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Defining a role for Hfq in Gram-positive bacteria: evidence for Hfq-dependent antisense regulation in *Listeria monocytogenes*

Jesper Sejrup Nielsen, Lisbeth Kristensen Lei, Tine Ebersbach, Anders Steno Olsen, Janne Kudsk Klitgaard, Poul Valentin-Hansen and Birgitte Haahr Kallipolitis*

Department of Biochemistry and Molecular Biology, University of Southern Denmark, Campusvej 55, DK-5230 Odense M, Denmark

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ABSTRACT

Small *trans*-encoded RNAs (sRNAs) modulate the translation and decay of mRNAs in bacteria. In Gram-negative species, antisense regulation by *trans*-encoded sRNAs relies on the Sm-like protein Hfq. In contrast to this, Hfq is dispensable for sRNA-mediated riboregulation in the Gram-positive species studied thus far. Here, we provide evidence for Hfq-dependent translational repression in the Gram-positive human pathogen *Listeria monocytogenes*, which is known to encode at least 50 sRNAs. We show that the Hfq-binding sRNA LhrA controls the translation and degradation of its target mRNA by an antisense mechanism, and that Hfq facilitates the binding of LhrA to its target. The work presented here provides the first experimental evidence for Hfq-dependent riboregulation in a Gram-positive bacterium. Our findings indicate that modulation of translation by *trans*-encoded sRNAs may occur by both Hfq-dependent and -independent mechanisms, thus adding another layer of complexity to sRNA-mediated riboregulation in Gram-positive species.

INTRODUCTION

During the last decades, the Gram-positive human pathogen *Listeria monocytogenes* has served as a model organism for the study of intracellular pathogenesis and bacterially induced actin-based movement (1). More recently, *L. monocytogenes* has been used as a model to study the role of the RNA-binding protein Hfq and small non-coding RNAs (sRNAs) in Gram-positive species (2–5). The sRNAs constitute a relatively novel group of gene regulators in prokaryotes (6). One important subgroup of sRNAs depends upon the RNA chaperone Hfq (7–9). These sRNAs regulate gene expression by binding in an antisense manner to one or more target mRNAs, usually in the vicinity of the start codon and/or Shine–Dalgarno sequence. More recently, some sRNAs have been reported to target more upstream regions (10) or even the coding sequence of target mRNAs (11,12). Binding of the sRNA generally serves to repress translation and/or promote mRNA degradation. However, examples do exist where an Hfq-binding sRNA stimulates translation by modulating the structure of the mRNA, thereby removing otherwise inhibitory secondary structures (6).

The Hfq protein is highly conserved in prokaryotes and belongs to the Sm protein family whose members are known to be involved in RNA transactions in both eukaryotes and prokaryotes (13–15). Hfq monomers form a doughnut-shaped homo-hexameric ring structure which contains at least two separate RNA binding sites: one located on the proximal side which binds AU-rich tracts (i.e. sRNAs as well as mRNAs) and one located on the distal side which binds polyA [i.e. mRNAs (16–19)]. The mechanism by which Hfq facilitates gene regulation by sRNAs has been the focus of many detailed studies in *Escherichia coli* and *Salmonella* and it appears that the role of Hfq in Gram-negative bacteria is multifaceted. Hfq stabilizes many sRNAs *in vivo* probably because Hfq-binding sites (AU-rich tracts) overlap with RNase E cleavage sites (20–22). Moreover, the protein promotes the formation of binary RNA–RNA complexes *in vitro* by increasing the on-rate of duplex formation (23–27). The precise mechanism by which this occurs is still a matter of dispute. Hfq may remodel the sRNA and/or mRNA to remove structures otherwise inhibitory to duplex formation or Hfq could simply serve as a docking platform to bring the sRNA and target mRNA in close proximity to each other. In addition, Hfq may promote targeted degradation of mRNAs through its

*To whom correspondence should be addressed. Tel: +45 6550 2372; Fax: +45 6550 2467; Email: bhk@bmb.sdu.dk
Present address: Tine Ebersbach, National Food Institute, Technical University of Denmark, Mørkhøj Bygade 19, 2860 Søborg, Denmark.

association with the scaffold part of RNase E (21,28,29) thus delivering the molecular machinery required for immediate degradation of the mRNA and in several cases also the sRNA.

Antisense regulation is well described for accessory elements such as plasmids, phages and transposons (30). In these cases, the antisense RNA and target RNA are encoded from the same chromosomal locus but in opposite direction. This ensures full complementarity and allows RNA base pairing to take place in the absence of accessory protein factors. In contrast, Hfq-binding sRNAs are encoded in *trans*, i.e. their genes are located at loci different from those encoding their targets. Consequently, the sRNA and target mRNAs exhibit only partial complementarity. This led to the early suggestion that most *trans*-encoded sRNAs could not function without the assistance of an RNA chaperone such as Hfq. However, the lack of corroborating data from species other than proteobacteria has upset this view.

In low GC Gram-positive bacteria, the function of Hfq is unclear. Although Hfq has been shown to interact with *Bacillus subtilis* SR1 and SR2 as well as *Staphylococcus aureus* RNAIII, it neither affects their stability nor facilitates the interactions between these antisense RNAs and their target mRNAs (31–37). Furthermore, a recent study from *S. aureus* failed to identify significant phenotypes for an *hfq* mutant strain (38). As a result of such observations, it has been speculated that Hfq is dispensable for riboregulation by sRNAs in Gram-positive bacteria, although several notions may question this view. First, Hfq homologues are present in one or more copies in several species belonging to the low GC Gram-positive bacteria (9,14). Importantly, many of the key amino acids involved in RNA binding are conserved. Second, the fact that some sRNAs are not destabilized in hfq^{-} strains should not necessarily be interpreted as a lack of association with Hfq, as exemplified by the *E. coli* sRNA OxyS (15). In addition, several sRNAs that are stabilized by Hfq in *E. coli* and *Salmonella* are degraded by RNase E, a ribonuclease not found in Gram-positive species (39). Finally, Hfq in *L. monocytogenes* contributes to stress tolerance and pathogenesis in mice and interacts with at least three sRNAs, suggesting a role for Hfq in sRNA-mediated riboregulation in this Gram-positive pathogen (2,40).

Here, we present the characterization of the Hfq-binding sRNA LhrA in *L. monocytogenes*. We show that LhrA is a regulatory RNA which inhibits translation of its target gene, *lmo0850*, at the post-transcriptional level, and that regulation requires specific base pairing between LhrA and a region upstream from the start codon of *lmo0850* mRNA. Importantly, the regulation exerted by LhrA is completely dependent on Hfq. Our data suggest that Hfq not only protects LhrA from degradation by an as yet unidentified ribonuclease, but also promotes duplex formation between LhrA sRNA and *lmo0850* mRNA. Collectively, the present study provides the first evidence for Hfq-dependent antisense regulation in Gram-positive bacteria.

MATERIALS AND METHODS

Bacterial strains and growth conditions

All *L. monocytogenes* strains used are derivatives of the EGDe serotype 1/2a (Supplementary Table S1). Unless otherwise stated, *L. monocytogenes* was grown in BHI medium (Oxoid) and *E. coli* was grown in LB medium at 37°C. Improved minimal media (IMM) was prepared as described (Phan-Than and Gormon 1997). When required, erythromycin or kanamycin was added at 5 μg/ml and 50 μg/ml respectively for *L. monocytogenes* and 175 μg/ml and 50 μg/ml for *E. coli*. For cloning of plasmids vectors, we used *E. coli* TOP10 (Invitrogen). For complementation of hfq^{-1} in *E. coli*, we used strain SØ928 and its isogenic hfq^{-1} (41,42).

Construction of recombinant *lhrA* strains

To generate an *lhrA* deletion strain, two PCR fragments of approximately 400 bp corresponding to sequences located upstream or downstream from *lhrA* was amplified and subsequently joined by PCR using the primers listed in Supplementary Table S2. The resulting DNA fragment was digested with XbaI and BamHI, cloned in the temperature sensitive plasmid pAULA (43) resulting in plasmid pAULA-Δ*lhrA*. The plasmid was transformed into competent EGD wild-type cells and the chromosomal deletion of *lhrA* was achieved by allelic exchange as previously described (40). Cells that had lost *lhrA* were identified by PCR and subsequently verified by DNA sequencing. The resulting strain is deleted for residues +10 to +218 of *lhrA* relative to the transcription start site, and thereby retains the promoter elements of the *lhrA* gene so as to minimize polar effects.

For the construction of strains carrying a mutated *lhrA* (*lhrA-Mut3**), two PCR fragments of approximately 400 bp corresponding to sequences upstream or downstream from the region to be mutated in *lhrA* were amplified and subsequently joined by PCR using the primers listed in Supplementary Table S2. The resulting DNA fragments were cloned into pAULA and subsequently transferred to EGD and Δ*hfq* cells, followed by allelic exchange, as described earlier. Cells containing the desired nucleotide substitutions in chromosomal *lhrA* were identified by PCR and subsequently verified by DNA sequencing.

Identification of LhrA targets

To identify putative antisense targets of LhrA we used TargetRNA (44) with the following sequence as query: TTATTTGTTTTCATTCATCTCATTG. We used the programs advanced mode with the following modifications: Hybridization seed = 9, search within −40 before and +20 after start codon, allow GU base pairing, $P < 0.01$.

Construction of *lacZ* fusions

For the construction of in-frame translational *lacZ* fusions, DNA fragments containing regions of the genes of interest were amplified by PCR using the primers listed in supplementary Table S2. In the case of *kdpB* which is

encoded from within a poly cistronic message, we constructed a chimeric fusion to a constitutive promoter as described previously (4). The resulting PCR fragments were digested with EcoRI and BamHI and ligated into pCK-*lac*, a derivative of pTCV-*lac* (45) containing a *lacZ* gene without a Shine–Dalgarno sequence or a start codon allowing for translational analysis of the gene in question. β-Galactosidase assay was carried out as described previously (40).

Construction of plasmids for expression of Hfq_{LMO} in *E. coli*

For the ectopic expression of Hfq_{LMO} in *E. coli*, *hfq* was cloned and inserted into the IPTG inducible vector pNDM220 (46). Plasmid pNDM-hfq_{LMO} was constructed by replacing a BamHI–EcoRI fragment of pNDM220 with a PCR-generated fragment, prepared using the primers listed in Supplementary Table S2 and *L. monocytogenes* chromosomal DNA as template.

Complementation of *E. coli* hfq^{-1}

Overnight cultures of SØ928 carrying the empty vector pNDM-220, and hfq^{-1} carrying pNDM-220, pNDM-hfq_{ECO} or pNDM-hfq_{LMO} were diluted in LB medium containing 30 μg/ml ampicillin and 1 mM IPTG to an OD_{450} of 0.02 and grown at 37°C. Growth was monitored by measuring OD_{450} every 30 min. At different stages of growth (exponential growth, transition phase and stationary phase), samples were drawn for western analysis. In parallel, cultures grown to $OD_{450} = 0.4$ were used for analysis of RyhB mediated *sodB* degradation as described earlier (47). Resistance to oxidative stress was analysed by disk diffusion assays. Overnight cultures grown in the presence of 1 mM IPTG were spread on agar plates containing 1 mM IPTG and 30 μg/ml ampicillin. Five millimetre paper disks were placed at the centre of the agar plates and 10 μl 30% H_2O_2 was added to the disks. The following day, the zone of inhibition was measured. The averages from three independent experiments each conducted in triplicate were used for statistical analysis using Students *t*-test. For western blot analysis, 1 ml cell samples were removed and equal amounts of total protein from each sample were separated on 12% SDS–polyacrylamide gels, and blotted onto an Immobilon-P membrane (Millipore). For detection of σ^S, the membrane was probed with anti-σ^S monoclonal antibodies (NeoClone) and subsequently with an anti-mouse alkaline phosphatase conjugate (Dako Cytomation). For the detection GroEL, the same membrane was stripped and subsequently probed with anti-GroEL monoclonal antibodies (Sigma). Western blots were developed using ECL Plus detection system (GE Healthcare).

RNA techniques and purification of Hfq_{LMO}

Total RNA was isolated from cells at the indicated time points. Approximately 1×10^9 cells were collected by centrifugation, snap frozen in liquid nitrogen and stored at −80°C until use. Frozen pellets were resuspended in 1 ml TRI reagent (MRCGENE) and cells lysed using a FastPrep instrument. RNA was subsequently isolated as recommended by the manufacturer. Quantification and RNA quality assessment was performed on a NanoDrop 2000. The integrity of the RNA was verified by agarose gel electrophoresis.

For northern blotting, ∼15 μg total RNA in loading buffer containing 95% formamide and 2 mM EDTA was separated on a 6% or 8% denaturing polyacrylamide gel and subsequently transferred to a Zeta probe nylon membrane by semi-dry electroblotting. For detection of RNA, the membranes were pre-incubated for 30 min in PerfectHyb hybridization buffer (Sigma-Aldrich) and then hybridized overnight with a specific ^{32}P-labelled DNA probe (for detection of LhrA sRNA and *lmo0850* mRNA, oligos LhrA3 and *lmo0850* NB probe was used, for detection of *Listeria* 5S rRNA, oligo 5S rRNA_LMO NB was used, for detection of RyhB sRNA and *sodB* mRNA, oligos RyhB and SodB were used respectively, for detection of *E. coli* 5S rRNA, oligo 5S rRNA_ECO NB was used, Supplementary Table S2). The membranes were washed as recommended by the manufacturer and subsequently visualized by autoradiography and/or phosphor imaging using a Typhoon scanner.

Hfq_{LMO} was purified using the Intein system (Impact-CN; New England Biolabs) as described previously (2). Templates for *in vitro* transcription of LhrA, LhrA-Mut3*, *lmo0850*, *lmo0850*-Mut3 and *lmo0850*-TOE were constructed by PCR using the primers listed in Supplementary Table S2. The templates contain a 5′-end T7 promoter. *In vitro* transcription was performed using the MegaScript kit as described by the manufacturer (Ambion). *In vitro* transcribed RNA was ethanol precipitated, resuspended in formamide loading dye and separated on an 8% denaturing polyacrylamide gel. The RNA was visualized by UV shadowing, excised from the gel and transferred to 300 μl 2 M NH_4Acetate. After overnight incubation at 14°C, the RNA was phenol extracted followed by ethanol precipitation. Quantification was performed on a NanoDrop 2000. *In vitro* transcribed RNA was 5′-end-labelled using the KinaseMax kit as described by the manufacturer (Ambion).

For gelshifts, 10 fmol 5′-end-labelled *lmo0850* or *lmo0850*-Mut3 RNA was incubated in a total of 10 μl with or without 10 or 100 nM unlabelled LhrA or LhrA-Mut3* in the absence or presence of 10 μM Hfq_{LMO} and 10 μg tRNA. The samples were incubated 20 min at 37°C followed by 10 min on ice and subsequently separated on a 5% non-denaturing polyacrylamide gel at 4°C. For time course experiments, 10 fmol 5′-end-labelled *lmo0850* RNA was mixed with 10 nM LhrA in the presence or absence of 10 μM Hfq_{LMO} and incubated at 37°C for 1, 2, 5 or 10 min followed by 30 s on ice. The samples were then loaded onto 5% non-denaturing polyacrylamide gel with the current running.

Toeprinting experiments were performed in 10 μl reactions with 0.4 μM *lmo0850*-TOE RNA (from +1 to +120, relative to the SigA-dependent transcription start site, see Supplementary Figures S2 and S3). The RNA was pre-incubated for 20 min with or without 4 μM or 40 μM *in vitro* transcribed LhrA or LhrA-Mut3

and subsequently mixed with 0.4 pmol of 5′-end-labelled *lmo0850* NB DNA probe in a buffer containing 60 mM NH_4Cl, 10 mM Tris–acetate (pH 7.5), 10 mM DTT, 1 µl RNAguard, 100 µM dNTP. The mixture was incubated 2 min at 94°C and then placed on ice for 5 min and 37°C for 5 min. A total of 0.4 pmol 30S ribosomes (*E. coli* MRE600, supplied by Professor Stephen Douthwaite, University of Southern Denmark) were added followed by 10 min incubation. The mixture was supplemented with 10 µM uncharged $tRNA^{fMet}$ (Sigma) followed by 15 min incubation after which, 2 U of AMV reverse transcriptase was added. The reaction was stopped after 30 min by the addition of 10 µl formamide loading dye. In parallel, a sequencing reaction was prepared using T7-*lmo0850*-TOE DNA as template. The resulting DNA was separated on an 8% denaturing polyacrylamide sequencing gel.

RESULTS

Characterization of a Δ*lhrA* mutant strain

In a previous study, we identified the LhrA RNA in *L. monocytogenes* EGD-e through its association with Hfq (2). The *lhrA* gene is highly conserved in *Listeria* species but seems not to be present in any other organisms sequenced so far (Figure 1 and Supplementary Figure S1). The sRNA is expressed throughout growth in both rich and minimal media and accumulates in an Hfq-dependent manner at the onset of stationary phase (2). The dependency on Hfq for stability is very strict: in wild-type cells the half-life of LhrA is ~30 min, whereas in the Δ*hfq* strain the half-life is <3 min (2).

To address its physiological role, we constructed a chromosomal deletion of *lhrA* in which the promoter and terminator sequence of *lhrA* is preserved. The Δ*lhrA* strain was tested in a number of different growth assays to identify putative phenotypes. Under all conditions tested (including BHI, BHI + 4% NaCl, BHI + 4% EtOH, IMM, IMM + 4% NaCl or IMM + 4% EtOH), we did not observe any differences in growth of the wild-type and Δ*lhrA* strain (data not shown). Likewise, we did not observe any differences in colony size or morphology on solid media. The lack of any readily apparent phenotype for the Δ*lhrA* strain is in contrast to the *L. monocytogenes* Δ*hfq* strain which was shown to be more sensitive to NaCl and EtOH in comparison to the wild-type strain (40).

Identification of LhrA base pairing targets

Since LhrA interacts with Hfq and depends on Hfq for stability *in vivo*, we hypothesized that LhrA acts as a regulatory sRNA that controls the expression of specific target genes by an antisense mechanism. To pursue this, we performed a computational analysis of the secondary structure of LhrA and searched for regions in LhrA that were likely to participate in base pairing with a target mRNA. As shown in Figure 1C, LhrA is predicted to be highly structured and contains four stem–loops. We noticed that stem–loops 2 and 3 are separated by a 21 nucleotide single stranded-spacer region which is preserved in all *Listeria* species (Supplementary Figure S1). Curiously, this pattern is similar to the GcvB sRNA in which highly conserved residues in single-stranded regions, flanked by less conserved stem–loop structures, were shown to be used for base-pairing with multiple target mRNAs (10). Thus, this region appeared to be an obvious candidate for base pairing to target mRNAs and we therefore used this 21 nucleotide sequence, including 4 upstream nucleotides, as a query to search for potential targets using TargetRNA (44). The top 5 candidates of our search are shown in Table 1. In all cases, LhrA is proposed to base pair with a region surrounding the start codon of the target mRNAs. Notably, two putative start codons have been suggested for *lmo0850* (Figure 1 and Supplementary Figure S2). The proposed base pairing between LhrA and the *lmo0850* mRNA takes place at the upstream start codon. The two putative start codons are conserved within *Listeria* species, although in *L. grayi* the downstream start codon is AUG instead of UUG. In all cases, the two putative start codons are located in-frame and separated by 30 nucleotides (Supplementary Figure S2).

LhrA represses translation of *lmo0850* mRNA by an Hfq-dependent base pairing mechanism

To test the effect of LhrA on the expression of the five genes listed in Table 1, in-frame translational fusions to *lacZ* were constructed in the vector pCK-*lac*. The resulting *lacZ* fusion vectors were transferred to EGD wild-type, Δ*hfq* and Δ*lhrA* strains, and β-galactosidase activity was assayed during growth in BHI medium as described earlier (40).

In the case of *lmo1883-lacZ* and *lmo1666-lacZ*, no detectable β-galactosidase activity was observed (data not shown) suggesting either that the expression of these genes under the conditions tested here is below the detection limit or that the genes are transcribed from upstream promoters not present in the *lacZ* fusion constructs. The chimeric *kdpB-lacZ* fusion as well as the *lmo1269-lacZ* fusion were expressed throughout growth, but no significant difference in β-galactosidase activity was observed between the wild-type and mutant strains tested here (data not shown).

In contrast, *lmo0850-lacZ* was highly expressed in exponentially growing cells and a significant difference in β-galactosidase activity was observed for wild-type cells in comparison to Δ*hfq* and Δ*lhrA* cells (Figure 2A). In fact, we observed a 3- to 4-fold higher expression of *lmo0850-lacZ* in the Δ*hfq* and Δ*lhrA* mutant strains relative to the wild-type strain, regardless of the growth phase tested (data not shown). We also assayed a transcriptional *lacZ*-fusion containing only the promoter region of *lmo0850* (pTCV-*lmo0850-lacZ*; the putative transcription start sites were mapped by primer extension, Supplementary Figure S3). We did not observe any difference in β-galactosidase activity between the wild-type, Δ*hfq* and Δ*lhrA* strains (Figure 2G) suggesting that the regulation of *lmo0850* by LhrA occurs at the post-transcriptional level.

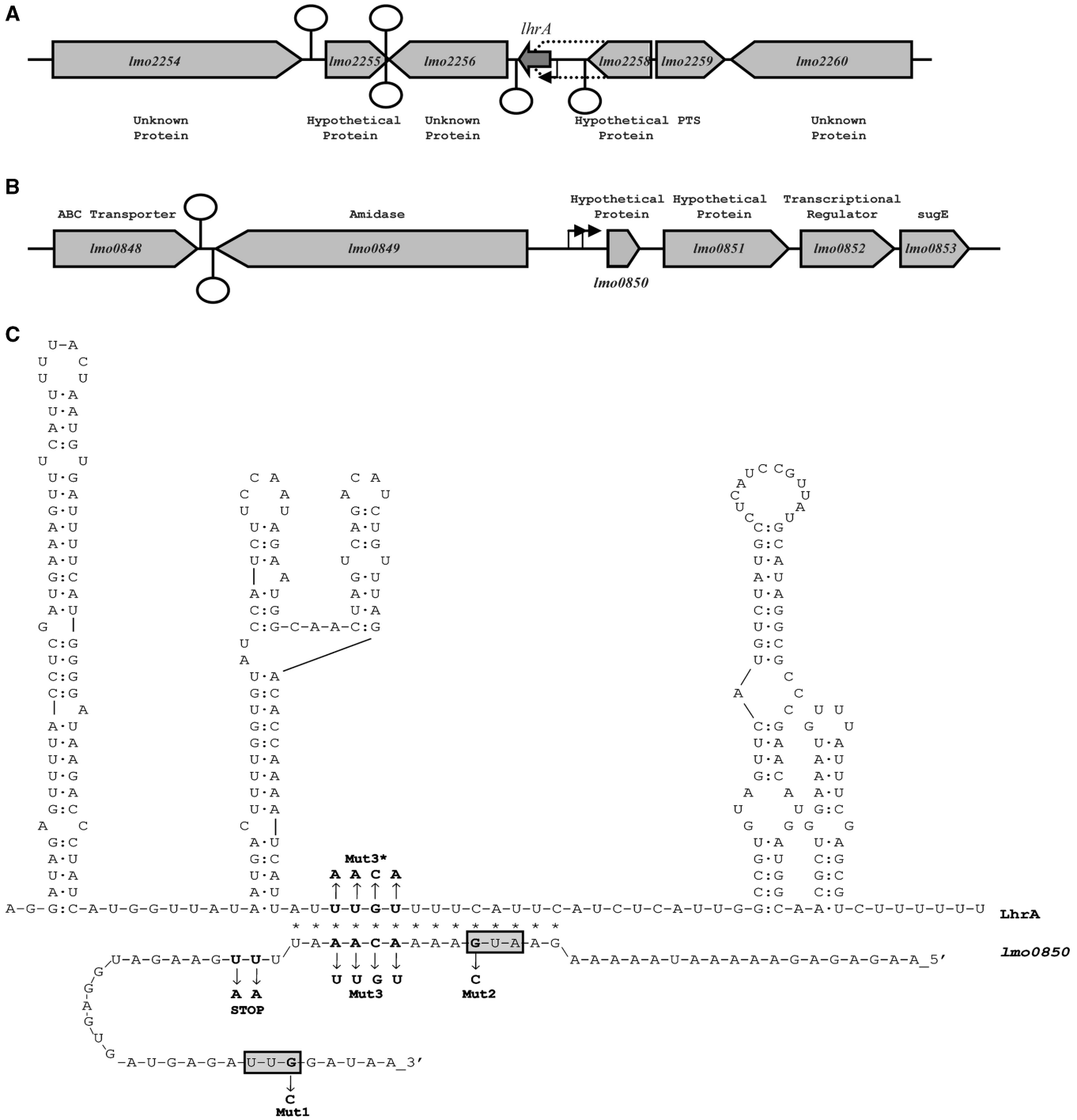

Figure 1. Genetic organization and putative secondary structure of LhrA and its target *lmo0850*. (**A**) Chromosomal location of *lhrA*. The upstream region of *lhrA* was found to overlap with another gene, *lmo2257* (dotted lines) encoding an unknown hypothetical protein. As discussed previously, the annotation of this open reading frame is highly questionable (2). (**B**) Chromosomal location of *lmo0850*, encoding a small hypothetical protein of unknown function. (**C**) MFOLD predicted secondary structure of LhrA RNA. The proposed interaction site with *lmo0850* is indicated with asterisks. The two putative start codons of lmo0850 are boxed. The G → C substitutions introduced to abolish translation initiation in *lmo0850-Mut1-lacZ* and *lmo0850-Mut2-lacZ* as well as the U → A substitutions introduced to create *lmo0850-STOP-lacZ* are indicated in bold. The nucleotide substitutions introduced in *lmo0850-Mut3-lacZ* and *lhrA*-Mut3* are also highlighted in bold.

Having identified *lmo0850* as a potential target gene for LhrA, we wanted to determine if translation of *lmo0850* initiates from the upstream or downstream start codon. To address this issue, we constructed a series of in frame translational *lacZ* fusions (Figure 2B–F). A construct, containing only the upstream start codon (Figure 2B, *lmo0850-START1-lacZ*), was not expressed under the conditions tested here, suggesting that the downstream

Table 1. Putative LhrA targets based on TargetRNA

Rank	Name	Score	Potential base pairing[a]
1	*lmo1883*—similar to chitinases	–82	5′_UUAUUUGUUUUC-AUUCAUCUCAUUG_3′ |||:|||||||| |||||||| 19 AAUGAACAAAAGGUAAGUAGA -2
2	*kdpB*—potassium-transporting atpase b chain	–76	5′_UUAUUUGUUUUCAUUCAUCUCAUUG_3′ |::|||:||||||:|||||| 4 AGUAAAGGUAAGUGGAGUAA -16
3	*lmo1666*—peptidoglycan linked protein (LPxTG)	–71	5′_UUAUUUG-UUUUCAUUCAUCUCAUUG_3′ ||||: |||||||||||:| 13 UAAAUGAAAAGUAAGUAGGG -7
4	*lmo0850*—hypothetical protein[b]	–70	5′_UUAUUUGUUUUCAUUCAUCUCAUUG_3′ |||||||||||||| -22 UAAACAAAAGUAAG -35
5	*lmo1269*—similar to type-I signal peptidase	–70	5′_AUUUGUUUUCA—UUCAUCUCAUUG_3′ ||||||||||| ||||| || || 19 UAAACAAAAGUGAAAAGUACAGAAA -6

[a]Start codons are indicated by a grey shade.
[b]Note that the start codon indicated does not correspond to the annotation on the Listilist webpage (http://genolist.pasteur.fr/ListiList/) but rather the more recent (http://cmr.jcvi.org/tigr-scripts/CMR/CmrHomePage.cgi).

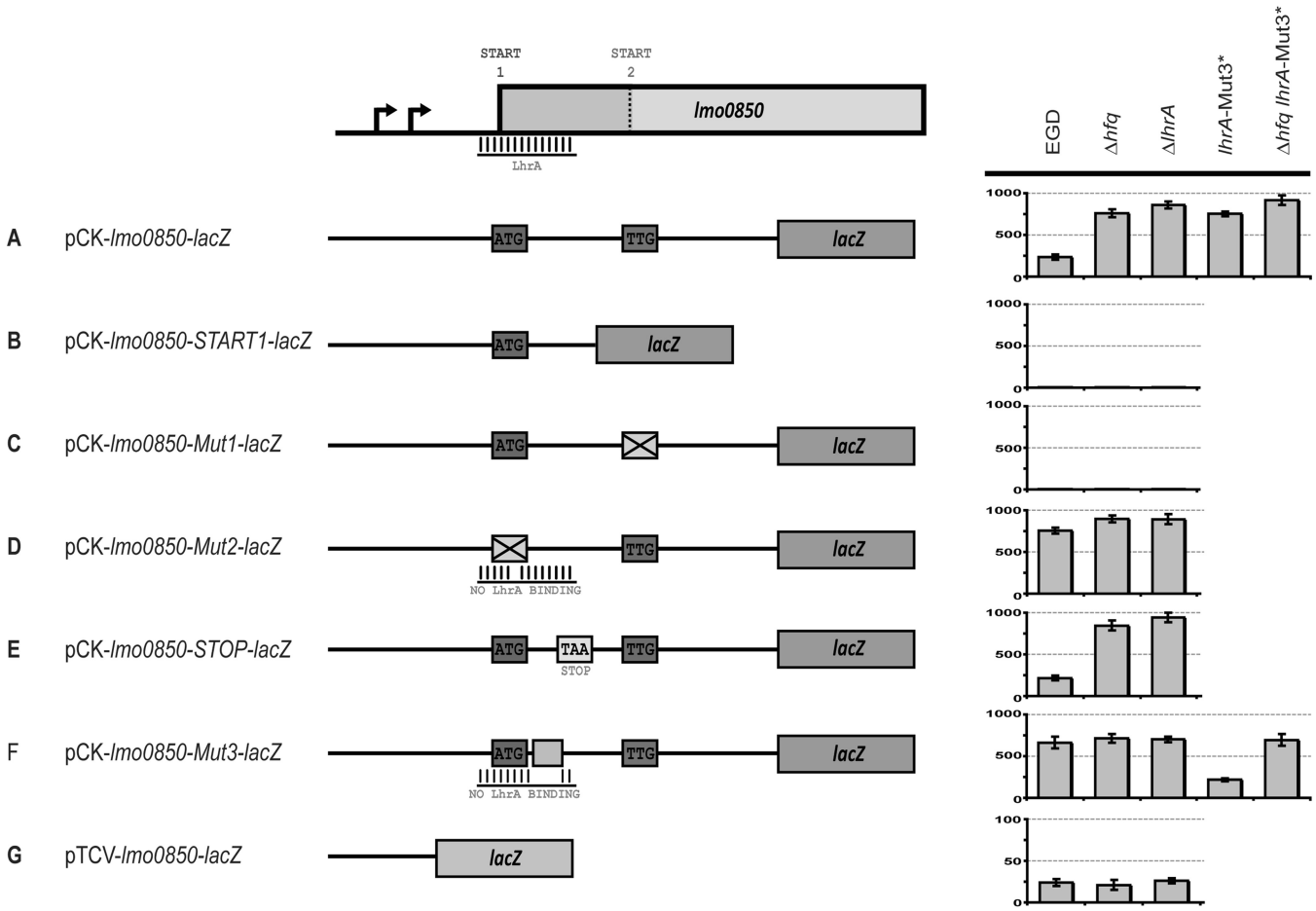

Figure 2. LhrA inhibits *lmo0850* translation in an Hfq-dependent manner. The left part shows a schematic overview of the seven *lacZ* fusions (**A–G**) tested by β-galactosidase assay in EGD wild-type, Δ*hfq* and Δ*lhrA* mutant strains. Two constructs (pCK-*lmo0850-lacZ* and pCK-*lmo0850-Mut2-lacZ*) were tested in EGD*lhrA-Mut3** and EGDΔ*hfq lhrA-Mut3** as well. The right part shows the corresponding specific β-galactosidase activity in cells harvested in the exponential growth phase. The presented activities are the averages of three independent experiments each conducted in duplicate.

start codon is used for translation of *lmo0850* mRNA. Next, we constructed *lacZ*-fusions containing either a G → C substitution in the 3rd position of each of the potential start codons (Figure 1C, Mut1 and Mut2), or a premature stop codon located in between the two putative start codons (Figure 1C, STOP). A point mutation in the downstream codon completely abolished translation (Figure 2C) while the G→C substitution of the upstream start codon resulted in a high level of expression and loss of regulation by LhrA and Hfq (Figure 2D). In contrast, the *lacZ* fusion containing the premature stop-codon was expressed in a fashion similar to the wild-type *lmo0850-lacZ* (Figure 2E). From these data we conclude that under the conditions tested here, *lmo0850* is exclusively expressed from the downstream start codon, resulting in the synthesis of a small highly basic protein of 48 amino acids.

The observation that a G → C substitution in the 3rd position of the upstream start codon completely abolished LhrA-mediated regulation (Figure 2D) suggested that the regulatory effect of LhrA indeed does rely on base pairing to this region. To expand on this, we constructed another mutant *lmo0850-lacZ* fusion in which four nucleotides predicted to be important for base pairing were substituted with their complementary bases (*lmo0850-Mut3-lacZ*). The four nucleotides correspond to residue − 27 to − 24 relative to the active start codon (Figure 1C, Mut3). When expression of *lmo0850-Mut3-lacZ* fusion was assayed, no significant difference was observed between the wild type strain and the Δ*hfq* and Δ*lhrA* strains suggesting that the four nucleotides are indeed important for LhrA-mediated regulation (Figure 2F).

To confirm the base pairing hypothesis, we exchanged the chromosomal copy of *lhrA* with a mutant version containing the four complementary nucleotide substitutions predicted to restore base pairing (Figure 1C, Mut3*). When expression of the wild type *lmo0850-lacZ* construct was assayed in this strain (EGD *lhrA-Mut3**) or in its isogenic Δ*hfq* strain (EGD Δ*hfq lhrA-Mut3**), no regulation was observed (Figure 2A, right). In contrast, when the *lmo0850-Mut3-lacZ* construct was assayed in the *lhrA-Mut3** strains, LhrA-mediated regulation was restored to wild-type levels in EGD *lhrA-Mut3**, while still impaired in the isogenic Δ*hfq* mutant background (Figure 2F, right).

Taken together, these results strongly suggest that LhrA regulates expression of *lmo0850* mRNA at the post-transcriptional level, by base pairing to a region located upstream from the active start codon. Importantly, this regulation requires Hfq.

Base pairing between LhrA sRNA and *lmo0850* mRNA specifically inhibits the formation of a translation initiation complex

Base pairing of a sRNA to a region in the vicinity of the start codon and/or Shine–Dalgarno sequence usually prevents ribosomes from associating with the mRNA and thus effectively blocks initiation of translation. Given that the proposed LhrA binding site in the 5′-leader of *lmo0850* mRNA is located upstream from the active start codon, we found it relevant to investigate if LhrA is able to inhibit the formation of a translation initiation complex on *lmo0850* mRNA. To address this issue, we conducted a toeprint experiment (Figure 3). An *in vitro* transcribed *lmo0850* mRNA fragment was incubated with a 5′-end-labelled primer complementary to the *lmo0850* coding sequence and incubated with 30S ribosomes in the absence or presence of uncharged $tRNA^{fMet}$ followed by primer extension. When $tRNA^{fMet}$ as well as 30S ribosomes were present, two distinct toeprint signals were observed exactly 13 nucleotides downstream from each of the two putative start codons (Figure 3, lane 3; Supplementary Figure S2). Thus, although translation only initiates from the downstream start codon *in vivo*, it appears that a translation initiation complex may indeed be formed at the upstream start codon as well. Furthermore, we observed that the formation of both toeprints was specifically inhibited by the addition of LhrA RNA (Figure 3, lanes 4 and 5) but not by LhrA-Mut3* RNA (Figure 3, lanes 6 and 7). Thus we conclude that binding of LhrA to *lmo0850* mRNA effectively blocks the formation of both the upstream and downstream translation initiation complex.

LhrA mediated post-transcriptional regulation of *lmo0850* translation leads to destabilization of *lmo0850* mRNA

In *E. coli* and *Salmonella*, sRNA mediated regulation of target mRNAs is often two-fold. In addition to

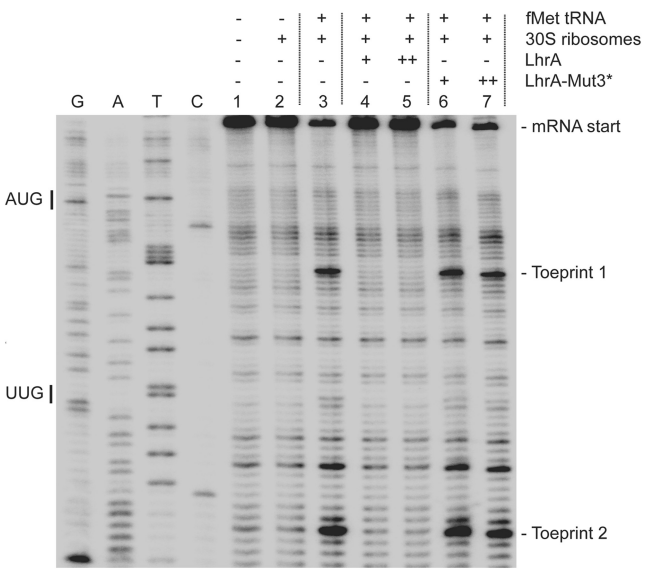

Figure 3. *In vitro* toeprint assay of *lmo0850* RNA in the absence or presence of LhrA. *In vitro* transcribed *lmo0850* RNA was incubated with 30S ribosomes in the absence or presence of excess levels of LhrA or LhrA-Mut3* RNA. Lanes 1 and 2: control reactions containing *lmo0850* RNA only, or *lmo0850* RNA together with 30S ribosomes. Lane 3: in the presence of 30S ribosomes and fMet tRNA, two distinct toeprint signals are observed precisely 13 nucleotides downstream from each of the two predicted start codons (AUG and UUG, respectively). Lanes 4 and 5: the addition of LhrA prevents formation of both toeprint signals. Lanes 6 and 7: the addition of LhrA-Mut3* does not prevent the formation of toeprint signals demonstrating that base pairing between LhrA and *lmo0850* RNA is essential for regulation.

post-transcriptional inhibition of translation, many Hfq-binding sRNAs also seem to mediate specific destabilization of the target mRNAs (21,48,49). To test whether LhrA affects the stability of *lmo0850* mRNA, we used northern blotting to compare *lmo0850* mRNA levels in wild-type, Δ*hfq* and Δ*lhrA* cells during growth in BHI. The results are presented in Figure 4A and show that the level of *lmo0850* mRNA is indeed affected by LhrA. Moreover, *lmo0850* mRNA levels are equally affected by Hfq. In both mutant strains, *lmo0850* mRNA levels are ~3- to 4-fold up regulated in exponentially growing cells and in early stationary phase cells. In overnight cultures, *lmo0850* mRNA is absent in all strains tested, suggesting an LhrA- and Hfq-independent down-regulation. It is also worth noting that in early exponential phase cells (i.e. the 4 h samples) the level of LhrA in Δ*hfq* cells is comparable to that found in wild type cells. Even so, the level of *lmo0850* mRNA in the Δ*hfq* strain is significantly higher than in the wild-type strain, suggesting a role for Hfq that extends beyond LhrA stabilization.

We also analysed the effect of *lhrA-Mut3** on the expression of *lmo0850* mRNA (Figure 4B). In wild type cells, *lmo0850* is only expressed at a relatively low level as compared to *lhrA-Mut3** cells, showing that the ability to down-regulate *lmo0850* has been compromised by the nucleotide substitutions in LhrA. Importantly, the level of LhrA-Mut3* is comparable to the level of LhrA in wild-type cells (Figure 4B).

Finally, we performed a complementation analysis. LhrA was expressed from a high copy number plasmid in the Δ*lhrA* mutant background and its effect on *lmo0850* mRNA levels was analysed (Figure 4C). The results show that ectopic expression of LhrA leads to reduced levels of *lmo0850* mRNA confirming that the observed effects are indeed caused by the lack of LhrA and is not due to polar effects of the chromosomal deletion.

Duplex formation between LhrA and *lmo0850* mRNA is stimulated by Hfq *in vitro*

Studies of the biochemical functions of Hfq in other organisms have demonstrated that one of the most important properties of Hfq is its ability to stimulate bi-molecular RNA-RNA interactions (23–27). To address this issue in *L. monocytogenes*, we turned to *in vitro* gel shift assays. We first conducted a standard electrophoretic mobility shift assay in which 5′-end-labelled *lmo0850* RNA was mixed with different concentrations of LhrA in the presence or absence of excess amounts of Hfq and incubated for a fixed amount of time (10 min at 37°C followed by 10 min at 4°C). The results are presented in Figure 5A, left panel. Both in the absence and presence of Hfq, duplex formation between *lmo0850* RNA and LhrA is observed (lanes 2–3 and 7–8). Notably, at the lowest concentration of LhrA, ~50% of the *lmo0850* RNA had shifted in the absence of Hfq, whereas in the

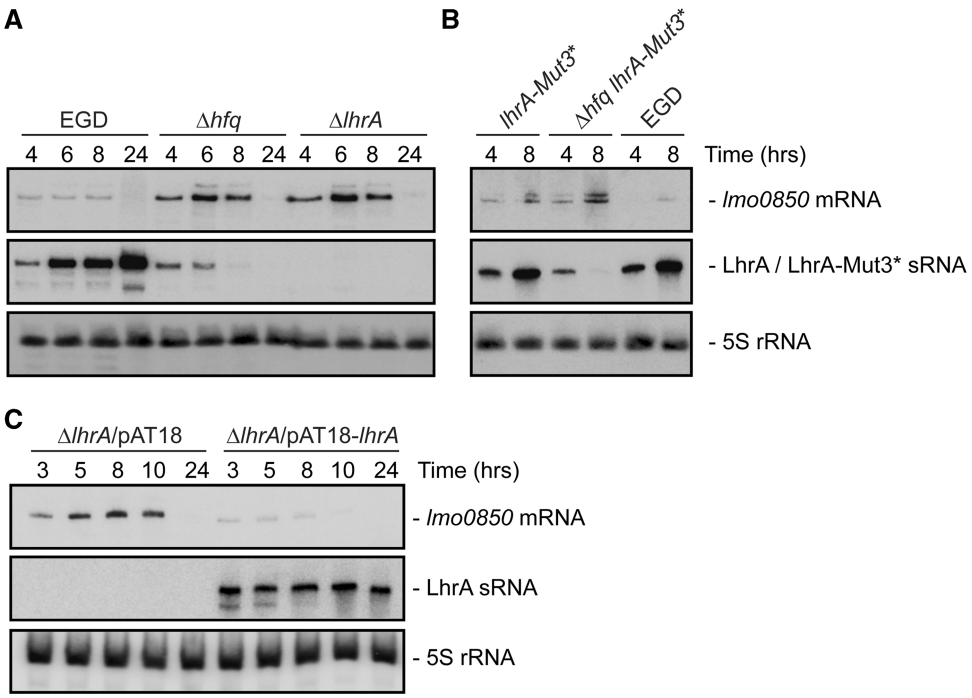

Figure 4. LhrA downregulates *lmo0850* transcript levels in an Hfq-dependent manner. (**A**) Northern blot showing the levels of *lmo0850* mRNA, LhrA and 5S rRNA in EGD wild-type, Δ*hfq* and Δ*lhrA* mutant strains at various time points during growth in BHI medium. (**B**) Northern blot showing the levels of *lmo0850* mRNA, LhrA/LhrA-Mut3* and 5S rRNA in EGD wild-type (to the right) as compared to an *lhrA-Mut3** strain (to the left) and the Δ*hfq lhrA-Mut3** strain (in the middle) at two different time points during growth in BHI medium. (**C**) Northern blot showing the effect of ectopic expression of LhrA from a high copy number plasmid. EGDΔ*lhrA* containing an empty vector (pAT18) or the LhrA-expression vector (pAT18-*lhrA*) was grown in BHI medium. At the indicated time points, cells were harvested and total RNA was prepared, and the levels of *lmo0850*, LhrA and 5S rRNA was determined by northern blotting.

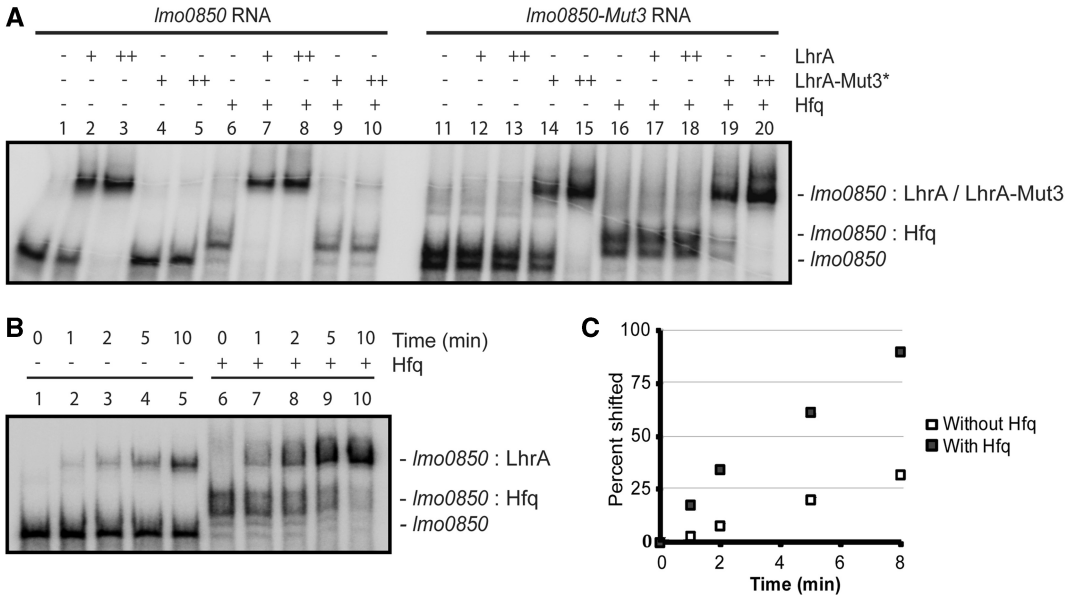

Figure 5. Hfq stimulates LhrA-*lmo0850* duplex formation. (**A**) *In vitro* binding assays of LhrA and *lmo0850* RNA in the absence (−) or presence (+) of Hfq. In the left part of the panel, an end-labelled wild-type *lmo0850* RNA fragment was used. In the right part of the panel, a *lmo0850-Mut3* RNA fragment was used. Where indicated, *in vitro* transcribed LhrA RNA or LhrA-Mut3* RNA was added at 10 (+) or 100 (++) fold excess of *lmo0850*. (**B**) Time course experiment with end-labelled *lmo0850* RNA fragment carried out in the absence (lanes1–5) or presence (lanes 6–10) of Hfq. The 5′-end-labelled *lmo0850* RNA fragment was mixed with 100-fold excess LhrA RNA and then incubated at 37°C for 0, 1, 2, 5 or 10 min, chilled for 30 s on ice, and then loaded onto a gel. The experiment was repeated twice with similar results. (**C**) Quantification of the time course experiment in (B).

presence of Hfq, almost 100% of the RNA had shifted (compare lanes 2 and 7). As shown in Figure 5A, the interaction between the two RNAs is strictly dependent on base pairing. Thus, the addition of even 100-fold excess LhrA-Mut3* does not result in a shift of *lmo0850* RNA in the binding assay. Similarly, LhrA does not associate with *lmo0850-Mut3* RNA (Figure 5A lanes 12, 13 and 17, 18). In contrast, *lmo0850-Mut3* readily forms a complex with LhrA-Mut3* (Figure 5A, lanes 14, 15 and 19, 20). Again, Hfq stimulates duplex formation in the presence of low levels of LhrA-Mut3* (compare lanes 14 and 19)

The standard gel shift analysis suggested that Hfq promotes the interaction between LhrA and *lmo0850* mRNA. To explore this further, we conducted a time course experiment in which a 5′-end-labelled *lmo0850* RNA fragment was mixed with 10-fold excess LhrA RNA and then incubated for a predetermined period of time. With this experimental setup, we can estimate the rate of association (on-rate) between the two RNAs. The results show that Hfq significantly increases the on-rate between *lmo0850* RNA and LhrA (Figure 5B and 5C).

The functional properties of Hfq_{LMO} are highly similar to Hfq_{ECO}

An *E. coli hfq* mutant strain displays broadly pleiotropic phenotypes, including decreased growth rate and increased sensitivity to various stress conditions such as oxidative stress (50,51). We have previously shown that *Listeria* Hfq (Hfq_{LMO}) is able to bind to *E. coli* sRNAs *in vitro* (2). To further explore the functional properties of Hfq_{LMO}, we tested whether Hfq_{LMO} could complement the growth defects and the increased sensitivity of the *E. coli hfq* mutant to oxidative stress. To this end, Hfq_{LMO} or Hfq from *E. coli* (Hfq_{ECO}) was expressed from an inducible plasmid in an *E. coli* mutant strain lacking functional Hfq. Indeed, we found that ectopic expression of Hfq_{LMO} in *E. coli* could restore the growth rate as well as resistance to oxidative stress, to nearly the same extent as Hfq_{ECO} (Figure 6A and B).

In *E. coli*, translation of *rpoS*, encoding the general stress sigma factor σ^S, is strongly dependent on functional Hfq, and so far three Hfq-dependent sRNAs (DsrA, OxyS and RprA) have been shown to target the *rpoS* mRNA (52). To further explore the functional properties of Hfq_{LMO}, we determined the levels of σ^S at different stages of growth by Western blotting (Figure 6C). The result of this experiment clearly shows that Hfq_{LMO} is able to stimulate the translation of *rpoS* in a manner similar to Hfq_{ECO}.

Finally, we examined whether Hfq_{LMO} can mediate sRNA-dependent degradation of a target mRNA in *E. coli*. To this end, we investigated the sRNA RyhB, which is known to down-regulate the synthesis of multiple iron-dependent proteins, including the *sodB*-encoded iron superoxide dismutase, in response to iron starvation (53). Specifically, Hfq_{ECO} has a stabilizing effect on RyhB *in vivo*, and is required for RyhB-mediated degradation of *sodB* mRNA. As shown in the northern blot experiments presented in Figure 6D, Hfq_{LMO} stabilizes RyhB and promotes degradation of *sodB* mRNA to the same extent as Hfq_{ECO} in response

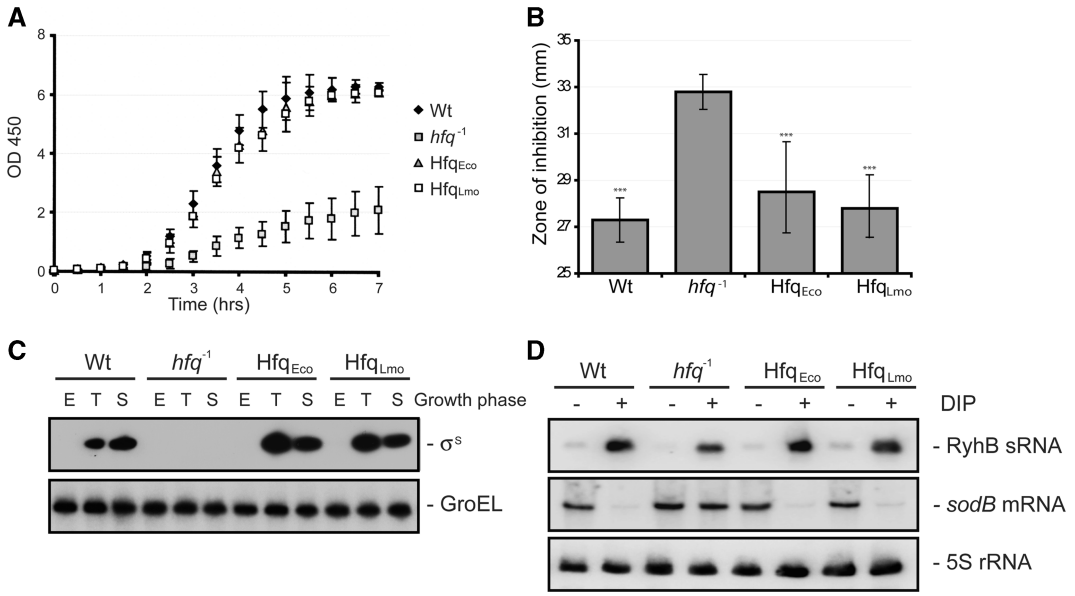

Figure 6. Hfq_{LMO} is able to restore several key defects associated with Hfq in *E. coli*. (**A**) Growth curves of *E. coli* wild-type (SØ928) carrying the empty vector pNDM-220, and *E. coli* hfq^{-1} carrying pNDM-220, pNDM-hfq_{ECO} or pNDM-hfq_{LMO}. Cells were cultivated in LB medium in the presence of 1 mM IPTG. The data shown are the result of three independent experiments each conducted in duplicate. (**B**) Resistance to oxidative stress. Overnight cultures were spread on agar plates and tested for their tolerance towards hydrogen peroxide by disk diffusion assay. Here, the averages of three independent experiments each conducted in triplicate are shown. The presence of three asterisks above a bar indicate a significant difference as compared to the hfq^{-} strain with $P < 0.001$. (**C**) Western blot analysis of the level of σ^S and GroEL (control). Cells were grown in LB medium containing 1 mM IPTG and cells were harvested at various time points during growth. E, exponential; T, transition phase; S, stationary phase. (**D**) Northern blot showing Hfq-dependent stabilization of RyhB and degradation of *sodB* mRNA. Cells were grown in LB medium containing 1 mM IPTG. At $OD_{600} = 0.4$, the cultures were split and 2,2′-Dipyridyl (DIP) was added to one of the cultures. After 10 min, cells were harvested for RNA extractions, and RhyB RNA, *sodB* mRNA and 5S rRNA levels were analysed by northern blotting.

to iron deprivation. Collectively, these results clearly suggest that Hfq_{LMO} has retained the functional properties of its *E. coli* counterpart.

DISCUSSION

During the last few years, 50 sRNAs have been discovered in *L. monocytogenes* by bioinformatics (3,4) and experimental approaches (2,5). Having identified these sRNAs, the major task now is to elucidate their biological role and mode of action. The present study focused on the mechanism by which the small RNA LhrA regulates gene expression in *L. monocytogenes* and the work presented here provides the first demonstration of a role for the bacterial Sm-like Hfq protein in sRNA-mediated gene silencing in Gram-positive bacteria.

By combining *in vitro* and *in vivo* analyses we first provided evidence that LhrA down-regulates the expression of the *lmo0850* gene at the level of translation initiation by an antisense mechanism. We hypothesized that pairing relied on a highly conserved region within LhrA and a region present in the translational initiation region of *lmo0850* mRNA. This prediction was verified by mutational analysis and a direct interaction between the sRNA and target RNA was further confirmed by the observation that compensatory mutations in LhrA (i.e. LhrA-Mut3*) specifically restores binding to and translational repression of a mutant version of *lmo0850* which cannot bind LhrA (i.e. *lmo0850-Mut3*). This suggests a model in which LhrA sequesters the *lmo0850* translation initiation region through complementary interactions. Curiously, two putative translation initiation sites have been predicted for *lmo0850* mRNA, resulting in polypeptides composed of 48 or 59 amino acids, respectively. Our *in vivo* studies indicated that only one site; the downstream (Figure 1C) is employed for translational initiation at the *lmo0850* message. However, our toeprint analysis revealed that translation initiation complexes may be formed at both sites. A likely explanation for this discrepancy is that we used 30S subunits from *E. coli* for toeprinting. Thus, it has been established that Gram-positive bacteria require a relatively strong Shine–Dalgarno sequence for translation initiation (54) which is only fulfilled for the downstream UUG initiation site. In any case, the toeprint results indicate that LhrA binding would prevent the formation of translation initiation complexes at either site. Thus, irrespectively of whether one or both start sites are being used *in vivo*, the presence of LhrA is predicted to interfere with translation initiation at the *lmo0850* transcript.

Our studies further established that LhrA-mediated regulation of *lmo0850* expression requires the presence of Hfq. The RNA chaperone acts in two ways to control the expression of *lmo0850*. Firstly, Hfq has a stabilizing effect on LhrA and appears to stimulate the LhrA-dependent degradation of *lmo0850* mRNA *in vivo* (Figure 4). The nature of these effects is not yet clear but is likely to involve specific ribonucleases, the exploration of

which may provide novel insights into the degradation pathways of RNA in Gram-positive species. Second, our *in vitro* binding studies showed that Hfq_{LMO} facilitates the association of LhrA with its target mRNA *in vitro* by accelerating the rate of RNA duplex formation (Figure 5). Since Hfq binds specifically to both RNA species, Hfq may accommodate both RNAs simultaneously thereby increasing their local concentrations. Alternatively, Hfq may act as an RNA chaperone to remodel LhrA and/or *lmo0850* mRNA into alternative structures more amendable to RNA duplex formation. Similar roles have been reported for Hfq in *E. coli* and *Salmonella* (8,19,55) and in line with this, we showed that ectopic expression of Hfq_{LMO} in an *E. coli hfq*-mutant strain could (i) restore growth and oxidative stress resistance to wild-type levels, (ii) promote translation of *rpoS* mRNA and (iii) stimulate RyhB-mediated degradation of *sodB* (Figure 6). These results demonstrate that Hfq_{LMO} and Hfq_{ECO} have a number of biochemical and biological properties in common. Regarding the ability to promote intermolecular base pairing, we note that Hfq_{LMO} has retained the conserved amino acids predicted to be involved in binding to poly(A) on the distal side of Hfq (16) as well as the amino acids involved in RNA binding on the proximal side of Hfq, as identified in the crystal structure of *S. aureus* Hfq bound to AU_5G (18). Curiously, our data also imply that Hfq_{LMO} is capable of participating in the RNase E-dependent degradation of target mRNAs in *E. coli*. RNase E is the primary endoribonuclease in *E. coli* and is part of the RNA degradosome, which is believed to act as a general RNA decay machine. Furthermore, RNase E, Hfq and sRNAs (such as RyhB) are able to form ribonucleoprotein complexes which act on target mRNAs (in this case, *sodB*) leading to translation inhibition and RNase E-dependent degradation of the target mRNA (29,56). Thus, despite the fact that RNase E is absent in *L. monocytogenes*, Hfq from this bacterium appears to have retained the ability to cooperate with RNase E in sRNA-mediated degradation of target mRNAs in *E. coli*. This finding supports the hypothesis that the interaction of Hfq and RNaseE in *E. coli* is mediated through RNA (57).

The significance of the above findings is stressed by the fact that no role for Hfq in riboregulation has been reported so far in other Gram-positive bacteria. In *S. aureus* and *B. subtilis*, the gene regulatory sRNAs studied so far all function without the requirement of Hfq (31,33–35,58–60). In *S. aureus*, Hfq is expressed at a very low level, and strains carrying deletions of *hfq* have no detectable phenotypes (38). Strikingly, Hfq proteins from different bacteria display a conserved common core (~aa residues 7–66) but considerable variation in length and sequence of their C-terminal ends, with the γ-proteobacteria possessing the longest C-terminal extensions (e.g. *E. coli* and *Salmonella* Hfqs of 102 aa residues). Based on the findings that an *hfq* variant of *E. coli* comprising the conserved core (Hfq_{65}) was impaired in binding to mRNAs and to support Hfq-mediated riboregulation in *E. coli*, it was predicted that an extended C-terminus was required for proper riboregulation [i.e. constitutes a hitherto unrecognized RNA interaction surface (61)]. However, like *S. aureus* and *B. subtilis* Hfqs, *Listeria* Hfq (77 aa residues) has a very short C-terminus, suggesting that the critical determinants required for Hfq_{LMO} to engage in riboregulation are located within the core structure. This notion is supported by several other studies which have shown that various Hfq variants with short C-terminal extensions can participate in riboregulation and substitute, at least in part, for *E. coli* Hfq [i.e. C-truncated *E. coli* Hfq; 75 aa residues (50,62), *Pseudomonas aeruginosa* Hfq; 82 aa residues (63), *Methanococcus jannaschii* Hfq; 71 aa residues (47), and *Aquifex aeolicus*; 80 aa residues (64)].

In summary, we conclude that Hfq in *L. monocytogenes* is a critical factor for sRNA-mediated riboregulation exerted by LhrA, and it is certainly conceivable that this mode of regulation is more widespread. Hence, lessons from *E. coli* and *Salmonella*, where multiple target regulation is a well known characteristic of Hfq-dependent sRNAs (65,66) hints that more mRNAs may be targeted by LhrA. Furthermore, additional Hfq-binding sRNAs in *L. monocytogenes* remain to be characterized with respect to their biological role and mode of action (2) and it seems safe to predict that some of those may act as Hfq-dependent antisense RNAs as well. In addition, we note that new powerful strategies, such as deep sequencing of Hfq-associated RNA (67) have not yet been carried out in low GC Gram positive bacteria. However, in line with observations in *B. subtilis* and *S. aureus*, the majority of the fifty sRNAs in *L. monocytogenes* appear not to require Hfq for stability or interaction with target mRNA *in vitro* (3–5). Intriguingly, a number of the *L. monocytogenes* sRNAs are predicted to be *cis*-acting or to encode small peptides (5). Thus, a highly complex picture of antisense regulation involving both *cis*-encoded and distinct classes of *trans*-encoded sRNAs is emerging in *L. monocytogenes* and related bacteria. One important direction for future studies is to clarify whether Hfq-independent *trans*-acting antisense RNAs act on their own or depend on other factors that substitute for the Hfq protein.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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