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Regulation and RNA-binding properties of Hfq-like RNA chaperones in *Bacillus anthracis*

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ABSTRACT

Background: Small RNAs (sRNAs) are important modulators of gene expression in bacteria. Regulation by sRNAs is yet to be established in *Bacillus anthracis*. Here, regulation and RNA-binding properties of Hfq-like RNA chaperones in *B. anthracis* are investigated.
Methods: Transcript levels were measured by RT-PCR. Proteins were cloned, purified, and their ability to bind sRNA was seen by EMSA. Regulators of Hfq1 were identified by *in silico* analysis and validated by EMSA. Conserved sRNAs were identified by homology search and their ability to bind Hfq1 was seen by EMSA. Residues crucial for sRNA binding were identified by mutational studies.
Results: hfq1 and *hfq3* showed expression during exponential phase on BHI medium, while *hfq2* showed no expression. Hfq1 and Hfq3 formed hexamer and sRNA–Hfq complex, not Hfq2. *In silico* prediction and EMSA confirmed AbrB binding to the promoter of Hfq1. Homology search identified 7 sRNAs in *B. anthracis*. The sRNA CsfG showed binding to Hfq1 via residues Y24, F29, Q30, R32, K56, and H57.
Conclusions: Hfq1 and Hfq3 can function as RNA chaperones in *B. anthracis*. The transition phase repressor AbrB might be responsible for the growth-dependent expression of Hfq1. The sporulation-specific sRNA CsfG binds to Hfq1 via its distal surface and requires an intact hexameric structure for forming CsfG-Hfq1 complex.
General significance: This is the first report demonstrating the regulation and functional properties of Hfq-like RNA chaperones in *B. anthracis* and paves the path towards establishing sRNA-based regulation in *B. anthracis*.

1. Introduction

Control of gene expression in bacteria occurs as a response to physiological and environmental signals. Small non-coding RNAs (sRNAs) are important mediators of post-transcriptional regulation in prokaryotes [1,2]. The RNA chaperone Hfq primarily functions as a post-transcriptional regulator by modulating translation and RNA degradation processes via the formation of sRNA–mRNA complexes [3–5]. Hfq was first reported as a protein required for the replication of bacteriophage Qβ in *Escherichia coli* [6]. Since then, Hfq-like proteins have been reported in many bacterial genomes including pathogens [7,8]. Hfq plays an important role as a major regulator of bacterial metabolism [9], growth [10], quorum-sensing [11], and stress response [12]. The role of Hfq in virulence like biofilm formation, production of toxins and virulence factors in several gram-negative and gram-positive pathogens have also been reported [13,14].

Hfq is a member of LSm protein superfamily, whose members are found in all three domains of life [15]. The bacterial LSm homolog Hfq mostly forms homo-hexamers [16], while those in eukaryotes and archaea form variable oligomeric structures [17]. Homologs of bacterial Hfq possess a rather conserved N-terminus that forms the core and a dispensable, variable length C-terminus whose function is yet to be identified [16,18]. Few crystal structure of Hfq from *E. coli*, *Bacillus subtilis*, and *Staphylococcus aureus* are available [19–21]. Hfq is shown to accomplish its diverse roles by means of crucial residues present on its three distinct RNA-binding surfaces namely the proximal, distal, and rim RNA-binding surfaces [22,23].

The regulatory roles of Hfq in gram-negative bacteria, mainly in *E. coli* and *Salmonella* spp., are well established along with their interacting sRNAs, corresponding target mRNAs and resulting physiological implications [24]. On the other hand, the function of Hfq in gram-positive bacteria in sRNA-based regulation is yet to be established, although numerous sRNAs have already been reported in these species [25,26]. In *Listeria monocytogenes*, although Hfq plays an important role in stress tolerance and virulence, its inactivation showed no effect on sRNA abundance [27,28]. Similarly, Hfq was not found essential for

Abbreviations: sRNAs, small RNAs; EMSA, electrophoretic mobility shift assay

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RNAIII function or sRNA stabilization in *S. aureus* [29–31]. A complete lack of Hfq-like proteins in the genome of few gram-positive organisms like lactococci, lactobacilli, and streptococci have also been reported [32].

B. anthracis is a gram-positive spore-forming bacterium and the etiological agent of anthrax. Anthrax is a fatal disease affecting both humans and animals, and can also be used as a major biological weapon [33]. *B. anthracis* possesses two extra-chromosomal plasmids—pXO1 and pXO2, which carry genes responsible for the formation of anthrax toxins and the poly-gamma-D-glutamate capsule [34]. Hfq is mostly found as a single copy except for bacterial species like *Magnetospirullum magnetotacticum*, *Burkholderia* genus, *Novosphingobium aromaticivorans*, and archeal species like *Methanobacterium thermoautotrophicum* and *Archaeoglobus fulgidus*. These organisms harbor two distinct copies of Hfq-like proteins in their genome [35]. In this study, *B. anthracis* was found to be unique among prokaryotes, especially gram-positive organisms, to possess three copies of Hfq-like proteins—two chromosomal (*hfq1* and *hfq2*) and one plasmid based (*hfq3*).

In this study, Hfq-like proteins were identified in *B. anthracis* and their regulatory and sRNA-binding properties were analyzed. Expression pattern of multiple Hfq paralogs on rich BHI medium were monitored by RT-PCR. Regulatory features were identified by in silico promoter analysis and then confirmed by EMSA. Oligomeric properties and the ability to form sRNA-protein complexes were also monitored. In contrast to previous studies that involved the use of RNA aptamers to study RNA-binding properties of Hfq, here sRNAs conserved in the genome of *B. anthracis* were first identified and then their ability to bind Hfq was assessed *in vitro*. Mutational analysis was further carried out to identify residues crucial for the formation of sRNA–Hfq complex. This is the first study to shed light on the regulatory and functional aspects of Hfq-like RNA chaperones in *B. anthracis*. The results obtained in this work will open avenues for in-depth studies aimed at understanding the sRNA mediated regulatory network in *B. anthracis* and its related gram-positive organisms.

2. Materials and methods

2.1. Bacterial strains and culture conditions

E. coli DH5α and BL21-DE3 strains were used as cloning and expression hosts, respectively. *B. anthracis* sterne strain was grown in BHI medium or sporulation medium (DIFCO) as appropriate. *E. coli* strains were maintained in LB (Luria Bertani) medium supplemented with 50 μg/ml of kanamycin as appropriate. Liquid cultures were grown at 37 °C with orbital agitation of 220 rpm.

2.2. Cloning and site-directed mutagenesis

Genes encoding *hfq1*, *hfq2*, *hfq3*, *abrB*, and *csfG* were PCR amplified using the *B. anthracis* DNA as template and the primers Hfq1 Fw/Rv, Hfq2 Fw/Rv, Hfq3 Fw/Rv, AbrB Fw/Rv, and CsfG Fw/Rv respectively (Table S1). The amplified product was further digested with *Nco*I and *Xho*I restriction enzymes. The plasmids pET-hfq1, pET-hfq2, pET-hfq3, pET-abrB, and pET-csfG were obtained by inserting the above digested fragments into a *Nco*I and *Xho*I-digested pET28a vector. Site-directed mutagenesis was performed using a QuickChange site-directed mutagenesis kit (Stratagene) as per manufacturer's instructions. Site-specific mutations were introduced into His-Hfq1 protein using the pET-hfq1 plasmid as template DNA and respective mutation specific primers (Table S1). All the clones were confirmed by DNA sequencing. The list of primers and oligonucleotides are listed in Table S1.

2.3. Protein overexpression and purification

All the clones were transformed into BL21-DE3 cells and protein expression was induced at OD_{600} ~ 0.6–0.8 with 0.5–1.0 mM IPTG at 18 °C for 16 hours. Protein purification was done using Ni^{+2}-NTA affinity chromatography (Qiagen) using standard protocol. The protein concentration was estimated using Bradford's reagent with BSA as standard.

2.4. Isolation and biotin labeling of mRNA/sRNA

Total RNA was isolated from liquid culture of *B. anthracis* using the Trizol method followed by RNAse free-DNase (Qiagen) treatment. A growth curve of *B. anthracis* was used to designate the different growth stages of bacteria. The sRNA fraction was isolated from total RNA using mirVana miRNA isolation kit (Ambion) according to manufacturer's instruction. RNA and sRNA quality was assessed by visual inspection on 3% agarose gel and Agilent 2100 Bioanalyzer system. The isolated sRNAs were 3'end labeled with biotin using biotin 3'end labeling kit from Pierce Biotechnology (Rockford, IL, USA) as per manufacturer's instructions.

2.5. Binding of Hfq-like proteins to sRNA by EMSA

Purified His-tagged Hfq proteins were incubated at room temperature with 2.75 μM of biotin-labeled sRNAs in 20 μL RNA-binding buffer

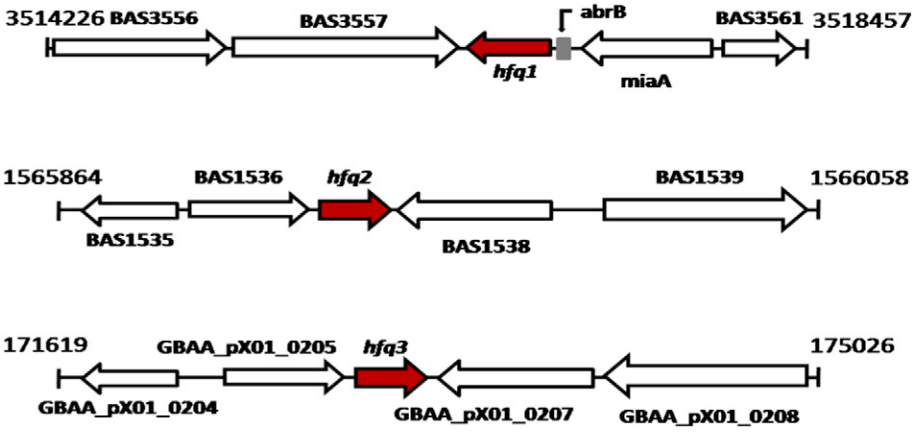

Fig. 1. Genetic organization of multiple Hfq-like RNA chaperones in *B. anthracis*. *hfq1*, *hfq2*, and *hfq3* are represented in red color. The predicted AbrB binding site in Hfq1 promoter region is also indicated.