Hfq Is a Global Regulator That Controls the Pathogenicity of Staphylococcus aureus

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Abstract
The Hfq protein is reported to be an RNA chaperone, which is involved in the stress response and the virulence of several pathogens. In E. coli, Hfq can mediate the interaction between some sRNAs and their target mRNAs. But it is controversial whether Hfq plays an important role in S. aureus. In this study, we found that the deletion of hfq gene in S. aureus 8325-4 can increase the surface carotenoid pigments. The hfq mutant was more resistant to oxidative stress but the pathogenicity of the mutant was reduced. We reveal that the Hfq protein can be detected only in some S. aureus strains. Using microarray and qRT-PCR, we identified 116 genes in the hfq mutant which had differential expression from the wild type, most of which are related to the phenotype and virulence of S. aureus. Among the 116 genes, 49 mRNAs can specifically bind Hfq protein, which indicates that Hfq also acts as an RNA binding protein in S. aureus. Our data suggest that Hfq protein of S. aureus is a multifunctional regulator involved in stress and virulence.

Introduction
Hfq protein was originally identified in E. coli as a host factor for the replication of the Qβ phage RNA [1] and is considered to be a multifunctional regulator of a variety of targets in bacteria [2]. Hfq regulates post-transcriptional gene expression in E. coli, specifically mRNA translation, stability or polyadenylation [3,4].

Recently most studies have focused on the RNA chaperone function of Hfq in mediating the interaction between sRNA(small RNA) and mRNA [5]. In E. coli, some known sRNAs can interact with Hfq [6]. In Salmonella, Hfq associates with almost half of the co-immunoprecipitated sRNAs [7]. It is reported that Hfq can influence the stability of several sRNAs [8–10]. Besides acting as an RNA chaperone, E. coli Hfq has ATPase activity and can mildly affect transcription and translation in vitro [11,12].

The inactivation of Hfq in E. coli can affect pleiotropic phenotypes, such as decreased growth rates, negative supercoiling of plasmids in stationary phase, increased carbon source oxidation, and increased sensitivity to ultraviolet light [13]. Hfq is also involved in stress response or virulence of a variety of pathogenic bacteria, such as Neisseria meningitides, Salmonella, Vibrio cholerae and Pseudomonas aeruginosa [7,14–19].

Staphylococcus aureus, a major Gram-positive pathogen, can cause a broad range of diseases, from minor skin infections to life-threatening diseases, such as toxic shock syndrome and septicemia [20]. Many reports identify that RNAIII can act as antisense RNA and regulate the expression of several virulence genes, such as spa (Staphylococcal protein A, an important virulence factor of S. aureus) and hla (alpha-toxin gene, a major virulence factor of S. aureus) [21]. There is also an Hfq-like protein (0.9kD) in S. aureus, which can form homo-hexamer [22]. The structure of Hfq-binding RNA has been identified [3,22]. Under in vitro condition, it is found that Hfq can specifically bind RNAIII and spa mRNA, which suggests that S. aureus Hfq can also act as an RNA chaperone [23]. However, Bohn et al. showed that Hfq did not play a crucial role in stress response, spa mRNA expression, or exoprotein expression in S. aureus RN6390, COL and Newman. They also tested ~2000 phenotypes in the RN6390 hfq mutant by the Phenotype Microarray (PM) Technology [24], and found Hfq was not involved in the stress response, resistance to several chemical agents, or metabolic pathways [24]. It was also reported that S. aureus Hfq could not affect SA1000 expression, which is a fibrinogen-binding protein and identified as a target of RNAIII [25]. Geisinger et al. suggested that the weak transcription of hfq was possibly due to the loss of the gene’s major promoter [26].

In this study, we found the carotenoid pigment increased in the hfq mutant of S. aureus 8325-4 (Δhfq-8325) and the mutant was less toxic during infection of MDMK cell and a mouse model of peritonitis than its parental strains. The results of the microarray studies showed that the expression of the 116 genes was altered in Δhfq-8325. We also found that 48 mRNAs of the 116 genes can specifically bind Hfq, which suggests that Hfq may be an RNA binding protein. We believe Hfq is a multifunctional regulator of gene expression and plays a major role in the infection of S. aureus. Our conclusion is contrary to the finding by Bohn et al. The difference of these findings may be due to differing expression patterns of the Hfq protein in different S. aureus strains.

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Results

Deletion of Hfq in S. aureus 8325-4 increased the carotenoid pigments

We constructed the hfq gene deletion mutant (Δhfq-8325) derived from S. aureus 8325-4. The result showed that Δhfq-8325 displayed high intensities of yellow pigmentation compared to the wild type strain. After the restoration of Hfq activity in Δhfq-8325, the color of the restored strain (rs-hfq-8325) was recovered (Figure 1A, 1B). The intensity of the yellow carotenoid pigment of S. aureus is one of the classical criteria for identification of this species. The genes of crtM and crtN, which encode dehydrospalen synthase and dehydrospalen desaturase respectively, are essential for the pigment’s synthesis of S. aureus. Both genes are located in the same operon [27]. We quantified the mRNA level of crtM in 8325-4, Δhfq-8325 and rs-hfq-8325 by qRT-PCR, and showed that the mRNA level of crtM increased in Δhfq-8325 (Figure 1C). This may explain why the carotenoid pigment of Δhfq-8325 was increased.

It was reported that the pigment acted as an antioxidant for S. aureus’ resistance to neutrophil killing [28]. We tested the susceptibility of Δhfq-8325 to oxidant, and showed that Δhfq-8325 was indeed more resistant to oxidant than the wild type (Figure 2A, 2B). In addition, we found that the number of the mutant bacterium survived in neutrophils was significantly larger than that of the wild type (Figure 2C). We also found that Δhfq-8325 didn’t display other phenotypic variations including biofilm formation, cell growth, surface charge and hydrophobicity (data not shown).

The bacterial strains used in the work of Bohn et al. were RN6390, COL and Newman, which were different from the ones in our work [24]. Although the mRNA of hfq could be detected in such strains by RT-PCR, we wondered whether this mRNA could be translated into the protein. So we extracted the same amount of total cellular proteins from 8325-4, RN6390 and other 11 strains, and detected the expression of Hfq by Western blot with the specific anti-Hfq antibodies prepared in our lab. The results revealed that Hfq protein could be found in 8325-4 and other 7 strains, but not in COL and RN6390 (Figure 3). The lack of Hfq may explain the non-detectable effect of Hfq in S. aureus COL and RN6390 in the work of Bohn et al. [24]. The sequences of all the genes encoding Hfq protein including their promoter regions in these strains are confirmed to be the same (data not shown). We also compared the transcription of hfq in 8325-4 and RN6390 by qRT-PCR using 16S rRNA as the control. No significant difference was observed (data not shown). This result suggested that there might be some post-transcription regulation of Hfq protein. Recently Becher et al. reported that mRNA of hfq (SACOL1324) could be detected in COL by DNA microarray, but the Hfq protein was undetectable [29]. This result is consistent with our observation. The different expression profiles of Hfq in S. aureus strains needs further investigation.

Δhfq-8325 was suscetive to the whole blood killing in spite of its increased golden pigments

The work of Liu et al. showed that the number of ActM-8325 survived in the whole blood decreased compared with WT because of reduced pigment synthesis [28]. We performed a similar experiment to verify whether Δhfq-8325 was more resistant to the whole blood than wild type. It was surprising that the number of surviving Δhfq-8325 cells was significantly less than the wild type (Figure 4). These results suggest that some other regulators in Δhfq-8325 might be more important than the pigments for S. aureus survival in the whole blood.

Figure 1. Effect of hfq deletion on pigmentation. S. aureus strains WT, Δhfq-8325 and rs-hfq-8325 were cultured for 12 h. The pigments of different strains were determined. The carotenoid pigment of Δhfq-8325 was increased (A) and showed the characteristic triple peak spectral profile of carotenoid (B). However, the color of rs-hfq-8325 was similar to the wild type. The expression level of crtM was detected by qRT-PCR. The results of qRT-PCR showed that the expression of crtM was increased in Δhfq-8325 and recovered in rs-hfq-8325 (C). The results shown were representative of three independent experiments (* P<0.01). WT, S. aureus 8325-4; Δhfq-8325, 8325-4 with an hfq::kan mutation; rs-hfq-8325, the restoration of Hfq activity in Δhfq-8325.

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The deletion of hfq decreased the toxicity of S. aureus

It is well known that the secreted toxins are important for infection caused by S. aureus. We compared the cytotoxicity of the hfq mutant with that of the wild type. After incubation with the supernatants from different strains, the survival cell numbers were quantified by CCK8 assay. We found that the toxicity of the supernatant of DΔhfq-8325 significantly decreased compared to its parental strain (Figure 5). However, the cytotoxicity of the supernatant of RN6390 and COL did not alter after the hfq gene deletion (Figure 5). In order to investigate if Hfq protein could influence the toxicity in other strains besides 8325-4, we constructed an hfq-deletion mutant from ATCC25923, which was an Hfq positive strain (Figure 3). Similarly, the survival percentage of cells after treatment of the supernatant of ATCC25923 was lower than that of the hfq mutant (Figure 5). The apoptosis and necrosis of MDBK cells induced by the supernatant was tested using flow cytometry. We found that the percentage of apoptosis and necrosis induced by the supernatant of DΔhfq-8325 was significantly lower than that of the wild type (Figure 6). In our further study, the pathogenicity of DΔhfq-8325 was assessed in a murine peritonitis model, with an equal number of bacterium from the wild type and DΔhfq-8325 injected.

Figure 2. Δhfq-8325 shows enhanced resistance to oxidant and neutrophil killing. The oxidant susceptibility assays were performed to detect the susceptibility of Δhfq-8325 to oxidative stress. S. aureus cells were incubated with oxidants or neutrophil. The survival number of bacteria was determined. The figures showed that Δhfq-8325 conferred enhanced oxidant and neutrophil resistance compared with the wild type (S. aureus 8325-4). The results shown were representative of three independent experiments (* p<0.01). A: the singlet oxygen assay; B: the double oxygen assay; C: neutrophil survival assays. The results shown were representative of three independent experiments. WT, S. aureus 8325-4; Δhfq-8325, 8325-4 with an hfq::kan mutation; rs-hfq-8325, the restoration of Hfq activity in Δhfq-8325.

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Figure 3. The detection of Hfq in the different S. aureus strains by Western blot. The total cellular proteins from the S. aureus cells at the same growth phase were extracted from different S. aureus strains, and the equal quantity of proteins was loaded to SDS-PAGE. The expression of Hfq was tested with the specific antibodies of Hfq by Western blot. The recombinant Hfq protein was used as a control. Lanes: 1, Molecular Weight Marker; 2, recombinant Hfq protein; 3, 040196; 4, 040188; 5, ATCC25923; 6, COL; 7, RN6390; 8, MRSA8963; 9, MRSA8972; 10, MRSA9004; 11, MRSA8973; 12, MRSA8979; 13, 8325-4; 14, Δhfq-8325; 15, rs-hfq-8325.

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intraperitoneally into the mice. The result showed that at the different time points (6 h, 10 h, 20 h, 24 h and 30 h), the survival number of the \( \textit{D} \text{hfq-8325} \) group was larger than that of the wild type group (Figure 7A), which was consistent with the results of the whole blood survival assay and the cytotoxicity assay. The pathogenicity of \( \textit{D} \text{hfq-25923} \) was also assessed in the same murine peritonitis model, and the result showed that the survival number of the \( \textit{D} \text{hfq-25923} \) group was larger than that of \( \textit{ATCC25923} \) group (Figure 7B).

Identification of the changed genes in \( \textit{?hfq-8325} \) using microarray assay

The phenotype and pathogenicity of \( \textit{D} \text{hfq-8325} \) were different from that of the wild type, so we analyzed the global change of gene expression in \( \textit{D} \text{hfq-8325} \) by microarray. The results revealed that the expression levels of 116 genes were altered in \( \textit{D} \text{hfq-8325} \) (fold change≥1.5), most of which were involved in the stress response and pathogenicity of \( \textit{S. aureus} \). Among the 116 genes, 33 had declined expression level (Table S1), while 83 had elevated expression level, compared to the wild type (Table S2). It was found that the level of \( \textit{cfrM} \) in \( \textit{D} \text{hfq-8325} \) was increased, which was consistent with the result of qRT-PCR. The expression of some virulence genes (such as serine protease, cysteine protease and staphylococcal nuclease) and global regulators (such as \( \textit{sarA} \)) was changed after \( \textit{hfq} \) deletion, which could affect the pathogenicity of \( \textit{D} \text{hfq-8325} \). In order to validate the microarray data, the level of four genes (two up-regulated genes and two down-regulated in \( \textit{D} \text{hfq-8325} \)) was tested. The results of qRT-PCR were consistent with the microarray data (Figure 8).

Although it remained controversial whether Hfq plays an important role in \( \textit{S. aureus} \), it has been confirmed that Hfq protein could specifically bind RNAIII [23]. We obtained the RNAs that could potentially bind Hfq protein by immunoprecipitation \textit{in vitro} and these RNAs were further analyzed by microarray. We identified about 300 mRNAs which were enriched in the pool of Hfq IP, including RNAIII (Table S3). Then we compared these RNAs with the 116 genes identified in \( \textit{D} \text{hfq-8325} \) (Table S1, S2), and we found that 49 of the 116 genes in \( \textit{D} \text{hfq-8325} \) were enriched in the pool of RNAs that bind Hfq protein. It is likely these genes may be regulated by Hfq.

Discussion

Hfq was discovered as an \( \textit{E. coli} \) host factor required for replication of RNA phage Qβ [1]. Hfq protein belongs to the large family of Sm and Sm like proteins. The sequence alignment shows that \( \textit{E. coli} \) Hfq protein and \( \textit{S. aureus} \) Hfq have the conserved Sm1 sequence motif, and the resolved structures show both of them can form homo-hexameric structures [3]. More recent studies show that Hfq protein can act as an RNA chaperone to mediate RNA-RNA interaction in \( \textit{E. coli} \) [10]. But it is controversial whether Hfq plays an important role in \( \textit{S. aureus} \). Many reports reveal that \( \textit{S. aureus} \) RNAIII acts as an antisense RNA to regulate its target genes expression [23,25,26], and it is a potential target of \( \textit{S. aureus} \) Hfq \textit{in vivo} [23]. However, Boisset et al. found that Hfq protein exerted no

![Figure 4. The survival number of different strains in whole blood.](image1)

S. aureus cells were incubated with human whole blood. The survival bacterial number was detected. Compared with its parental strain, the \( \textit{D} \text{hfq-8325} \) strain was more sensitive to whole blood killing. The results shown were representative of three independent experiments (* p<0.01). WT, \( \textit{S. aureus} \) \( 8325-4 \); \( \textit{D} \text{hfq-8325}, \) \( 8325-4 \) with an hfq::kan mutation; \( \textit{rs-hfq-8325}, \) the restoration of Hfq activity in \( \textit{D} \text{hfq-8325} \). doi:10.1371/journal.pone.0013069.g004

![Figure 5. Detection of the percentage of survival cell using CCK8 assay.](image2)

The supernatants from different strains were collected by centrifugation and incubated with MDBK cells for 12 h at 37 °C. Cell survival was determined by CCK8 assay. The supernatant of \( \textit{D} \text{hfq-8325} \) was less cytotoxic than that of wild type (* p<0.01). doi:10.1371/journal.pone.0013069.g005
major effect in vivo on SA1000, SA2333, rot mRNA levels or RNAIII stability in S. aureus RN6390 [25]. Furthermore Bohn et al. reported that the deletion of Hfq had no noticeable effect on virulence gene expression nor was this protein highly expressed in S. aureus RN6390 [24].

In our work, we found that the deletion of Hfq can increase the pigments synthesis in 8325-4, while the color of the bacteria was recovered after the hfq gene was restored in Δhfq-8325. These results suggest that Hfq can influence the phenotype of S. aureus. The reason why our conclusion is different from that of Bohn et al. may be due to the different strains used in the experiments. Although S. aureus 8325-4 and RN6390 both originate from S. aureus 8325, the RN6390 has been generated by three rounds of transduction from 8325-4, and they have been validated to be different strains [30–32]. We also found that the Hfq protein can be detected in 8325-4 and other strains, but not in RN6390. The lack of Hfq expression may explain that there were no detectable changes induced by inactivation of Hfq in some strains. The reason why Hfq can not be detected in some strains is not clear and is now being investigated in our lab.

Although the pigments increased in Δhfq-8325, we found the survival number of Δhfq-8325 in the whole blood decreased significantly and the Δhfq-8325 was less toxic to mammalian cells and mice. These results suggest that the pigments are just one factor of S. aureus pathogenicity and there may be some other more crucial factors regulated by Hfq. We then compared the gene expression profile of Δhfq-8325 with that of wild type. The transcription analysis showed that the expression of many genes was altered. Several serine protease and cysteine proteases (such as sspA, sspB and splC) were down-regulated in Δhfq-8325 and identified as the important factors for S. aureus infection. A transposon mutant of the ssp operon had an attenuated virulence in three separate animal models (mouse abscess, bacteremia and wound infection) [33]. This may explain why the pathogenesis of Δhfq-8325 was decreased in the animal model.

Some recent researches have suggested that the studies defining the regulatory roles of sarA and agr using RN6390 are not always representative of the events that occur in clinical isolates of S. aureus [34,35]. So we constructed an hfq deletion mutant from ATCC25923, which is a clinical isolate, to investigate the

Figure 6. Flow cytometric analysis of the percentage of apoptosis and necrosis of cells. The apoptosis and necrosis of MDBK cells treated with the supernatant of S. aureus was detected by flow cytometry. The figure shows the summary data of the percentage of normal, apoptosis and necrosis of MDBK cells after the challenge of supernatant of Δhfq-8325 was lower than wild type. The final data represented the mean ± SD for three independent experiments (* p<0.01).
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Figure 7. The pathogenicity of the hfq deletion mutant was decreased in the animal model. The pathogenicity of the hfq deletion mutants was assessed in a murine peritonitis model. S. aureus cells were injected intraperitoneally into the mice (10 mice per group). Survival of mice was recorded at the different time points. The result showed that the survival number of the hfq deletion mutants group was larger than that of the wild type group at each time point.
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biological function of Hfq. Our results showed that the cytotoxicity and pathogenesis of the Δhfq-25923 was significantly decreased as compared to its parental strain which suggests that Hfq can influence the toxicity of lab strains and clinical isolates.

The RNA chaperone activity of S. aureus Hfq has not been confirmed [23–25]. Although Hfq protein of E. coli has a longer C terminal-tail than that of S. aureus, the tail appears to be dispensable in the RNA binding function of Hfq [3]. Here we obtained the RNAs that bind Hfq in S. aureus 8325-4 by immunoprecipitation. The binding RNAs were then identified using microarray technology. We found that about 40% of the mRNAs of the changed genes in Δhfq-8325 could specifically bind Hfq protein. The results suggest that the Hfq protein in S. aureus can also act as an RNA binding protein such as in E. coli. We are investigating the mechanism how these genes can be regulated by Hfq directly or indirectly.

Materials and Methods

Ethics Statement

All animal experimental protocols of the study are in accordance with the national guidelines for the use of animals in scientific research. It’s also approved by Animal Care and Use Committee of Beijing Institute of Basic Medical Sciences, with the approval number BMS-081210.

Bacterial strains and growth conditions

The strains used in this study are listed in Table 1. Strains were grown in 5 ml of brain heart infusion (BHI, BD) or Luria-Bertani (LB) medium at 37°C for 12 h with shaking at 200 rpm in a 25-ml test tube. Cells from 1 ml of pre-culture were transferred to 100 ml of BHI or LB medium in a 500-ml flask and incubated at 37°C on a rotary shaker at 200 rpm. S. aureus strains were routinely grown in BHI and E. coli strains were grown in LB medium either with no antibiotics, or with 20 μg/ml erythromycin, 100 μg/ml ampicillin and 50 μg/ml kanamycin.

Expression and purification of Hfq protein

The hfq gene was amplified from the genome of S. aureus 8325-4 with the primers (hfq NcoI and hfq XhoI) in Table 2. The PCR product was cloned into the expression vector pET28(a) as an NcoI-XhoI fragment, generating pET28(a)-hfq, which was subsequently transformed into E. coli strain BL21 (DE3). Expression of the recombinant Hfq protein was induced by adding 0.1 mM isopropyl β-D-thiogalactopyranoside (IPTG) to a growing culture at OD₆₀₀ = 0.4. Purification of recombinant protein on Q Sepharose Fast Flow and SP Sepharose Fast Flow columns (Amersham Biosciences) was carried out according to the manufacturer’s instructions.

Production of polyclonal antibodies against Hfq

Female New Zealand White rabbits were first immunized by subcutaneous injection with 1 mg of Hfq protein in Phosphate Buffered Saline (PBS) mixed with complete Freund’s adjuvant in a total volume of 1 ml. And the subsequent booster injections, i.e., 1 mg of Hfq in PBS emulsified in incomplete Freund’s adjuvant, were administered at 3 and 6 weeks after the primary immunization. At the eighth week, the sera were collected and the antibody titers were determined by ELISA.

Figure 8. qRT-PCR quantification of the expression of sspB, nuc, sarA and capJ. The expression level of sspB, nuc, sarA and capJ was detected by qPCR. The results of qRT-PCR were accordant to the microarray data. The results shown were representative of three independent experiments (* P<0.01).
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Construction of \textit{hfq} deletion mutant

The mutant was constructed using the method described previously [36] with some modifications. In order to create a deletion mutant of \textit{hfq} in the chromosome of 8325-4, two regions of DNA flanking the \textit{hfq} gene were amplified by PCR using the primers (Up-\textit{hfq} F-EcoRI and Up-\textit{hfq} R-KpnI; Down-\textit{hfq} F-KpnI and Down-\textit{hfq} R-SalI) with restriction sites listed in Table 2. The upstream fragment (659 bp) was digested with EcoRI and KpnI, and the downstream fragment (680 bp) was digested with KpnI and SalI. The two fragments were cloned together into pMD19T digested with EcoRI and SalI. The resulting construct was digested with KpnI, and then a 1.6-kb kanamycin cassette which was amplified from the plasmid of pTZ-TRAP::kan provided by Dr. Balaban N was inserted. The resulting plasmid was digested with EcoRI and SalI, and a fragment harboring kanamycin resistance between the upstream and downstream fragments was ligated into pAUL-A digested with EcoRI and SalI to create plasmid pAUL-A-\textit{hfq}. pAUL-A has a temperature-sensitive origin of replication that is active in \textit{S. aureus} at 30\degree C but not at 42\degree C. The recombinant plasmid initially isolated from \textit{E. coli}, was introduced into \textit{S. aureus} RN4220 by electroporation and colonies resistant to kanamycin and erythromycin were selected after growth at 30\degree C. The resistant clones were subjected to a temperature shift to 42\degree C to select the plasmid integration into the chromosome. Bacteria resistant to kanamycin but sensitive to erythromycin were selected. The mutation was confirmed by PCR, and followed by transduction into strains 8325-4, RN6390, COL and ATCC25923 with phage \textit{W}11 to create the mutant strains from which the coding region of \textit{hfq} (234 bp) was deleted (Table 1).

Restoring Hfq Activity

Primers rs-\textit{hfq}F and rs-\textit{hfq}R (listed in Table 2) were designed to PCR-amplify a 1406 bp fragment encompassing the gene encoding for Hfq, the promoter region and termination site with SalI/EcoRI sites. The PCR product was digested and cloned into the SalI/EcoRI digested pMAD, which could replicate in \textit{E. coli} but not in \textit{S. aureus}. The resulting plasmid was used to transform into \textit{E. coli} DH5\textalpha. Cells were selected on LB plates containing 100 \mu g/ml ampicillin. Plasmid was isolated from positive clones and used to transform

| Strain or plasmid | Comments | Source or reference |
|-------------------|----------|---------------------|
| **Strain** | | |
| 8325-4 | Wild-type, rsbU’ | [30] |
| \textit{hfq}8325 | 8325-4 with an \textit{hfq}::kan mutation | This study |
| rs-\textit{hfq}8325 | \textit{hfq}8325 restoring Hfq activity | This study |
| ATCC25923 | clinical isolate | [42] |
| \textit{hfq}25923 | ATCC25923 with an \textit{hfq}::kan mutation | This study |
| COL | Highly methicillin resistant clinical isolate | Dr. William M. Shafer |
| \textit{hfq}– COL | COL with an \textit{hfq}::kan mutation | This study |
| RN6390 | Laboratory virulent strain derived from 8325 | [31] |
| \textit{hfq}–RN6390 | RN6390 with an \textit{hfq}::kan mutation | This study |
| RN4220 | Restriction-negative strain, 8325 derivative | [43] |
| MRSA 8963 | Methicillin resistant clinical isolate | professor Jingui Cao |
| MRSA 8972 | Methicillin resistant clinical isolate | professor Jingui Cao |
| MRSA 8973 | Methicillin resistant clinical isolate | professor Jingui Cao |
| MRSA 8979 | Methicillin resistant clinical isolate | professor Jingui Cao |
| MRSA 9004 | Methicillin resistant clinical isolate | professor Jingui Cao |
| 040188 | Laboratory strain | stored in our lab |
| 040196 | Laboratory strain | stored in our lab |
| **E. coli** | | |
| DH5\textalpha | A host strain for cloning | Transgene |
| BL21(DE3) | A host strain for protein expression | Transgene |
| **Plasmids** | | |
| pMD19T | \textit{E. coli} cloning vector, ampR | TaKaRa |
| pMD20T | \textit{E. coli} cloning vector, ampR | TaKaRa |
| pET28 (a) | \textit{E. coli} expression vector, kanR | Novagen |
| pAUL-A | Temperature-sensitive \textit{S. aureus} suicide vector; EmR | [44] |
| pAUL-A-\textit{hfq} | pAUL-A containing whole \textit{hfq} gene | This study |
| pMAD | Temperature-sensitive \textit{S. aureus} suicide vector; EmR | [45] |
| pMAD-\textit{hfq} | pMAD containing whole \textit{hfq} gene for restoring Hfq activity | This study |

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**Table 2. Sequences of forward and reverse primers used in this study.**

| Primer/sequencer | Oligonucleotide sequence (5’ to 3’) |
|------------------|-----------------------------------|
| hqf NcoI         | CATGCACTGGCAGTATGGCGCAAGAACAT     |
| hqf XhoI         | CCGCCTGGATATCTTCCTTCACTAGTATG     |
| 16S F            | GCCTAATACGCAAGT                  |
| 16S R            | CATTTGATCCGGAAGTAAAGGG           |
| Up-hqf F-EcoRII  | CATTCCGAAATTCACTCAATGAGTTGAAC    |
| Up- hqf R-KpnII  | TCATTGCTTACCTGGCGACTTTTACT       |
| Down- hqf F-KpnII| CGACAGGTTAACAAGTGGAAGAATTTGAA    |
| Down- hqf R-Sall | ACAGCGCTTGACAAATAGGTTGACTTTAC    |
| rs-hqF           | TCTAGATGTCGACTATTACCTAACAAG      |
| rs-hqR           | GATATCGATGATCTCACTGAAGAATTTTTAT  |
| crtM RT primerF  | GACTTGGTGTAAGTGGTC                |
| crtM RT primerR  | CTTGATGATGATGTTGTC               |
| sspB RT primerF  | AAAGCGGAAGACAAAGAGA              |
| sspB RT primerR  | CTTGATGATGAGAAGGTTG              |
| nuc RT primerF   | GTAGCCCATATATTTGAG               |
| nuc RT primerR   | CCAAGGATGGTACCTTTG               |
| sarA RT primerF  | CTCAAGAAGATTTCTGCAG              |
| sarA RT primerR  | GCTGATGATTCGGTTA                 |
| capL RT primerF  | ATATCTCAAAGGGTGAAC               |
| capL RT primerR  | CTTATCAAGGCAATAGGA               |

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**aurus RN4220. The transformants were selected on tryptic soy agar plates containing 10 μg/ml erythromycin at 30°C.** Then the plasmid was isolated from the positive clone (RN4220 containing pMAD-rs-hfq) and transformed to Δhqf-8325 by electroporation. The transformants were selected on tryptic soy agar plates containing 10 μg/ml erythromycin at 30°C for 48 h, and then transferred to 42°C for 12 h. The reconstituted colonies (rs-hqf-8325) were confirmed by PCR and RT-PCR analysis.

**Oxidant susceptibility assay**

The assay to test susceptibility to oxidants was performed as previously described [37] with some modifications. For hydrogen peroxide susceptibility assay, *S. aureus* cells were grown to early exponential phase and harvested by centrifugation. Hydrogen peroxide (H$_2$O$_2$) was added at 1.5% final concentration to the bacteria (2 x 10$^7$/ml) and incubated at 37°C for 1 h. Then, 1,000 U/ml of catalase (Sigma-Aldrich) was added to quench residual H$_2$O$_2$. Dilutions were plated on tryptic soy agar plates for enumeration of surviving cells.

The assay to test susceptibility to singlet oxygen was performed as previously described [37] with some modifications as well. *S. aureus* strains were grown to early exponential phase and harvested by centrifugation. The bacteria (10$^7$/ml) were incubated at 37°C in a 24-well culture plate. 200 μl of 0.1 μg/ml methylene blue was added to the wells and the plate was situated at exactly 10 cm from a 100-W light source. The viable cells were assessed after 1.5 h by plating dilutions on tryptic soy agar plates.

**Neutrophil intracellular survival assay**

Neutrophils for intracellular survival assays were purified from healthy human volunteers as previously described [37]. Bacteria were washed twice in PBS, diluted to a concentration of 4.5 x 10$^6$ CFU (colony forming unit) in 100 μl RPMI1640 with 10% fetal calf serum and mixed with 3 x 10$^7$ neutrophils in the same media. The mixture was centrifuged at 700 g for 5 min, and then incubated at 37°C in a 5% CO$_2$ incubator. After 10 min gentamicin was added to a final concentration of 400 μg/ml for killing the extracellular bacteria. Then, the contents of sample wells were withdrawn and centrifuged to pellet the neutrophils. Neutrophils were washed twice, and then lysed in 0.02% Triton X-100. CFU was calculated by serial dilution plated on Todd-Hewitt agar (THA; 1.5% agar in Todd-Hewitt broth) plate.

**Whole-blood killing assay (WBKA)**

*S. aureus* strains were grown for 12 h and harvested by centrifugation (10,000 g, 1 min). Bacteria were washed twice in PBS, and then 1 x 10$^8$ cells were diluted to the volume of 25 μl in PBS, and mixed with 75 μl freshly drawn mouse blood in heparinized tubes. The tubes were incubated at 37°C for 4 h with agitation, and then surviving cells were enumerated by plating the dilutions on THA plates.

**Cytotoxicity assay**

The supernatant of *S. aureus* was collected as described [38,39] with some modifications. Briefly, *S. aureus* cells were grown to stationary phase at 37°C. Growth culture was centrifuged at 6,000 g for 10 min at 4°C. The supernatant was collected and filtered through a 0.22 μm filter to remove residual cells.

Madin-Darby Bovine Kidney (MDBK, ATCC-CCL22) cells were resuspended at a concentration of 1 x 10$^5$ cells/ml and added to a 96-well plate (100 μl/well) at 37°C in a 5% CO$_2$ incubator for 6 h. The supernatant of *S. aureus* strains (5 μl/well) was added, with BHI broth as the control. The CCK8 essay was used to test the number of survival cells as per the protocol (Dojindo) after 12 h incubation. The cell viability percentage was calculated as: Viability percentage (%) = (Absorption value of supernatant of treatment group)/Absorption value of supernatant of control group) x 100%.

For flow cytometric analysis, the MDBK cells were resuspended at a concentration of 1 x 10$^5$ cells/ml and added to a 12-well plate (1 ml/well) at 37°C in a 5% CO$_2$ incubator for 6 h. The cultivated cells were incubated with BHI or with supernatant (100 μl/well) of *S. aureus* strains for 1 h. Prior to harvesting, the cells were washed twice with PBS, trypsinized, and pelleted. Then cells were resuspended at a concentration of 1 x 10$^6$ cells/ml in Binding Buffer (0.01 M HEPES/NaOH, pH 7.4, 14 mM NaCl, 0.25 mM CaCl$_2$). 500 μl aliquots of cells were added into FACS tubes and mixed with 25 ng/ml fluorescein isothiocyanate–labeled annexin V and 10 mg/ml propidium iodide (PI) to incubation for 15 min at room temperature in the dark. Then the cells were analyzed immediately by flow cytometry.

**RNAs binding to Hfq were obtained by immunoprecipitation**

The immunoprecipitation assays were prepared as described previously [23] with some modifications. The purified Hfq protein (100 μg) was incubated with 5 ml rabbit immune serum containing *S. aureus* Hfq-specific antibodies or rabbit normal serum at 37°C for 30 min. The captured antibodies were then bound to 1 ml of protein A sepharose CL-4B (Sigma-Aldrich) at 37°C for 30 min. After four washes with 2 ml washing buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.5% Triton X-100), the total RNA of *S. aureus* was added and incubated at 37°C for 30 min. After another four washes with 2 ml washing buffer, the binding complexes were eluted from protein A sepharose with 1 ml of 0.1 M Glycine–HCl, pH 2.5, and neutralized with 100 ul
of 1 M Tris–HCl, pH 8.0. Then the mixture was extracted by phenol, followed by RNA precipitation and the RNAs which were bound to Hfq were analyzed by microarray. Calculation of enrichment factor was performed as follows: enrichment factor = signal intensities of Hfq IP/signal intensities of control IP.

Transcriptional microarray analysis
Gene expression profiles of the Ahfq-8325 and wild type strains were analyzed by using Affymetrix S. aureus arrays as described previously [40] with some modifications as follows. The S. aureus array contains probe sets to over 3,300 previously [40] with some modifications as follows. The S. aureus genomic sequences of N315, Mu50 and COL [41]. Total (ORFs) and 4,800 intergenic region sequences based on the updated S. aureus genomic sequences of N315, Mu50 and COL [41]. Total bacterial RNA was extracted from S. aureus which were grown for 6 h with shaking at 37°C using Trizol (Invitrogen) as previously described [41]. DNase digestion of 80 μl of total RNA was performed with 10 U of RNase-free DNase I (Promega) and 10 μl of the 10 × reaction buffers in a total reaction volume of 100 μl for 30 min at 37°C. The RNA (10 μg) was reversely transcribed to cDNA by using M-MLV reverse transcriptase (Promega) and random primers (Promega) for 1 h at 42°C. The fragmented cDNAs were then directly labeled with biotin by using a biotin-ddUTP kit (Affymetrix). Biotinylated cDNA was hybridized to the GeneChips. Microarrays were scanned using an Affymetrix GeneChip Scanner 3000 and image data were extracted using GeneChip Operating Software (GCOS) Version 1.4. Two independent labeling reactions and hybridizations were carried out for each RNA sample. This experiment was completed by Bioassay Laboratory of CapitalBio Corporation. Normalization and expression analysis were performed with DNA-chip analyzer (dChip). Invariant set normalization was used to normalize arrays at the probe level. Genes with variations (n-fold $1.5) were collected. All data is MIAME compliant, and the raw data has been deposited in the database ArrayExpress (No. E-TABM-1005).

Acute murine peritoneal infection model
Groups (n=10) of 6- to 8-week-old, male Balb/c mice were injected intra-abdominally with 500 μl of different strains. The injected cell number of Ahfq-8325 or S. aureus 8325-4 was 1×10⁶ CFU per mouse. And the injected cell number of Ahfq-8259 or ATCC25923 was 5×10⁶ CFU per mouse. The survival number of mice was recorded at the different time points (6 h, 10 h, 20 h, 24 h and 30 h) post challenge. Survival outcomes in the wild type or the Ahfq-8325 groups were compared.

Preparation of S. aureus total proteins and Western blot
The cell extract was prepared as described [41] with some modifications as follows. Cells were grown for 6 h with shaking at 37°C. Equal numbers of cells (1×10⁶ CFU) were collected and then resuspended in 1 ml PBS buffer containing lysostaphin (100 μg/ml, Sigma-Aldrich). After incubation for 30 min at 37°C, the mixture was centrifuged at 13,000 g for 10 min and the precipitate was resuspended in 1 ml PBS. The mixture was sonicated on ice three times for 30 s each and centrifuged at 13,000 g for 10 min. The supernatant was precipitated by adjusting filtered supernatants to 10% trichloroacetic acid (TCA) and incubated at 4°C for 4 h. After centrifugations at 12,000 g for 10 min, precipitated proteins were washed twice in ice-cold 96% ethanol, and air dried. The proteins were resolved in an appropriate volume of a solution containing 7 M urea and 2 M thiourea. The samples were then subjected to 15% SDS-PAGE and the proteins were blotted onto Hybond-ECL nitrocellulose membrane (Amerham Biosciences). The membrane was blocked in 5% non-fat dry milk at 37°C for 2 h, probed with 1:500 diluted polyclonal rabbit anti-Hfq antibodies (prepared by ourselves) for 1 h at room temperature, and washed twice in PBS with 0.5% Tween 20 (PBST). Then the membrane was incubated in a 1:5,000 solution of HRP-conjugated goat anti-rabbit secondary antibody (Jackson) at room temperature for 1 h. After further washing with PBST, the membrane was assayed by the enhanced chemiluminescence (ECL) Western blotting detection system (Pierce).

Quantitative reverse transcription PCR (qRT-PCR)
For cDNA synthesis, 1 μg of total RNA was mixed with 500 ng of random hexamer (Promega). Samples were incubated at 65°C for 10 min with 5 μl of 5× first-strand buffer, 2 μl of 5 mM dNTP, 2 μl of RNasin (Takara), 1 μl of M-MLV reverse transcriptase (Promega) and distilled water to a total volume of 25 μl. The qPCR reaction mixture contained 12.5 μl of 2×SYBR green PCR mix (GenePharma), 0.3 μM of gene-specific forward and reverse primers, and 1 μl of template, made up to a final volume of 25 μl with distilled water. The primers are shown in Table 2. Cycling parameters were set as follows: initial activation step at 95°C for 10 min, denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 40 s. Melting curve analysis was performed at the range of 65°C to 95°C with stepwise fluorescence acquisition at every 1°C to 5°C. Melting curves of each gene were confirmed to correspond to the correct amplicon size by agarose gel electrophoresis of the PCR products. The levels of gene expression were calculated by relative quantification using 16S rRNA as the endogenous reference gene. All samples were amplified in triplicate and the data analysis was carried out using the MxPro qPCR system software (Stratagene).

Statistical analysis
All quantitative data were analyzed using Student t-tests. P<0.05 was considered to be statistically significant.

Supporting Information
Table S1  Genes down-regulated in Ahfq-8325. Found at: doi:10.1371/journal.pone.0013069.s001 (0.07 MB DOC)
Table S2  Genes up-regulated in Ahfq-8325. Found at: doi:10.1371/journal.pone.0013069.s002 (0.14 MB DOC)
Table S3  Genes which were enriched in the pool of Hfq IP compared to the negative control. Found at: doi:10.1371/journal.pone.0013069.s003 (0.43 MB DOC)

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Author Contributions
Conceived and designed the experiments: GY. Performed the experiments: YL NW JD YG. Analyzed the data: XZ. Contributed reagents/materials/analysis tools: CM. Wrote the paper: NS GY.

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