction directly mediated by the master regulator of the general stress response RpoS.

**SraL sRNA down-regulates the expression of tig mRNA**

Although there are some studies about SraL sRNA, the biological function of this sRNA was not yet revealed. To identify SraL targets, we analyzed the proteome in *S. Typhimurium* strains expressing different levels of SraL. We performed this analysis using cells in stationary phase of growth (OD2 + 6 h), the condition in which this sRNA is more expressed (see Fig. 1C; Viegas et al. 2007). We have constructed a *sraL* null mutant strain (in which we deleted the entire sequence of the gene) and an overexpressing strain in which the SraL region was cloned into a constitutive expression plasmid. We proceeded with analyses at the RNA level (see Supplemental Fig. S2). Through the results obtained by reverse transcription (RT) PCR, three of the 10 putative targets matched the proteomic results (NuoG, RfbH, and Tig). Moreover, by using the IntaRNA algorithm (http://www.bioinf.uni-freiburg.de/Software/) (Busch et al. 2008), all of these three putative targets were predicted to base pair with SraL sRNA. Out of these three candidates, we proceeded with the analysis of TF, encoded by the *STM0447* (*tig*) gene, since it appeared to be the most consistent putative target to pursue (Supplemental Fig. S2). At the protein level, there was difference of about twofold between the *sraL* deletion mutant and the SraL overexpressing strain, and the same tendency was obtained at the RNA level (see Supplemental Fig. S2).

TF is found in all eubacteria and is the first chaperone encountered cotranslationally by most of the nascent chains since it is localized at the exit of the ribosome tunnel (Stoller et al. 1995). This localization enables its binding to nascent polypeptides and prevents improper intra- and/or intermolecular interactions of the chains emerging on the surface of the ribosome (Valent et al. 1995). TF was also shown to be a peptidyl-prolyl cis/trans isomerase (PPIase) and therefore accelerates proline-limited steps in protein folding with a very high efficiency (Stoller et al. 1995; Hesterkamp et al. 1996).

We proceeded with a Northern blot analysis of the same three strains that were analyzed by proteomic and RT-PCR. Two specific *tig* transcripts were detected in the *tig*+ strains that were absent in the *tig* deletion mutant. The larger transcript was detected near the 16S RNA (∼1.5 kb in size; used as loading control) (Mattattall and Sanderson 1996). The other *tig* transcript was smaller in size. In *E. coli* two different *tig* promoters were described that originate two transcripts with ∼1.5 kb and 1.37 kb (Aldea et al. 1989; Mendoza-Vargas et al. 2009), which seem to match with the sizes of *tig* transcripts detected in *Salmonella*. The quantification of the transcripts obtained by Northern blot analysis revealed that when SraL is absent, *tig* mRNA levels increase about twofold compared with the wild-type (Fig. 4, upper and middle panels). Moreover, there was a 50% reduction of the *tig* mRNA levels when SraL is transcribed from an overexpressing plasmid. Hereupon, SraL seems to negatively control either directly or indirectly the *tig* mRNA levels in the conditions tested.

**SraL base pairs with tig**

To further investigate the role of SraL sRNA in the regulation of *tig* mRNA, we performed a bioinformatic prediction to
was then blotted to a Hybond-N+ membrane and hybridized with the micrograms of RNA was separated on a 6% PAA/8.3 M urea gel. The gel variations in the amount of RNA used in each sample. (Rehmsmeier et al. 2004). Both algorithms were able to predict an imperfect SraL–tig interaction composed by two short segments (7 and 3 bp) (Fig. 5A). Additionally, the predicted interaction between the sRNA and its target responds to a well-conserved region in both RNAs (Figs 1A; Supplemental Fig. S2). To test whether pairing was direct and whether the predicted region was required for the interaction, three base changes were introduced in sraL chromosomal region (SraL*) in the predicted base-pairing site with the tig mRNA (Fig. 5A). We ensured by bioinformatic predic-
tions that these mutations do not modify the structure of the sRNA (cf. Figs. 1B and 5B). These mutations in the interaction site of the sRNA should prevent the regulation of SraL over tig mRNA. The effect of these point mutations on the down-regulation of tig mRNA was tested by Northern blot, and in fact, the point mutations in the sRNA (SraL*) abolished the repression of tig mRNA (Fig. 5C, upper panel). To validate the previous result, we introduced three point mutations in the target (tig*) at the positions corresponding to the mutations in the sRNA, such that full complementarity would be restored when combining both mutations. The results obtained show that wild-type SraL is only able to repress wild-type tig mRNA and not tig* mRNA (Fig. 5C, upper panel, cf. lanes 1 and 4). Additionally, SraL* efficiently down-regulates tig*, but it does not down-regulate the wild-type tig mRNA (Fig. 5C, upper panel, cf. lanes 5 and 3). Thus, the down-regulation of tig* can only be restored when SraL carries the corresponding compensatory mutations enabling the base-pairing between both RNAs. These results provide important additional evidence that confirms that SraL negatively regulates TF directly, by interacting with its mRNA.

SraL sRNA forms a duplex with tig RNA in vitro

To examine whether SraL binds to tig mRNA, we investigated the duplex formation between the two RNAs in vitro by performing gel mobility shift assays. A fixed concentration of [32P]-labeled tig RNA was incubated with increasing concentrations of unlabeled SraL RNA for 1 h. The duplex formation was analyzed by gel electrophoresis on a native polyacrylamide gel. When wild-type tig RNA was incubated with wild-type SraL, the formation of a retarded SraL–tig RNA complex was obtained with the increasing concentration of SraL sRNA (Fig. 6, left panel). We have also tested the effect of the introduction of mutations in the base-pairing region on the duplex formation. When SraL* RNA was incubated with wild-type tig RNA, the formation of a retarded SraL–tig RNA complex was obtained with the increasing concentration of SraL sRNA (Fig. 6, left panel). We have also tested the effect of the introduction of mutations in the base-pairing region on the duplex formation. When SraL* RNA was incubated with wild-type tig RNA duplex formation was no longer observed (Fig. 6, middle panel), while the tig* RNA (carrying the compensatory mutations) restored the duplex formation with SraL* RNA (Fig. 6, right panel). These data are consistent with the in vivo results, indicating that SraL sRNA directly interacts with the tig mRNA through the predicted interaction region.

FIGURE 4. Regulation of tig mRNA by SraL sRNA. Total cellular RNA was extracted from the S. Typhimurium strains indicated grown in LB at 37°C until 6 h after OD600 of 2. (Upper panel) Twenty micrograms of total RNA was separated on a 1.3% formaldehyde/Agarose gel. The gel was then blotted to a Hybond-N+ membrane and hybridized with the corresponding tig riboprobe. Full-length transcripts were quantified using a Molecular Dynamics PhosphorImager. The amount of RNA in the wild-type was set as one. The ratio between the amounts of RNA of each strain and the wild-type is represented (relative levels). A representative membrane is shown, and the values indicated correspond to the average of several Northern blot experiments with RNAs from at least two independent extractions. The membrane was stripped and then probed for 16S rRNA as loading control. (ND) Nondetectable. (Middle panel) Reverse transcription (RT) PCR experiments were carried out with specific primers for tig using 75 ng of total RNA extracted from the wild-type and derivatives, as indicated in each lane. RT-PCR primers specific for 16S rDNA show that there were not significant variations in the amount of RNA used in each sample. (Lower panel) Fifteen micrograms of RNA was separated on a 6% PAA/8.3 M urea gel. The gel was then blotted to a Hybond-N+ membrane and hybridized with the corresponding SraL riboprobe. Probing for 5S rRNA confirmed equal loading.

DISCUSSION

Trans-encoded sRNAs are known to regulate several genes involved in stress responses. Computational and experimental methodologies have allowed the association of several of these sRNAs with important regulons of both E. coli and Salmonella. The RpoS regulon includes genes with functions in carbon metabolism, stress resistance, cell envelope integrity, morphology, stationary phase, and virulence (Dong and Schellhorn 2010; Battesti et al. 2011). In this report we have included the SraL sRNA in the RpoS regulon since...
SraL was shown to be directly regulated by this σ factor. An RpoS-recognized promoter is normally identified by a series of characteristic features (Typas et al. 2007). The predicted SraL promoter region (Argaman et al. 2001) presents several of these features, namely, the −35 and −10 box and the extended −10 motif TAA. Moreover, these features are also present in the several enteric bacteria analyzed. Accordingly, it is possible to admit that besides its expression in several other enterobacterial species, this sRNA is also directly transcribed by RpoS in these bacteria. There are only a few studies reporting the control of other sRNAs by RpoS (Opdyke et al. 2004; Padalon-Brauch et al. 2008). However, up to now there is only the case of SdsR sRNA (that controls the synthesis of the major Salmonella porin OmpD) that is controlled by RpoS and is conserved in a broad range of enteric bacteria (Fröhlich et al. 2012). Therefore, SraL constitutes

**FIGURE 5.** Analysis of the interaction between SraL sRNA and tig mRNA. (A) Predicted interaction region between SraL sRNA and tig mRNA. The Shine-Dalgarno region and the start codon of tig are indicated. Chromosomal point mutations to generate SraL∗ and tig∗ alleles are indicated. (B) S. Typhimurium SraL∗ sRNA structure predicted by Mfold program (Zuker 2003). (C) Mutations in SraL and tig in the interaction region between both RNAs validate SraL–tig interaction. Total cellular RNA was extracted from the S. Typhimurium strains indicated grown in LB at 37°C until 6 h after OD600 of 2. (Upper panel) The expression level of tig mRNA was determined by using a 1.3% formaldehyde/Agarose gel. The amount of RNA in wild-type was set as one. The ratio between the RNA amount of each strain and the wild-type is represented (relative levels). A representative membrane is shown, and the values indicated correspond to the average of several Northern blot experiments with RNAs from at least two independent extractions. The membrane was striped and then probed for 16S rRNA as loading control. The symbol ¥ in the picture indicated the position of the 16S rRNA. (Lower panel) Fifteen micrograms of RNA was separated on a 6% PAA/8.3 M urea to determine the expression level of both SraL and SraL∗; probing of 5S rRNA was used as a loading control.

**FIGURE 6.** Analysis of the duplex formation between tig RNA and SraL RNA by gel mobility shift assays. [32P]-labeled tig RNA (0.015 pmol) was incubated at 37°C for 1 h with increasing concentrations of unlabeled SraL RNA. The duplex formation was monitored by gel mobility shift assay on native polyacrylamide gels. The concentration range of unlabeled SraL RNA was 0, 22, 44, 88, 175, 350, 700, and 1400 nM. (Left panel) [32P]-labeled tigWT RNA was incubated with SraLWT RNA. (Middle panel) [32P]-labeled tigWT RNA was incubated with SraL∗ RNA. (Right panel) [32P]-labeled tig∗ RNA was incubated with SraL∗ RNA.
the second example of a conserved sRNA that is controlled by RpoS. In previous work, we had shown that SraL is post-transcriptionally controlled by PNPase, the degradosome complex, and also by polyadenylation (Viegas et al. 2007). Therefore, after this report we can conclude that SraL is a tightly regulated sRNA both at transcriptional and post-transcriptional levels.

After the discovery of MicF sRNA and subsequent unraveling of its function (Mizuno et al. 1984), more than 100 sRNAs were identified. However, the biological function of many of these sRNAs is still unknown. In this study we present for the first time a target for SraL sRNA. We show that SraL contributes to the regulation of the expression of the chaperone TF in late stationary phase. SraL inhibits tig expression at the post-transcriptional level by an antisense mechanism that implicates the base-pairing between a region in the 5’-end of SraL and a few nucleotides before the ribosome binding site (RBS) of the tig mRNA. This interaction region between the sRNA and its target was confirmed through the introduction of point mutations in both RNAs. The mutated version of SraL was not able to down-regulate the expression of the wild-type tig mRNA. Nevertheless, the insertion of compensatory mutations in a mutated version of tig mRNA restored the regulation of SraL over tig mRNA. These results were also confirmed in vitro by gel mobility shift assays. The wild-type tig RNA was not able to interact with the mutated version of SraL. Conversely, we observed the formation of a duplex between the mutated tig RNA and the mutated version of SraL.

In a previous work, we studied the influence of the chaperone Hfq in the stability of SraL at stationary phase and concluded that in the absence of this chaperone SraL has a faster decay rate (Viegas et al. 2007). In the present work, we have confirmed that the steady-state levels of SraL are lower in the hfq deletion mutant strain both at early stationary phase (OD2) and at stationary phase (OD2 + 6 h) (Supplemental Fig. S4, lower left panel). On the other hand, a deep sequencing analysis study reported that at the early stationary phase reported that at the early stationary phase (OD600 of 2), tig mRNA levels are also affected by the absence of Hfq (3.5-fold higher in the hfq deletion mutant strain) (Sittka et al. 2008). We have confirmed this result by Northern blot analysis (Supplemental Fig. S4, upper left panel). However, at the stationary phase (OD2 + 6 h), the absence of Hfq did not show a strong effect in the expression of tig mRNA, even though there is a slight increase in tig mRNA levels probably due to the lower levels of SraL sRNA. Moreover, we have seen that at stationary phase in the double sraL−/hfq− mutant, the levels of tig mRNA are very low compared with those of the SraL mutant (see Supplemental Fig. S4, right panel). Therefore, it seems to exist at stationary phase another level of tig mRNA regulation by Hfq, direct or through an additional pathway/sRNA.

Unlike what happens in many cases of riboregulation, the region of interaction between SraL sRNA and tig mRNA does not overlap the RBS and the tig mRNA start codon. However, interactions involving nucleotides in the mRNA leader in the vicinity of the RBS and/or the start codon have been also shown to inhibit translation (Liu et al. 1997; Babitzke and Gollnick 2001; Chen et al. 2004). Therefore, it is plausible to assume that it is also the case in this regulation. Based on the results obtained with the proteomic analysis, the level of TF protein was also shown to be higher in the absence of SraL in the cell.

TF is one of the three major chaperones (along with DnaK and GroEL) that cooperate in the folding of the newly synthesized cytosolic proteins (Lecker et al. 1989; Kandror et al. 1995; Stoller et al. 1995; Deuerling et al. 1999). Moreover, it was very recently reported that this chaperone can also unfold preformed structures and reverse premature misfolds, giving nascent chains a new opportunity for productive folding (Hoffmann et al. 2012). It possesses PPIase activity and accelerates proline-limited steps in protein folding with a very high efficiency (Stoller et al. 1995). This reaction is often a rate-limiting step in the folding of certain polypeptides. Even though it is dispensable for growth, TF is a very important protein since it is the first chaperone encountered by the majority of nascent peptide chains due to its location in contact with the large subunit of the ribosomes (Stoller et al. 1995). Therefore, this protein associates cotranslationally with most of the nascent polypeptides. TF competes with DnaK in the chaperoning of newly synthesized peptides (Deuerling et al. 1999; Teter et al. 1999), which explains why it is not an essential protein. The importance of TF in bacterial metabolism is indicated by the presence of a tig gene in Mycoplasma genitalium (Bang et al. 2000). This bacterium is believed to be free from genetic redundancy and thus contains only the minimal set of genes required for life. This chaperone appears to be the only PPlase of this organism (Bang et al. 2000).

This study presents for the first time a regulatory role of SraL sRNA in S. Typhimurium. Despite some significant differences over the sequences of both SraL and tig genes in Enterobacteriaceae, the interaction region between the two RNAs corresponds to a region very well conserved. Thus, it is possible that this regulation of SraL sRNA over tig mRNA also occurs in many other enteric bacteria. The biological significance of the regulatory pathway involving SraL and TF is not totally clear. During the stationary phase, the overall rate of protein synthesis is reduced compared with an exponentially growing culture (Albertson et al. 1990; Kuzj et al. 1998), concomitant with a decrease in the levels of ribosomes (Lambert et al. 1983). This happens because the cell avoids the production of unnecessary proteins when cells are not growing. Since TF is associated with the ribosomes and plays a key role in the folding of nascent peptides, it is possible that it is less required in stationary phase. In fact, results from our laboratory have shown that tig mRNA levels are higher at exponential phase (data not shown). Since TF is constitutively expressed in the cell, the RpoS induction of SraL sRNA under stationary phase seems to occur to avoid the superfluous production of this chaperone.
Since both SraL and TF are very well conserved in enterobacteria, this report will have a significant impact in the field. Moreover, this study constitutes the first report connecting small RNAs with protein folding.

**MATERIALS AND METHODS**

**Oligonucleotides**
All oligonucleotides used in this study are listed in Supplemental Table S1 in the Supplemental Material and were synthesized by STAB Vida and Sigma-Aldrich.

**Bacterial strains and plasmids**
All bacterial strains and plasmids used in this study are listed in the Tables 1 and 2, respectively. All *Salmonella* strains used are isogenic derivatives of the wild-type *S. Typhimurium* strain SL1344. The *sraL* (CMA-651) and *tig* (CMA-652) null mutants were constructed using the primer pairs pIS-001/pIS-002 and pIS-005/pIS-006, respectively, and following the λ-red recombine method described previously (Datsenko and Wanner 2000) with few modifications, as previously described (Viegas et al. 2007). All chromosomal mutations were subsequently transferred to a fresh genetic background (SL1344 strain) by P22 HT105/1 int-201 transduction (Schmieger 1971). The chloramphenicol-resistance (CmR) cassette of plasmid pKD3 replaces nucleotides –9 to +120 of the *sraL* gene and –16 to +1303 of *tig*. The gene deletions were verified by colony PCR using the primer pair pIS-003/pIS-004 for *sraL* and pIS-007/pIS-008 for *tig*. The *S. Typhimurium* *rpoS* null mutant (CMA-653) was obtained by P22 transduction from the SV4210 strain (Tirer and Garcia-del Portillo 2004).

The chromosomal mutants with substitutions/point mutations in *sraL* (CMA-655) and *tig* (CMA-657) were constructed by a multiple-step PCR process. The strain CMA-654 was constructed by inserting a CmR cassette 59 nt upstream of the *sraL* gene and 65 nt upstream of the *tig* transcription start site, in the intergenic region between the *bolA* and *tig* genes. The CmR cassettes were amplified from plasmid pKD3 using the primer pairs pIS-025/pIS-026 for *sraL* and pIS-007/pIS-008 for *tig*. The *S. Typhimurium* rpoS null mutant (CMA-653) was obtained by P22 transduction from the SV4210 strain (Tirer and Garcia-del Portillo 2004).

For construction of pISVA-001 plasmid expressing SraL, a PCR fragment containing the entire *sraL* sequence was amplified from SL1344 chromosome using the primer pair pIS-009/pIS-010. The resultant PCR fragment carrying a 5'-phosphate at one end was cleaved with KpnI and ligated into the constitutive pZE12luc plasmid (blunt/KpnI site) (Lutz and Bujard 1997). In this plasmid, the initiation site of the encoded RNA lies at position +1 of the constitutive PLaCo promoter of pZE12luc plasmid.

For the *rpoS* complementation plasmid pISVA-002, a PCR fragment containing the entire *rpoS* sequence was amplified from SL1344 chromosome using the primer pair pIS-012/pIS-013 and was cloned into the XbaI and HindIII sites of the plasmid pWSK29 (Wang and Kushner 1991).

For the construction of plasmid pISVA-003 (P~sraL::lacZ~), a fragment of the 5'-UTR region of *sraL* gene, including promoter signals, was amplified by PCR with primers pIS-014 and pIS-015 (containing the restriction sites for XbaI and BamHII, respectively). Both the insert and pSP417 vector were digested with XbaI and BamHII enzymes and ligated. The cloned sequence includes a region of 195 bp before the start of the gene (including promoter signals) and 9 bp of *sraL* sequence.

Competent *E. coli* DH5α cells (New England Biolabs) were used for cloning procedures during plasmid construction.

**Bacterial growth**
All strains were grown in Luria-Bertani (LB) broth at 37°C and 220 rpm throughout this study. SOC medium was used to recover transformants after heat shock (in the case of *E. coli*) or electroporation (in the case of *Salmonella*), before plating. Conditions indicated as SPI-1 and SPI-2 inducing conditions corresponded to growth in high-salt (0.3 M NaCl) LB medium with low oxygen in sealed Falcon tubes, as described for SPI-1 induction (Sittka et al. 2007), and in PCN minimal medium (1 mM phosphate buffer at pH 5.8) as described for SPI-2 induction (Lober et al. 2006).

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**TABLE 1.** List of strains used in this work

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant markers/genotype</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. Typhimurium</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SL1344</td>
<td>Str&lt;sup&gt;R&lt;/sup&gt;hisG&lt;sup&gt;G&lt;/sup&gt; rpsL&lt;sup&gt;L&lt;/sup&gt; syl&lt;sup&gt;S&lt;/sup&gt;</td>
<td>Hoiseith and Stocker</td>
</tr>
<tr>
<td>CMA-651</td>
<td>SL1344 sraL&lt;sup&gt;(AsraL::Cm&lt;sup&gt;R&lt;/sup&gt;)&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>CMA-652</td>
<td>SL1344 tig&lt;sup&gt;(ΔsraL::Cm&lt;sup&gt;R&lt;/sup&gt;)&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>CMA-653</td>
<td>SL1344 rpoS&lt;sup&gt;(ΔsraL::Cm&lt;sup&gt;R&lt;/sup&gt;)&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>CMA-654</td>
<td>SL1344 [sraL-STM4267]&lt;sup&gt;G&lt;/sup&gt;:Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>CMA-655</td>
<td>SL1344 [sraL-STM4267]&lt;sup&gt;G&lt;/sup&gt;:Cm&lt;sup&gt;R&lt;/sup&gt;sraL&lt;sup&gt;(C25T/G28C/C33G)&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>CMA-656</td>
<td>SL1344 [bolA-tig]&lt;sup&gt;Δ&lt;/sup&gt;[:Cm&lt;sup&gt;R&lt;/sup&gt;]</td>
<td>This study</td>
</tr>
<tr>
<td>CMA-657</td>
<td>SL1344 [bolA-tig]&lt;sup&gt;Δ&lt;/sup&gt;[::Cm&lt;sup&gt;R&lt;/sup&gt;tig&lt;sup&gt;G114C/C119G/G123A&lt;/sup&gt;]&lt;sup&gt;G&lt;/sup&gt;]</td>
<td>This study</td>
</tr>
<tr>
<td>CMA-658</td>
<td>SL1344 [bolA-tig]&lt;sup&gt;Δ&lt;/sup&gt;[::Cm&lt;sup&gt;R&lt;/sup&gt;sraL&lt;sup&gt;(C25T/G28C/C33G)&lt;/sup&gt;]&lt;sup&gt;G&lt;/sup&gt;]</td>
<td>This study</td>
</tr>
<tr>
<td><em>E. coli</em> DH5α</td>
<td>recA1 endA1 gyrA96 thi-1 hsdR17</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td></td>
<td>supE44 relA1 ΔlacZYA-arg FU169 F80ΔlacZD15</td>
<td></td>
</tr>
</tbody>
</table>
Growth medium was supplemented with the following antibiotics when appropriate: ampicillin (150 µg/mL), chloramphenicol (25 µg/mL), and streptomycin (90 µg/mL). For heat shock treatment, cells grown at 30°C to an OD600 of 0.5 were transferred for 15 min to 42°C. For cold shock treatment, cultures at an OD600 of 0.5 were transferred from 37°C to 10°C for 30 min and 4 h.

To apply osmotic shock, cells were grown at 37°C to an OD600 of 0.3. NaCl was added to the culture at a final concentration of 0.5 M.

RNA extraction, Northern blot, and RT-PCR analysis

Overnight cultures were diluted 1/100 in fresh medium and grown to the indicated cell densities at OD600 (growth medium and conditions are detailed in the respective figure legends). Culture samples were collected, mixed with 1 volume of stop solution (10 mM Tris at pH 7.2, 25 mM NaNO3, 5 mM MgCl2, 500 µg/mL chloramphenicol), and harvested by centrifugation (10 min, 6000 × g). RNA was isolated using the phenol/chloroform extraction method, precipitated in ethanol, resuspended in water, and quantified on a Nanodrop 1000 machine (Nanodrop Technologies).

For Northern blot analysis, 15 µg of total RNA was separated under denaturing conditions either by 8.3 M urea/6% polyacrylamide gels, transfer of RNA onto Hybond-N+ membranes (GE Healthcare) was performed by electroblotting (1 h 50 min, 24 V, 4°C) in TAE buffer. For Agarose gels, RNA was transferred to a nitrocellulose filter using a semi-dry apparatus (Biorad) according to the method previously described (Viegas et al. 2007). The riboprobes and oligoprobes were performed according to the method previously described (Viegas et al. 2007).

In vitro transcription and gel mobility shift assay

DNA templates for the in vitro transcription of the substrates were generated by PCR using chromosomal DNA from SL1344 wild-type strain for the wild-type transcripts, and the chromosomal DNA from CMA-655 and CMA-657, in the case of SraL and tig, respectively, for the transcripts with the point mutations. sraL was amplified with the primer pair pIS-037/pIS-038 and tig with pIS-039/pIS-040. For the synthesis of internally labeled tig and nonlabeled sraL transcripts, in vitro transcription was carried out using the purified PCR products using equimolar concentrations of all four ribonucleotides with the Riboprobe in vitro Transcription System (Promega) and T7 RNA polymerase. The internally labeled tig transcripts were purified by electrophoresis on an 8.3 M urea/5% polyacrylamide gel. The gel slices were crushed, and RNA was eluted with elution buffer purified by electrophoresis on an 8.3 M urea/6% polyacrylamide gel. The RNA was ethanol precipitated and resuspended in water, and quantified on a Nanorad 1000 machine (Nanodrop Technologies).

RT-PCR reactions were performed using total RNA with the OneStep RT-PCR kit (Quiagen). Reactions were mainly carried out according to the supplier’s instructions. Modifications were introduced regarding the amount of RNA and number of PCR cycles, depending on gene expression levels. The primer pair pIS-016/pIS017 was used to analyze tig expression. As a control, 16S rRNA was amplified with specific primers pIS-018/ pIS-019. Prior to RT-PCR, all RNA samples were treated with Turbo DNA free Kit (Ambion). Control experiments, run in the absence of reverse transcriptase, yielded no product.

Hybridization probes

Primer sequences are listed in Supplemental Table S1. Labeling of the riboprobes and oligoprobes were performed according to the method previously described (Viegas et al. 2007). The riboprobes were obtained using the primer pair pIS-021/pIS-022 for SraL riboprobe and pIS-017/pIS-020 for tig riboprobe. 5S rRNA and 16S rRNA were detected by the 5′-end-labeled oligonucleotides pIS-023 and pIS-024, respectively.

TABLE 2. List of plasmids used in this work

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Comments</th>
<th>Origin/marker</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pKD3</td>
<td>Template for mutants construction; carries chloramphenicol-resistance cassette</td>
<td>oriR6K/AmpR</td>
<td>Datsenko and Wanner (2000)</td>
</tr>
<tr>
<td>pKD46</td>
<td>Temperature-sensitive X-red recombinase expression plasmid</td>
<td>oriR101/AmpR</td>
<td>Datsenko and Wanner (2000)</td>
</tr>
<tr>
<td>pZEl21uc</td>
<td>P lacO promoter; constitutive expression plasmid</td>
<td>ColE1/AmpR</td>
<td>Lutz and Bujard (1997)</td>
</tr>
<tr>
<td>pSP417</td>
<td>lacZ transcriptional fusion vector</td>
<td>pBR322/AmpR</td>
<td>Podkovyrov and Larson (1995)</td>
</tr>
<tr>
<td>pISVA-001</td>
<td>pZEluc derivative; P lacO promoter; constitutive plasmid expressing SraL</td>
<td>ColE1/AmpR</td>
<td>This study</td>
</tr>
<tr>
<td>pISVA-002</td>
<td>pWSK29 derivative; constitutive expression plasmid Rp05</td>
<td>pSC101/AmpR</td>
<td>This study</td>
</tr>
<tr>
<td>pISVA-003</td>
<td>Transcriptional sraL-lacZ fusion</td>
<td>pBR322/AmpR</td>
<td>This study</td>
</tr>
</tbody>
</table>
Protein extraction and Western Blot analysis

Bacteria were grown in the appropriate volume of Laemmli sample buffer (1.3% SDS, 10% [v/v] glycerol, 50 mM Tris/HCl, 1.8% β-mercaptoethanol, 0.02% bromophenol blue at pH 6.8) to get ∼10^9 bacteria per microliter. RpoS protein was detected using the mouse monoclonal anti-σ S 1RS1 antibody (Santa Cruz Biotechnology) at 1:5000 dilution in antibody dilution buffer (50 mM Tris-HCl at pH 7.5, 0.1% Tween-20, 3% BSA, 1 mM sodium azide) and a goat anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (Bio-Rad Life). For recognition of the chaperonin GroEL, an anti-GroEL rabbit polyclonal antibody was used (dilution 1:10,000, Sigma) and a goat anti-mouse HRP-conjugated secondary antibody (Bio-Rad Life). Membranes were developed with 1/10 diluted ECL prime reagent (GE Healthcare) and visualized using the ChemiDoc XRS+ imaging system and the Quantity One software (Bio-Rad Life).

β-Galactosidase assays

β-Galactosidase activity was determined essentially according to the method first described by Miller with minor modifications (Maloy 1990). In brief, 100 μL of culture was added to 655 μL of cold buffer Z (100 mM Na2HPO4/NaH2PO4 at pH 7, 10 mM KCl, 1 mM MgSO4, 50 mM β-mercaptoethanol), and chloroform-SDS was used to permeabilize the cells. The reaction was started by the addition of the chromogenic substrate ortho-Nitrophenyl-β-D-galactoside to a final concentration of 0.8 mg/mL, conducted until it reaches a pale yellow color at 30°C, and stopped with Na2CO3. Prior to recording absorbance at 420 nm, samples were cleared by centrifugation. Optical density of the bacterial culture was also recorded at the time of the extraction of the sample. β-Galactosidase activity in Miller units was calculated as follows: (1,000 × A420)/(t × ν × OD600), where t corresponds to the reaction time in minutes and ν to the sample volume in milliliters.

ChIP assays

Ten milliliters of overnight grown wild-type SL1344 and isogenic rpoS mutant cultures was exposed to 150 μg/mL rifampicin for 30 min to trap RNA polymerase at gene promoters. Cells were subjected to chemical crosslinking in vivo by adding formaldehyde and phosphate buffer (pH 7.6) to a final concentration of 1% and 10 mM, respectively. Noncrosslinked control samples of both strains were processed in parallel. Crosslinking was left to proceed for 30 min at 37°C with shaking and then quenched with 100 mM glycine for 30 min at 4°C. Bacteria were recovered by centrifugation and washed twice with cold PBS. Pelletular bacteria were resuspended in 1 mg/mL lysozyme in 0.2× IP buffer containing EDTA-free protease inhibitors cocktail (Roche) and maintained for 10 min at 37°C. One volume of 2× IP buffer (200 mM Tris-HCl at pH 8.0, 600 mM NaCl, 4% Triton X-100) was added, and the samples were sonicated in a B. Braun sonifier (Labsonic U model; duty cycle 0.7, output 0.49). DNA in cleared lysates was further digested with 0.1 unit of micrococal nuclease (New England Biolabs) and 0.5 μg of RNAse A in the presence of 5 mM CaCl2 and 0.1 mg/mL BSA for 10 min at 37°C. The digestion was stopped with 10 mM EDTA. DNA shearing was followed by Agarose electrophoresis after reversing the crosslinking of an aliquot for 6 h at 65°C.

Prior to immunoprecipitation, 1/10 volume of the total extract was taken to be used as input sample control. The extracts were then precleared with 20 μL of a 50% slurry containing 1:1 mix of protein-A and protein-G Sepharose (Sigma) in 1× IP buffer for 4 h at 4°C with rotation. Immunoprecipitation was carried out with 2 μL of monoclonal mouse anti-σ S 1RS1 antibody (Santa Cruz Biotechnology) overnight at 4°C. All samples (no-antibody or pre-clearing controls and IPs) were washed once with LiCl wash buffer (250 mM LiCl, 100 mM Tris-HCl at pH 8, 2% TritonX-100), twice with 0.6 M NaCl buffer (100 mM Tris-HCl at pH 8, 600 mM NaCl, 2% TritonX-100), twice with 1× IP buffer, and once with TE buffer (10 mM Tris-HCl at pH 8, 1 mM EDTA). To elute complexes from the protein-A and -G Sepharose, beads were resuspended in 30 μL of ChiP elution buffer (50 mM Tris-HCl at pH 8, 10 mM EDTA, 1% SDS) and incubated for 30 min at 65°C. The complexes were then incubated 6 h at 65°C to reverse crosslinking. Half of the sample was used to assess the efficiency of the immunoprecipitation by Western blot. DNA was obtained from the other half of the sample by phenol/chloroform extraction and precipitated with isopropanol using 20 μg of glycogen (Roche) as a carrier, and the pellet was dissolved in 12 μL of nuclease-free water.

For real-time quantitative PCR analysis of target DNA enrichment, a 1/50 dilution sample of IP and no-antibody control were used as template. In the case of input and flow-through samples, we used a 1/200 dilution. Reactions were performed with the Power Sybr Green PCR master mix (Applied Biosystems) in a 10 μL final volume and run in an ABI Prism 7,500 instrument (Applied Biosystems) using standard reaction conditions recommended by the manufacturer (10 min at 95°C; 45 cycles of 15 sec at 95°C, and 1 min at 60°C; dissociation curve of 15 sec at 95°C, 1 min at 60°C, and a progressive temperature increase until 95°C). Each sample was run in triplicate. Oligonucleotides osmY-F, osmY-R, sraL-F, sraL-R, rnpB-F, rnpB-R, 16S-F, and 16S-R were used to amplify the corresponding target DNA at 0.5 μM final concentration and are included in Supplemental Table S1. For data analysis, the mean Ct value of technical replicates showing a standard deviation below 0.1 for target DNA was normalized to the mean Ct for rrs (16S) in the same sample (Ct_target−Ctinfile). These values were referred to wild-type input sample and the anti-logarithm calculated.

Proteomic analysis

For the proteomic analysis, the cells were grown in the same conditions as for the RNA extraction (see above). Then, 2 OD units of each culture were transferred to a tube containing 0.2 volumes of stop solution (5% phenol/95% ethanol) and kept on ice for 30 min. Cells were spun down by centrifugation for 10 min at 3200 g 4°C, washed with 1:5 diluted stop solution, centrifuged again, and stored at −80°C. Pellets were lysed in Laemmli sample buffer without bromophenol blue dye (see above), and the total protein estimated using the Bradford reagent (Bio-Rad). Approximately 30 μg of total protein (corresponding to ∼0.3 ODs) was run in a SDS-PAGE 12% gel. Loading equivalence among the samples was confirmed by checking GroEL levels by Western blot analysis. Five slices from mid-run gels covering molecular masses from ∼150 kDa to 10 kDa were submitted to in-gel tryptic digestion. The tryptic peptide mixtures were processed for protein identification by liquid chromatography in a C-18 reversed-phase nano-column (100-μm inner diameter × 12 cm, Mediterranea Sea, Teknokroma) and real-
time ionization and peptide fragmentation on an LTQ-Orbitrap XL ETD mass spectrometer (Thermo Fisher Scientific). For protein identification, tandem mass spectra were analyzed using SEQUEST (Thermo Fisher Scientific, versión 1.0.43.2) and X! Tandem (The GPM, versión 2007.01.01.1) using SALTY proteome (UniProtKB, Taxon nr.99287) as reference. Scaffold (version Scaffold_3_00_03, Proteome Software) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at >95.0% probability. Protein identifications were accepted if they could be established at >95.0% probability and contained at least two identified peptides. For a further description of the experimental details of the procedure, see García-del Portillo et al. (2011).

Sequence retrieval and alignments

BlastN was used for sequence alignments (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi) of the following genome sequences: S. Typhimurium LT2 (NC_003197), Salmonella enterica serovar Typhi Ty2 (NC_004631), Salmonella bongori NCTC 12419 (NC_015761), Shigella boydii CDC 3083-94 (NC_010658), Shigella flexneri 2a str. 301 (NC_004337), Shigella dysenteriae Sd197 (NC_007606), Escherichia coli K12 (NC_000913), Citrobacter rodentium ICC168 (NC_013716), Citrobacter koseri ATCC BAA-895 (NC_009792), Enterobacter sp. 638 (NC_009436), and Klebsiella pneumoniae 342 (NC_011283). Alignments were made using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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An RpoS-dependent sRNA regulates the expression of a chaperone involved in protein folding

Inês Jesus Silva, Álvaro Darío Ortega, Sandra Cristina Viegas, et al.

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