

## The Hfq Homolog in *Legionella pneumophila* Demonstrates Regulation by LetA and RpoS and Interacts with the Global Regulator CsrA

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**A gene in *Legionella pneumophila* that has significant homology to published *hfq* genes demonstrated regulation by RpoS and the transcriptional regulator LetA. Additionally, Hfq has a positive effect on the presence of transcripts of the genes for CsrA and the ferric uptake regulator Fur. Mutants lacking *hfq* demonstrate defects in growth and pigmentation and slight defects in virulence in both amoeba and macrophage infection models. Hfq appears to play a major role in exponential-phase regulatory cascades of *L. pneumophila*.**

The pleiotropic regulator Hfq (host factor 1, Q $\beta$  phage replication) was first characterized in *Escherichia coli* as necessary for replication of the Q $\beta$  phage (33). Hfq has been shown to contribute to virulence in *Yersinia enterocolitica*, *Pseudomonas aeruginosa*, and *Vibrio cholerae* (7, 23, 31); to adaptation to the intracellular milieu in *Brucella* species; and to stress response in *Listeria monocytogenes* (6, 26). Many of the defects seen in the *hfq* mutants concern efficient translation of the stationary-phase sigma factor RpoS through interaction with various small RNAs (sRNAs) (5, 6, 16, 25, 38). One functional mechanism is that the interaction with Hfq leads to posttranscriptional gene expression modulation through complementary base pairing of the sRNA with *rpoS* mRNA (4). The interaction of Hfq with sRNA in the regulation of *rpoS* mRNA is apparently essential for stress responses in *E. coli* and *P. aeruginosa* (31, 33). In *L. monocytogenes*, Hfq is transcriptionally regulated through the functional homolog to RpoS, the alternative stress sigma factor  $\sigma^B$  (6), while in *V. cholerae*, the sigma factor RpoE and not RpoS interacts with Hfq (7). Additionally, Hfq binds directly to other mRNAs, affecting stability (20, 34) and stimulating elongation of poly(A) tails (11). Hfq is capable of stabilizing RNA molecules through protection of the molecule from RNase E degradation (9, 18, 21) or stimulating decay of the mRNA (36).

Legionellae are intracellular pathogens that require a host cell in order to replicate. When *Legionella* bacteria enter a host cell, it becomes essential to switch off survival and transmission genes and turn on those for replication. In *Legionella pneumophila*, the species responsible for most cases of Legionnaires' disease, RpoS regulation and function have been shown to diverge from the characteristic RpoS stationary-phase trait induction as found in the enterobacteria (1, 12). Recently it was reported that RpoS transcripts in legionellae appear mainly during exponential-phase growth, disappearing as the bacteria enter stationary phase (2). RpoS functions within a network of regulatory genes, including those encoding the two-

component system LetA/S and the global regulatory protein CsrA. All four of these genes have been shown to be important for growth phase-dependent cellular processes (8, 14, 17, 22). The global regulator CsrA is essential for replication in macrophages and for repressing stationary-phase traits, such as flagellar expression, during replication (8, 10, 22). As nutrients are depleted, external signals call for the relief of CsrA repression, accomplished by LetA, allowing transmission and survival trait expression (13, 14). Both CsrA and LetA also affect RpoS expression (10, 17). In an attempt to further elucidate the regulatory system in legionellae, we identified a gene with significant homology to the well-described gene *hfq*. By using an *hfq* deletion mutant, we characterized the relationship of Hfq with the regulatory proteins CsrA, LetA, and RpoS, as well as the effects of this deletion on the growth and infectivity of the bacteria.

**Transcription and expression of the *Legionella* Hfq homolog.** The *Legionella* homolog of Hfq lies in open reading frame (ORF) lpg0009 of the Legionella Genome Project (<http://legionella.cu-genome.org/>; last accessed November 2004). The *Legionella* Hfq protein is 64% identical to that in *E. coli* and 67% identical to that found in *P. aeruginosa*. Further analysis of the amino acid sequence revealed conservation of the amino acids at positions 8 (Gln), 42 (Tyr), 56 (Lys), and 57 (His), which have been proposed to be necessary for RNA binding (28, 32). However, the conserved chromosomal gene arrangement found in *E. coli*, *V. cholerae*, and other organisms is only partially found in *L. pneumophila*. In *E. coli*, the *hfq* gene is part of a multigene operon in which *hfq* is preceded by the gene *miaA* and followed by *hflX*, *hflK*, and *hflC*. The *Legionella hfq* gene is followed by an ORF with homology to the *hflX* gene, as seen in *E. coli*; however, these are the only two genes from the operon that are present in this region of the *Legionella* genome. Upstream of the *L. pneumophila hfq* gene is an ORF, running in the transverse direction, with homology to a *pspA* gene. Downstream of the *hflX* homolog ORF are two ORFs, again with transverse orientation, the first being a thioredoxin homolog and the further one being a putative phospholipase C protein. With the QIAGEN One-Step reverse transcription-PCR kit, experiments with JR32 wild-type exponential-phase cultures grown at 37 and 30°C determined that

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TABLE 1. PCR Primers used in this study

Primer	Sequence	Reference
Hfq M F1	5'-ACATGCATGCCTCCTGAACGATCTTGAGCAGGA-3'	This study
Hfq M R1	5'-GGGGTACCCAGGTACCTTTTCCTTGCGC-3'	This study
Hfq M F2	5'-GGGGTACCCAGTTGTTCCCTTCGAATGGTC-3'	This study
Hfq M R2	5'-CGCGGATCCCAAGGTTGCAAACAATTGATCAGC-3'	This study
Hfq ORF F <sup>b,c</sup>	5'-GGGGTACCCTCCTGAACGATCTTGAGCAG-3'	This study
Hfq ORF F2 <sup>a</sup>	5'-CATCAGTAGCCTGATTCTAGATGTC-3'	This study
Hfq ORF R <sup>a,c</sup>	5'-AAACTGCAGCACCGCCTTGTGGACGTT-3'	This study
RT F2 <sup>b</sup>	5'-TCAGTGTTCCTGGTCAATGGT-3'	This study
RT R1 <sup>b</sup>	5'-GTCTGCCACAGTTCCTTCT-3'	This study
RT R2 <sup>b</sup>	5'-CCGTTTTTCATCTACAGACTC-3'	This study
csrA uni <sup>a</sup>	5'-TTGATTTTGACTCGGCGTATAG-3'	10
csrA rev <sup>a</sup>	5'-GATTCTTTTTCTTGTGTATGCGTA-3'	10
fliA U3 <sup>a</sup>	5'-TTAGTGTACTCTGTTTG-3'	17
fliA R5 <sup>a</sup>	5'-TTTATTCCGGTAATCTTGATC-3'	17
flaA uni <sup>a</sup>	5'-GTAATCAACACTAATGTGGC-3'	17
flaA rev <sup>a</sup>	5'-GTTGCAGAATTTGGTTTTTGGTC-3'	17
rpoS F <sup>a</sup>	5'-AAAACGTCTTTCGATAACCTG-3'	4
rpoS R <sup>a</sup>	5'-CCAAGCGAGGATCCGTTTTTC-3'	4
Fur Luc F	5'-CGCGGATCCCCAGGTTACCTTTCCTCG-3'	This study
Fur Luc R	5'-CCCAAGCTTCTGTAACGAATTCGAGC-3'	This study
Fur N F <sup>a</sup>	5'-CACATTACCTCGTATCAAGG-3'	This study
Fur N R <sup>a</sup>	5'-GTCATGATGTTTCGCCTTGAG-3'	This study

<sup>a</sup> Used for single-stranded DNA probe synthesis.

<sup>b</sup> Used for reverse transcription-PCR.

<sup>c</sup> Used for complementation.

the *hfq-hflX* genes are cotranscribed (data not shown). In the *hfq* mutant background, no signal was obtained from the primers spanning the region of the *hfq* gene into the *hflX* gene (RT F2 and RT R2). Experiments with a primer 168 nucleotides upstream of the *hfq* gene (Hfq ORF F2) and a primer near the end of the *hfq* gene (RT R1) resulted in no product. Northern blot analysis, conducted as previously described (10), of *hfq* transcript expression in wild-type strain JR32, a serogroup 1 isolate, demonstrated strong expression during exponential-phase growth (optical density [OD] = 1.2 and 1.5) at 30 and 37°C. No transcripts were detected in stationary-phase bacteria (OD = 1.8) (data not shown). All primers used in this work are listed in Table 1.

#### Hfq interaction with exponential-phase genes *csrA* and *fur*.

An *hfq* mutant was constructed through homologous recombination in wild-type strain JR32 and verified by standard Southern blot analysis. Cultures for determination of growth and pigmentation kinetics were grown in either standard *Legionella* BCYE (buffered charcoal-yeast extract) medium or chemically defined medium (24). Experiments were conducted as previously described (10). The *hfq* mutant revealed defects in growth at both 37°C (Fig. 1a) and 30°C (Fig. 1b) in standard BYE growth medium, as well as in chemically defined minimal medium (Fig. 1c). Pigmentation, a virulence-associated trait, was reduced only when bacteria were grown at 30°C (Fig. 1d). Interestingly, the growth and pigmentation defects seen in the *hfq* mutant were similar to defects seen in the JR32 *csrA* mutant at 30°C (10). Under all conditions, the growth deficit appeared to be a delayed lag phase rather than a deficit in replication ability. Growth and pigmentation defects could be complemented by introduction of plasmid pBCKS carrying the *hfq* gene under the control of its own promoter region (pBC-hfq).

As *hfq* expression appeared to be growth phase dependent

and mutants exhibited growth and pigmentation defects similar to those seen in the *csrA* mutant, we examined the relationship of *hfq* with the global regulatory protein CsrA. We examined *csrA* transcript expression in the *hfq* mutant in comparison with wild-type expression through standard Northern blot analysis. CsrA is normally expressed in replicative-phase cells during early and mid-exponential-phase growth and is necessary for replication in amoebae and macrophages (10, 22). In comparison with those in JR32 wild-type cells, *csrA* transcripts were present but reduced in the *hfq* mutant (Fig. 2a). Complementation of the mutant with pBC-hfq resulted in a wild-type level of *csrA* transcript expression. Experiments carried out at 30 and 37°C revealed the same expression pattern among the wild type, the mutant, and the complemented strain (*hfq/C*).

A *csrA* deletion mutant is characterized by premature expression of transmission and stationary-phase traits. *fliA* and *flaA* transcript expression at mid-exponential phase, as well as production of the flagellar protein FlaA, is seen in the *csrA* deletion mutant (10, 22). Analysis of these traits revealed no defects in the *hfq* mutant compared to the wild type (data not shown) at either 30 or 37°C. Western blot analysis with a *Legionella*-specific FlaA antibody (15) provided by K. Heuner (Würzburg University) also revealed no differences from wild-type expression. Because of this flagellar phenotypic difference in the *hfq* and *csrA* mutants, CsrA protein levels were examined in the *hfq* mutant. Western blot assays with an *E. coli* CsrA-specific antibody (provided by T. Romeo, Emory University) revealed no significant difference in CsrA protein levels between the wild type and the *hfq* mutant (Fig. 2b). In both strains, as well as an *rpoS* mutant strain, CsrA protein was present during exponential-phase growth and at the beginning of the stationary phase. For comparison, our regulatory *csrA* mutant, which still contained minute amounts of *csrA* mRNA, did not produce detectable amounts of the corresponding pro-

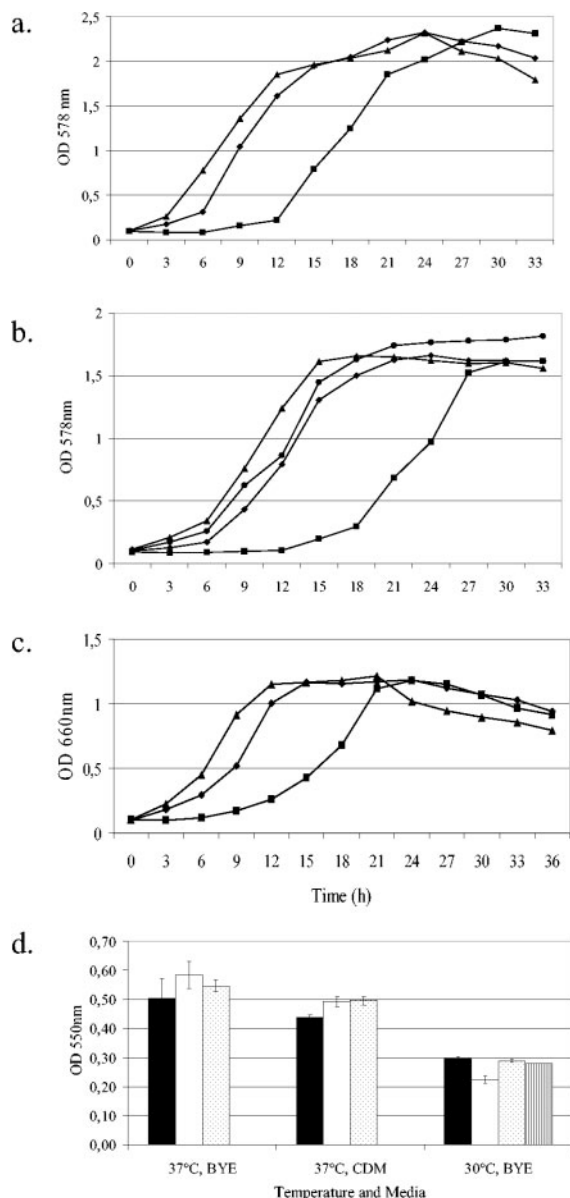


FIG. 1. Growth curves of the wild-type (◆), *hfq* mutant (■), and *hfq/pBC-hfq* (▲) strains in BYE medium at 37°C (a), BYE medium at 30°C (b), with additional curves for *hfq* pMMB2002*csrA* (●) and CDM at 37°C (c), demonstrate growth defects of the *hfq* mutant. (d) Pigment production by wild-type strain JR32 (■), the *hfq* mutant (□), and the *hfq/C* strain (▨) at 37°C in BYE and CDM and 30°C in BYE medium (with pigment production from *hfq/pMMB2002csrA* (▩)).

tein. Therefore, although *csrA* mRNA levels were reduced in the *hfq* mutant, the amount present was capable of being translated to produce levels of CsrA protein necessary for repression of stationary-phase traits. We therefore tested if we could complement the growth and pigmentation defects of the *hfq* mutant by expressing *csrA* from a plasmid. With the expression of *csrA* from plasmid pMMB2002 (27), we were able to restore normal growth and pigment production to the *hfq* mutant at 30°C, demonstrating that these defects are at least in part due to the interaction of Hfq with *csrA* RNA (Fig. 1b).

In the process of identifying genes whose expression is af-

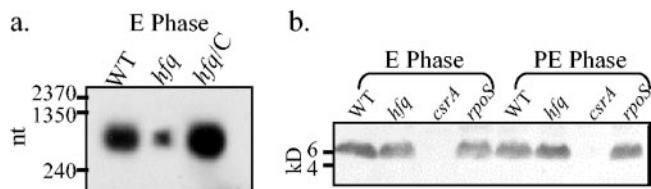


FIG. 2. (a) Determination of *csrA* transcription by RNA Northern blot assay. Total RNA (10 µg) isolated from the wild-type (WT), *hfq* mutant, and *hfq/C* strains at an OD at 578 nm of 1.2 was applied to a membrane and hybridized with a *csrA*-specific probe. Strains were grown at 30°C. (b) CsrA protein levels determined by Western blot assay. Ten-microgram samples of protein from exponential (E)- and postexponential (PE)-phase cultures of the wild-type, *hfq* mutant, *csrA* mutant, and *rpoS* mutant strains were analyzed. Experiments were performed four times with independent RNA and protein samples. nt, nucleotides; kD, kilodaltons.

ected by loss of the Hfq protein, we also examined the transcription kinetics of the *fur* gene and the translation efficiency of the *fur* promoter region in an *hfq* mutant background. The interaction of the Hfq protein with the ferric uptake regulator has been described in *E. coli*, functioning through the *rhyB* sRNA (35). Although not as prominent as the reduction of *csrA*, the amount of *fur* transcript was reduced in the *L. pneumophila* *hfq* mutant and clearly complemented to more than wild-type levels with the introduction of the *hfq* gene in *trans* on multicopy plasmid pBCKS (Fig. 3a). Further analysis at 30 and 37°C with a luciferase reporter gene fusion to the *fur* promoter region revealed high activity of the *fur* promoter in wild-type cells during exponential-phase growth, as expected. Luciferase assays were conducted as previously described (17). In comparison, the same pattern of expression was present but clearly reduced in the *hfq* mutant background. Interestingly, this same reduction in expression was also seen in the *csrA* and *rpoS* mutants (Fig. 3b).

In order to determine if the defect in Fur expression was a cause for the growth defect, we examined the growth kinetics of the wild type, the mutant, and the complemented strain in BYE medium with twice the normal amount of iron supplement (120 µg) added, as well as in CDM without iron. Experiments were conducted at 30°C, as this is the temperature at which the greatest variation between the wild type and mutant exists. Interestingly, in medium without an iron source the JR32 and *hfq/C* strains were able to reach OD levels seen in complete CDM but took longer to reach this level. The *hfq* mutant, however, grew poorly in this medium (Fig. 3c). In medium containing excess iron, the *hfq* mutant was able to grow at wild-type rates; however, pigment production remained lower than that of the wild type (data not shown). These data point to a role for Hfq in the iron uptake and storage system of *L. pneumophila*.

**Regulation of *hfq* by RpoS and LetA.** LetA has been well characterized as a activator of stationary-phase phenotypes through relief of CsrA repression (3, 14). We therefore examined the relationship of *hfq* and *letA*. *hfq* expression levels of *letA* mutant cultures grown at 30°C to the early exponential, mid-exponential, and early stationary phases were compared to wild-type expression levels. At mid-exponential phase, when *hfq* transcripts were at their highest in wild-type cells, practically no transcripts were found in the *letA* mutant (Fig. 4a).

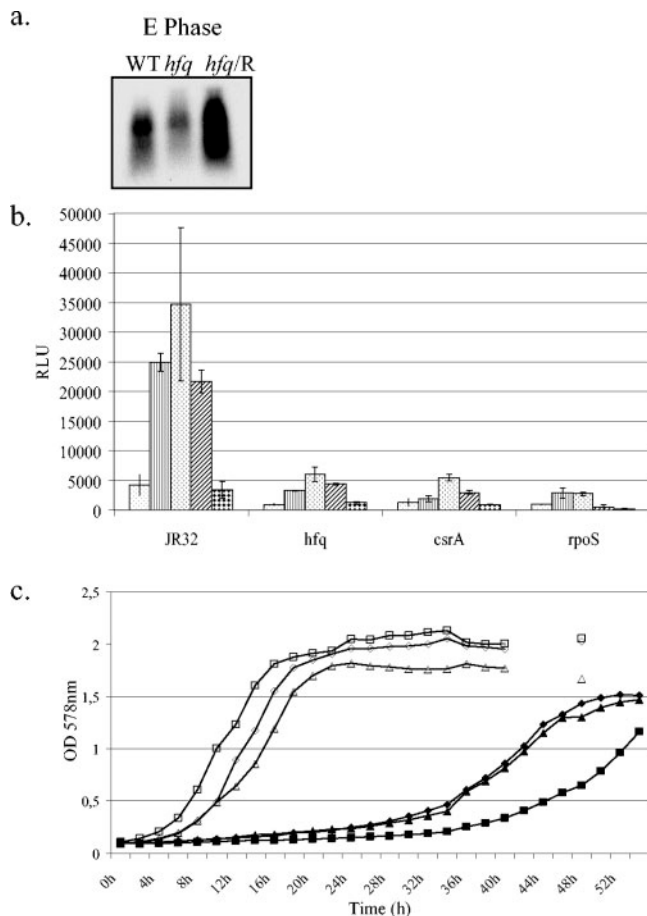


FIG. 3. (a) Determination of *fur* transcription by RNA Northern blot assay. Total RNA (30  $\mu$ g) isolated from the wild-type (WT), *hfq* mutant, and *hfq/C* strains at an OD at 578 nm of 1.2 was applied to a membrane and hybridized with a *fur*-specific probe. Bacteria were grown at 30°C. Experiments were performed three times with independent RNA isolates. E, exponential. (b) Luciferase production (relative light units [RLU]) of the JR32, *hfq* mutant, *csrA* mutant, and *rpoS* mutant strains containing a *fur*-luciferase fusion. Bacteria were grown in BYE medium at 30°C, and samples were taken every 2 h for determination of OD at 578 nm and luciferase activity. Shown are luciferase activities at ODs of 0.5 ( $\square$ ), 1.2 ( $\blacksquare$ ), 1.5 ( $\square$ ), and 1.8 ( $\blacksquare$ ) and in the late postexponential phase ( $\blacksquare$ ). Averages of two experiments with the standard deviations are presented. (c) Growth kinetics of the JR32 ( $\blacklozenge$ ), *hfq* ( $\blacksquare$ ), and *hfq/C* ( $\blacktriangle$ ) strains at 30°C in BYE with excess iron (open symbols) and in chemically defined medium with no iron supplement (closed symbols).

This defect could be complemented when the *letA* gene was introduced into the mutant on plasmid pMMB-*letA* (17). Interestingly, at stationary phase, when *hfq* transcripts are no longer detectable in wild-type cells, we could detect more *hfq* transcripts in the *letA* mutant than seen during the exponential phase (Fig. 4b). It has been previously shown that RpoS is reduced in a *letA* mutant background (17). Therefore, we were interested in determining if the reduction in *hfq* transcripts in the *letA* mutant was due solely to the loss of LetA or perhaps to the reduction of RpoS. Investigations of transcript expression in an *rpoS* mutant (generously provided by H. Shuman, Columbia University) showed a very low or no signal of *hfq* transcripts at both 30 and 37°C (Fig. 4c). The increase in *hfq*

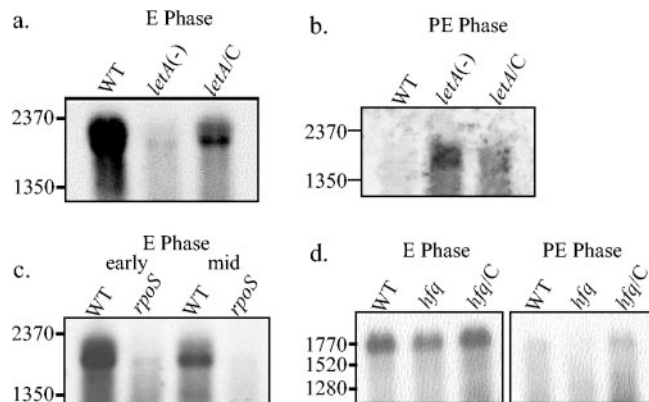


FIG. 4. Determination of *hfq* transcription by RNA Northern blot assay. Total RNA (10  $\mu$ g) isolated from the wild-type (WT), *letA* mutant, and *letA*/pMMB-*letA* strains at an OD at 578 nm of 1.5 (a) or 1.8 (b) was applied to a membrane and hybridized with an *hfq*-specific probe. Bacteria were grown at 30°C. Experiments were performed three times with independent RNA isolates. The experiment shown in panel c was performed with total RNA from the wild type and the *rpoS* mutant at an OD at 578 nm of 1.2 or 1.5. (d) Determination of *rpoS* transcription by Northern blot assay. Total RNA (10  $\mu$ g) from the wild type, the *hfq* mutant, and the *hfq/C* strain were probed with an *rpoS*-specific probe. Strains were grown at 30°C, and the experiment was repeated twice with independent RNA samples. E, exponential; PE, postexponential. The values on the left are RNA sizes in nucleotides.

transcripts seen in the *letA* mutant at stationary phase was not seen in the *rpoS* mutant at that time point. Therefore, *hfq* transcription appears to be negatively regulated by the LetA protein in the stationary phase but positively regulated in the exponential phase by the stationary-phase sigma factor RpoS.

Since Hfq has been shown to be necessary for RpoS translation in various other bacterial species (5, 29, 38), we wanted to determine if that was the case for *L. pneumophila*. We examined *rpoS* RNA and protein expression in the *hfq* mutant background in comparison to that in wild-type cells at 30 and 37°C. *rpoS* mRNA in *L. pneumophila* strain LP02 has been reported to be expressed during exponential-phase growth but not during stationary phase (2). We confirmed this expression pattern to also be the case in *L. pneumophila* JR32 (Fig. 4d). Additionally, comparison of *rpoS* transcripts of the *hfq* mutant to those of our wild type showed no significant differences in the expression pattern. Further analysis of the translation of RpoS with luciferase assays (17) also demonstrated no differences between the wild type and the *hfq* mutant (data not shown). This was not caused by a necessary secondary structure of the *rpoS* mRNA for Hfq function, as a reporter gene fusion vector that included the first 20 nucleotides of the coding region also led to no difference in luciferase expression levels between the wild type and the *hfq* mutant. In *L. pneumophila*, *hfq* is apparently regulated by RpoS and is not, under these growth conditions, necessary for RpoS expression. Thus, the regulation is similar to the regulation of *hfq* found in *L. monocytogenes*, where *hfq* expression is regulated by the RpoS functional homolog  $\sigma^B$  (6). In gram-negative bacteria such as *E. coli* and *P. aeruginosa*, an inverse direction of regulation has been observed (29, 30). Along with this difference in Hfq function in comparison with the *E. coli* *hfq* gene, the *Legionella* *hfq* gene is also not able to complement an *E. coli* mutant for

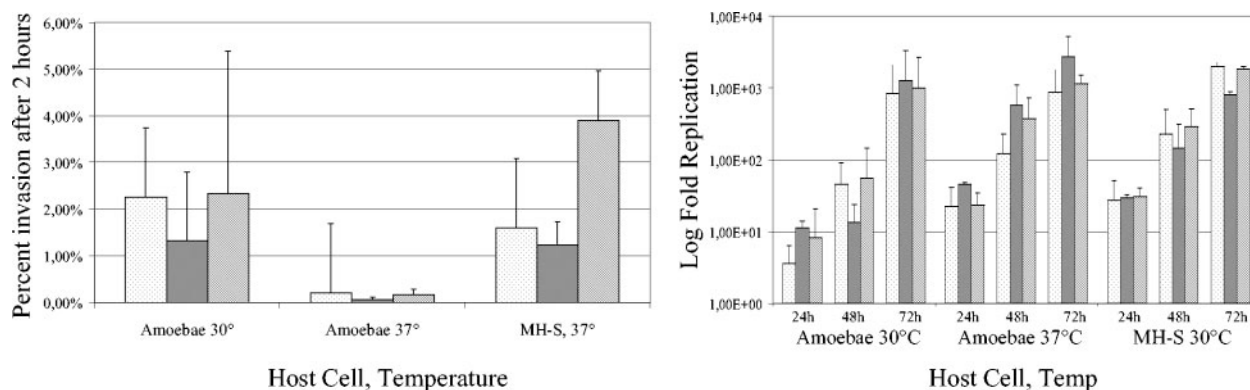


FIG. 5. (a) Invasion capabilities of the *hfq* mutant (■) are slightly less than those of the JR32 (□) and *hfq/C* (▨) strains in both *A. castellanii* amoebae and macrophage cell line MH-S. The invasion capability of all of the strains was less in host amoebae when they were grown at 37°C. (b) No significant differences exist between the replication ability of the JR32 (□), *hfq* (■), and *hfq/C* (▨) strains in amoebae infected at either 30 or 37°C. In infected macrophages, after 72 h the *hfq* mutant has a slight defect in comparison to wild-type replication ability.

Q $\beta$  phage replication (30), demonstrating further functional differences in this protein for the two species (data not shown).

Hfq has been shown to be capable of stabilizing mRNA transcripts by protecting the transcripts from RNase E degradation (21). Most likely, the interaction of Hfq with *csrA* and *fur* is necessary for stability during the replicative cycle. Analyses of both the *csrA* and *fur* gene sequences demonstrate the presence of AU-rich areas that are possible targets for both RNase E and Hfq binding. From the growth curves we could see that the *hfq* mutant demonstrated more of a prolonged lag phase but, once replication began, was capable of replication at a similar rate and reached the same levels as the wild type. Perhaps this prolonged lag phase is due to degradation of *csrA* transcripts where at the beginning of the replication cycle the first transcripts are immediately degraded. However, over time a threshold level of *csrA* transcripts can be reached where more transcripts are produced than can be simultaneously degraded. The excess of transcripts would then allow translation of the CsrA protein, which is then sufficient for replication and repression of stationary-phase traits. The growth and pigmentation defects found in the *hfq* mutant could then be due at least in part to the reduction in *csrA* or by other CsrA-independent functions. This theory is supported by the ability of plasmid-expressed *csrA* to cure the growth and pigment defects of the *hfq* mutant, as well as by the growth kinetics of *hfq* and *hfq/C* in medium with excess iron or without iron.

A proposed scheme of *hfq* regulation is that in exponential phase RpoS induces the expression of Hfq, which contributes to the stability of exponential-phase RNA, allowing the bacteria to quickly adapt to and efficiently use the replicative environment. Then, when cells respond to signals of nutrient deprivation and must transcend to an infectious, virulent form, LetA, in its role as an inducer of stationary-phase traits (2, 3), either directly or indirectly turns off *hfq* transcription. This turning off of transcription occurs through an RpoS-independent pathway, as *hfq* transcripts are not found in *rpoS* mutants at stationary phase.

Although much about the function of the RNA binding Hfq protein has been published, only few studies have examined its regulation. Our studies open a new area for Hfq research in examining the regulation by the LetA transcriptional activator,

as well as the stationary-phase sigma factor RpoS. Additionally, Hfq is well known to be essential for several regulatory mechanisms through binding with sRNAs. To date, no sRNAs have been identified in legionellae. BLAST searches for homology to the known sRNAs from *E. coli*, salmonellae, or yersiniae have produced no hits in the *Legionella* genome (data not shown). Through the use of Hfq, it may now be possible to identify these molecules and determine what role they may play in regulating the biphasic life cycle of legionellae. Further studies should also concentrate on determining the mechanism behind the proposed stabilization of *csrA* and *fur* mRNA by Hfq.

**Infection assays for virulence in *A. castellanii* und MH-S macrophages.** Because of the pleiotropic effects of a deletion of *hfq*, we were interested to see if this also translates into defects in multiplication in living cells. Therefore, *Acanthamoeba castellanii* host cells were infected with the JR32, *hfq*, and *hfq/C* strains at a multiplicity of infection of 10 in accordance with previously published protocols (17). Although no significant differences were found between the wild type and the *hfq* mutant, the trend demonstrated that the *hfq* mutant invaded with slightly less efficiency than the wild type (Fig. 5a) at both 30 and 37°C. Replication in the amoebae was not affected at 37°C, but at 30°C it demonstrated a lag in replication reminiscent of that seen in the in vitro growth curves (Fig. 5b). The host cells responsible for supporting human infection are alveolar macrophages. The MH-S mouse alveolar macrophage cell line has been demonstrated to be a competent model for *Legionella* infection of the lung (19, 37). Infection assays were conducted by standard protocols. Briefly, MH-S cells ( $10^6$ ) in six-well plates were infected in duplicate at a multiplicity of infection of 10 with either the JR32, *hfq*, or *hfq/C* strain. Bacterial suspensions were plated on BCYE to determine accurate bacterial counts. Plates were centrifuged briefly in order to ensure *Legionella* contact with the macrophages and then incubated for 2 h. Cells were then washed twice with phosphate-buffered saline and incubated for a further 60 min in culture medium with gentamicin (200  $\mu$ g/ml). Cells were then again washed twice in phosphate-buffered saline and incubated until the appropriate time point for analysis. To determine invasion ability, the first set of cells was imme-

diately lysed and bacteria were plated on BCYE agar to determine CFU. The percentage of bacteria capable of invading was calculated as the number of CFU at 2 h divided by the number of CFU in the initial bacterial suspension. Replication ability was calculated as the log of the number of CFU at each time point divided by the number of CFU at 2 h. Each experiment was done with duplicate wells for each time point, and the experiment was conducted three times. The macrophage infection assays again showed no significant differences in invasion between the wild type and the mutant (Fig. 5a) but revealed the same trend seen in the amoeba infections. (Fig. 5b). After 72 h of intracellular growth in macrophages, the *hfq* mutant had a significant decrease in number compared to the JR32 and *hfq/C* strains.

We therefore conclude that *hfq* plays a crucial role in the regulatory network of legionellae that is different from that in other gram-negative bacteria. Despite this, *hfq* deletions only slightly affect the ability of legionellae to invade cells and multiply intracellularly. It remains to be examined if the defect results in alterations in virulence in an in vivo animal model.

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