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A Unique cis-Encoded Small Noncoding RNA Is Regulating *Legionella pneumophila* Hfq Expression in a Life Cycle-Dependent Manner

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**ABSTRACT** *Legionella pneumophila* is an environmental bacterium that parasitizes protozoa, but it may also infect humans, thereby causing a severe pneumonia called Legionnaires’ disease. To cycle between the environment and a eukaryotic host, *L. pneumophila* is regulating the expression of virulence factors in a life cycle-dependent manner; replicating bacteria do not express virulence factors, whereas transmissive bacteria are highly motile and infective. Here we show that Hfq is an important regulator in this network. Hfq is highly expressed in transmissive bacteria but is expressed at very low levels in replicating bacteria. A *L. pneumophila hfq* deletion mutant exhibits reduced abilities to infect and multiply in *Acanthamoeba castellanii* at environmental temperatures. The life cycle-dependent regulation of Hfq expression depends on a unique cis-encoded small RNA named Anti-hfq that is transcribed antisense of the *hfq* transcript and overlaps its 5’ untranslated region. The Anti-hfq sRNA is highly expressed only in replicating *L. pneumophila* where it regulates *hfq* expression through binding to the complementary regions of the *hfq* transcripts. This results in reduced Hfq protein levels in exponentially growing cells. Both the small noncoding RNA (sRNA) and *hfq* mRNA are bound and stabilized by the Hfq protein, likely leading to the cleavage of the RNA duplex by the endoribonuclease RNase III. In contrast, after the switch to transmissive bacteria, the sRNA is not expressed, allowing now an efficient expression of the *hfq* gene and consequently Hfq. Our results place Hfq and its newly identified sRNA anti-*hfq* in the center of the regulatory network governing *L. pneumophila* differentiation from nonviral to virulent bacteria.

**IMPORTANCE** The abilities of *L. pneumophila* to replicate intracellularly and to cause disease depend on its capacity to adapt to different extra- and intracellular environmental conditions. Therefore, a timely and fine-tuned expression of virulence factors and adaptation traits is crucial. Yet, the regulatory circuits governing the life cycle of *L. pneumophila* from replicating to virulent bacteria are only partly uncovered. Here we show that the life cycle-dependent regulation of the RNA chaperone Hfq relies on a small regulatory RNA encoded antisense to the *hfq*-encoding gene through a base pairing mechanism. Furthermore, Hfq regulates its own expression in an auto-regulatory loop. The discovery of this RNA regulatory mechanism in *L. pneumophila* is an important step forward in the understanding of how the switch from inoffensive, replicating to highly virulent, transmissive *L. pneumophila* is regulated.

In recent years, the discovery of a class of regulatory elements, called small noncoding RNAs (sRNAs) revealed a high complexity of posttranscriptional gene regulation in prokaryotes and eukaryotes (1). sRNAs were reported to exert a wide range of cellular functions in bacterial physiology, in which rapid and fine-tuned adaptations in response to environmental changes are required (2, 3). sRNAs are classified as **cis**- or **trans**-
encoded sRNAs that modulate gene expression through complementarity to their adjacent or distant mRNA targets, respectively. In bacteria, trans-encoded sRNAs commonly require the assistance of the RNA chaperone Hfq to promote their interaction with the cognate mRNA targets. Although cis-encoded sRNAs share extended base pairing complementarity to their counterpart mRNAs, in a few cases, Hfq is required for their function (4). First identified in Escherichia coli as a host factor essential for the replication of the Qβ RNA phage, Hfq is now recognized as a global regulator of gene expression present in a wide variety of bacteria that impacts many molecular processes in bacterial physiology, stress response, and virulence (5, 6). The importance of the RNA-binding protein Hfq was uncovered by the characterization of hfq null mutants in diverse bacterial pathogens (7, 8). Further detailed research in its function in different bacteria showed that Hfq is a key posttranscriptional regulator, stabilizing sRNAs or facilitating sRNA/mRNA interactions that inhibit or enhance translation initiation. Furthermore, Hfq can act independently to modulate gene expression by affecting mRNA translation (for reviews, see references 6 and 9). Although deep sequencing approaches have revealed a high number and broad spectrum of sRNAs in diverse pathogens, such as Salmonella enterica serotype Typhimurium (10), Pseudomonas aeruginosa (11), Yersinia pseudotuberculosis (12), or Legionella pneumophila (13), the extent of Hfq-mediated riboregulation is highly complex and variable for each RNA type and in each organism. Furthermore, Hfq-associated sRNAs have been reported to control gene expression of multiple targets, thus regulating diverse cellular pathways, such as biofilm formation (14), catabolite repression (15), quorum sensing (16), or the control of transcriptional factors (17). Hfq is closely related to the Sm family of RNA-binding proteins in archaea and eukaryotes and phylogenetically widespread among bacteria, as about half of the sequenced bacterial genomes harbor at least one copy of the hfq gene (4, 18).

Legionella pneumophila is an intracellular bacterium that inhabits environmental aquatic systems, like lakes and rivers where it replicates in aquatic protozoa, but it can also infect humans to cause a severe pneumonia, and it also carries a gene that encodes Hfq (19, 20). However, little is known about the role of Hfq in the L. pneumophila life cycle or its regulatory function. The change between extra- and intracellular life and between replication in a host (replicative phase) and transmission to a new host (transmissive/virulent phase) demands a highly fine-tuned regulatory network (21). Indeed, the life cycle switch from replicative to transmissive/virulent L. pneumophila is governed through the function of several key regulators. Probably the most important ones are the two-component system (TCS) LetA/LetS (Legionella transmission activator and sensor, respectively) that induces traits necessary for efficient host transmission (22–24) and CsrA (carbon storage regulator) that is a posttranscriptional regulator, repressing transmissive/virulence traits during replication of L. pneumophila and releasing them in later stages of infection (25, 26; T. Sahr, C. Rusniok, F. Impenes, G. Oliva, O. Sismeiro, J. Y. Coppee, and C. Buchrieser, unpublished data). Moreover, the three sRNAs RsmX, RsmY, and RsmZ that are sequestering CsrA in transmissive phase to allow virulence traits to be translated are indispensable in this regulatory cascade (27, 28).

Here we report that L. pneumophila Hfq is regulated in a life cycle-dependent manner by a unique sRNA, named Anti-hfq that is transcribed in the early phase of the L. pneumophila life cycle. Our data support a complex model of regulation of the hfq transcript by the Anti-hfq sRNA, in which the Hfq chaperone together with RNase III are engaged to ensure the growth phase-dependent expression of this RNA-binding protein. Moreover, our results show that Hfq affects intracellular multiplication in amoebae, and consequently L. pneumophila virulence.

**RESULTS**

Hfq is highly conserved within the genus Legionella and other bacterial species. In L. pneumophila, Hfq is an 85-amino-acid protein encoded by the gene lpp0009. The hfq gene is organized in an operon with the putative GTP-binding protein HflX encoded by gene lpp0010 (Fig. 1A). Although the L. pneumophila hfq gene shares the conserved chromosomal gene arrangement typical of other organisms like E. coli or...
Vibrio cholerae only partly, it shows high nucleotide and amino acid identity with Hfq of many Gram-negative bacteria (up to 70%) and Gram-positive bacteria (up to 50%). Furthermore, all residues that contribute to RNA binding are conserved in L. pneumo-

phila (Fig. 1B). Comparison of the Hfq amino acid sequence among more than 300 L. pneumophila strains sequenced in the last years (19, 29–32) revealed that Hfq is 100% conserved across the different L. pneumophila strains. Analyses of four non-

pneumophila Legionella species (33) showed that Hfq is 80% conserved (Fig. 1C).

**Hfq is highly expressed during postexponential/transmissive growth phase.** In several pathogens, the level of expression of Hfq is growth phase dependent. In order to assess the transcriptional and posttranscriptional level of Hfq at different growth phases, we performed Northern and Western blot analyses of total RNA and whole protein lysates obtained from cultures of L. pneumophila (wild type [wt]) grown in liquid medium at 37°C. Northern blots using an hfq-specific probe showed very low hfq transcripts during exponential growth (optical density at 600 nm [OD600] of 2), but high transcript levels upon entry into postexponential growth (OD600 of 4). Protein expression followed the same pattern as shown by immunoblotting using anti-Hfq antibodies (Fig. 2A).

**Hfq is necessary for efficient intracellular replication at environmental temperatures.** In order to analyze the role and regulation of Hfq of L. pneumophila, we constructed an hfq deletion mutant (Δhfq) by the insertion of an in-frame apramycin resistance cassette (Fig. 2B). The resistance cassette used does not contain a transcriptional terminator; thus, transcription of the downstream gene, hflX, was not negatively affected as verified by transcriptome analyses (described below). Furthermore, the Δhfq mutant strain was completely sequenced using the Illumina technique, which ascertained that no secondary mutations had been introduced during the mutant construction. Analyses of the Δhfq mutant confirmed that the expression of Hfq was indeed abolished (Fig. 2C). To complement the Δhfq mutant, a plasmid harboring the entire hfq gene and its own promoter was transformed into the Δhfq mutant, generating the

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**FIG 1** Legionella Hfq is conserved across the genus and other bacterial species. (A) Schematic organization of the L. pneumophila hfq locus. TSS, transcription start site; aa, amino acids. (B) Alignment of the L. pneumophila Hfq protein sequence with other bacterial Hfq protein sequences reveals high sequence and RNA binding site conservation. (C) Alignment of the L. pneumophila Paris Hfq protein sequence with the Hfq protein sequences from different L. pneumophila strains and other Legionella or Legionella-like species. Amino acids involved in RNA binding are boxed. Conserved amino acid residues (asterisks) and semiconservative substitutions (dots) and conservative substitutions (colons) are indicated. The bars above the sequence alignment indicate the sequence percentage of sequence conservation.
complemented strain Δhfq pBChfq. Western blot analyses using anti-Hfq antibodies confirmed the expression of Hfq in Δhfq pBChfq (Fig. 2D). In contrast to a previous report where the Δhfq mutant in another L. pneumophila strain showed a prolonged lag phase (20), the growth pattern of the Δhfq mutant analyzed here was very similar to that of the wt strain at 37°C (see Fig. S1A in the supplemental material) and at 20°C (Fig. S1B), indicating that the growth defect of a strain lacking Hfq is due to the intracellular environment in amoeba, and not to a general growth defect at lower temperatures.

To learn whether Hfq is implicated in virulence of L. pneumophila as reported for other bacterial pathogens, we compared the ability of the wt L. pneumophila and Δhfq mutant to infect and multiply in Acanthamoeba castellanii and in the human monocyte-derived cell line THP-1. Similarly to what was reported previously, the Δhfq mutant strain showed only a minimal growth defect in A. castellanii and THP-1 cells at 37°C (Fig. 3A and B). In contrast, when replication in A. castellanii was monitored at 20°C, the Δhfq mutant showed a clear replication defect compared to the wt strain (Fig. 3C). Furthermore, complementation of the Δhfq mutant restored the intracellular replication pattern (Fig. 3D). Taken together, our data imply that Hfq plays a role in intracellular replication in amoeba at environmental temperatures and thus on the virulence of L. pneumophila.

**Hfq expression is affected by RpoS and LetA.** The activation of virulence traits of L. pneumophila is highly regulated at the transcriptional and posttranscriptional levels. Major regulators implicated are the sigma factor RpoS and the two-component system LetA/LetS (21) (Fig. 4D). Hfq is another candidate, as the mutant showed a replication defect (Fig. 3C). To determine the role and place of Hfq in this regulatory network, we analyzed the hfq transcript and protein levels in rpoS and letA mutants. Northern blot analysis showed that hfq transcripts were abolished in ΔrpoS and ΔletA mutants, confirming that RpoS and LetA are implicated in the regulation of hfq expression (Fig. 4A). This was also reflected in the protein level, as observed by immunoblot analysis, where Hfq expression in the ΔrpoS and ΔletA mutants was strongly decreased compared to the Hfq levels in the wt strain (Fig. 4B). Thus, RpoS and LetA are strongly
implicated in the regulation of Hfq expression at the transcript and protein levels. Flagella and consequently motility are hallmarks of the transmissive/virulent phase of L. pneumophila. We thus analyzed FlaA expression in the /H9004 hfq mutant strain and the /H9004 rpoS and /H9004 letA mutants in which FlaA expression is known to be reduced. As expected, FlaA was highly expressed in the late postexponential phase in the wt but strongly reduced in the /H9004 hfq mutant strain, suggesting its involvement in the regulatory cascade governing L. pneumophila differentiation, motility, and virulence (Fig. 4C).

Taken together, the expression of Hfq in L. pneumophila is regulated in a growth phase-dependent manner and is influenced by RpoS and LetA. Furthermore, Hfq itself seems to be implicated in the activation of traits typical of the transmissive/virulent phase of L. pneumophila.

Transcriptome analyses of the Δhfq mutant strain reveal only few changes in gene expression. To analyze which genes Hfq is affecting that may lead to the decreased intracellular replication, transcriptome analysis at postexponential growth (OD$_{600}$ of 4 grown in vitro in BYE medium and in vivo after 96 h of infection of A. castellanii) when Hfq is expressed the highest was performed. The comparison of the wt and Δhfq mutant transcriptomes in vitro identified only 18 differentially expressed genes (Table S1). This is in accordance with an in vitro transcriptome analysis of an hfq mutant in strain L. pneumophila JR32, where only a few genes and a mobile genetic element that excised upon the deletion of hfq were differentially expressed (34). In vivo, 74 genes were differentially transcribed due to the loss of Hfq, the majority of which (69 genes) was upregulated in the absence of Hfq, whereas only five genes were downregulated (Table S2). Interestingly, CsrA (0.43×), a major regulator of metabolic and regulatory functions during replication (Sahr et al., unpublished) was downregulated in

FIG 3 Efficient intracellular replication of L. pneumophila in A. castellanii and THP-1 macrophages is dependent on functional Hfq. (A) THP-1 cells were infected with wt and Δhfq mutant strains at an MOI of 10 at 37°C. The number of intracellular bacteria was monitored for 72 h, revealing a slightly diminished replication of the Δhfq mutant compared to the wt. (B and C) Monolayers of A. castellanii were infected with wt and Δhfq strains at an MOI of 0.1 at 37°C (B) and at an MOI of 1 at 20°C (C), showing a slight growth defect of the Δhfq mutant at 37°C but a clear defect at 20°C. (D) Infection of A. castellanii with the complemented Δhfq pBC_hfq strain at an MOI of 1 at 20°C, showing complementation of the growth phenotype. The wt strain carrying plasmid pBC-KS, the Δhfq strain carrying the empty plasmid, and complemented strain Δhfq pBC_hfq were examined. The number of intracellular bacteria was determined by recording the number of CFU per milliliter. Results are expressed at log$_{10}$ ratio of CFU at T/t. Each time point represents the mean ± standard deviation (SD) (error bar) from at least three independent experiments.
In contrast, no effect of Hfq on other important regulators like RpoS or the two-component system LetA/LetS was seen on the transcript level, indicating no direct feedback cascade for this regulatory pathway. In total, eight genes were upregulated both in vitro and in vivo. Two of these genes are involved in flagellar assembly and motility (flgG and flgH), and two are coding for the enhanced entry protein EnhA (lpp2693) and EnhB (lpp2694), which are implicated in host cell infection (35). Additionally, the macrophage infectivity potentiator Mip, at least four Dot/Icm effector proteins, transcriptional regulators Fis1 and Fis2, and the DNA-binding protein HU-beta are differentially transcribed in the Δhfq mutant during in vivo growth. These data might suggest a direct influence of Hfq on virulence formation as seen in infection of A. castellanii.

An antisense RNA is present in the 5′ untranslated region of hfq. We had previously established a complete transcriptional map of the L. pneumophila genome that revealed the presence of a dynamic pool of sRNAs regulated in a growth phase-dependent manner (13). Among these sRNAs, we identified a transcriptional start site (TSS) of a noncoding gene located in the reverse strand of the 5′ untranslated region (5′ UTR) of the hfq gene (Fig. 5A). In order to confirm experimentally the presence of a sRNA, we performed 3′ rapid amplification of cDNA ends (RACE), which yielded only a single band around 100 bp from RNA samples isolated from a culture grown at the early exponential phase (OD600 of 2). Cloning and sequencing of this cDNA amplifier that we named Anti-hfq showed that the noncoding RNA is 101 bp long (Fig. 5B and Fig. S2A). Using the program Mfold (36), the anti-hfq secondary structure was predicted to be composed of a duplex, with a 5′ overhang of 1 nucleotide (5′ C) and a 3′ overhang of 3 nucleotides (3′ UUA) containing a putative Rho-independent terminator identified by FindTerm (Softberry) (Fig. 5C). Although other programs did not confirm this terminator structure, the RACE PCR results showed that the transcript terminated at 101 bp where FindTerm predicted the terminator; hence, under the given conditions, Anti-hfq is indeed an sRNA. Bioinformatic analysis revealed the presence of an identical
Anti-hfq sequence among all *L. pneumophila* strains investigated. Anti-hfq homologues were also found among other *Legionella* species with a sequence identity of at least 80%, but no homologous sequences were found in other bacterial genomes. Thus, Anti-hfq represents a unique sRNA element within the genus *Legionella*.

Anti-hfq is expressed at the early exponential phase of the *Legionella* growth cycle. To determine the pattern of the Anti-hfq transcripts during the *L. pneumophila* life cycle, total RNA was extracted at exponential growth (OD$_{600}$ of 1) and postexponential growth (OD$_{600}$ of 4) of wt *L. pneumophila* grown in liquid BYE medium. The total RNA was reverse transcribed, and quantitative PCR (qPCR) analysis on the obtained cDNA was performed. We used different primer pairs: primer pair 1 (hfq-qPCR-F [F stands for forward] and hfq-qPCR-R [R stands for reverse]) exclusively recognizing the hfq mRNA and primer pair 2 (anti-hfq-qPCR-F and anti-hfq-qPCR-R) recognizing both the hfq and Anti-hfq RNAs, as these two transcripts entirely overlap (Fig. 5A). To confirm the growth phase-dependent expression of Anti-hfq, we calculated the ratio between the hfq and Anti-hfq transcript levels in the two growth phases. This showed that in the exponential phase, the Anti-hfq transcript was expressed about 1.5-fold higher than the hfq transcript, whereas its expression levels decreased to 0.05-fold compared to hfq in the postexponential phase (Fig. 5D). This alternative expression of either hfq or Anti-hfq suggests a regulation in which the expression of the Anti-hfq transcript might inhibit the expression of the sense transcript due to the cis regulatory function of the Anti-hfq sRNA.

Anti-hfq affects intracellular replication. To analyze whether the Anti-hfq sRNA indeed impacts Hfq expression levels, we first constructed a strain overexpressing

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**FIG 5** A small noncoding RNA named Anti-hfq is expressed antisense to hfq and influences Hfq expression and intracellular replication. (A) Schematic organization of the chromosomal organization of the *L. pneumophila* hfq and anti-hfq locus. (B) 3’ RACE PCR product in a 2% agarose gel obtained from exponentially grown wt *L. pneumophila* confirms the presence of an sRNA of 101 bp, named Anti-hfq. (C) Structure of the Anti-hfq sRNA of *L. pneumophila* as predicted by the program FindTerm. (D) qPCR analyses of the expression of Anti-hfq in the wt strain grown to exponential (E) phase and to postexponential (PE) phase, showing that Anti-hfq is expressed about 1.5 times in the E phase and 0.05 in the PE phase normalized to an OD$_{600}$ of 1. gyrB and tldD were used as internal controls for normalization. Each time point represents the mean plus standard deviation from three independent experiments. The means for the wt strain at the E and PE phases were statistically significantly different (*P* < 0.05) by the *t*-test as indicated by the bar and asterisk. (E) The anti-hfq sRNA influences Hfq and FlaA protein expression as evaluated by Western blotting analysis using the anti-Hfq or anti-FlaA antisera and lysates of wt and Anti-hfq-overexpressing (pMMBanti-hfqOE) strains grown to an OD$_{600}$ of 4. Membrane (Mb) signals are shown as loading controls. (F) Infection of *A. castellanii* with the pMMBanti-hfqOE strain shows a similar growth defect as the hfq mutant strain, indicating a role in intracellular replication. Monolayers of *A. castellanii* were infected with wt and the pMMBanti-hfqOE strain at an MOI of 1 at 20°C. Intracellular replication was determined by recording the number of CFU per milliliter. Results are expressed in log$_{10}$ ratio CFU T$_n$/T$_0$. Each time point represents the mean ± SD from three independent experiments.
Anti-hfq sRNA, in which the anti-hfq gene was cloned under the control of an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter. Upon induction with IPTG, the overexpression of the Anti-hfq sRNA decreased Hfq expression levels compared to the wt (Fig. 5E, top blot), supporting the idea that Anti-hfq sRNA is able to directly regulate Hfq expression. As the deletion of hfq resulted in a strongly decreased flagellin expression (Fig. 4C), we postulated that the overexpression of the Anti-hfq sRNA should also impact flagellin expression via the repression of Hfq. Indeed, when the Anti-hfq sRNA was overexpressed, the expression of FlaA was strongly reduced compared to the wt strain (Fig. 5E, bottom blot), further suggesting a cis regulatory function of the Anti-hfq sRNA on Hfq expression. This result is consistent with a model in which an antisense sRNA regulates the transcription of its sense protein-coding gene, here hfq.

The L. pneumophila Δhfq mutant is attenuated in intracellular growth of A. castellanii (Fig. 3C), and flagellin is less well expressed in comparison to the wt strain (Fig. 4C). Thus, to test whether Anti-hfq has a role in intracellular replication of L. pneumophila, we infected A. castellanii with the strain overexpressing anti-hfq. At 72 h postinfection, 10-fold fewer intracellular bacteria were recovered from amoeba infected with the Anti-hfq sRNA-overexpressing strain (pMMBantihfqOE) compared to the wt, similar to the replication rate seen for the Δhfq mutant strain (Fig. 5F and 3C). Thus, Anti-hfq sRNA plays a role in intracellular replication of L. pneumophila.

**Hfq expression is regulated by the Anti-hfq sRNA.** In the Δhfq mutant used until now, the anti-hfq gene was still intact (Fig. 2B). Thus, to further study the function of Anti-hfq sRNA, we constructed a second mutant containing a larger deletion as the entire region spanning hfq and anti-hfq (Δhfq Δanti-hfq(-10)) was replaced with an apramycin cassette (Fig. 6A). By complementing this mutant with the plasmid pBCanti-hfq (-10) in which two single mutations in the anti-hfq −10 box had been introduced, we were able to study the role of the Anti-hfq sRNA without disturbing Hfq expression. This complemented strain was named the Δanti-hfq(-10) strain (Fig. 6A). When analyzing the Hfq expression levels in the Δanti-hfq(-10) mutant, the Hfq expression pattern differed...
compared to the wt strain, as the expression of the *hfq* transcripts started already during exponential growth of *L. pneumophila* (Fig. 2A, bottom blot, and Fig. 6B), indicating that Anti-hfq sRNA indeed represses *hfq* transcripts in exponential growth. In contrast, in the complemented mutant strain (Δhfq Δanti-hfq pBCffq), Hfq expression was restored to wt levels (Fig. 6C), whereas in the control strain (Δhfq Δanti-hfq pBC), no expression of Hfq was seen, as expected (Fig. 6C). Thus, the antisense RNA Anti-hfq regulates Hfq expression levels in a growth phase-dependent manner by functioning as a *cis*-complementary sRNA.

The *hfq* and Anti-hfq RNA transcripts interact in vitro. Our previous results suggest a regulation of the *hfq* transcript through binding of its Anti-hfq antisense sRNA. To investigate a direct interaction of Anti-hfq and *hfq* mRNA in vitro, we performed electrophoretic mobility shift assays (EMSAs). Incubation with a radioactively labeled Anti-hfq RNA probe and increasing concentrations of cold *hfq* mRNA resulted in a slower-migrating complex, suggesting a direct interaction of the two RNA molecules (Fig. 7A). In contrast, when the EMSA was performed with Anti-hfq sRNA and a truncated *hfq* mRNA probe spanning the nucleotides 78 to 255 missing the 5′ UTR region and the first 26 codons (*hfq OUT*), no changes in terms of migration were observed, consistent with the absence of formation of a complex (Fig. 7A). Similar results were obtained when using the mRNA of an unrelated gene (*lpp0644* RNA probe) as a second negative control (Fig. S2B). Thus, Anti-hfq forms an RNA duplex with the *hfq* mRNA and most likely regulates *hfq* mRNA expression by direct binding due to complementarity.

Purified Hfq binds *hfq* and Anti-hfq sRNA with different affinity. Although the Hfq protein is known to facilitate the interaction between *trans*-encoded sRNAs and their mRNA targets, the Hfq chaperone may also function to stabilize/destabilize *cis*-encoded sRNAs and their complementary mRNA targets. Thus, we sought to determine whether the Hfq protein might be able to form complexes either with the *hfq* mRNA or with the Anti-hfq sRNA. The analysis of the *hfq* and anti-*hfq* sequences revealed the presence of (AAN)₃ triplets and AU-rich regions, which could be Hfq binding regions, further suggesting the hypothesis of an Hfq autoregulatory loop. To assess the ability of Hfq to bind *hfq* and Anti-hfq transcripts separately, we evaluated binding in vitro by EMSAs using recombinant Hfq protein. As shown in Fig. 7B and C, Hfq interacts with both RNA molecules but with different affinities.

To study the inhibitor complex formed by the *hfq* mRNA, Anti-hfq, and the Hfq protein in more details, we employed a gel-shift kinetic assay (Fig. 7D). A radioactively labeled Anti-hfq RNA probe was incubated with 25 nM of cold *hfq* mRNA in the absence (Fig. 7D, lanes 1, 3, and 5) or presence (Fig. 7D, lanes 2, 4, and 6) of Hfq protein for 0.5 (lanes 1 and 2), 1.5 (lanes 3 and 4), and 2.5 (lanes 5 and 6) minutes. As shown above, the two RNA molecules were able to interact. Additionally, we detected a strong band corresponding to the formation of a ternary complex already after 0.5 min of incubation. Moreover, the intensities of the shifted bands indicated that the affinity of Hfq for the RNA-RNA complex might be much stronger than for the single RNAs alone. The super shift and thus the formation of the ternary complex was increasing with longer incubation time (after 1.5 and 2.5 minutes). To test the specificity of this complex, radioactively labeled *hfq* or Anti-hfq probes were incubated alone in parallel with increasing amounts of Hfq confirming that Hfq is indeed able to bind each of the RNA molecules separately (Fig. 7D, lanes 7 to 9 and 10 to 12). Therefore, although Anti-hfq is complementary to its own target and thus it should not require Hfq for binding, Hfq is able to bind the two RNA molecules, forming a ternary complex.

RNase III might participate in the double-strand RNA (dsRNA) regulation. One of the regulatory functions of the Hfq RNA chaperone is the recruitment of RNases for the degradation of sRNA and/or mRNA targets. Thus, we wondered whether RNases might be involved in the degradation of the ternary complex in *L. pneumophila*. To answer this question, we performed an RNA stability assay in the wt and an RNase III gene (*lpp1834*) deletion mutant that we constructed. Analysis of the *hfq* mRNA levels,
after the addition of rifampin, showed a half-life of 4.1 min in the wt strain and of 8.2 min in the RNase III deletion mutant (Fig. 7E). In contrast, when the half-life of the glyceraldehyde-3-phosphatedehydrogenase (GAPDH) transcript was determined in the same conditions, no significant differences were observed in the relative mRNA levels between the wt and the RNase III deletion mutant (Fig. 7F). This strongly suggests that RNase III is involved in the cleavage of the hfq–Anti-hfq RNA duplex and hence, affects the stability of the hfq mRNA, closing the Hfq regulation loop.

**DISCUSSION**

*Legionella pneumophila* needs to adapt to many different environmental conditions, including low-temperature and nutrient-poor aquatic and hostile intracellular environ-
ments of protozoa or human macrophages. To regulate the transition from one environment to another environment, *L. pneumophila* has evolved a complex regulatory cascade allowing it to switch from a replicative stage to a transmissive/virulent stage (21). This regulatory network is comprised of many global regulators like the RNA-binding protein CsrA and its small noncoding RNAs RsmX, RsmY, and RsmZ, the TCS LetA/LetS, and the stress sigma factor RpoS (22–28; Sahr et al., unpublished). Here we demonstrate that the RNA chaperone Hfq is another major player in the regulation of the switch to transmissive/virulent *L. pneumophila* and that life cycle-dependent Hfq expression is regulated by an antisense RNA named Anti-hfq.

Comparative sequence analyses showed that Hfq is highly conserved and present in all *L. pneumophila* strains sequenced thus far (Fig. 1B). Our observation that the *hfq* transcript and the Hfq protein are barely expressed at early stages of growth but highly expressed at the postexponential phase of growth (Fig. 2A) establishes Hfq as a growth phase-dependent regulated protein and suggests its implication in the regulation of the expression of virulence traits, a feature of postexponential bacteria. Interestingly, in 2005, McNealy and colleagues (20) had reported that Hfq of *L. pneumophila* JR32 is expressed in the exponential phase of growth and is positively regulated by the stationary-phase sigma factor RpoS. Furthermore, they proposed that upon entry into stationary phase, Hfq expression is abolished through the regulatory function of the two-component regulator LetA, thereby ensuring that *hfq* transcripts are off when the infectious traits need to be activated (20). The differences from our results might be due to the different strains used or perhaps to the excision of the 100-kb plasmid pL100 when *hfq* is deleted, as reported by Trigui and colleagues (34). However, our results are in agreement with the *hfq* expression pattern observed in several other bacterial pathogens such as *P. aeruginosa* and *Listeria monocytogenes* (37, 38) but also with the life cycle of *L. pneumophila* (21, 39). The regulation of virulence traits by Hfq, which demands its expression in the postexponential growth phase, is supported by the observation that the *hfq* mutant is defective in intracellular growth, a characteristic also reported by McNealy and colleagues, and the transcriptome results identifying virulence genes and virulence gene regulators to be differentially expressed upon deletion of *hfq* (see Table S1 and S2 in the supplemental material). By analyzing the protein and transcript levels of Hfq in different regulatory mutants, we show that Hfq expression is influenced by the stationary sigma factor RpoS and the response regulator LetA during the postexponential phase, as both directly or indirectly turn on *hfq* transcription (Fig. 4A and B). Thus, Hfq plays an important role in the regulatory cascade governing the switch to the transmissive phase of *L. pneumophila* (Fig. 4D).

In agreement with the position of Hfq in this regulatory network, the loss of Hfq impaired intracellular replication at 20°C, the optimal growth temperature of *A. castellanii* and a temperature that is close to environmental conditions (Fig. 3C). The transcriptome analysis of the Δhfq mutant during infection of *A. castellanii* supported this finding, as several secreted effector proteins, the enhanced entry proteins EnhABC, the global DNA-binding transcriptional regulators Fis1 and Fis2, and the DNA-binding protein HU-beta were differentially regulated in the *hfq* mutant. Moreover, the above-mentioned regulators are all related to environmental adaptation, virulence, and stress response regulation and fitness in different pathogenic bacteria (40). Furthermore, a hallmark of transmissive/virulent *L. pneumophila*, the expression of flagellar protein FlaA that is intimately linked to virulence, was strongly reduced in the Δhfq mutant at an OD600 of 4, similar to what is seen in LetA and RpoS mutants (Fig. 4C). Collectively, these results indicate that *L. pneumophila* requires Hfq to promote motility and to efficiently multiply within *A. castellanii* at environmental temperatures.

Most studies of Hfq analyzed its role in the regulation of sRNAs and their mRNA targets, but not how Hfq expression itself is regulated. *L. pneumophila* Hfq is clearly growth phase-dependently regulated, as transcript and protein levels are low during replicative/exponential growth but are strongly expressed in transmissive/postexponential growth (Fig. 2A). This growth phase-dependent regulation is achieved by an sRNA that we named Anti-hfq as it is transcribed on the antisense strand of the *hfq*
gene overlapping its 5’ UTR (Fig. 5A). Anti-hfq is a 101-bp long sRNA that is highly expressed during exponential growth, but its expression is strongly decreased upon entry into the transmissive/postexponential growth phase. These opposite expression patterns of the hfq and Anti-hfq transcripts together with the fact that the sRNA is encoded antisense to hfq suggested that it has a role in regulating hfq expression. Furthermore, the identification of a partly conserved LetA binding site (two mismatches) suggested that the growth phase-dependent expression of Anti-hfq sRNA might be regulated by LetA. However, we could not firmly establish a specific interaction; thus, this regulatory pathway remains to be analyzed in the future. A detailed analysis of the anti-hfq sequence revealed the presence of a putative Rho-independent transcriptional terminator as described in a large part of functional Hfq binding modules of sRNAs (41). Furthermore, the ARN or ARNN (R is purine, and N is any nucleotide) motifs that are preferentially bound in the distal site of the Hfq homohexameric (42) were also present in the proximity of the hfq ribosome binding site (RBS), and we showed that Anti-hfq sRNA binds the complementary region of the hfq mRNA. Furthermore, Hfq is able to interact separately with both RNA molecules, hfq and Anti-hfq (Fig. 7B and C), but it also forms a ternary complex, suggesting an autoregulatory circuit (Fig. 7D). Finally, the riboendonuclease RNase III takes part in the regulation of Hfq probably cleaving the double-strand RNA as suggested by RNA stability measurements in an RNase III mutant strain (Fig. 7E). Several studies of E. coli had suggested that Hfq binds two distinct sites of the 5’ UTR of its own mRNA, hindering the formation of the translation initiation complex and thus negatively regulating its own expression. In E. coli, RNase E is recruited to exert its RNase function to degrade hfq mRNA (43). Thus, collectively, the data suggest that binding of the cis-encoded Anti-hfq sRNA obstructs Hfq translation in exponential growth (Fig. 7A).

The regulation of Hfq by a cis-encoded sRNA is an unusual feature. We propose that binding of the cis-encoded Anti-hfq sRNA to hfq mRNA in exponential growth leads to low translation of Hfq, whereas when the expression of Anti-hfq sRNA decreases in the transmissive phase, high expression of Hfq is possible. This leads to the expression of several Dot/Icm secreted substrates, global regulators like Fis1 and Fis2 that are implicated in the regulation of virulence traits (44) and probably of several of the many growth phase dependently regulated sRNAs that we identified earlier (13) (Fig. 8). Thus, L. pneumophila is equipped with a highly sophisticated regulatory mechanism further fine-tuning the regulation of the reciprocal expression of distinct sets of genes under different environmental conditions.

MATERIALS AND METHODS

Bacterial strains, growth media, and culture conditions used. The bacterial strains used in this study are listed in Table 1. L. pneumophila strain Paris and its derivatives were cultured in N-(2-acetamido)-2-aminoethanesulfonic acid (ACES)-buffered yeast extract broth (BYE) or on ACES-buffered charcoal-yeast (BCYE) extract agar (45), and E. coli was grown in Luria-Bertani (LB) broth and agar. All strains were grown at 37°C. For the construction of knockout mutants and complementation plasmids, antibiotics were used at the following concentrations: ampicillin at 100 mg/ml, kanamycin at 50 mg/ml, and chloramphenicol at 20 mg/ml for E. coli; and kanamycin at 10 mg/ml, chloramphenicol at 20 mg/ml, and apramycin at 15 mg/ml for L. pneumophila. A. castellanii ATCC 50739 was cultured in PYG 712 medium (2% proteose peptone, 0.1% yeast extract, 0.1 M glucose, 4 mM MgSO4, 0.4 M CaCl2, 0.1% sodium citrate dihydrate, 0.05 mM Fe(NH4)2(SO4)2 · 6H2O, 2.5 mM NaH2PO4, 2.5 mM K2HPO4) at 20°C. THP-1 human monocytes were grown in RPMI 1640 GlutaMAX medium (Gibco) supplemented with 10% fetal bovine serum at 37°C and 5% CO2.

Mutant and plasmid constructions. The plasmids and oligonucleotide primers used in this study are listed in Table 1 and 2, respectively. Mutant strains of L. pneumophila were constructed as previously described (39, 46). In brief, the gene of interest was inactivated by introduction of an apramycin resistance (Apr’) cassette. The mutant alleles were constructed using a three-step PCR. For the construction of the Δhfq deletion mutant strain, three overlapping fragments (lpp0009 upstream region primers hfq-Mut_F and hfq-apra_R, antibiotic cassette-primers apra_F and apra_R, lpp0009 downstream region primers hfq-apra_F and hfq-Mut_R; Table 2) were amplified independently and purified on agarose gels. The three resulting PCR products were mixed at the same concentration (15 nM), and a second PCR with flanking primers (primers hfq-Mut_F and hfq-Mut_R) was performed. This PCR product, the resistance marker cassette flanked by 300-bp regions homologous to lpp0009 was introduced into the L. pneumophila Paris strain by natural competence (47). Strains that had undergone allelic exchange were selected by plating on BCYE containing apramycin, and the mutant was verified by PCR and sequencing. For the
construction of the Δhfq Δanti-hfq double mutant strains and the RNase III mutant, the same cloning strategy was used, and the primers are listed in Table 2.

For complementation experiments, the region, including lpp0009 and lnc0003 was PCR amplified with primers containing HindIII and SalI restriction sites at their ends (Hfq_compl_F and Hfq_compl_R) and ligated to the pBC-KS plasmid, previously digested with the two restriction enzymes. The resulting plasmid, named pBC hfq, was introduced into the Δhfq and Δhfq Δanti-hfq deletion mutant strains by electroporation. The wild-type (wt) L. pneumophila Paris, the Δhfq and the Δhfq Δanti-hfq deletion

### TABLE 1 Bacterial strains and plasmids used in the study

| Strain or plasmid | Description* | Reference or source |
|-------------------|--------------|---------------------|
| **Strains**       |              |                     |
| L. pneumophila CIP 107629 | L. pneumophila serogroup 1 strain Paris (LpP) | 19 |
| L. pneumophila pBC  | LpP carrying pBC-KS | 54 |
| L. pneumophila pMMB207C | LpP carrying pMMB207C | 28 |
| L. pneumophila Δhfq | LpP hfq::Aprr | This study |
| L. pneumophila Δhfq pBC | LpP hfq::Aprr carrying pBC-KS | This study |
| L. pneumophila Δhfq pBChfq | LpP hfq::Aprr carrying pBChfq | This study |
| L. pneumophila Δhfq Δanti-hfq | LpP hfq anti-hfq::Aprr | This study |
| L. pneumophila Δhfq Δanti-hfq pBC | LpP hfq anti-hfq::Aprr carrying pBChfq | This study |
| L. pneumophila Δhfq Δanti-hfq pBChfq | LpP hfq anti-hfq::Aprr carrying pBChfq | This study |
| L. pneumophila Δanti-hfq (-10) | LpP hfq anti-hfq::Aprr carrying pBChfq (−10) | This study |
| L. pneumophila pMMBanti-hfqOE | LpP carrying pMMBanti-hfqOE | This study |
| L. pneumophila ΔletA | LpP letA::Kmr | 28 |
| L. pneumophila ΔrpoS | LpP rpoS::Kmr | 13 |
| L. pneumophila Δrnaselli | LpP carrying the RNase III gene fused to the Aprr cassette | This study |
| E. coli DHSα | F− Δ800dlacZΔM15 Δ(lacZYA-argF)U169 deor recA1 endA1 gyrA96 relA1 hsdR17 (rK K1 mK phoA supE44) Δthi-1 gyrA96 relA1 | Invitrogen |
| **Plasmids**      |              |                     |
| pGEM-T Easy       | Cloning of PCR products; Amp' | Promega |
| pBC-KS            | Expression vector; Cm' | Stratagene |
| pMMB207C          | Legionella expression vector; ΔmobA; Cm' | 55 |
| pBChfq            | pBC-KS containing hfq and anti-hfq genes; Cm' | This study |
| pMMBanti-hfqOE    | pMMB207C containing anti-hfq gene under the ptac promoter; Cm' | This study |
| pBChfq (−10)      | pBChfq mutated in the −10 upstream region of anti-hfq; Cm' | This study |

*Abbreviations: Amp', ampicillin resistance; Apr, apramycin resistance; Cm', chloramphenicol resistance; Kmr, kanamycin resistance.
TABLE 2 Primers used in this study

| Primer                  | Primer sequence (5’ – 3’) | Purpose                                                                 | Reference |
|------------------------|---------------------------|------------------------------------------------------------------------|-----------|
| hfq-Mut_F              | AAGAATTGATCGAGCCGCTGTC    | Deletion of the hfq gene                                               | This study|
| h fq-Mut_R             | CCCGCGATGCGTAATGGGA       | Deletion of the hfq gene                                               | This study|
| apra_F                 | TTATGCTGAGCCTCATCAGC      | Deletion of the hfq gene                                               | This study|
| apra_R                 | GAGGGGATCCGGGATGTGCTCT    | Deletion of the hfq gene                                               | This study|
| hfq-apra_R             | GCTGAGGAGCTGCAGATGGAATT   | Deletion of the hfq gene                                               | This study|
| hfq-anti-hfq1_F        | ACACCCAAAGGCGGCGGCGTG     | Deletion of the hfq and anti-hfq genes                                 | This study|
| hfq-anti-hfq2_R        | GCTGAGGAGCTGCAGATGGAATT   | Deletion of the hfq and anti-hfq genes                                 | This study|
| hfq-anti-hfq2_F        | GAGGGGATCCGGGATGTGCTCT    | Deletion of the hfq and anti-hfq genes                                 | This study|
| Hfq_compole_R          | AAAGCTGGCCAGCTCAATGGAATT  | Deletion of complementation and hfq                                    | This study|
| Hfq_compole_R          | GTCGACTTGATTCTGCACAGTTCC  | Complementation of hfq and anti-hfq                                    | This study|
| M-10anti-hfq_R         | ATGGACCAAGAATCACTAGAAGG   | Deletion of the –10 promoter of anti-hfq                               | This study|
| anti-hfq_3’ Inv_F      | TCTAGAGCGCAACTTATTAGAAGAGG | Overexpression of anti-hfq                                              | This study|
| anti-hfq_3’ Inv_R      | TTCGAGAAACCCAGCGGCTAGAAATAC | Overexpression of anti-hfq                                              | This study|
| anti-hfq_3’ RACE_R      | TATGAAAGGGCTGGTTGATGAAATG | 3’ RACE anti-hfq                                                        | This study|
| anti-hfq_3’ RACE_F      | AATTAGTAGATACCCGTTTTGCCC | 3’ RACE anti-hfq                                                        | This study|
| maselli_Mut_F          | ATGGCCGCTGCAATTAGAATAGG   | Deletion of the RNA3 gene                                               | This study|
| maselli_Mut_R          | TCTGTCGTCGATGAGGCTGATG    | Deletion of the RNA3 gene                                               | This study|
| maselli_Inv_F           | GAGGGGATCCGGGATGTGCTCT    | Deletion of the RNA3 gene                                               | This study|
| maselli_Inv_R           | GCTGAGGAGCTGCAGATGGAATT   | Deletion of the RNA3 gene                                               | This study|
| anti-hfq RNA_T7_F       | TAATACGACGCTCATATAGGGAAGAGG | In vitro transcription of anti-hfq                                      | This study|
| anti-hfq RNA_T7_R       | TATGAGTACGCCCTTTTGGCC     | In vitro transcription of anti-hfq                                      | This study|
| hfq-mRNA_T7_F           | TAATACGACGCTCATATAGGGAAGAGG | In vitro transcription of hfq mRNA                                      | This study|
| hfq-mRNA_T7_R           | TTGTTAGCTGCACAGCTCC      | In vitro transcription of hfq mRNA                                      | This study|
| lpp0644_T7_F            | TAATACGACGCTCATATAGGGAAGAGG | In vitro transcription of lpp0644 mRNA                                | This study|
| lpp0644_T7_R            | TCCAGCTGCTGCAGCCCATCC    | In vitro transcription of lpp0644 mRNA                                | This study|
| hfqOUT_T7_F             | TAATACGACGCTCATATAGGGAAGAGG | In vitro transcription of hfq mRNA                                 | This study|
| anti-hfq_qPCR_F         | TTTAGAAGAGGCTGCTTGTAGAA   | qPCR analysis of the anti-hfq region overlapping hfq mRNA              | This study|
| anti-hfq_qPCR_R         | AATAGTTAGATACCCGTTTTGCC  | qPCR analysis of the anti-hfq region overlapping hfq mRNA              | This study|
| tldD_qPCR_F             | AATCGGGACGCTGATGAGCTG    | qPCR analysis of the tldD mRNA                                           | This study|
| tldD_qPCR_R             | ATCCTACCCCTTTATACAGAG    | qPCR analysis of the tldD mRNA                                           | This study|
| gyrB_qPCR_F             | GACGGTGAAGCGCCAGTTAGA    | qPCR analysis of the gyrB mRNA                                           | This study|
| gyrB_qPCR_R             | TGATGACCAAGGCGGCTTACAT   | qPCR analysis of the gyrB mRNA                                           | This study|
| hfqRNA_NB_F             | TAATACGACGCTCATATAGGGAAGAGG | Northern blot analysis of hfq mRNA                                      | This study|
| hfqRNA_NB_R             | GTTACAGTGTCTGGCTCAATGG   | Northern blot analysis of hfq mRNA                                      | This study|
| hfq_qPCR_F              | TCAGGTCTTCTCTCTCAATGG    | Determination of hfq mRNA half-life                                     | This study|
| hfq_qPCR_R              | AACGACGTGGATAMGGCTGCTT   | Determination of hfq mRNA half-life                                     | This study|
| gapDH_qPCR_F            | GTATGATGACAGGCTTCTAGTGG  | Determination of GAPDH RNA half-life                                    | This study|
| gapDH_qPCR_R            | CATGAGCACGCTTCTCAAGGCC   | Determination of GAPDH RNA half-life                                    | This study|
| 165_qPCR_F              | TTGCTGACGCTGCTGCTACAGATG | Determination of 16S half-life                                         | This study|
| 165_qPCR_R              | AGCTTCTGCTTCTAGCAGATTG   | Determination of 16S half-life                                         | This study|

mutant strains containing the empty plasmid pBC-KS were used as control. For constructing the Anti-hfq mutant strain, site-directed mutagenesis of anti-hfq was performed on pBChfq plasmid as the template using the QuikChange site-directed mutagenesis kit (Qiagen) following the manufacturer’s instructions. Two mutations were introduced in the –10 promoter region of the anti-hfq gene using the primers M-10anti-hfq_F and M-10anti-hfq_R. The resulting plasmid, pBCh-anti-hfq(–10), was introduced into the Δhfq Δanti-hfq deletion mutant, creating the Δanti-hfq(–10) mutant strain.

For overexpression of Anti-hfq sRNA in L. pneumophila, we used the pMMB207C (derived from pTS-10, kindly provided by H. Hilbi [48]). The anti-hfq gene was amplified using primers containing XbaI and PstI restriction sites (anti-hfq OE_F and anti-hfq OE_R primers) and ligated into pMMB207C, linearized using the same restriction enzymes. The resulting plasmid (pMMBanti-hfqOE) and the control plasmid pMMB207C (here named pMMB207C) were introduced via electroporation into wt L. pneumophila Paris strain. For overexpression, IPTG (0.5 mM) was added at an OD600 of 0.8.

**Sequencing of the Δhfq mutant strain.** For whole-genome sequencing, paired-end sequences and a read length of 100 bases were obtained from an Illumina HiSeq platform (Biomics pole Institut Pasteur).
Sequence reads were mapped to a reference genome using SMALT v0.7.4, and single nucleotide polymorphisms (SNPs) were searched for using a standard approach.

**A. castellanii and THP-1 infection assay.** Infection of *A. castellanii* with *L. pneumophila* Paris and its derivatives was done as described previously (49). In brief, *A. castellanii* were washed once with infection buffer (PYG 712 medium without protease peptone, glucose, and yeast extract) and seeded at a density of 4 × 10^6 cells per 25-cm² flask. Wild-type and mutant strains of *L. pneumophila* were grown on BCYE agar to stationary phase, diluted in infection buffer, and mixed with *A. castellanii* at a multiplicity of infection (MOI) of 0.1 or 1 (as indicated in the figure legends). Intracellular multiplication was monitored by plating a 100-µl sample that was centrifuged at 14,500 rpm and vortexed to break up amoeba, at different time points on BCYE plates. The number of bacteria recovered was counted as CFU. In THP-1 cell infection assays, cells were seeded in 12-well tissue culture trays (TPP) at a density of 2 × 10^5 cells/well. THP-1 cells were pretreated with 10 to 80 µM phorbol 12-myristate 13-acetate (PMA) (Sigma) for 72 h to induce differentiation into macrophage-like adherent cells. Stationary-phase *L. pneumophila* bacteria were resuspended in serum-free medium and added to cells at an MOI of 10. After 2 h of incubation, cells were washed with phosphate-buffered saline (PBS) before incubation with serum-free medium. At 2, 24, 48, and 72 h, the supernatant was collected and the cells were lysed with PBS–0.1% Triton X-100. The infection efficiency was monitored by determining the CFU of the different *L. pneumophila* strains recovered on BCYE agar plates. Each infection was carried out in triplicate.

**RNA isolation and Northern blot analysis.** Total RNA was extracted as previously described (50). Wild-type and mutant *L. pneumophila* Paris strains were grown in BYE medium and harvested for RNA isolation at exponential phase (OD_{600} of 1.0 and 2.0) and postexponential phase (OD_{600} of 3 and 4). Total RNA was treated with DNase I and purified using columns (Qiagen). Ten micrograms of total RNA were treated with DNase I and purified using columns (Qiagen). Ten micrograms of total RNA isolated from different conditions (see above) were size separated on 10% denaturing polyacrylamide gels containing 8 M urea (Bio-Rad) and transferred onto positively charged nylon membranes (BrightStar-Plus; Ambion). The membranes were photographed under UV light to capture ethidium bromide staining of rRNA bands for loading controls. RNA was cross-linked to membranes by exposure to UV light for 2 min, and membranes were prehybridized in UltraHyb buffer (catalog no. AM8670; Ambion) for 1 h. RNA probes radioactively labeled with[^32P]UTP (catalog no. BLU007X500UC; PerkinElmer) were generated using the T7 Maxiscript kit (catalog no. AM1314; Ambion), and PCR templates were amplified from genomic DNA using primers listed in Table 2. The membrane was then hybridized at 65°C by adding the radiolabeled probes overnight. Blots were washed twice at the hybridization temperature in 2× SSC–0.1% SDS (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and then washed twice in 0.1× SSC–0.1% SDS. Membranes were wrapped in Saran Wrap and subsequently used to expose films (catalog no. 28906844; GE Healthcare).

**RNA isolation, labeling, and microarray hybridization.** For total RNA extraction, wild-type Paris and the ∆hfq mutant strains were grown in BYE medium *in vitro* and harvested for RNA isolation at postexponential growth phase (OD_{600} of 4). For *in vivo* experiments, *A. castellanii* amoebae were infected with wt or ∆hfq mutant at an MOI of 100 as described above. Cells were cultivated at 20°C and harvested for RNA isolation after 96 h. RNA was prepared in biological triplicates for *in vitro* and biological duplicates for *in vivo* experiments as described above, and all samples were hybridized twice to the microarrays (dye swap). RNA was reverse transcribed with Superscript indirect cDNA kit (Invitrogen) and labeled with Cy5 or Cy3 (Amersham Biosciences, Inc) according to the supplier's instructions. The design of microarrays containing gene-specific 70-mer oligonucleotides based on all predicted genes of the genome of *L. pneumophila* strain Paris (CR628336) and its plasmid (CR628338) was previously described (39). Hybridization was performed following the manufacturers' recommendations (Corning) using 250 pmol of Cy3- and Cy5-labeled cDNA. Slides were scanned on a GenePix 4000A scanner (Axon Instruments). Laser power and/or the photomultiplier tube (PMT) were adjusted to balance the two channels, and the resulting files were analyzed using GenePix Pro 4.0 software. Spots were excluded from analysis in case of high local background fluorescence, slide abnormalities, or weak intensity.

Data normalization and differential analysis were conducted using the R software ([http://www.R-project.org](http://www.R-project.org)). No background subtraction was performed, but a careful graphical examination of all the slides was conducted to ensure a homogeneous, low-level background in both channels. A loess normalization (51) was performed on a slide-by-slide basis (BioConductor package marray; [https://www.bioconductor.org/packages/release/bioc/html/marray.html](https://www.bioconductor.org/packages/release/bioc/html/marray.html)). Differential analysis was carried out separately for each comparison between two time points, using the VM method ([VarMix package (52)](https://www.bioconductor.org/packages/release/bioc/html/VarMix.html)), together with the Benjamini and Yekutieli P value adjustment method (53). Empty and flagged spots were excluded from the data set, and only genes with no missing values for the comparison of interest were analyzed.

**Determination of RNA half-life and quantitative RT-PCR.** Wild-type and RNase III gene deletion mutant strains of *L. pneumophila* were grown to an OD_{600} of 2.5 in BYE medium. Cells were subsequently treated with rifampin (final concentration of 500 µg/ml). Aliquots were removed at time zero (just before treatment) or after 5, 10, or 20 min of treatment. Cells were harvested by centrifugation in a tabletop centrifuge at 13,000 rpm for 1 min. Pellets were flash frozen in liquid nitrogen, and subsequently RNA was isolated as described above. Quantitative reverse transcription-PCR (qPCR) was then performed as described previously (39) at CDNA concentrations ranging from 5 ng to 5 × 10^{-3} ng. Primers used are listed in Table 2. Primer efficiencies were evaluated by generating a standard curve with serial dilutions, which indicated an efficiency of 90% to 110% for all primers used. The specificity of the amplified product and primer dimer formation was verified for each primer set by the presence of a single peak in a dissociation step carried out after each run. The absence of contaminating DNA was verified using control samples for each RNA sample for which no prior reverse transcription reaction had been carried out.

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**Table 2**

| Experiment | Description |
|------------|-------------|
| RNA isolation and Northern blot analysis | Total RNA was extracted as previously described (50). Wild-type and mutant *L. pneumophila* Paris strains were grown in BYE medium and harvested for RNA isolation at exponential phase (OD_{600} of 1.0 and 2.0) and postexponential phase (OD_{600} of 3 and 4). Total RNA was treated with DNase I and purified using columns (Qiagen). Ten micrograms of total RNA isolated from different conditions (see above) were size separated on 10% denaturing polyacrylamide gels containing 8 M urea (Bio-Rad) and transferred onto positively charged nylon membranes (BrightStar-Plus; Ambion). The membranes were photographed under UV light to capture ethidium bromide staining of rRNA bands for loading controls. RNA was cross-linked to membranes by exposure to UV light for 2 min, and membranes were prehybridized in UltraHyb buffer (catalog no. AM8670; Ambion) for 1 h. RNA probes radioactively labeled with[^32P]UTP (catalog no. BLU007X500UC; PerkinElmer) were generated using the T7 Maxiscript kit (catalog no. AM1314; Ambion), and PCR templates were amplified from genomic DNA using primers listed in Table 2. The membrane was then hybridized at 65°C by adding the radiolabeled probes overnight. Blots were washed twice at the hybridization temperature in 2× SSC–0.1% SDS (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and then washed twice in 0.1× SSC–0.1% SDS. Membranes were wrapped in Saran Wrap and subsequently used to expose films (catalog no. 28906844; GE Healthcare).
| RNA isolation, labeling, and microarray hybridization | For total RNA extraction, wild-type Paris and the ∆hfq mutant strains were grown in BYE medium *in vitro* and harvested for RNA isolation at postexponential growth phase (OD_{600} of 4). For *in vivo* experiments, *A. castellanii* amoebae were infected with wt or ∆hfq mutant at an MOI of 100 as described above. Cells were cultivated at 20°C and harvested for RNA isolation after 96 h. RNA was prepared in biological triplicates for *in vitro* and biological duplicates for *in vivo* experiments as described above, and all samples were hybridized twice to the microarrays (dye swap). RNA was reverse transcribed with Superscript indirect cDNA kit (Invitrogen) and labeled with Cy5 or Cy3 (Amersham Biosciences, Inc) according to the supplier's instructions. The design of microarrays containing gene-specific 70-mer oligonucleotides based on all predicted genes of the genome of *L. pneumophila* strain Paris (CR628336) and its plasmid (CR628338) was previously described (39). Hybridization was performed following the manufacturers' recommendations (Corning) using 250 pmol of Cy3- and Cy5-labeled cDNA. Slides were scanned on a GenePix 4000A scanner (Axon Instruments). Laser power and/or the photomultiplier tube (PMT) were adjusted to balance the two channels, and the resulting files were analyzed using GenePix Pro 4.0 software. Spots were excluded from analysis in case of high local background fluorescence, slide abnormalities, or weak intensity.
| Determination of RNA half-life and quantitative RT-PCR | Wild-type and RNase III gene deletion mutant strains of *L. pneumophila* were grown to an OD_{600} of 2.5 in BYE medium. Cells were subsequently treated with rifampin (final concentration of 500 µg/ml). Aliquots were removed at time zero (just before treatment) or after 5, 10, or 20 min of treatment. Cells were harvested by centrifugation in a tabletop centrifuge at 13,000 rpm for 1 min. Pellets were flash frozen in liquid nitrogen, and subsequently RNA was isolated as described above. Quantitative reverse transcription-PCR (qPCR) was then performed as described previously (39) at CDNA concentrations ranging from 5 ng to 5 × 10^{-3} ng. Primers used are listed in Table 2. Primer efficiencies were evaluated by generating a standard curve with serial dilutions, which indicated an efficiency of 90% to 110% for all primers used. The specificity of the amplified product and primer dimer formation was verified for each primer set by the presence of a single peak in a dissociation step carried out after each run. The absence of contaminating DNA was verified using control samples for each RNA sample for which no prior reverse transcription reaction had been carried out.
Western blot analysis. Samples were denatured at 90°C for 10 min and separated on a 4 to 20% gradient SDS-PAGE gel (Bio-Rad) and transferred using a Trans-Blot Turbo transfer system (Bio-Rad). The membrane was stained with black amide or red ponceau solutions for loading controls and blocked in 1.2% bovine serum albumin (BSA) in Tris-buffered saline with Tween 20 (TBS-Tween) for 1 h at room temperature. Membranes were incubated overnight at 4°C with anti-Hfq or anti-FlaA primary antibodies that we generated. Briefly, Hfq and FlaA 6×His protein production was induced at an OD_{600} of 0.5 by 0.4 mM IPTG at 37°C for 4 h. Hfq and FlaA-6x-His proteins were purified using nickel-nitrilotriacetic acid (Ni-NTA) agarose beads and a Poly-Prep chromatography column. The resulting proteins were injected into rabbits, and crude sera were recovered 90 days later (Thermo Fisher Custom Antibody Services). Specific immunoglobulins were purified from serum samples using a 1.0-ml Hitrap affinity NHS column (GE Healthcare) according to the manufacturer’s instructions. The antibody specificity and purity were assessed by Western blotting against the purified proteins. Membranes were incubated overnight at 4°C with Hfq or FlaA primary antibodies (diluted 1:2,000). The membranes were washed three times for 5 min each in TBS–0.5% Tween at room temperature. The membrane was incubated for 1 h at room temperature with the secondary antibody, horseradish peroxidase (HRP)-labeled anti-rabbit (Dako) in TBS–0.5% Tween before the membrane was washed as described above. Signals were visualized using the ECL2 prime Western blot detection kit (Pierce) and the G-Box imaging system (Syngene).

Rapid amplification of the 3’ end of cDNA (3’ RACE). Amplification of the 3’-end region of anti-hfq was performed using total RNA purified from wt L. pneumophila Paris strain in the early exponential growth phase (OD_{600} of 2) as described above. Total RNA was treated with DNase I (Roche), incubated at 37°C with 10 U tobacco acid pyrophosphatase (TAP) (Epicentre) as previously described (28), subjected to phenol-chloroform-isooamyl alcohol (IAA) extraction (25:24:1), and precipitated overnight at −20°C with 10% 3 M sodium acetate (pH 5.2), 2% glycogen (20 mg/ml), and 2.5 volume of ethanol. For the 3’ adapter ligation, a mix of 3’ RNA adapters P-UCGUAGCGUCUCCUGCUUG-UIdT (100 μM) was ligated to the processed RNA using the T4 RNA ligase (Epicentre) according to the manufacturer’s instructions. cDNA was then synthesized as described above, and amplification (primers anti-hfq_3’RACE_F and anti-hfq_3’RACE_R) products were fractionated in a 2% agarose gel. After staining with ethidium bromide, the sole band obtained of about 100 nucleotides (nt) was cut from the gel and purified using the NucleoSpin Extract kit (Macherey-Nagel). The purified size-selected cDNA fragment was cloned into the pGEM-T Easy (Promega) plasmid, and the cloned fragment was sequenced.

RNA in vitro transcription and labeling. Anti-hfq (103-nt), hff mRNA (335-nt), lpp0644 (137-nt), and hffOUT (180-nt) genes used for electrophoretic mobility shift assay (EMSA) gel mobility assays and hff mRNA (128 nt) used for Northern blot analyses were amplified from bacterial DNA with primers containing the T7 promoter at the 5’ end of the Anti-hfq transcript and the first 26 codons of the hff transcript, and lpp0644 as a control. Briefly, 25 nM anti-hfq, together with 0, 10, 15, 30, or 50 nM hff full transcript or 0, 15, 30, or 50 nM hffOUT probes was incubated with buffer containing 10 mM Tris-CL (pH 8.3) and 0.1 mM EDTA, denatured at 70°C for 5 min, and cooled down for 15 min at room temperature. In vitro formation of complexes between Hfq and hff mRNA or Anti-hfq sRNA (25 nM) in vitro was analyzed by EMSA using 0.05, 0.08, 0.1, 0.16, 0.22, 0.27, 0.5, and 1 μM His-tagged Hfq (Hfq6XHis) and supplemented with 5× structure buffer (50 mM Tris-Cl [pH 8.0], 250 mM NaCl, 250 mM KCl, 200 ng/ml tRNA) and incubated at 37°C for 15 min.

For the formation of ternary complexes, 25 nM radioactively labeled Anti-hfq and 25 nM cold hff mRNA probes were incubated alone or with 1 μM His-tagged Hfq (Hfq6XHis) protein for 0.5, 1.5, or 2.5 min and supplemented with 5× structure buffer (50 mM Tris-Cl [pH 8.0], 250 mM NaCl, 250 mM KCl, 200 ng/ml tRNA). For a control, 25 nM radioactively labeled Anti-hfq or hff mRNA probes were incubated with 0.5 and 1 μM (Hfq6XHis) for 10 min at room temperature. Prior to loading, reactions were mixed with native loading buffer, and samples were loaded onto 6 or 8% polyacrylamide 1× Tris-acetate-EDTA gel in 1× Tris-acetate EDTA running buffer. Following electrophoresis at 4°C, the gels were wrapped in Saran Wrap and subsequently exposed to films (GE Healthcare).

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FIG S1, TIF file, 3.2 MB.
FIG S2, TIF file, 9.4 MB.
TABLE S1, DOCX file, 0.1 MB.
TABLE S2, DOCX file, 0.1 MB.
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