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Research paper

Lack of interchangeability of Hfq-like proteins

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ABSTRACT

Hfq is an RNA-binding protein that participates in the regulatory activity of small non-coding RNAs (sRNAs) in many species of bacteria. Hfq protein was first crystallized from *Staphylococcus aureus* and this crystal structure constitutes a hallmark for bacterial Sm-like proteins. Paradoxically, however, the functional relevance/role of *S. aureus* Hfq (Hfq_{SA}) remains uncertain, as growing evidence suggests that the hfq_{SA} gene is expressed at very low levels or unexpressed in many *S. aureus* strains. To gather further insight, in the present work we exchanged the structural portion of the *hfq* gene of *Salmonella enterica* serovar Typhimurium (hfq_{STM}) with hfq_{SA} and analyzed the effects of the replacement on various Hfq-related phenotypes. Our results show that the replacement strain – in spite of expressing Hfq_{SA} at levels comparable to Hfq_{STM} in wild-type *Salmonella* – behaves as an *hfq* null mutant in three discrete small RNA-mediated regulatory responses. These defects correlate with an abrupt reduction in the intracellular concentration of sRNAs, as observed in an *hfq* null mutant. Failure of Hfq_{SA} to protect *Salmonella* sRNAs from degradation suggests that Hfq_{Sa} does not bind to these sRNAs. A parallel study with the *Borrelia burgdorferi hfq* gene (hfq_{BB}) gave essentially identical results: when made from a single copy chromosomal gene, Hfq_{BB} fails to substitute for Hfq_{STM} in sRNA-mediated regulation.

1. Introduction

Base pairing between regulatory RNAs and complementary sequences in messenger RNAs is a highly conserved mechanism controlling gene expression at the post-transcriptional level in all forms of life. In bacteria, best-studied regulatory small RNAs (sRNAs) are encoded at separate locations from their target genes and interact with target mRNAs through short and imperfect stretches of complementarity (reviewed in [1]). In some bacterial species, like *Escherichia coli*, *Salmonella enterica* and *Vibrio* species, these *trans*-encoded sRNAs require chaperon protein Hfq for activity. Discovered in the late nineteen sixties as a host factor needed for in vitro replication of RNA phage Qß [2], Hfq was later found to be a key player in a number of RNA transactions (reviewed in [3–5]). In particular, Hfq participates in sRNA-mediated regulation by binding both sRNAs and cognate mRNAs and stimulating their association. For many sRNAs, Hfq-binding is also essential to confer protection against degradation by ribonucleases [6]. Hfq and Hfq-like proteins belong to the Sm-like (Lsm) family of RNA-binding proteins characterized by a ring-shaped multimeric architecture. X-ray crystal structure analysis of the Hfq-like protein from *Staphylococcus aureus* (Hfq_{SA}) and of *E. coli* Hfq (Hfq_{EC}) showed that both proteins assemble in homohexameric rings approximately 70 Å in diameter [7–9]. A short U-rich synthetic RNA co-crystallized with Hfq_{SA} was found to circle around the positively charged central pore of the torus on the so-called proximal face [9]. In contrast, A-containing RNA oligomers bind to the distal face of Hfq_{EC} [7]. The notion of opposite Hfq surfaces having different ligand specificities is independently supported by mutational studies [10]. It should be noticed, however, that while similar on the proximal face, Hfq from *S. aureus* and *E. coli* differ sharply in their charge distribution on the distal face and in the trough that connects proximal and distal faces. This latter region has a positively charged surface in Hfq_{EC} and a negatively charged surface in Hfq_{SA} [3]. Finally, while Hfq-like proteins have an evolutionarily conserved core of 65 amino acids, the C-terminus is variable in length, leading to a controversy about its function [11,12]. The Hfq extended C-terminus is found in γ- and β-proteobacteria whereas in the case of Gram-positive bacteria such as in Hfq_{SA}, extensions are short.

In most bacteria, loss of Hfq function, albeit not a lethal event, causes a variety of pleiotropic defects and renders strains particularly susceptible to environmental stress [13]. Many of these phenotypes result from the loss of sRNA activities. For example, the σ^S-dependent stress response is poorly induced in *hfq* mutants due

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to the lack of sRNA-mediated activation of *rpoS* mRNA translation (reviewed in [14]). At the same time, the σ^E-dependent envelope stress response is chronically induced due to over-accumulation of outer membrane proteins that are normally downregulated by sRNAs [15–20].

Genes encoding Hfq-like proteins are found in about half of the sequenced genomes [21]. In many pathogens, they are required for virulence and were shown to participate in sRNA-mediated regulatory processes (reviewed in [22]). The functional proficiency of Hfq homologues was inferred from their ability to complement the loss of Hfq in *E. coli*. For example, *Pseudomonas aeruginosa* Hfq, which shares 92% identity with Hfq_{EC} in the initial 68 amino acids, fully replaced Hfq_{Ec} in terms of its requirement for Qβ replication and *rpoS* expression [23]. Likewise, the *Moraxella catarrhali hfq*-like gene, in spite of being twice the size of the hfq_{EC} (but highly similar in the N-terminal encoding domain) partially complemented the growth defect and the stress sensitivity of an *E. coli hfq* mutant [24]. Intriguingly, even a protein with very limited sequence relatedness to Hfq (only 12% identity), encoded by *Borrelia burgdorferi* (Hfq_{BB}), was recently reported to complement an *E. coli hfq* mutant [25]. On the other hand, Hfq-like proteins from *Neisseria meningitides* and *Aquifex aeolicus,* and from archaeon *Methanocaldococcus jannaschii* did not reverse the chronic σ^E induction of a *Salmonella* strain lacking Hfq [26]. However, these proteins were capable of binding some *Salmonella* sRNAs and also caused specific RNA processing defects [26].

The function of Hfq-like proteins remains unclear for some bacterial species. For example, deletion of the *Bacillus subtilis hfq*-like gene (*ymaH*) does not affect growth rate, stress adaptation, or activities of all sRNAs tested [27–29]. Similar results were reported for *S. aureus*. Deletion of the hfq_{SA} gene in several pathogenic isolates did not impair or in any way impact their physiology [30] possibly because hfq_{SA} is poorly expressed or not expressed in the strains used for these studies [30,31](see also [32]). On the other hand, in *S. aureus* strains where Hfq is detected, deletion of its coding gene reportedly resulted in decreased toxicity and virulence, suggesting that Hfq is a global regulator [33].

To gather insight into the functional status and regulatory properties of *S. aureus* Hfq, in the present study, we introduced the sequence encoding this protein in place of the endogenous *hfq* gene in the *Salmonella* chromosome. (Hfq_{STM} is 100% identical to Hfq_{EC} in the initial 78 amino acids). In parallel with the above work, a similar exchange was performed using a DNA fragment spanning the *Borrelia hfq*-like gene. Both constructs were made with surgical precision replacing only protein coding portions – *i.e*., the segment between initiation and termination codons – to allow the heterologous sequences to fall under the control of signals normally devoted to hfq_{STM} expression. We show below that Hfq_{SA} and Hfq_{BB} in spite of being made at levels comparable to Hfq_{STM}, fail to replace the latter in sRNA-mediated regulation as well as in protecting representative sRNAs from degradation.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Strains used in this study, listed in Table 1, were all derived from *S. enterica* serovar Typhimurium strain MA3409, a derivative of strain LT2 cured for the Gifsy-1 prophage [34]. Bacteria were cultured at 37 °C in liquid media or in media solidified by the addition of 1.5% (w/v) Difco agar. LB broth [1% bacto tryptone (w/v), 0.5% Difco yeast extract (w/v), 0.5% NaCl (w/v)] was used as complex medium. When needed, LB medium was supplemented with 0.2% (w/v) arabinose. Antibiotics (Sigma–Aldrich) were included at the following final concentrations: chloramphenicol, 10 μg/ml; kanamycin monosulphate, 50 μg/ml; sodium ampicillin 100 μg/ml; tetracycline hydrochloride, 25 μg/ml. Liquid cultures were grown in New Brunswick gyratory shakers and growth was monitored by measuring the optical density at 600 nm with a Shimazu UV-mini 1240 spectrophotometer.

Table 1
Salmonella enterica serovar Typhimurium strains used in this work.

Strain[a]	Genotype	Source or reference
MA3409	wild-type	[34]
MA7455	wild-type/pKD46	[18]
MA8028	*eptB115*::MudK	[18]
MA8029	*eptB115*::MudK Δ*hfq67*::*cat*	[18]
MA8149	*katE561*::MudK	[18]
MA8679	*katE561*::MudK Δ*hfq67*::*cat*	[18]
MA9132	*chiP91*::pCE40(*lac*)	[43]
MA10675	Δ*hfq116*::*tetAR*	this work
MA10740	Δ*hfq116*::*tetAR*/pKD46	this work
MA10741	Δhfq_{STM}::hfq_{SA}	this work
MA10744	*chiP91*::pCE40(*lac*) Δ*hfq116*::*tetAR*	this work
MA10746	*eptB115*::MudK Δhfq_{STM}::hfq_{SA}	this work
MA10747	*chiP91*::pCE40(*lac*) Δhfq_{STM}::hfq_{SA}	this work
MA11042	Δhfq_{STM}::hfq_{BB}	this work
MA11043	*eptB115*::MudK Δhfq_{STM}::hfq_{BB}	this work
MA11044	*chiP91*::pCE40(*lac*) Δhfq_{STM}::hfq_{BB}	this work
MA11054	*hfq*-3xFLAG-*aph* (KnR)	this work
MA11055	Δhfq_{STM}::hfq_{SA}-3xFLAG-*aph* (KnR)	this work
MA11056	Δhfq_{STM}::hfq_{BB}-3xFLAG-*aph* (KnR)	this work
MA11057	*katE561*::MudK Δhfq_{STM}::hfq_{SA}	this work
MA11058	*katE561*::MudK Δhfq_{STM}::hfq_{BB}	this work

[a] All strains are derived from *Salmonella enterica* serovar Typhimurium strain MA3409. The latter is a derivative of strain LT2 cured for the Gifsy-1 prophage [34].

2.2. Enzymes and chemicals

Restriction enzymes, T4 polynucleotide kinase and Taq DNA polymerase were from New England Biolabs, Pfu-Turbo DNA polymerase was from Stratagene. DNA oligonucleotides were obtained from Sigma–Aldrich. Acrylamide-bisacrylamide (30%, 29:1) and other electrophoresis reagents were from Bio-Rad. Hybond-N^+ membranes and hybridization buffer used for Northern blot analysis were from GE Healthcare and from Applied Biosystems-Ambion, respectively.

2.3. Genetic techniques

Generalized transduction was carried out using the high frequency transducing mutant of phage P22, HT 105/1 *int-201* [35]. "λ Red"-mediated chromosomal recombineering was carried out by the method of Datsenko and Wanner [36] implemented as in [37]. Donor DNA fragments were generated by the polymerase chain reaction (PCR) using plasmid or chromosomal DNA templates. DNA oligonucleotides used as primers for PCR amplification are listed in Table 2. Amplified fragments were electroporated into the appropriate strains using a Bio-Rad MicroPulser under the conditions specified by the manufacturer. Constructs were verified by PCR and DNA sequence analysis (performed by GATC company).

2.4. Construction of relevant strains

Salmonella strains carrying the structural portions of the *hfq*-like genes from *S. aureus* or *B. burgdorferi* were constructed with a two-step recombineering procedure as described [38]. Firstly, a *tetAR* module (amplified with primers ppH71 and ppH72) was inserted in the *hfq* gene in the *Salmonella* chromosome. Subsequently, the entire *hfq*::*tetAR* was crossed out selecting for the loss of tetracycline resistance [39] using DNA fragments amplified from chromosomal DNA of *S. aureus* RN4220 and *B. burgdorferi* clinical isolate 28354 with primer pairs ppI06/ppI07 and ppJ44/ppJ45, respectively