Requirement of upstream Hfq-binding (ARN)$_x$ elements in *glmS* and the Hfq C-terminal region for GlmS upregulation by sRNAs GlmZ and GlmY[†]

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ABSTRACT

Hfq is an important RNA-binding protein that helps bacteria adapt to stress. Its primary function is to promote pairing between *trans*-acting small non-coding RNAs (sRNAs) and their target mRNAs. Identification of essential Hfq-binding motifs in up-stream regions of *rpoS* and *fhlA* led us to ask the question whether these elements are a common occurrence among other Hfq-dependent mRNAs as well. Here, we confirm the presence of a similar (ARN)$_x$ motif in *glmS* RNA, a gene controlled by two sRNAs (GlmZ and GlmY) in an Hfq-dependent manner. GlmZ represents a canonical sRNA:mRNA pairing system, whereas GlmY is non-canonical, interfacing with the RNA processing protein YhbJ. We show that *glmS* interacts with both Hfq-binding surfaces in the absence of sRNAs. Even though two (ARN)$_x$ motifs are present, using a *glmS:gfp* fusion system, we determined that only one specific (ARN)$_x$ element is essential for regulation. Furthermore, we show that residues 66–72 in the C-terminal extension of *Escherichia coli* Hfq are essential for activation of GlmS expression by GlmY, but not with GlmZ. This result shows that the C-terminal extension of Hfq may be required for some forms of non-canonical sRNA regulation involving ancillary components such as additional RNAs or proteins.

INTRODUCTION

Small non-coding RNAs (sRNAs) are used to modulate a wide variety of cellular responses in both bacterial and eukaryotic systems (1–4). In eukaryotes microRNAs and siRNAs (∼21 nt) often regulate translation and RNA turnover and defend the genome from invasive nucleic acids (3). Similarly, in bacterial systems, two main classes of sRNAs have been identified based on the origin of the sRNAs relative to their mRNA target. *Cis*-acting sRNAs base pair extensively with mRNAs as they are transcribed from the opposite strand of their target RNA (4). These systems are often used by mobile genetic elements such as bacteriophages, transposons and plasmids to regulate translation, RNA processing and RNA decay (5,6). Riboswitches represent another ubiquitous class of *cis*-acting RNAs. Riboswitches respond to cellular stimuli by refolding the mRNAs via their 5′-UTRs to trigger translation regulation or transcription termination (7). In contrast, *trans*-acting RNAs are synthesized from different genetic loci than their targets and are widely used to modulate stress responses in bacteria. Unlike *cis*-RNAs, *trans*-acting RNAs can accommodate imperfect base pairing with their target RNAs and often require the RNA-binding protein Hfq to facilitate complex formation.

Hfq was first identified as a host factor involved in the replication of phage Qβ RNA (8). Hfq belongs to the Sm/Lsm family of proteins and associates into a homo-hexameric structure to form the functional nucleic acid-binding core. In general, the nucleic acid-binding Sm1 and Sm2 domains are highly conserved across bacterial species (7–66 amino acids) and form two distinct RNA-binding surfaces, often called the proximal and distal surfaces, respectively (9). These two RNA-binding surfaces are used to promote sRNA–mRNA base pairing by increasing the local concentration of the two binding partners. Bacterial strains lacking Hfq exhibit increased sensitivity to stress and reduced pathogenesis among other phenotypes (10–14). One of the most intriguing features of Hfq-mediated gene regulation is the network of regulatory responses that allows a single sRNA to trigger a multi-dimensional response to stress (15–17). For instance, the sRNA RyhB regulates multiple genes (including *sodB*, *ftnA*, *bfR*, *acnA* and *sdhC*) to help the organism respond to low iron concentration (18). While

the use of Hfq in many other stress pathways such as cold shock (DsrA) (19), oxidative stress (OxyS) (20) and sugar stress (SgrS) (21,22) are well established, a common mechanism describing how Hfq controls all these simultaneously is lacking.

The recent discovery of Hfq-binding elements, so-called $(ARN)_x$ motifs, found in upstream regions of those mRNAs that are regulated by sRNAs has provided new clues for how Hfq helps mediate gene regulation. In both *rpoS* and *fhlA* mRNAs, these Hfq-binding sequences are essential for regulation (23–25) and structural data confirmed that the $(ARN)_x$ motif specifically interacts with the distal RNA-binding surfaces of Hfq hexamers (26). If these upstream Hfq-binding motifs are a common feature of regulated messages, this could help explain how Hfq rapidly targets specific mRNAs for regulation during stress. In theory, as described in the *fhlA* work, Hfq could pre-form complexes with relevant mRNAs and could promote an efficient stress response upon sRNA synthesis if and when it is induced by environmental stimuli (25). Furthermore, recent work on the DsrA-*rpoS* system has shown that Hfq binding to *rpoS* refolds the mRNA for a favorable interaction with the sRNA (27). These observations, although currently limited to a few systems, help explain the complex manner by which Hfq facilitates integrated responses to environmental stress.

To further understand the importance of upstream $(ARN)_x$ motifs in Hfq-dependent gene regulation, the *glmS* system was studied. *glmS* encodes an essential enzyme glucosamine-6-phosphate synthase (GlcN-6-P) (28). In many Gram-positive bacteria (such as *Bacillus subtilis)* the expression of GlmS is tightly regulated by a self-cleaving ribozyme located within *glmS* that catalyzes cleavage in response to high GlcN-6-P concentrations (29). However, in Gram-negative species, GlmS expression is regulated by two sRNAs GlmZ and GlmY, a dual regulatory system that requires Hfq for activity (30). In addition to Hfq and RNase E, YhbJ, a predicted NTPase, was shown to be involved in this regulatory network in *Escherichia coli* (31) (Figure 1). In brief, GlmS is encoded as a poly-cistronic message that also includes GlmU, a gene for a bifunctional enzyme that produces UDP-GlcNAc. In order to individually regulate GlmS expression, RNase E cleaves the *glmU–glmS* transcript at the stop codon of *glmU* to produce a monocistronic *glmS* transcript that is translationally repressed by its 5′-UTR structure (30). GlmZ interacts with *glmS* to unmask the ribosome-binding site (RBS) and promote translation. Additional modulation is provided by YhbJ, a protein involved in GlmZ turnover and thus negatively regulates GlmS expression by decreasing the amount of GlmZ available to activate *glmS* (30). GlmY is a second sRNA involved in this pathway. GlmY has significant sequence and structural homology to GlmZ such that when it is expressed, GlmY acts as a decoy, recruiting YhbJ away from GlmZ (32). Thus, GlmY prevents GlmZ degradation increasing GlmZ concentration and allowing more *glmS*-GlmZ adduct formation, with a net result of enhancing GlmS expression.

In this work, we show that the *glmS* interaction with Hfq is similar to what was previously reported with *fhlA,*

binding both the proximal and distal surfaces of Hfq. Structural characterization of *glmS* revealed two potential $(ARN)_x$ motifs similar to those found in *fhlA* and *rpoS*. Using a GFP fusion system we show that one of the two upstream $(ARN)_x$ elements is essential for Hfq-mediated regulation of *glmS* by both GlmZ and GlmY whereas the other is dispensable. Furthermore, we probed the requirement of the variable C-terminal region of Hfq for regulation—a topic that has been very controversial. We show that the Hfq C-terminus is not required for the classical Hfq regulation involving GlmZ pairing with GlmS. However, at least seven residues of the C-terminal extension (residues 65–72) were essential for GlmY activation of GlmS expression mediated through YbhJ and GlmZ. Thus the C-terminal extension may be involved in systems where ancillary RNA or protein partners (such as YhbJ) must be recruited to the complex during regulation.

MATERIALS AND METHODS

DNA oligonucleotides

The complete list of DNA oligonucleotides used for cloning is provided as Supplementary Table S1.

### Bacterial strains, media and growth conditions

The *E. coli* strain Top10 (Invitrogen) was used for cloning, GFP fusion expression analysis and in all that involved coexpression of GFP fusions with sRNAs and Hfq variants. The Δhfq strains were also derived from Top10 cells and were generated as described previously by replacing *hfq* with chloramphenicol or kanamycin resistance cassettes using the Quick and Easy Conditional Knockout Kit (Gene Bridges) (33). In order to comply with the usage of antibiotics, the chloramphenicol resistance cassette was removed from the $hfq::Cam^R$ strain as described in the Quick and Easy Conditional Knockout Kit (Gene Bridges) operational manual. The successful removal of the Cam^R cassette was verified by antibiotic screening followed by PCR.

Growth in Luria–Bertani (LB) broth or on LB plates at 37°C was used throughout this study. Antibiotics were applied at the following concentrations: 100 µg/ml ampicillin, 100 µg/ml spectinomycin, 34 mg/ml chloramphenicol and 30 mg/ml kanamycin.

Plasmids

Fusion plasmids

All GFP fusion systems were constructed starting from the plasmid pBacEmGH [gift of the Cunningham Lab (34)]. In brief, a *glmS* mRNA fragment from −162 to +21 relative to the translation start site was cloned behind a pBAD promoter to prepare pNS9006 (Supplementary Figure S2). Primers NS20 and NS21 were used to amplify the *glmS* fragment from genomic DNA and inserted into pBacEmGH using NotI and NheI restrictions endonucleases. The plasmid was then verified using DNA sequencing. Preparation of ARN mutants in *glmS* was carried out by synthesizing *glmS* using primer extension where mutations were introduced by the primers that were used. To
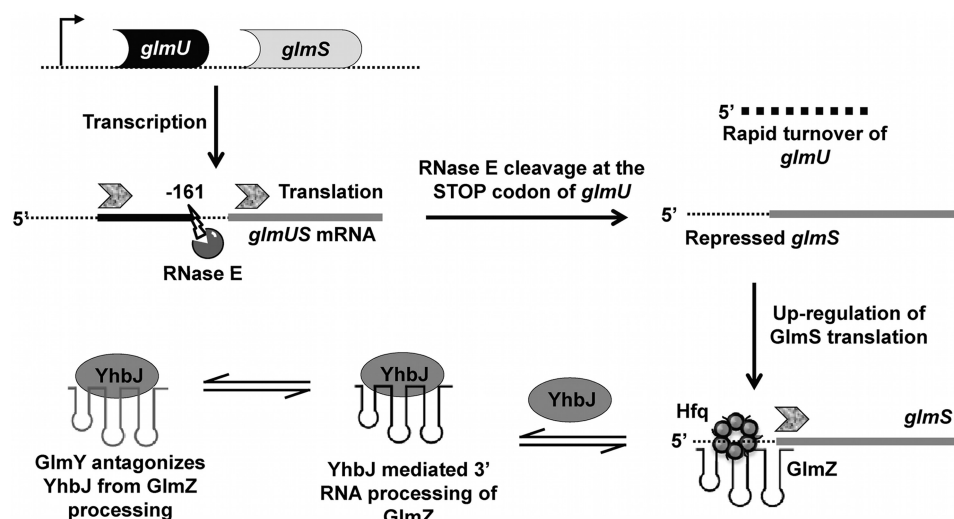
Figure 1. Mechanism of Hfq-mediated regulation of GlmS with sRNAs, GlmZ and GlmY [adapted from (31)]. *glmS* is transcribed as a poly-cistronic message that also includes *glmU*. Upon transcription, RNase E cleaves at the stop codon of *glmU* (at −161 nt position with respect to the translation start site of *glmS*) that separates the translational control of GlmS and GlmU genes. The RNase E cleaved *glmS* transcript is repressed for translation by its 5′-UTR structure that masks the RBS. Upon synthesis of GlmZ sRNA, GlmS translation is activated in an Hfq-dependent manner. Here the GlmZ–*glmS* interaction relieves the inhibitory structure of *glmS* for translation. YhbJ, a predicted NTPase negatively regulates GlmS expression by processing GlmZ sRNA. GlmY, a second sRNA which has structural and sequence homology to GlmZ, antagonizes YhbJ from GlmZ, upregulating GlmS expression. Overall, the GlmS expression is activated by two homologous sRNAs GlmZ and GlmY in a coordinated manner in the presence of Hfq.

construct pNS 9009 that contains the ARN Δ1 mutation in *glmS*, five cycles of PCR were performed using primers NS23, NS24 and NS27 where the desired mutation was introduced in NS24. The PCR amplification was performed using Taq polymerase according to standard procedure. After the first five cycles, the PCR product was diluted 100-fold into a fresh PCR reaction to amplify the 5′- and 3′-regions of *glmS* using primers NS22 and NS21. The amplified PCR fragment was then subjected to NotI/NheI restriction digestion before cloning into pBacEmGH. Similarly pNS9010 (derivative of pNS9006::ARNΔ2, prepared using NS23, NS25 and NS27) and pNS9011 (derivative of pNS9006::ARNΔ1Δ2, prepared using NS23, NS26 and NS27) were constructed. Successful construction of the plasmid was validated by DNA sequencing.

sRNA plasmids
sRNA plasmids, pNS9007 (GlmZ) and pNS9008 (GlmY) were based on pNM12 (35). sRNA fragments for GlmZ (NS37/NS38) and GlmY (NS35/NS36) were amplified using DNA from TOP10 *E. coli* cells. The amplified fragments were then inserted into pNM12 after restriction digestion using MscI/EcoRI. The plasmids were verified by DNA sequencing.

Hfq plasmids
All Hfq constructs were based on the plasmid ptac-KanL94P (Addgene (34) plasmid 15927) (36). Hfq wild-type (pNS9012, NS42/NS43), Hfq65 (pNS9013,NS42/NS44), Hfq72 (pNS9014, NS42/NS45), Hfq87 (pNS9015, NS42/NS46) were amplified using Top10 *E. coli* genomic DNA. Hfq mutants Y25A Hfq (pNS9017), K56A Hfq (pNS9020) and H57A Hfq

(pNS9016) were amplified using a plasmid as template that was prepared previously (9). *Clostridium perfringens* Hfq (pNS9018, NS47/NS48) and *Clostridium difficile* Hfq (pNS9019, NS49/NS50) were amplified using *C. perfringens* and *C. difficile* genomic DNA. The PCR fragments were then restriction digested with BamHI and HindIII and inserted into ptac-KanL94P. The plasmids were verified by DNA sequencing.

Fluorescence data collection

GFP expression of fusion constructs was measured after lysis of cells grown to early stationary phase. All cultures were grown at 37°C in LB media with appropriate antibiotics at concentrations listed above. Bacterial cultures were generated by diluting overgrown overnight cultures. After 3 h of growth, the cultures were induced with 0.1% of arabinose. Cells were then allowed to grow for another 2.5 h. For Hfq complementation studies using the pTac-plasmid system, Hfq expression was induced using 1 mM IPTG after 1 h of growth of diluted cultures before inducing the expression of GlmS-GFP fusion proteins at 3 h. Cells were grown for an additional 2.5 h, before the absorbance was measured at 600 nm to monitor the growth of cells. 3.0 ml of cells were then harvested by centrifuging at 10 000 rpm for 4 min. The cells were then resuspended in 200 µl of lysis buffer (50 mM Tris–HCl pH 7.5, 25 mM NaCl, 2 mM EDTA). To lyse cells 15 µl of lysozyme (20 mg/ml, Fisher), 30 µl of protease inhibitor solution (complete EDTA-free protease inhibitor tablet dissolved in 8 mL Roche) and 30 µl of 1% TritonX-100 was added followed by an incubation of 30 min at 37°C while shaking. The lysed cells were then centrifuged briefly. An amount of 200 µl of the supernatant was

then withdrawn to measure the fluorescence intensities of GFP in a 96-well flat bottom black plate (Corning®). Fluorescence measurements were made at an excitation wavelength of 485 nm and at an emission wavelength of 535 nm using a Tecan GENios Plus multi label plate reader. The detector gain values were consistently set between 95 and 103. All fluorescence measurements were normalized to an identical culture where the expression of GFP was not induced by arabinose to account for cellular fluorescence. All measurements were at least in triplicate and error values for each experiment were based on the standard deviation between independent trials.

RNA preparation for SHAPE and gel shift analysis

A_{18} RNA was purchased from Thermo Scientific Dharmacon Technologies (Lafayette, CO) and deprotected following the manufacturer's protocol. RNA quality was assessed using denaturing PAGE and gel purified. For *in vitro* binding analysis, *glmS* mRNA was transcribed using a DNA fragment that amplifies the *glmS* from −161 to +21 in *E. coli* XL-10 cells using primers NS33 and NS34 and digested with Ssp I prior to transcription. For SHAPE analysis, *glmS* mRNA was *in vitro* transcribed using a DNA template that was amplified from XL-10 cells using primers NS31 and NS32, that includes a structure cassette in the 5′- and 3′-regions as previously described (37). *In vitro* transcription was performed after digesting the amplified product with Ssp I. DsrA was obtained by runoff transcription of pBAU10301 that was digested by Ssp I (38).

Electrophoretic mobility shift assays

All binding reactions were performed in 50 mM Tris–HCl pH 7.5, 100 mM KCl and 10 mM MgCl₂ at room temperature. Prior to any interactions all RNAs were annealed at 90°C for 3 min in buffer, and cooled to room temperature for 30 min. For all reactions 10 μl aliquots were loaded after diluting with loading buffer (10% (w/v) sucrose, xylene cyanol, bromophenol blue) under a power of 5 W on native 5–7% polyacrylamide (37:1) gels in 1× TBE. Dried gels were visualized by phosphorimaging using a Typhoon 9210 imaging system (GE LifeSciences). Quantification was done using ImageQuant 5.1 (Molecular Dynamics) and Kaleidagraph 3.0 (Synergy). Data were fit using nonlinear least-square analysis to a cooperative binding model shown below [Equation (1)]. Here, *L* is the ligand concentration and the cooperatively is indicated by n. Typical values for *n* ranged from 1.5 to 2.1.

$$Q_{\text{Fraction}} = \frac{(L)^n}{K_D + L^n} \tag{1}$$

In the case of A_{18}, DsrA competition assays, the *glmS*•Hfq complex was pre-formed and A_{18} and DsrA was titrated at varying concentrations from 0 to 30 μM.

Chemical SHAPE analysis and footprinting

The method for using SHAPE technology to determine the secondary structure of an mRNA 5′-UTR and the footprinting of Hfq on the folded RNA has been described previously (25,37,39).

Western blot analysis

Total cellular extracts were collected from appropriate strains and were lysed using sonication. Total protein was separated on 15% (w/v) polyacrylamide gels and electroblotted to a PVDF membrane. The membrane was probed with rabbit anti-Hfq polyclonal anti-sera. The antigen–antibody complex was visualized using goat anti-rabbit immunoglobin horseradish peroxidase conjugated antibody (Super Signal West Pico Chemiluminescent Substrate, PIERCE).

## RESULTS

glmS interaction with Hfq

Previous work performed on the *glmS* regulatory system in *E. coli* showed the requirement of Hfq for GlmZ- and GlmY-mediated activation of *glmS* (31,32). While the basic outline of the regulatory network was established, no molecular level understanding was developed. Based on our previous work with OxyS/*fhlA* (25) and DsrA/*rpoS* (24) we asked whether Hfq pre-binds the *glmS* mRNA and how similar the *glmS*–Hfq interaction is relative to other systems. The *glmS* mRNA fragment used throughout this study was 182 nt in length. This segment extends from position −161 nt upstream of the GlmS start codon, which is the RNase E processing site that separates the *glmU–glmS* bicistronic transcript (Figure 1), and extends +21 nt into the coding region. We used this construct to test whether *glmS* behaves in a manner similar to *fhlA* (25), and to determine whether one or both RNA-binding surfaces were involved in the interaction with Hfq (Figure 2).

To measure the binding constant between *glmS* and Hfq, [5′-³²P] *glmS* mRNA was incubated with varying Hfq concentrations from 0 nM to 2 μM (hexamer). The *glmS*–Hfq complex was resolved on native gels to provide a K_D of ~30 nM (Figure 2A). This value was comparable to the interaction between *fhlA*–Hfq and is typical of RNA–Hfq interactions measured previously (9,24,25,40).

To determine which binding surfaces of Hfq interact with Hfq, a competition gel shift assay was carried out where the *glmS**–Hfq complex was pre-formed and competed against *bona fide* distal (A_{18} RNA) and proximal (DsrA) site binders of Hfq. As observed in Figure 2B, the *glmS**–Hfq complex could only be displaced by the simultaneous addition of both DsrA and A_{18} showing that GlmS interacts with both faces of Hfq similar to *fhlA* and *rpoS* (25).

Secondary structure of *glmS* mRNA and Hfq-binding sites probed using SHAPE

SHAPE (37,41) was carried out to determine the secondary structure of *glmS* mRNA to identify its functional elements. Figure 3 depicts the secondary structure for *glmS* mRNA derived from SHAPE constraints,
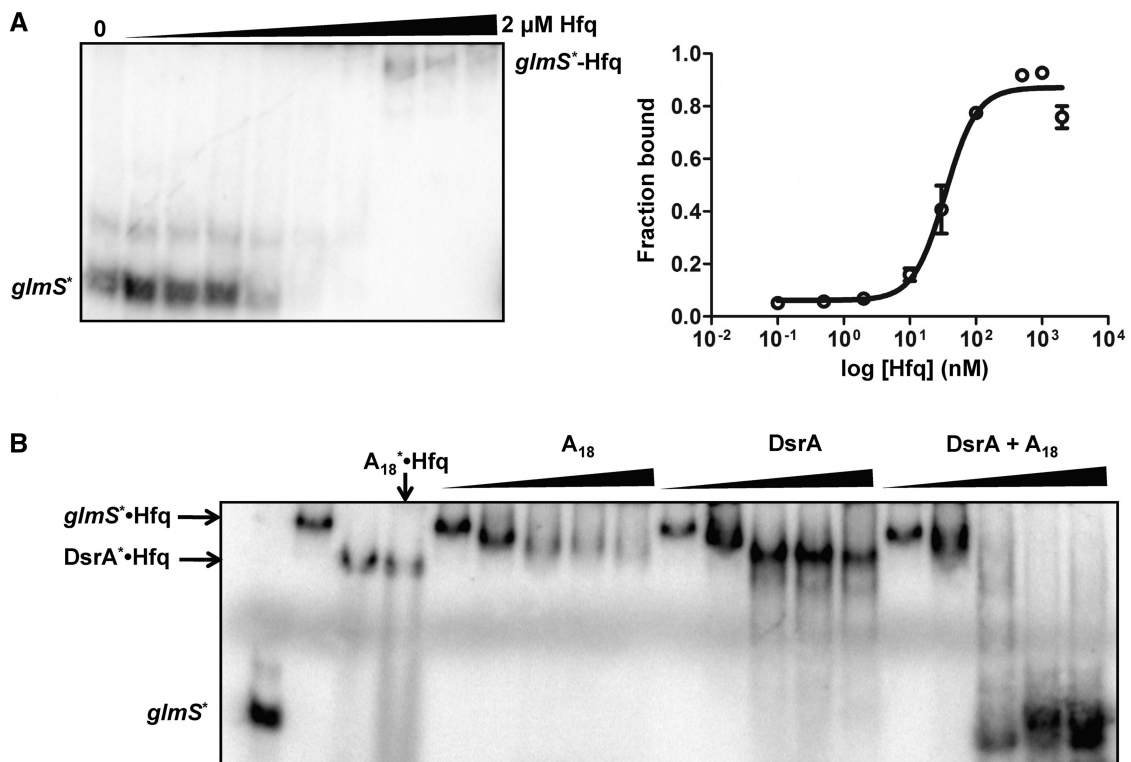
Figure 2. *glmS* interaction with Hfq. (**A**) Analysis of *glmS* interaction with Hfq (left). Gel shift experiment showing the binary complex between Hfq and *glmS* mRNA. ^{32}P-labeled *glmS** was titrated with varying concentrations of Hfq ranging from 0 to 2 µM. Quantitative analysis of the *glmS*–Hfq gel (right). As described in materials and methods, thermodynamic constants were determined by non-linear least squares analysis fitted to a cooperative binding model. (**B**) Competition gel-binding experiment to determine the Hfq-binding surface to *glmS*. The *glmS**–Hfq complex was pre-formed and incubated with increasing amounts of either distal (A$_{18}$ RNA), proximal (DsrA) or both (A$_{18}$ and DsrA) binding RNAs (0–30 µM) to compete *glmS** away from Hfq.

superimposed with the modification intensity data. The functional elements in *glmS* mRNA such as the potential (ARN)$_x$ motifs, GlmZ-binding site, RBS and translation start sites are also depicted in Figure 3. According to the proposed *glmS* structure, two potential (ARN)$_x$ motifs can be identified as candidates that could serve as upstream Hfq-binding sites. To confirm Hfq-binding regions in *glmS*, SHAPE footprinting was used as described previously (25) and the results are indicated in Figures 3 and Supplementary Figure S1. As expected, both (ARN)$_x$ regions footprinted with Hfq among other probable bindings sites indicated in Figure 3.

Measuring translational regulation of GlmS using a GFP fusion system

A GFP fusion system was used to measure the Hfq-dependent translational regulation of GlmS by sRNAs GlmZ and GlmY. A schematic diagram of the fusion expression system is shown in Supplementary Figure S2. As described above, a *glmS* fragment spanning the region of −161 to +21 was N-terminally fused to GFP under the transcriptional control of a pBAD promoter. The fusion system is constructed in a pBAC vector backbone (kindly provided by the Philip Cunningham Lab, Wayne State University, Biological Sciences) which is based on the F-plasmid *ori2*/RepE

replicon, that allows stable maintenance of large genomic fragments as very low or single copy plasmids (42). In addition, the vector also contains an *oriV* replication origin that can be induced by L-arabinise to produce high copy numbers of the plasmid in an *oriV* specific manner when expressed in a host strain that can supply the replication initiation protein TrfA in *trans* (43). However, throughout this study a low copy number of fusion plasmids were maintained since TrfA is not present in these strains.

To monitor expression of GlmS in the presence of sRNAs, GlmZ or GlmY were coexpressed together with the GlmS:GFP reporter. Here vectors pNS9007 and pNS9008, derivatives of the pBAD plasmid series, were used to express GlmZ and GlmY. Therefore coexpression of the GFP fusion and sRNAs were both under the control of a pBAD promoter induced with 0.1% arabinose. The coordinated transcription of mRNAs and their cognate sRNAs is required for efficient Hfq-mediated regulation *in vivo* (15,16).

To confirm the proper functioning of the fusion construct and coexpression conditions, GlmS:GFP expression was measured to obtain previously validated observations on the GlmS regulatory system (31,32) and the results are shown in Figure 4. To measure GFP fluorescence, liquid cultures were grown from overgrown overnight cultures.
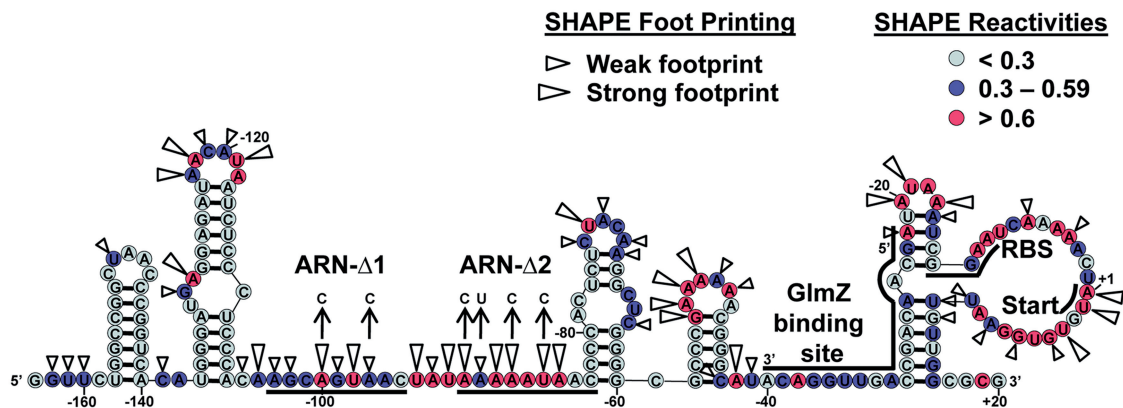
Figure 3. SHAPE-derived secondary structure model for *glmS* mRNA. The NMIA reactivity for each nucleotide corresponds to a given color, nucleotides with reactivities >0.6 are shown in red, from 0.3 to 0.59 in purple and <0.3 in grey. Hfq footprinting data in the presence and absence of 0.5 μM Hfq hexamer are superimposed on the structure. Large wedges indicate where there was a strong protection (>0.6) from wild-type Hfq observed and small wedges where a weak protection (0.3–0.59) was observed. The $(ARN)_x$ sites, glmZ-binding site, RBS and the start codon are also shown.

New cultures were then grown at 37°C for 3 h while shaking before inducing with 0.1% L-arabinose. The cultures were left to grow for another 150 min upon induction before the cells were harvested. The growth of each individual culture was monitored by its OD_{600} absorbance. Cells were consistently collected at their early stationary phase throughout (OD_{600} ∼1.4 ± 0.2). Lower dilutions from overnight cultures were made for slow growing strains, such as *Δhfq* cells to reach early stationary phase within the time frame of the assay. Fluorescence was measured upon lysis of 3.0 ml of cell culture (see 'Materials and Methods' section). The fluorescence data measured from liquid cultures are shown in Figure 4, where GFP expression is normalized to an identical un-induced culture to correct for cellular fluorescence and used to calculate the fold expression of GlmS.

Basal levels of GlmS expression in the absence of either sRNA are ∼2-fold that of an identical culture that was not induced with arabinose. The presence of either GlmZ or GlmY leads to an additional 2- to 3-fold increase in GlmS:GFP expression. The ability to upregulate GlmS expression is lost in the absence of Hfq, even in the presence of sRNAs. These data correlate nicely with previous observations of this regulatory system (31,32). They show that the GFP fusion construct and the assay conditions are able to capture the effects of sRNAs and Hfq-mediated regulation of GlmS.

To test whether the translational fusion can recognize defective Hfq species that are incapable of regulating gene expression, proximal and distal mutants of Hfq were used to measure GlmS activation (Figure 5). Y25A Hfq and K56A Hfq mutants have been shown previously to diminish nucleic acid binding at distal and proximal RNA-binding sites, respectively, (9,25) and the H57 proximal residue has been implicated to be crucial for hexamer organization in *Pseudomonas aeruginosa* (44) and *Salmonella typhimurium* (45). Data presented in Figure 5, indicates that either proximal (K56A) or distal (Y25A) mutants are incapable of facilitating GlmS upregulation in the presence of sRNAs. However, H57A
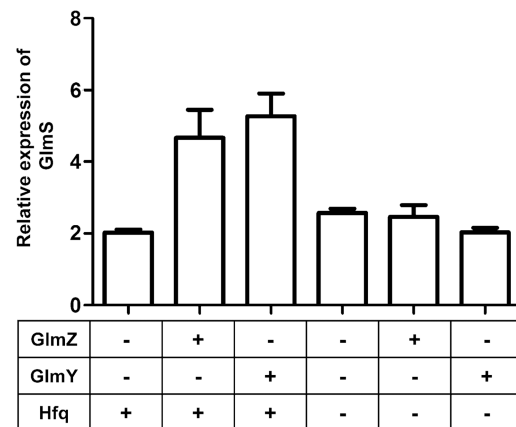

Figure 4. Expression of GlmS:GFP measured using GFP fluorescence. Relative expression levels of GlmS are measured in response to sRNAs GlmZ, GlmY and Hfq. Fluorescence was measured from *E. coli* cells that were grown in liquid media according to the conditions described in materials and methods. The growth of cells was controlled and monitored using the cell density (OD_{600}) where all cultures were harvested upon reaching early stationary phase (OD_{600} 1.4 ± 0.2). Cells were lysed before fluorescence was measured using a Tecan GENios Plus multi label plate reader. All fluorescence measurements were at least triplicated using independent inoculations. To calculate relative expression levels of GlmS, the fluorescence levels of each sample was compared to an identical uninduced culture to correct for cellular fluorescence. GlmS:GFP and sRNA synthesis was controlled using a pBAD promoter inducible by L-arabinose. To measure the Hfq dependence on GlmS expression, experiments were carried out in a *Δhfq* background.

Hfq shows a weak facilitation towards GlmS expression compared to wild-type Hfq. These data indicate the proper functioning of the GlmS:GFP fusion system, and that it is sensitive to sRNAs (GlmZ/GlmY) and Hfq.

Upstream Hfq-binding $(ARN)_x$ elements are essential for regulation

$(ARN)_x$ motifs in upstream regions are essential for Hfq-mediated gene regulation of *fhlA* (25) and *rpoS* (23,24).
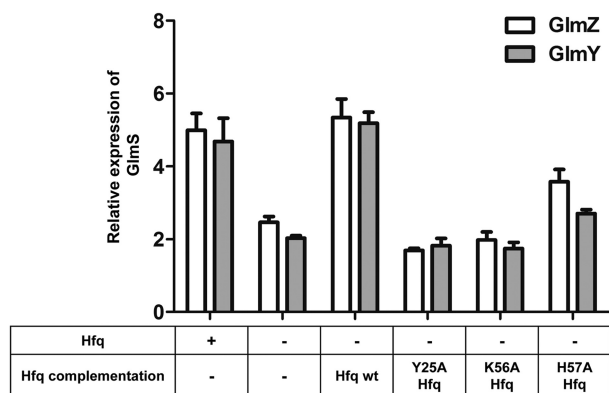
Figure 5. GlmS:GFP expression levels in the presence of mutant Hfq variants. To further characterize the functionality of the fusion system, GlmS expression levels were monitored in the presence of sRNAs with mutant Hfq variants that were previously shown to deter Hfq function. External Hfq for these experiments was supplemented using a plasmid system where the synthesis of Hfq was under the control of a pTac promoter that is inducible by IPTG. Here Hfq expression was induced prior to GlmS:GFP and sRNA synthesis to allow efficient regulation.

Structural evidence also indicates the preferential binding of $(ARN)_x$ motifs at the distal site of Hfq (26). Furthermore, a recent study showed the specific enrichment of these sequence elements in genomic SELEX experiments that were performed to identify Hfq binders (46). The above observations lead to a model where, the presence of upstream $(ARN)_x$ motifs could be a common occurrence in Hfq-dependent mRNAs. The secondary structure model together with Hfq footprinting data for *glmS*, indicated the presence of two potential $(ARN)_x$ motifs either of which could be required for regulation (Figure 3). The GFP fusion system was used to identify $(ARN)_x$ element(s) that are essential for GlmS activation *in vivo*. Both potential ARN motifs were mutated serially (*glmS* Δ1 and *glmS* Δ2) and simultaneously (*glmS* Δ1Δ2) to identify the role of each in *glmS* regulation (also see Figure 3).

The expression of GlmS Δ1:GFP, GlmS Δ2:GFP and GlmS Δ1Δ2:GFP was monitored in the presence of GlmZ (Figure 6A) and GlmY (Figure 6B). Data in Figure 6A show that, sRNA GlmZ is unable to activate GlmS expression of the *glmS* Δ2 mutant. However GlmS expression is higher than wild-type levels for the *glmS* Δ1 mutant. The overactivation caused by *glmS* Δ1 was absent when both ARN motifs were mutated (Δ1Δ2). To test whether *glmS* Δ1 activation is Hfq dependent, the regulation was monitored in the absence of Hfq. Data from this experiment reveals that the Δ1 mutation did not change the Hfq-dependence of GlmS activation by GlmZ. However, neither ARN mutant (Δ1 nor Δ2) were able to upregulate GlmS expression in the presence of GlmY (Figure 6B), implying that the Δ1 mutation only affects the GlmZ pathway. These results indicate the requirement of $(ARN)_x$ elements for Hfq-mediated regulation of GlmS and that ARN-2 is the most essential Hfq-binding motif for both GlmZ and GlmY pathways.

Requirement of the C-terminal domain of Hfq for regulation of GlmS by sRNAs GlmZ and GlmY

Hfq is present in most Gram-positive and Gram-negative bacteria. In all cases the nucleic acid-binding Sm1 and Sm2 motifs are evolutionary conserved across species which spans positions 7 through 66 based on the *E. coli* numbering. The greatest variability is observed at the C-terminus of Hfq where *E. coli* and *Salmonella enterica* have fairly long tails (∼102 amino acids) while species such as *C. difficile* and *C. perfringens* have shorter C-terminal extensions. The functional importance of the C-terminus of Hfq for regulation is unclear and conflicting reports have been presented previously (47–49). Therefore, the necessity of the C-terminal region of Hfq for GlmS activation by sRNAs, GlmZ or GlmY was tested using the GFP fusion system.

Several C-terminal truncations of *E. coli* Hfq were coexpressed together with the GlmS:GFP fusion protein and sRNAs in a *Δhfq* background to identify if the C-terminal amino acids are required for regulation. In addition to wild-type Hfq that consists of 102 amino acids, shortened C-terminal constructs of Hfq87 (87 amino acids), Hfq72 and Hfq65 were prepared. Here the Hfq65 variant can essentially be regarded as the tail-less version of Hfq since it only consists of the core Sm1 and Sm2 motifs. All C-terminal variants of Hfq were constructed using the ptac-KanL94P plasmid as the parent vector (36), that is under the transcriptional control of an IPTG inducible pTac promoter. The assay conditions to measure the GFP expression were similar to those described earlier, although Hfq induction was carried out using 1 mM IPTG, 1 h before arabinose induction to allow synthesis of Hfq prior to sRNA regulation. Relative expression levels of GlmS were monitored as the ability to upregulate GlmS synthesis individually by sRNAs GlmZ and GlmY with varying C-terminal extensions of Hfq (Figure 7).

From data shown in Figure 7, it is clear that plasmid borne and native Hfq (Hfq wt) regulate GlmS expression at comparable levels regardless of whether GlmZ or GlmY is used as the activator. GlmS regulation by GlmZ was mostly independent of the length of the Hfq C-terminal extension as Hfq72 and Hfq87 showed identical levels of GlmS expression to wild-type Hfq. Hfq65 on the other hand showed reduced levels of GlmS expression implying a possible effect caused by shortening the Hfq C-terminal tail. Semi-native western blot analysis of cellular Hfq65 showed multiple Hfq conformations relative to wild-type Hfq implying possible destabilization of the Hfq functional core in the absence of C-terminal residues (Supplementary Figure S3). However, all Hfq C-terminal variants (including Hfq65) were better at upregulating GlmS expression than a *Δhfq* strain in the presence of GlmZ, indicating at least some functional hfq hexamer is present *in vivo* (Figure 7). Conversely, for GlmY-mediated activation, Hfq65 was unable to upregulate GlmS above basal levels while Hfq72 and Hfq87 were proficient. These data show that the absence of amino acid residues from at least 66–72 affected GlmY's ability to upregulate GlmS in a manner distinct
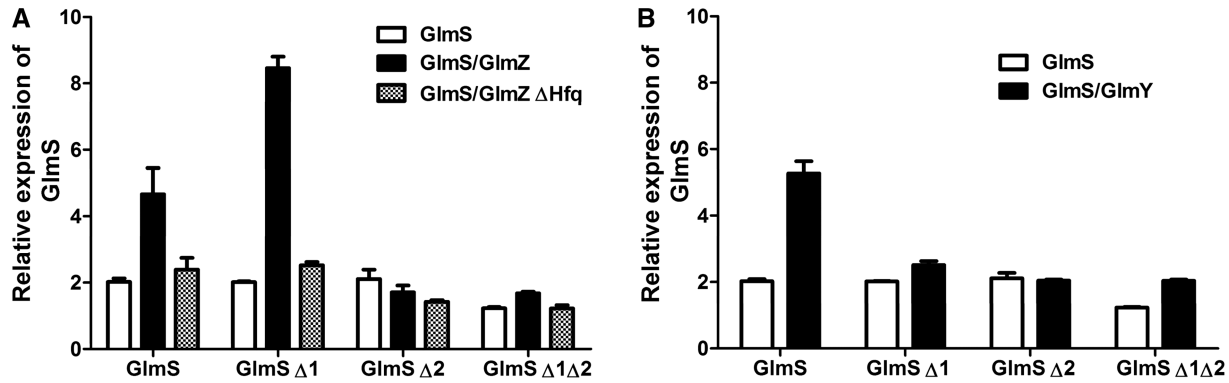
Figure 6. Effect of upstream Hfq binding $(ARN)_x$ elements in GlmS activation by GlmZ and GlmY. (**A**) Relative GlmS:GFP expression levels of *glmS* ARN mutants measured in the presence of GlmZ. Here mutations to *glmS* ARN elements were made as indicated in above Figure 2. Since two potential ARN motifs were detected they were removed sequentially and simultaneously to produce *glmS* Δ1, *glmS* Δ2 and *glmS* Δ1Δ2 respectively. The expression level of GlmS was measured as indicated in material and methods. The relative upregulation of GlmS was compared to expression levels when in the absence of GlmZ and Hfq. (**B**) Upregulation of *glmS* ARN mutants were measured with GlmY and compared to wild-type *glmS* expression levels.
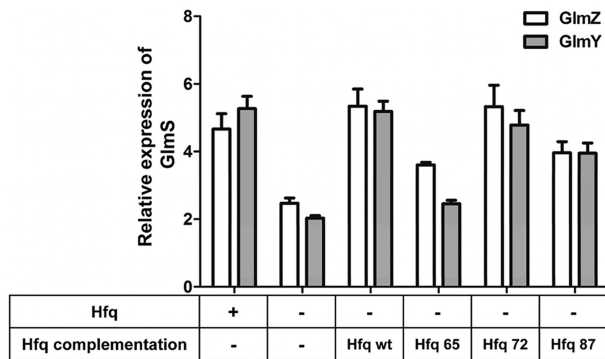
Figure 7. Requirement of Hfq C-terminal domain for GlmS:GFP upregulation by GlmZ and GlmY. Activation of GlmS:GFP expression by sRNAs was measured in the presence of Hfq that consists of varying C-terminal extension lengths. In addition to Hfq wild-type (102 amino acids), Hfq65 (65 amino acids), Hfq72 and Hfq87 were tested for its ability to perform regulation. Hfq65 consists only the nucleic acid-binding Sm1 and Sm2 domains while Hfq72 and Hfq87 have 7 aa and 22 aa extensions off the Sm cores. All externally supplemented *hfq* was under the transcriptional control of a pTac promoter that was induced using 1 mM IPTG. Coexpression and induction conditions of GlmS:GFP, sRNA and Hfq plasmid systems are described in material and methods.

from GlmZ regulation. However, as described above, GlmZ and GlmY act in a hierarchical manner during regulation. Therefore a loss of GlmY's ability to recruit YhbJ for self-processing would affect GlmZ's ability to activate GlmS synthesis. These data provide sufficient evidence to suggest the importance of at least the first seven amino acids of the C-terminal tail of Hfq for some *in vivo* functions.

Clostridium perfringens and *C. difficile* Hfq variants are proficient in GlmZ but not GlmY-mediated regulation of GlmS in *E. coli*

To validate these observations that the first 7-amino acids of the C-terminal tail of Hfq were essential for the GlmY pathway, Hfq variants belonging to Gram-positive bacteria

C. difficile and *C. perfringens* were used to substitute for *E. coli* Hfq to activate GlmS synthesis. Both Hfq^{Cd} and Hfq^{Cp} variants have similar nucleic acid-binding regions (Sm1/Sm2) compared to *E. coli*. However, not much sequence homology is observed beyond the Sm regions (Figure 8A). Therefore, the nucleic acid-binding properties should be conserved in Hfq^{Cd} and Hfq^{Cp} to promote GlmZ–*glmS* base pairing. However, if there is a need for the C-terminal extension to facilitate regulation, Hfq^{Cp} and Hfq^{Cd} may not be able to cross complement GlmY native functions.

GlmS activation was monitored in the presence of Hfq^{Cd} and Hfq^{Cp} variants with either GlmZ or GlmY sRNAs in a *Δhfq* background. The results are shown in Figure 8B. Hfq^{Cd} and Hfq^{Cp} both successfully promote GlmZ-dependent GlmS activation. In fact, increased upregulation of GlmS is observed for Hfq^{Cp} in the presence of GlmZ. These data fundamentally confirm that the GlmZ–*glmS* interaction, which revolves around the ability of Hfq to facilitate base-paring between a sRNA and its target mRNA, can be achieved so long as the core nucleic acid-binding domain is present. However, Hfq^{Cd} and Hfq^{Cp} were unable to upregulate GlmS expression via the GlmY pathway. Gel shift assays confirmed that GlmY binds Hfq^{Ec} and Hfq^{Cp}, although its affinity for Hfq^{Cp} is about 10-fold weaker than for Hfq^{Ec} (Supplementary Figure S4). In contrast, GlmZ binds Hfq^{Cp} and Hfq^{Ec} with only a slight loss of affinity implying some differences in the manner with which GlmZ and GlmY bind Hfq despite their sequence and structural homology. Since the only significant difference between the three Hfq variants are at the C-terminal region, a potential role of the C-terminus is implied for GlmY activation, where additional protein–protein or protein–RNA interactions are likely required.

DISCUSSION

Being able to adapt to environmental stress is vital for the survival and propagation of bacteria. The RNA-binding
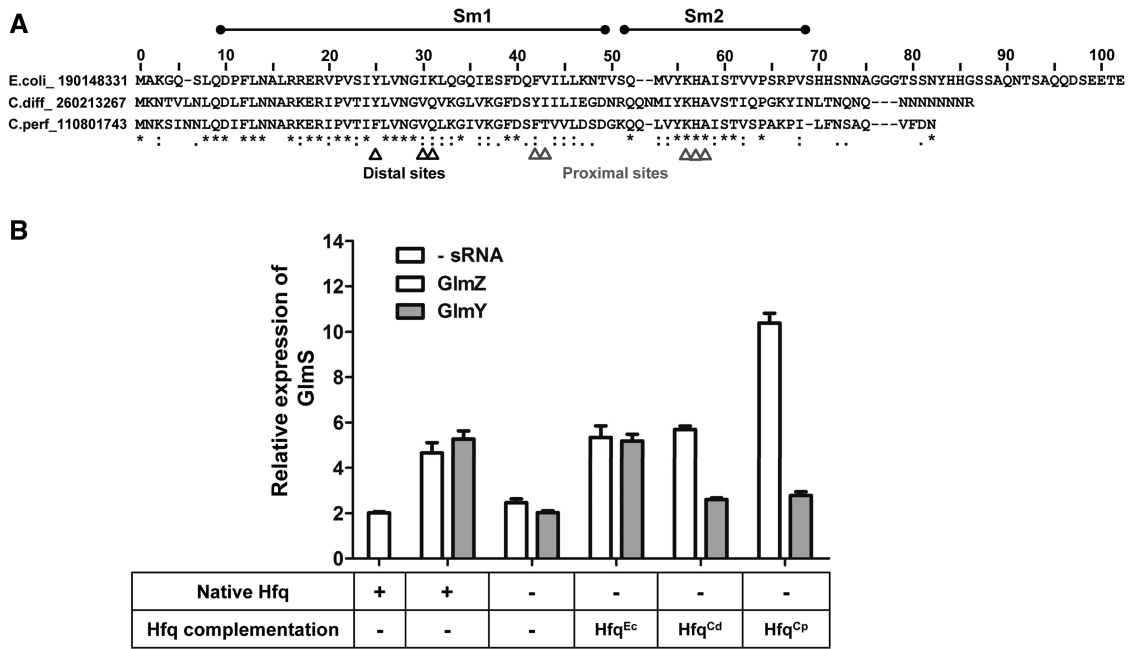
Figure 8. GlmS:GFP upregulation using *C. perfringens* and *C. difficile* Hfq variants by GlmZ and GlmY. **(A)** Sequence homology between *E. coli*, *C. difficile* and *C. perfringens* Hfq variants. Nucleic acid-binding Sm1/Sm2 domains and important amino acid residues in proximal and distal RNA-binding surfaces required for regulation are indicated. **(B)** Cross-complementation for Hfq^{Ec} using Hfq^{Cp} and Hfq^{Cd} homologues for GlmS upregulation. The ability to activate GlmS expression by GlmZ and GlmY was measured when Hfq^{Cd} and Hfq^{Cp} was supplemented in *E.coli Δhfq* cells.

protein Hfq plays a key role in modulating stress responses in bacteria by regulating gene expression in a sRNA-dependent manner. Hfq functions primarily in promoting sRNA–mRNA base pairing by increasing the local concentrations of sRNAs and target mRNAs through its distal and proximal RNA-binding surfaces. In addition, Hfq acts as a chaperone to trigger structural transitions in RNAs to initiate recognition between cognate sRNA–mRNA pairs (27), or partners directly or indirectly with processing enzymes such as RNase E (50,51), Poly A polymerase (52), PNPase (50), RNA helicases (53,54), RelA (55) and YhbJ (30) to aid stringent gene regulation. These diverse functions enable Hfq to be involved in a cascade of regulatory events that control multiple stress responsive pathways. Since all Hfq networks involve sRNAs to initiate gene regulation at the post-transcriptional level, the sRNA–mRNA interaction can be regarded as the main trigger for downstream regulatory outcomes.

A number of recent reports have indicated that upstream (ARN)$_x$ sequence motifs are common among Hfq-dependent mRNAs and help recruit Hfq to these mRNAs (23,25,56). Structural evidence shows that (ARN)$_x$ motifs interact with the distal RNA-binding site of Hfq while the proximal site harbors sRNAs during regulation (26). In this work, (ARN)$_x$ motifs were identified and characterized within *glmS* mRNA, a message regulated by sRNAs GlmZ and GlmY in an Hfq-dependent manner. The interaction between Hfq and *glmS* is similar to what was previously observed for *fhlA* (25) where the mRNA uses both RNA-binding surfaces to interact with Hfq in the absence of sRNAs.

This mode of interaction between mRNAs and Hfq was attributed to a model wherein, the complexes could serve as a precursor RNP complex that enables a rapid response to stress in bacteria.

Examining the proposed secondary structure of *glmS* and Hfq footprinting data obtained using SHAPE, it was apparent that two potential ARN motifs (ARN-1 and ARN-2) were present upstream of the RBS (Figure 3). To identify which ARN element(s) were essential for regulation, *glmS* mRNA was fused N-terminally to GFP to measure GlmS expression levels. GlmS translation was upregulated in the presence of either sRNA in an Hfq-dependent manner confirming the proper functioning of the fusion system. Of the two potential ARN motifs, ARN-Δ2 had the most adverse effects on regulation in the presence of either sRNA. However, the loss of ARN-1 caused a hyper-activation of GlmS only in the presence of GlmZ. Nonetheless, upregulation of *glmS* ARN-Δ1 was Hfq dependent, implying that the regulatory node was still intact despite the changes in *glmS* functional elements. These results indicate that ARN-2 is the essential (ARN)$_x$ motif in *glmS* required for Hfq-mediated regulation. While we do not completely understand why *glmS*-ARN-Δ1 led to over-activation, it might result from structural changes in *glmS* due to the sequence modifications required to ablate the Hfq binding site. Alternatively, it could also be explained by a partitioning model. If Hfq must be present at ARN-2 for glmZ activation, and Hfq can bind at either ARN-1 or ARN-2, destruction of ARN-1 would lead to an increased population of the active adduct (Hfq-binding to ARN-2) triggering a more favorable response than wild-type *glmS*. The key

point however, is that, as observed in previous cases such as in *rpoS, rprA* (23) and *fhlA* (25), Hfq-mediated regulation of *glmS* is absolutely dependent on the presence of an upstream $(ARN)_x$ element, adding to the list of networks that rely on such sequence motifs for function and may indicate that this is a global requirement within mRNAs that are destined for Hfq-dependent regulation.

The manner by which Hfq recognizes target mRNAs could be important for understanding the mechanism of stress regulation in bacteria. This interaction could allow Hfq●mRNA complexes to form prior to stress, thus identifying those messages that might be regulated during a time-sensitive response. While we have no definitive data to confirm this hypothesis, this model would explain Hfq's ability to handle multiple regulatory networks using multiple sRNAs. Recent evidence also suggests that Hfq can be a limiting factor in cells and that a coordinate expression of sRNA and mRNAs is required for the proper functioning of regulatory nodes (15). This implies the intense competition between RNAs to acquire Hfq which in turn explains the need for expressed mRNAs to capture Hfq to facilitate their own regulation. Other functions of this interaction could include initiation of structural rearrangements in the mRNA to facilitate the interaction with sRNA as was observed in the case of DsrA and *rpoS* (27). However, the structural transitions that are triggered by the mRNA–Hfq interaction alone are insufficient to affect basal translation levels. The *glmS*–Hfq interaction alone had no effect in GlmS expression, for instance (data not shown). Similar observations were reported for *sodB, rpoS, ompC* and *fhlA*, all having only minimal effects on translation upon binding to Hfq (15).

The role of Hfq in stress regulation throughout the bacterial kingdom is well established. Comparison of Hfq variants across species has confirmed the presence of an evolutionary conserved nucleic acid-binding core (Sm1 and Sm2) and a variable C-terminal region that is less well preserved. While the requirement of the Sm core for Hfq function is clear, involvement of the C-terminal region of Hfq in regulation is still controversial (49,57).

To gain more information regarding the function of the C-terminal region of Hfq during regulation, the *glmS* system was used. A clear advantage of using this system to address this problem stems from the presence of two regulatory layers that are directed by GlmZ and GlmY in an Hfq-dependent manner. While the role of Hfq in facilitating the GlmZ–*glmS* interaction is well established, the requirement of Hfq by GlmY in YhbJ processing is unclear. Since an interaction between GlmY and *glmS* is not established, (31) YhbJ could be recruited by a GlmY–Hfq complex potentially through the C-terminal region of Hfq.

When the requirement of the C-terminal region of Hfq for GlmS activation was measured, data indicated that both Hfq72 and Hfq87 were equally proficient at assisting GlmY and GlmZ upregulate GlmS. However, when only the Sm core of Hfq was present (Hfq65), only GlmZ allowed significant upregulation of GlmS expression relative to a *Δhfq* strain. Although crystallographic data show that C-terminal residues are not essential for hexamer association (58), semi-native western blot

analysis of Hfq65 indicates some issues associated with speciation or reduced stability of the functional Hfq hexamer (Supplementary Figure S3) as previously postulated (13). This result explains the reduced activity of GlmZ-mediated upregulation of *glmS* expression in the presence of Hfq65. However, the inability of GlmY to utilize Hfq65 to any appreciable extent remains puzzling since a significant amount of functional hexamer is still present in these cells based on western blot analysis.

Hfq^{Cp} and Hfq^{Cd} were used to cross complement for Hfq^{Ec} in order to completely rule out the potential differences in Hfq utilizations by sRNAs during regulation. Hfq variants from *C. perfringens* and *C. difficile* fully complemented the *Δhfq* strain when measured based on GlmZ activation of GlmS. GlmY, however, was unable to take advantage of cross-species complementation. Sm cores in Hfq^{Cp} and Hfq^{Cd} are highly conserved compared to Hfq^{Ec} and successfully promote GlmZ–*glmS* base pairing that represents the classical Hfq mediation process through its nucleic acid-binding domains. The inability of GlmY to use Hfq65, Hfq^{Cp} and Hfq^{Cd} to upregulate GlmS suggests that the Sm core alone is insufficient for regulation in this pathway. Since the primary functional role of GlmY is to self-direct the processing complex that utilizes YhbJ and polyA polymerase (PAP I) (31), Hfq C-terminal region may facilitate the assembly of this complex through additional RNA and/or protein interactions with Hfq.

The Hfq C-terminal region has long been predicted as a landing pad for either protein or nucleic acid-binding partners. In fact, recent structural data compares the Hfq C-terminal regions to intrinsically disordered proteins (IDPs) that could potentially facilitate intermolecular interactions (47,57). Pull down experiments carried out to map Hfq-protein complexes indicate that Hfq interacts directly or indirectly with proteins in the bacterial RNA degradosome such as RNase E, polynucleotide phosphorylase (PNPase), poly A polymerase (PAP I) (59,60) and a variety of regulatory proteins in bacteria (T. Lee and A. Feig, unpublished data). Furthermore, a recent study by Prévost *et al.* proposed a model for the physiological relevance of an RNase E–Hfq interaction during RNA degradation triggered by the sRNA–mRNA recognition (61,62). Since no detectable protein interactions have been reported within the Sm cores, these complexes should be accommodated at the C-terminal region of Hfq. Whether Hfq recruits YhbJ as part of a novel processing complex that affects sRNA turnover or whether it promotes an RNA interaction with GlmY through its C-terminal that is essential for GlmY turnover needs to be tested further.

Overall, this work further illustrates the requirement for Hfq to bind mRNAs through upstream $(ARN)_x$ motifs during sRNA-dependent gene regulation. In addition, these studies show that a non-canonical sRNA pathway requires additional Hfq interactions traced to the C-terminal extension that are not essential for the canonical sRNA–mRNA pairing behavior typically carried out by Hfq. C-terminal amino acids 66–72 from *E. coli* are essential for GlmY-mediated regulation of GlmS, implying a conditional requirement for the C-terminal region depending on the mode of sRNA recruitment and

may relate to additional RNA or protein-binding interactions.

SUPPLEMENTARY DATA

FUNDING

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