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Original article

hfq regulates acid tolerance and virulence by responding to acid stress in *Shigella flexneri*

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Abstract

Shigella flexneri is an important etiological agent of bacillary dysentery in developing countries. The Hfq protein is thought to play a major regulatory role in various cellular processes in this organism. However, the roles of Hfq in stress tolerance and virulence in *S. flexneri* in response to environmental stress have not been fully studied. In this study, *hfq* was highly expressed when *S. flexneri* was exposed to low pH. Growth retardation was observed in the *hfq* deletion mutant at pH values ranging from 5.0 to 7.0 and the survival rate of the mutant strain was reduced by 60% in acidic conditions (pH 3.0) compared with the wild-type strain. Additionally, competitive invasion assays in HeLa cells and lung invasion assays showed that the virulence of the *hfq* deletion mutant was significantly decreased. An evaluation of the mechanism revealed that, along with the expression of the Type III secretion system genes, acid resistance genes were also increased with acid stress. Interestingly, a statistically strong linear correlation was observed between the expression of *hfq* and Type III secretion system genes, as well as between *hfq* and acid resistance genes, under various pH conditions. In this study, we provide evidence that Hfq regulates genes related to acid resistance for survival under acid stress and controls virulence through the positive regulation of Type III secretion systems. Importantly, we propose that *hfq* is a key factor in maximal adaptation to host acid stress during infection, regulating acid stress tolerance and virulence in response to acid stress in *S. flexneri*.

Keywords: *Shigella flexneri*; *hfq*; Acid tolerance; Virulence

1. Introduction

Shigella is a Gram-negative bacterium that causes bacillary dysentery and gastroenteritis symptoms known as shigellosis. Both diseases occur predominantly in developing countries and mostly among children aged <5 years. It is estimated that *Shigella* causes 160 million shigellosis cases and hundreds of thousands of deaths annually. Of the four known *Shigella* species, *Shigella flexneri* is the most frequently isolated in developing countries and the most common serotype is 2a [1]. *S. flexneri* organisms invade the colonic and rectal epithelia of primates and humans and cause intense mucosal inflammation, which is characteristic of shigellosis [2].

The pathogenic processes used by *S. flexneri* strains to cause disease are complex. *S. flexneri*, like other enterobacteria, is normally ingested orally and must survive in the acidic environment of the stomach (pH 2–3) before reaching the colon, where it must then colonize the epithelium in an acidic pH (pH 5.5–7.0) [3]. Therefore, the adaption of *S. flexneri* to the acid stress environment of its host is crucial for its survival and pathogenesis. *Shigella* spp. use two acid

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resistance (AR) mechanisms for survival in low pH conditions: AR1 requires the oxygenation of cultures and AR2 is the glutamate-dependent acid resistance (GDAR) system. GDAR is an effective pathway for resisting stomach acid stress. The GDAR pathway is a glutamate decarboxylase system consisting of two homologous decarboxylase enzymes, GadA and GadB and an antiporter, GadC [4,5]. *S. flexneri* possesses several periplasmic chaperones that are major acid resistance proteins encoded by the *hdeA*, *hdeB* and *hdeD* genes. *hdeB* encodes HdeB, which is thought to form a heterocomplex with HdeA [6]. The genes of these periplasmic proteins are localized in the acid resistance island, which is a gene cluster that encodes several proteins involved in acid stress resistance [7].

Similarly, virulence regulators are central to pathogenesis in *S. flexneri*. The virulence of this bacterium is dependent on Type III secretion systems (T3SSs), which consist of the invasion plasmid antigens IpaABCD and *mxi-spa*-encoded secretion and molecule chaperones; together, these form injection devices that deliver effector proteins into host cells [8,9]. These factors are indispensable for the virulence of *S. flexneri*.

The Hfq protein is an RNA binding protein that plays several physiological roles in bacteria. According to previous studies, Hfq regulation is important in a diverse population of bacteria, including *Escherichia coli*, *Salmonella* and *Yersinia* [10–12]. Hfq was initially identified in *E. coli* and is a member of the Sm/Sm-like protein family. It can form ring-shaped hexameric structures that are known to bind single-stranded RNA molecules [13–15]. The role of *hfq*-mediated regulation at the translational or RNA level involves at least five mechanisms, including translation inhibition, translation promotion, small RNAs protection, cleavage of some small regulatory RNAs and their target mRNAs and induction of mRNA degradation [16]. It has been reported that the functions of Hfq focus mainly on growth-dependent metabolism, resistance to stress, modulation of virulence and drug resistance [14,17–20]. For example, in *Francisella novicida*, Hfq was found to have a role in the stress response to osmotic changes, low pH, heat shock and oxidative stress [21]. The Hfq protein of enterohemorrhagic *E. coli* (EHEC) had an impact on virulence, cell growth and acid stress processes [22]. In other pathogenic bacteria like *Brucella abortus* [23], *Burkholderia cepacia* [24], *Francisella tularensis* [25] and *Yersinia pestis* [26], *hfq* mutants exhibited a defect in stress tolerance and virulence. Additional analyses showed that other bacterial species regulated virulence and stress tolerance through Hfq-dependent gene expression. For example, Hfq in *Salmonella* positively regulated the target of the *hilD* mRNA to affect secretion through a T3SS [20]. However, the Hfq protein in EHEC, *Vibrio* spp. and *Pseudomonas* negatively regulated the T3SS [27,28].

Although the roles of Hfq in other bacteria have been largely studied, the structure and functions of Hfq are unique to each bacterial species. A few studies [27,29–33] have investigated the function of Hfq in *Shigella*. Mitobe et al. showed that Hfq was involved in osmotic response and virulence in *Shigella sonnei* [29]. Sharma and Payne reported that the deletion of *DksA*, which is required for the transcription of *hfq*, resulted in decreased expression of T3SS virulence genes in *S. flexneri* [30]. Oglesby et al. showed that a small RNA (sRNA), *ryhB*, which binds to *hfq*, suppressed acid resistance in *S. flexneri* [31]. However, some essential unsolved problems need further verification. First, the function of Hfq in the acid response and enhanced acidic resistance in *S. flexneri* has not been examined. In addition, little is known about how Hfq acts as a regulatory protein that participates in the response to environmental stress and how it influences virulence. We performed a systematic study of the roles of Hfq in *S. flexneri*. We also created the *hfq* deletion and *hfq*-complemented strains of *S. flexneri* to measure their response to acid stress and their virulence; the relationship between the stress response and virulence was also determined.

2. Materials and methods

2.1. Bacterial strains and growth media

S. flexneri 301 is a serotype 2a strain; this wild-type strain, its isogenic *hfq* deletion mutant (Δ*hfq*) and an *hfq*-complemented derivative strain (Δ*hfq* + p*hfq*) were routinely cultured at 37 °C with aeration in Luria–Bertani (LB) medium (1% tryptone, 0.5% yeast extract and 1% NaCl), or on tryptic soy agar (TSA) containing 0.01% (w/v) Congo red. When required, ampicillin, kanamycin and chloramphenicol were added to a final concentration of 50 μg/mL.

2.2. Construction of an S. flexneri 301 hfq deletion strain and its isogenic plasmid-complemented strain

The λ-Red-mediated recombination method [34] was used to construct a Δ*hfq* strain by replacing the *hfq* gene with *kana*, encoding kanamycin resistance. pKD46 plasmid contains the λ-RED system which used to replace the target gene with kanamycin resistance. Briefly, PCR was used to amplify regions of the sequences upstream and downstream of the *hfq* gene, using primer pairs (Table 1). Primers Hfq-U-F and Hfq-U-R amplify the 441-bp region upstream of *hfq* gene and primers Hfq-D-F and Hfq-D-R amplify the 523-bp region downstream of *hfq* gene. Primer Hfq-U-R and Hfq-D-F have 20 nucleotides of overlapping sequence, resulting in fusion of hfq upstream, downstream and kanamycin genes after fusion PCR. Two DNA fragments of 441 and 517 bp, containing an overlapping sequence from the *kana* gene, were fused together by PCR with a complementary *kana* PCR fragment, resulting in the replacement of *hfq* with *kana*. The resulting PCR product was gel-purified using a gel extraction kit (Promega, Madison, WI, USA). The fused PCR fragment was electroporated into the wild-type *S. flexneri* strain 301 carrying pKD46. The Δ*hfq* strains were identified by screening transformants on LB agar plates containing kanamycin (50 μg/mL). The deletion mutant was further confirmed by RT-PCR.

To construct the complementing plasmid p*hfq*, the *hfq* coding region and 200 bp upstream of the transcriptional start site were amplified from *S. flexneri* strain 301 using primers

Table 1
Primers used in this study.

Name	Primer sequence (5′–3′)
hfq-U-F	AGCCGTGAACTGCTCCAT
hfq-U-R	GACATTCATCCCAGGTGGCAAAGATTGCCCCTTAGCC
hfq-D-F	TCTGGGGTTCGAAATGACCGGAATAAGGTTTCGGG CTGTT
hfq-D-R	CTTTCTGTCTTTCAAGGTGG
hfq-RT-F	TCCAGTTTCTATTTATTTGGTG
hfq-RT-R	TCTTCGCTGTCCTGTTGC
kana-F	GCCACCTGGGATGAATGTC
kana-R	CGGTCATTTCGAACCCCAGA
phfq-F	TCTAGACCACGAGACAGTTGGCGAAGCGGCA (*Xba*I)
phfq-R	GCATGCTTATTCGGTTTCTTCGCTGTCCTGT (*Sph*I)
ipaA-F	GACAAAAACTCCTCAATAC
ipaA-R	GCCTGTGCGTCAACTAAAT
ipaB-F	GATTGGCTCTATTCTGGGGG
ipaB-R	AAGGTCTGTGAGGGTTTTAC
ipaJ-F	AAGTAACATTTTTTCGCAAG
ipaJ-R	AGGTCTACACAACACCCAGT
ipaH4.5-F	CAGAACTCTCCATTTTGT
ipaH4.5-R	GGTCATTTGCTGTCACTC
ipaH9.8-F	AACCCATTATCATCCCAT
ipaH9.8-R	TTCTGTTGTCCGTCACTC
ipaH1.4-F	TGGAAACAGATTAGACAG
ipaH1.4-R	TTATTCATTAGCACAGCC
ipaH7.8-F	TGAGTGACGGACAACAGA
ipaH7.8-R	GCAGCAACAGCGAAAGAC
ipaH2.5-F	GGAGTGAATGGGAAAGGA
ipaH2.5-R	CGGGTAAGGCAGGTAAGC
ipgH-F	GTTTCAGTTTTGGTTTTTTC
ipgH-R	AGAGTCTCGGACCTTGTTCA
ipgD-F	TGATGAGGGGAAGGAAAT
ipgD-R	GCGTCAGAAGAGAAGTCG
mxiC-F	CTTCCGCTCTTTCGTCAT
mxiC-R	TTTCATCTTCTTCCCCAT
mxiA-F	TATTTGGGAATCCTTTTG
mxiA-R	TTCTTTTTCTACGACCTT
mxiI-F	CAAATAATGGAGATGGTAAG
mxiI-R	TTGAGAGCGTCGTCTGTAAC
mxiM-F	TGTATTAGTGGTAAGGCGGT
mxiM-R	TAGCTATCTTTTGTGCATCC
mxiE-F	TTGGTTCTTTATCTTCTG
mxiE-R	GTGATTGTTTGATTGTGC
spa15-F	TCAATGAGCAGGTTATGT
spa15-R	AACAATCCCATCGTGAAC
spa13-F	AGGCATTAGATAAAAGGATT
spa13-R	TCGTTCAGACTGATATTGGA
spa33-F	CGAATGAAATCCAGTATG
spa33-R	GCAATGAGTCAAGAGAAA
spaP-F	CGAATGAAATCCAGTATG
spaP-R	GCAATGAGTCAAGAGAAA
spa9-F	CTCAACTCCAAGAGCAGA
spa9-R	GACAATAAAACCTCACCA
gadB-F	CAAACCAACGGATAAACC
gadB-R	CGAAAGTCGGCACCACGC
gadA-F	CAAACCAACGGATAAACC
gadA-R	CGAAAGTCGGCACCACGC
hdeB-F	TGTAGCGGCTTTGTCACT
hdeB-R	TTCTGCGGGTTTTTCTTA
hdeA-F	TGGTGGTCTGCTTCTTCTGC
hdeA-R	TTGTCCCATTCGCCTTTAAC
hdeD-F	TCTGGAGATGCTGAAAAA
hdeD-R	GCACGGATGAAGAAATAG

phfq-F and phfq-R. These primers were designed to include unique restriction enzyme sites (*Xba*I and *Sph*I) so that when the PCR product was digested with *Xba*I and *Sph*I, it could be ligated with the similarly digested plasmid pACYC184 in an orientation-specific manner. The recombinant DNA products were verified by sequencing. The resulting plasmid pACYC184-*hfq* was used to transform the Δ*hfq* strain by electroporation, with selection by chloramphenicol. The constructed strain containing the complementing plasmid was named Δ*hfq* + p*hfq*. The transcription of *hfq* in the Δ*hfq* + p*hfq* strain was further confirmed by RT-PCR.

2.3. Growth curve and stress tolerance assays

The responses of the wild-type, Δ*hfq* and Δ*hfq* + p*hfq* strains to different environmental stresses were measured by the changes in their growth curve profiles. The growth curve experiments were initiated by using an overnight culture of bacteria (grown in LB medium or LB medium containing kanamycin or chloramphenicol at 37 °C) that had been diluted with LB to an optical density at 600 nm (OD_{600}) of 0.1. The diluted cultures were supplemented with the appropriate stress inducer before incubation at 37 °C with aeration (160 rpm). The bacterial response to changes in pH (5.0, 5.5, 6.0 and 7.0) was determined. Because of the limited bacterial growth at pH values below 4.5, the growth curve experiments were restricted to a pH range from 5.0 to 7.0. Bacterial growth was monitored at 2-h intervals by measuring the OD_{600} [35].

For stress tolerance assays, the wild-type and Δ*hfq* strains were exposed to acid stress conditions. Bacteria were inoculated into LB medium and grown to the early logarithmic phase (about 10^6 CFU/mL) at 37 °C. To determine the response to acid stress, the log-phase cells were harvested and transferred to LB medium at pH 3.0 and then incubated at 37 °C for 30 min. After treatment, the cells were diluted and plated on LB plates to determine the number of colony forming units (CFUs) [36]. Results represent the mean from at least three separate experiments.

2.4. The expression of hfq under acidic conditions

To study *hfq* expression under exposure to different acid stress conditions, 50 μL of an overnight culture of *S. flexneri* strain 301 were inoculated into 5 mL of LB broth and grown to the exponential phase (OD_{600} = 0.8) at 37 °C. The bacterial culture was harvested and centrifuged at 8000 × *g* for 5 min. The bacteria were resuspended in LB medium (control, pH 7.0) or LB medium at pH values ranging from 2.0 to 6.5, at intervals of 0.5 units, for 30 min; then, the bacterial cells were collected by centrifugation. Total RNA was extracted using Trizol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA) and quantified on a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). cDNA synthesis was performed using a reverse transcription kit (Promega). Quantitative reverse transcription PCR (qRT-PCR) was performed in 25-μL reaction volumes containing 12.5 μL of 2× SYBR Green I Master

Mix (Takara Biochemicals, Dalian, China), 100 nM of each primer and 1 μL of cDNA sample. The thermal cycling conditions were as follows: 10 min at 95 °C for pre-incubation, followed by 45 cycles of amplification (95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s). The relative transcriptional level was determined using the $2^{-\Delta\Delta Ct}$ method as described previously [25]. Briefly, the relative fold change (treatment/control) = $2^{-\Delta\Delta Ct}$, where ΔCt (gene of interest) = Ct (gene of interest) − Ct (reference gene of the same sample) and ΔΔCt (gene of interest) = ΔCt (treatment) − ΔCt (control). 16S rRNA was used as a reference gene to normalize expression data for the target gene.

2.5. qRT-PCR analysis of acid resistance genes and T3SS transcripts

Overnight cultures of the wild-type and the Δ*hfq* strains were diluted 1:100 in LB broth and LB broth containing kanamycin, respectively. The cells were grown at 37 °C to an OD_{600} value of 0.8 and then adjusted to 10^6 CFU/mL in LB. To further study the role of the *hfq* product in response to acid stress, the wild-type and Δ*hfq* cells were resuspended in LB broth (adjusted to a range of pH values from 2.0 to 6.5, increasing in increments of 0.5 units) and cultured for 30 min and then were harvested by centrifugation. RNA was extracted and used for qRT-PCR following the procedures described previously. The expression levels of *S. flexneri* acid resistance genes (*gadB*, *gadA*, *hdeB*, *hdeA* and *hdeD*) and the T3SS genes (*ipaA*, *ipaB*, *ipaJ*, *ipaH4.5*, *ipaH9.8*, *ipaH1.4*, *ipaH7.8*, *ipaH2.5*, *ipgH*, *ipgD*, *mxiC*, *mxiI*, *mxiM*, *spa15*, *spa13*, *spa33*, *spaP* and *spa9*) were monitored.

2.6. HeLa cell invasion assays

To further verify the influence of *hfq* on virulence in *S. flexneri*, a competitive invasion assay was performed using HeLa cells. Wild-type and Δ*hfq* cells were respectively grown to the exponential phase, adjusted to the same concentration and then mixed at a 1:1 (v/v) ratio (i.e., these two strains were mixed equally in the in vitro HeLa cell invasion assay). The mixed strains were suspended in Dulbecco's modified Eagle's medium (DMEM: Gibco, Carlsbad, CA, USA) and then added to HeLa cells at a multiplicity of infection of 100:1. After infection for 4 h, the infected HeLa cells were washed three times with phosphate-buffered saline (PBS) and three times with DMEM, and then DMEM containing 50 μg/mL gentamicin was added [34] (Gentamicin kills extracellular bacteria, while intracellular bacteria are protected.). Following incubation at 37 °C for 24 h, the cell cultures were washed with PBS and then harvested and lysed, and cellular contents were cultured on LB plates and LB plates containing kanamycin. The competitive index (CI) was calculated, which represents the ratio of recovered Δ*hfq* CFU to wild-type CFU (normalized for the input ratio) and Student's *t* test was used to determine the P-value [26]. This assay was repeated three times and the mean was reported as the final result.

2.7. Animal model

Six-week-old BALB/c female mice weighing approximately 20 g were used for in vivo experiments. The animals were obtained from the laboratory animal center (Academy of Military Medical Sciences). The experimental protocol was approved by the Ethics Committee for Animal Experimentation of the Academy of Military Medical Sciences. Mice were housed in individual cages and provided with water and food throughout the experiment.

Mice were anesthetized with diethyl ether. A bacterial suspension (25 μL) was applied drop-wise on the external nares of each mouse using a syringe. Groups (eight mice) were challenged with 10^6 CFU/animal. After inoculation for 24 h, mice were sacrificed and lungs were aseptically removed, washed with sterile PBS and homogenized in a blender. The concentration of *S. flexneri* strain 301 and its isogenic *hfq* mutant was determined by plating serial dilutions on brain–heart infusion plates with and without kanamycin. Results are given as the total CFU and the CI as described above.

3. Results

3.1. Expression and role of hfq in S. flexneri in response to acid stress

The *hfq* gene from different organisms is differentially expressed in response to various environmental conditions [20,25–29,34]. To examine the role of the *S. flexneri hfq* gene in response to acid stress, the expression of *hfq* was examined using qRT-PCR assays, with the expression level of 16S rRNA used as the internal control (Fig. 1A). Under these experimental conditions, *hfq* had higher expression in the pH range from 2.0 to 4.0 than at pH 7.0; there was a significant declining trend in the observed expression as the pH became more basic (Fig. 1A). The linear correlation between the pH value and the expression of *hfq* was also statistically significant ($R = -0.806$, $P = 0.005$).

Enteropathogenic bacteria can adapt to extreme acid stress environments such as that in the stomach. Changes in acidic pH are an important signal for the bacterium, triggering the induction of many virulence genes [36]. To further study the mechanism of this phenomenon in *S. flexneri*, qRT-PCR was performed to identify changes in the expression of selected virulence and acid resistance genes with exposure to acid stress. All acid resistance and virulence-associated genes were upregulated in extreme acidic conditions, pH 2.0–4.0, but the changes in expression were not significantly different in the pH range from 4.5 to 6.5 (Fig. 1B and C). Interestingly, a significant correlation between the expression of *hfq* and the T3SS genes ($R = 0.830$, $P = 0.003$), as well as between the expression of *hfq* and acid resistance genes ($R = 0.879$, $P = 0.001$), was identified. These observations suggest that the *hfq* gene product is important for the *S. flexneri* response to acid stress.

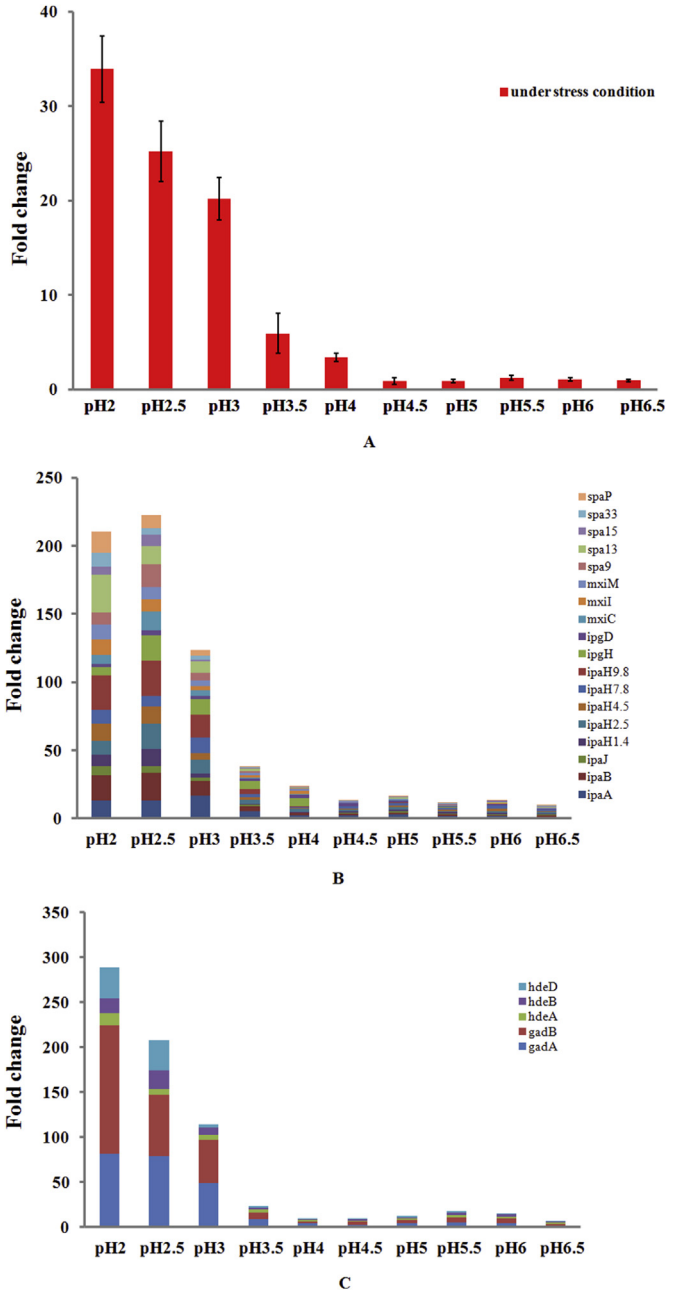

Fig. 1. Expression of the *hfq*, Type III secretion system (T3SS) and acid resistance genes in *S. flexneri*. The relative transcription levels in *S. flexneri* strain 301 were examined after exposure of cells to a range of pH conditions for 30 min. Strain 301 was cultured in Luria–Bertani broth buffered to a range of pH values. (A) Expression of *hfq*. The red bars indicate fold changes, which were calculated as means from triplicate experiments, representing the ratios of the levels of *hfq* under various pH conditions relative to expression at pH 7.0. The standard deviation is indicated by error bars. Statistical analysis was performed with Student's *t* test. (B) T3SS gene expression was determined by quantitative reverse transcription PCR (qRT-PCR). (C) Acid resistance gene expression was determined by qRT-PCR. Statistical analysis was performed with correlation analysis (for panels B and C). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. *Construction and verification of the* S. flexneri *Δ*hfq *strain and growth characteristics*

To further explore the role of the *hfq* gene in *S. flexneri*, a deletion mutant was created in strain 301. The *hfq* gene was identified between nucleotide coordinates 4505106 and 4505414 in the genome of *S. flexneri* strain 301 in the *miaA-hfq-hflX* cluster (Fig. 2A). Deletion of the *hfq* gene was verified by PCR and sequence analysis (Fig. 2B). A plasmid was constructed consisting of the wild-type *hfq* gene on plasmid pACYC184; this construct was transformed into the Δ*hfq* strain to assess the complementation of function *in trans*. The *hfq* was detected in the Δ*hfq* + p*hfq* strain but not in the Δ*hfq* strain (data not shown).

3.3. *Importance of* hfq *for acid tolerance in* S. flexneri

For stress growth curve experiments, the wild-type, Δ*hfq* and Δ*hfq* + p*hfq* strains were grown in LB broth buffered to a range of pH values and their growth curves were determined. Because of the lack of growth with prolonged exposure to pH <5.0 (data not shown), assays were performed in the pH range from 5.0 to 7.0. When exposed to the pH 5.0 environment, the Δ*hfq* mutant had a significant lengthening of its lag phase and a significant reduction of its entire growth phase compared with the wild-type strain ($P < 0.05$); at pH 5.5, slower growth was also evident in the mutant, but was less pronounced (Fig. 3A and B). Interestingly, at pH 6.0 and 7.0, the Δ*hfq* strain entered the exponential phase in a similar manner as the wild-type strain; however, the mutant was unable to maintain its survival in the stationary phase (Fig. 3C and D). No significant difference was observed between the wild-type and Δ*hfq* + p*hfq* strains under acidic conditions.

For *S. flexneri* to successfully invade the human intestinal epithelium, it must be able to survive the stomach's acidity. To determine whether *hfq* is important in the stress response of *S. flexneri*, stress tolerance of the wild-type, Δ*hfq* and Δ*hfq* + p*hfq* strains was measured using survival assays. The survival rate of the wild-type 301 strain was 68% at pH 3.0, in which 6.13×10^8 cells were added at the beginning of the assay, but only 2.19×10^8 cells were recovered. Compared with the wild-type strain, the survival of the Δ*hfq* mutant was significantly decreased (60% loss in CFU) at pH 3.0 (Fig. 4).

3.4. *HeLa cell invasion of the Δ*hfq *mutant in vitro*

A 1:1 (v/v) mixture of wild-type and Δ*hfq* cells was tested in an in vitro HeLa cell invasion assay. The median total CFU recovered after gentamicin treatment was 1.9×10^6 for the wild-type cells, which was significantly higher ($P < 0.01$) than that of the kanamycin-resistant Δ*hfq* strain (5×10^5) (Fig. 5A). The calculated CI median value was 0.03 (Fig. 5B). The CI value is calculated from the following formula: (mutant number after invasion/wild-type strain number after invasion)/(mutant number before invasion/wild-type strain

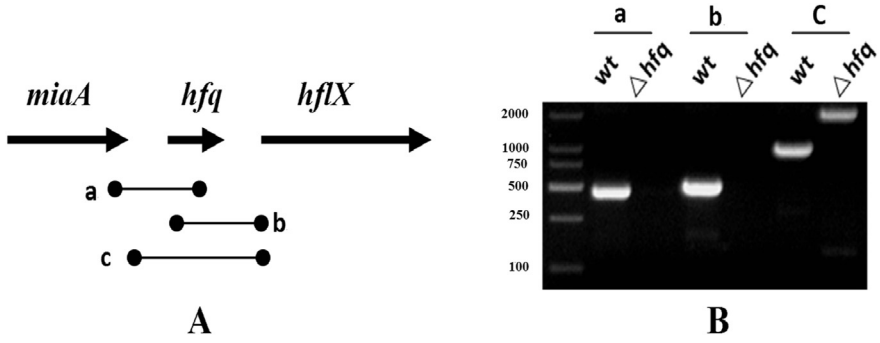

Fig. 2. Chromosomal position and detection of the *S. flexneri* strain 301 *hfq* gene by PCR. (A) Genetic organization of the *hfq* locus in *S. flexneri*. Lines a–c represent regions of the locus amplified by PCR as shown in B. (B) Verification of the *S. flexneri* strain 301 *hfq* gene and subsequent deletion/insertion mutation with a kanamycin-encoding gene cassette by agarose gel electrophoresis. Amplification of regions shown in (A) from wild-type *S. flexneri* strain 301 (lanes 2, 4 and 6) and the isogenic Δ*hfq* mutant (lanes 3, 5 and 7). Lane 1 contains a molecular weight marker (DL2000, Takara).

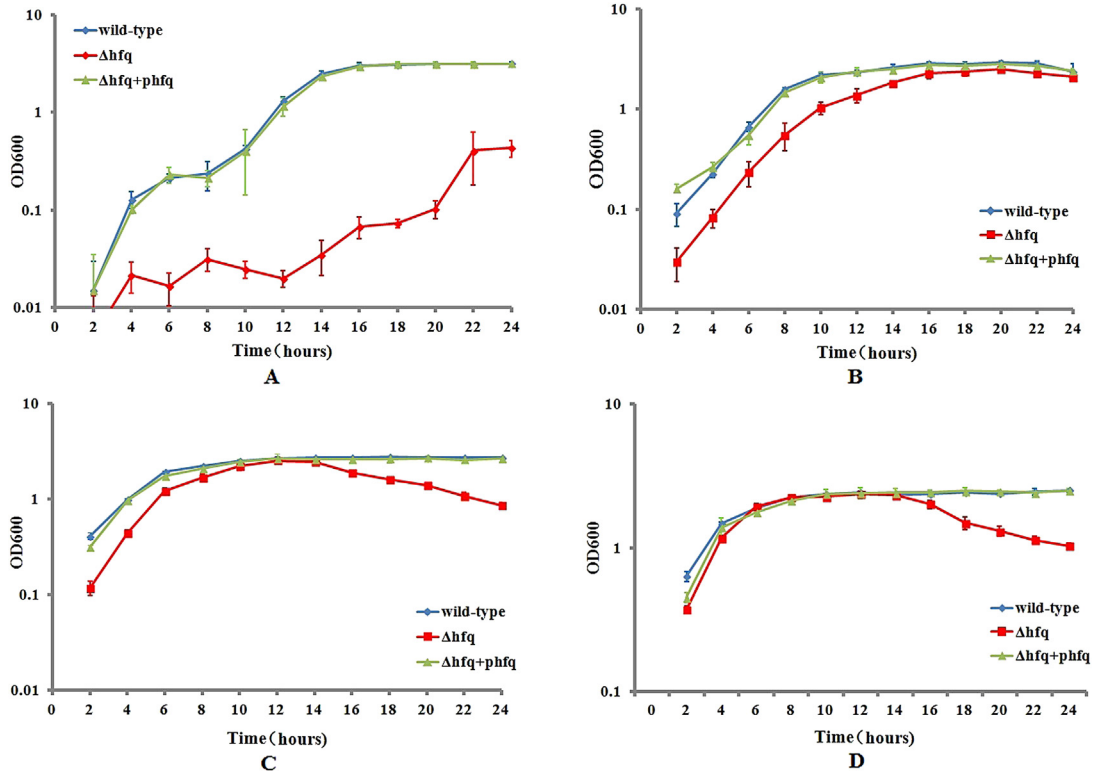

Fig. 3. Acid stress tolerance growth curves of *S. flexneri* strain 301 (wild-type), its isogenic *hfq* deletion mutant (Δ*hfq*) and the *hfq*-complemented strain (Δ*hfq* + p*hfq*). Growth characteristics of the three strains were examined in Luria–Bertani broth buffered to the following pH values: (A) pH 5.0, (B) pH 5.5, (C) pH 6.0 and (D) pH 7.0. Error bars indicate standard deviations based on triplicate experiments.

number before invasion). A ratio of 1 means that there are no significant changes in the numbers between the mutant and wild-type strains after invasion and represents an equal capacity for invasion by the mutant and wild-type strains. The CI value calculated in this study was significantly less than 1 (Student's *t* test, $P < 0.05$), strongly suggesting that *hfq* deletion has an impact on the ability of *S. flexneri* to effectively invade cultured cells in vitro.

3.5. Altered virulence of the hfq *mutant in vivo*

We assessed whether the disease-causing ability of the *hfq* deletion mutant was impacted in an animal model compared with the wild-type *S. flexneri* strain 301. BALB/c mice were infected intranasally with wild-type *S. flexneri* strain 301 and its isogenic *hfq* mutant. The total CFUs representing *S. flexneri* and the *hfq* mutant recovered from mice lungs were 5.7×10^4 and 8.36×10^2, respectively, representing a significant difference (Fig. 5C). Similarly to in vitro cell invasion assays, the calculated CIs were less than 1 and were significantly different (student's *t* test, $P < 0.05$) (Fig. 5D). The *hfq* mutant showed decreased ability to invade nasopharyngeal and endothelial cells. These results suggest that the decreased virulence associated with the *hfq* mutant strain is due to lack of expression of the Hfq protein, indicating that *hfq* is important for *S. flexneri* virulence in vivo.

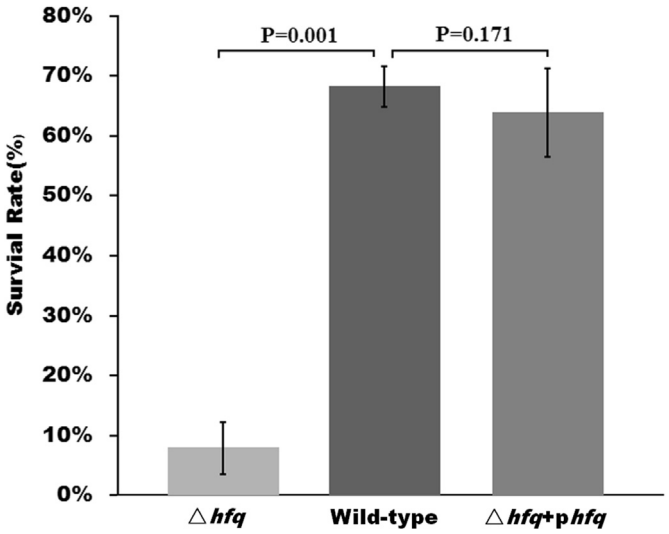

Fig. 4. Survival of wild-type 301, Δ*hfq* and Δ*hfq* + p*hfq* strains under acid stress conditions. For the acid stress resistance assay, the wild-type 301, Δ*hfq* mutant and *hfq*-complemented (Δ*hfq* + p*hfq*) strains were grown in Luria–Bertani (LB) broth to the logarithmic phase and then subcultured to acid stress condition (pH = 3.0). After treatment, surviving bacteria were numbered by plating serial dilutions onto LB plates. Bars represent the mean percentage survival compared with untreated controls and error bars represent standard errors of percentage survival from three replicates. Significant differences between the mutant and wild-type strains are indicated as follows: *P < 0.05.

3.6. Role of Hfq in T3SS and acid resistance

According to the results of the previous assays (Fig. 1), Hfq may regulate T3SS and acid resistance genes in *S. flexneri*. To assess whether *hfq* regulates virulence through the T3SS, gene expression was analyzed in the wild-type, Δ*hfq* and Δ*hfq* + p*hfq* strains by qRT-PCR to identify genes coding for T3SS that are required to invade the colorectal epithelium. The *ipaA*, *ipaB*, *ipaJ*, *ipaH4.5*, *ipaH9.8*, *ipaH1.4*, *ipaH7.8*, *ipaH2.5*, *ipgH*, *ipgD*, *mxiC*, *mxiI*, *mxiM*, *spa15*, *spa13*, *spa33*, *spaP* and *spa9* genes were monitored by qRT-PCR. The expression levels of these T3SS genes were almost undetectable in the Δ*hfq* strain, and weakly expressed relative to the wild-type strain (Fig. 6A). The exception was *ompA* (encoding an out membrane protein), the expression of which, in Δ*hfq*, showed no significant difference between that of the wild-type and Δ*hfq* + p*hfq* strains (the median of relative expression of *ompA* in Δ*hfq*, wild-type and Δ*hfq* + p*hfq* was 0.83, 1, 0.85). These results indicated that Hfq positively regulates T3SS genes in *S. flexneri*.

As described above, the *hfq* gene is important for acid resistance in *S. flexneri*. Therefore, we examined the expression of acid resistance genes in the Δ*hfq* mutant. qRT-PCR results indicated that the expression levels of *gadB*, *gadA*, *hdeB*, *hdeA* and *hdeD* were significantly decreased in the Δ*hfq* mutant relative to the wild-type strain. In the Δ*hfq* strain, the relative expression of *gadB*, *gadA*, *hdeB* and *hdeA* was 100-fold lower than in the wild-type strain and that of *hdeD* was 30-fold lower (Fig. 6B). These results indicated that the *hfq* gene positively regulates acid resistance genes in *S. flexneri*.

4. Discussion

In the present work, we report the ability of *hfq* to accommodate for environmental stress and affect virulence in *S. flexneri*. This was the first study to explore expression of the *hfq* gene under extreme acid stress and it confirmed that Hfq is an essential acid stress response factor in *S. flexneri*. Our results also showed for the first time that Hfq enhances acid resistance in *S. flexneri* by regulating acid resistance genes (*gadB*, *gadA*, *hdeB*, *hdeA* and *hdeD*). Using in vitro and in vivo virulence assays, we further verified that the *hfq* gene has a strong effect on the virulence of *S. flexneri*. We also provide direct evidence that *hfq* is required for positive regulation of T3SS gene expression, which influences the virulence of the organism. Importantly, the expression levels of genes related to acid resistance and T3SS were markedly increased by acidic conditions, and these expression levels exhibited a strong linear correlation with *hfq* expression under acid stress. These significant findings demonstrated that Hfq plays a key role in the regulation of stress tolerance and virulence in *S. flexneri* in response to environmental stress.

In general, *S. flexneri* must pass through the stomach's acidic environment while intact so as to invade epithelial cells of the colon and cause diarrheal disease [36]. To survive in these extremely hostile environments, *S. flexneri* must acclimatize to environmental changes and respond quickly by adjusting the expression of stress- and virulence-associated genes [26]. However, the specific regulatory networks of these characteristics in *S. flexneri* are not clear. Our research provided evidence that *hfq* has a profound effect on the connection between adaptation to the environment and pathogenesis. We demonstrated that the *hfq* gene acts as a stress response factor that senses changes in environmental stress in the stomach tract. The higher expression of *hfq* in response to environmental stress might be a trigger that increases acid stress tolerance by enhancing the expression of acid resistance genes in *S. flexneri*. The higher expression of *hfq* also activated increased virulence by enhancing the expression of T3SS genes in *S. flexneri*.

About 30–35% of sRNAs are Hfq-binding sRNAs in *E. coli* and *Salmonella enterica* Typhimurium [37]. Hfq is regarded as an RNA chaperone because it interacts with and stabilizes many sRNAs to facilitate sRNA-mRNA base pairing. Regulation by Hfq, in combination with sRNAs, is a means of controlling expression of environmental resistance-specific regulators [38]. For instance, Repoila et al. and Lease and Belfort demonstrated that the sRNA *dsrA*, which is mediated by Hfq, stimulates *rpoS* translation and decreases *hns* mRNA stability. RpoS and H-NS regulators are important for the general stress response in *E. coli* [38,39]. Opdyke et al. showed that the Hfq-associated sRNA *gadY* positively regulates *gadX* mRNA stability at low pH [40]. Therefore, we infer that binding of Hfq with the associated sRNA leads to activation of acid-resistant mRNA translation, which induces altered expression of corresponding proteins to improve survival under acid stress. In *Shigella dysenteriae*, *ryhB* sRNA is involved in the response to environmental iron levels, and it

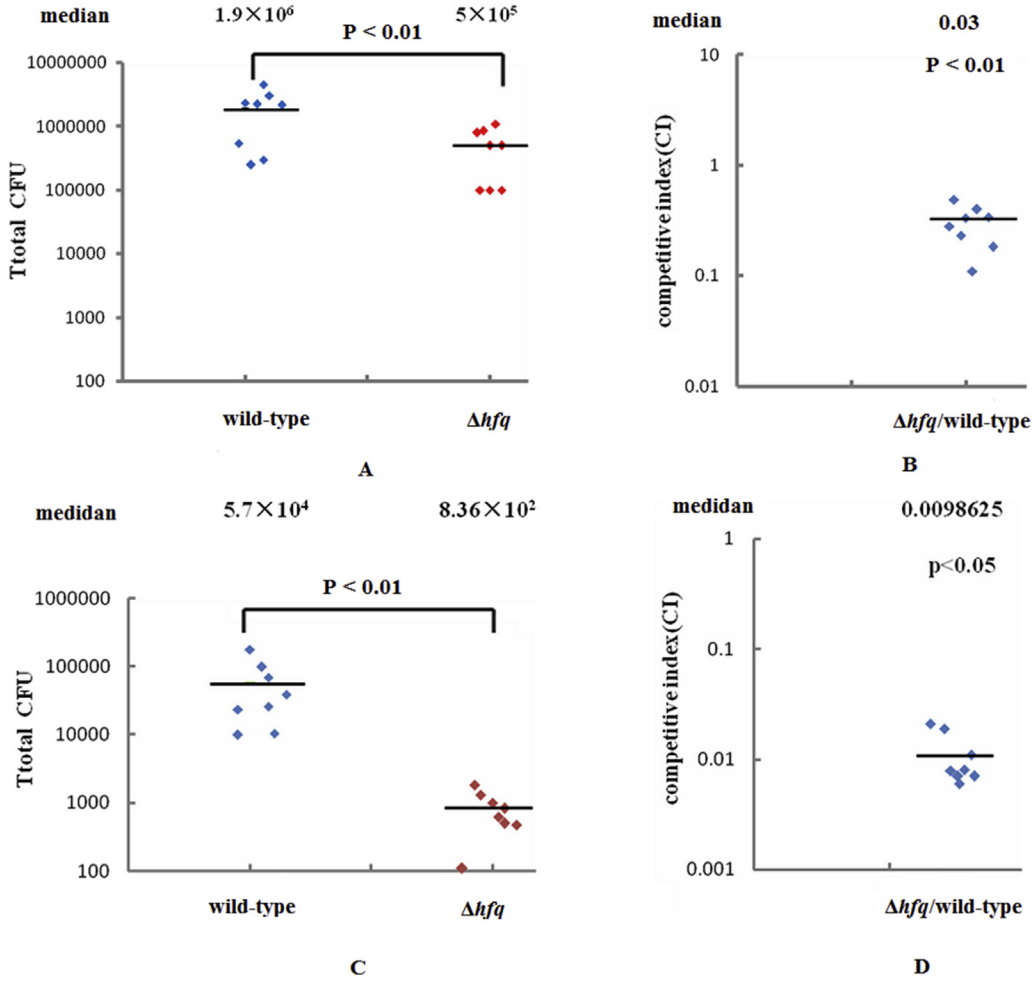

Fig. 5. HeLa cell competitive invasion assays of *S. flexneri* strain 301 (wild-type) and its isogenic *hfq* deletion mutant (Δ*hfq*). The results show the competitive index (CI) of the Δ*hfq* strain. (A) Total CFUs in HeLa cells are reported ($P < 0.01$, two-sample *t* test). (B) CI of the Δ*hfq* strain was significantly lower ($P < 0.05$) than that of the wild-type strain. (C and D) BALB/c mice were infected by *S. flexneri* strain 301 and its isogenic *hfq* deletion mutant; results were assessed using the CI model. (C) The total mean CFU recovered from mice lungs was significantly different ($P = 0.016359$, two-sample *t* test). (D) The CI of the wild-type strain and *hfq* mutant was less than 1 ($P < 0.05$).

regulates expression of T3SS via repression of the transcriptional VirB. RyhB, an iron environmental factor, participates in the complex virulence regulation of *Shigella* [41]. Thus, we infer that Hfq likely binds with *ryhB* to regulate T3SS genes and affects the virulence of *Shigella*. Further studies will be necessary to determine how *ryhB* functions in the control of virulence by binding to Hfq.

Our ongoing work with high-throughput sequencing revealed that about 39% of sRNAs can interact with Hfq in *S. flexneri* (data not shown). The functions of these sRNAs include acid resistance and virulence in *S. flexneri*. Previous studies showed that expression of virulence genes in *Shigella* is regulated in response to environmental changes such as temperature and osmotic stress [26,42,43]. However, acid-dependent regulation of virulence gene expression in *Shigella* was unclear. Our data indicate that the increased expression of T3SS genes is in response to the higher expression of *hfq* with acid stress. A previous study in *S. enterica* Typhimurium established that Hfq and sRNAs affect the major activator of the SPI-1-encoded T3SS and effector genes *hilA* [44]. Likewise, in *Vibrio cholerae*, Hfq, in conjunction with sRNAs, affects the virulence gene master regulator HapR [45]. In high-throughput sequencing analysis of *S. flexneri*, we also found that a few of the Hfq-binding sRNAs can regulate virulence (data not shown). Therefore Hfq control of T3SS gene expression is required for sRNA regulation, which is associated with the binding of Hfq to target T3SS mRNA genes. Currently, we are identifying the specific Hfq-binding sRNAs that are involved in pathways for intercellular stress survival and pathogenesis. Our observations are also consistent with previous findings indicating that bacterial virulence is not only influenced by dedicated virulence factors, but is also dependent on protein expression suited to specific environmental stresses [6,46,47].

This study provides information that will help us to understand the complex regulatory networks used by *S. flexneri* when exposed to acid stress. The characterization of *hfq* in this process will provide significant insight into the pathogenesis and infection of *S. flexneri* and other enteropathogens. This study also provides insight into the mechanism used by

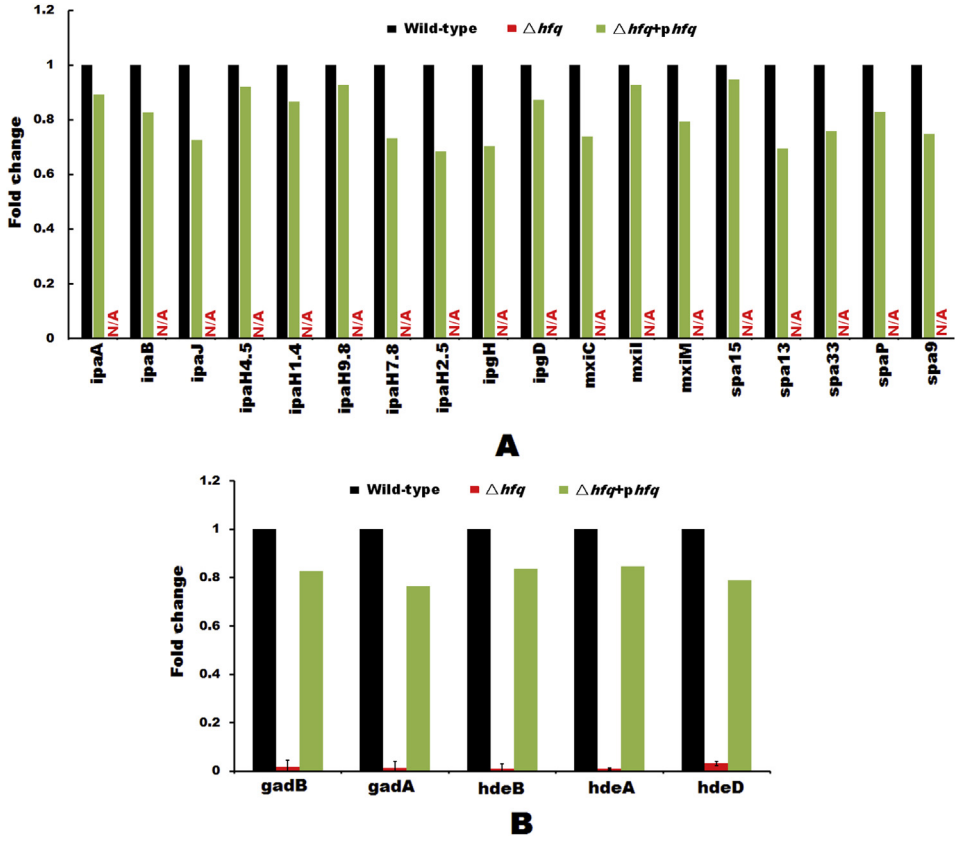

Fig. 6. qRT-PCR measurement of transcript levels of the Type III secretion system (T3SS) and acid-resistance-related genes in *S. flexneri* strain 301 (wild-type) and its isogenic *hfq* deletion mutant (Δ*hfq*) and *hfq*-complemented (Δ*hfq* + p*hfq*) strains. The standard deviation is indicated by error bars. Statistical analysis was performed by Student's *t* test. Data are representative of three independent experiments. A twofold or greater difference in expression was considered a significant difference. (A) T3SS genes. (B) Acid resistance genes. "N/A" indicates that T3SS gene values were undetectable.

pathogens to invade and replicate within certain microenvironments. Accordingly, *S. flexneri* has evolved elaborate acid stress response mechanisms that require the *hfq* gene product to facilitate survival, aid in colonization and enhance pathogenicity. In particular, our study lends support to the notion that bacteria have the ability to tolerate stress and cause infection in response to the host's stressful environment. Given the unique role of *hfq* in host survival and pathogenesis, *hfq* represents an important target for preventing infection caused by *S. flexneri*. At present, we simply suggest a possible mechanism in which an interaction between Hfq and an as-yet-unidentified sRNA is involved in acid stress response and virulence. To date, the specific molecular mechanisms of the relationship between stress response and virulence have not been completely identified. Further analysis will reveal the mechanism by which Hfq and interacting sRNAs mediate these effects upon the biology and virulence of *S. flexneri*.

Authors' contributions

LW, HS, WL and GY designed the research, assessed and interpreted the results and prepared the manuscript. GY, YW, PL, SQ, RH and ZW carried out data analysis and designed experiments. JZ, CY and JW assisted in the experiments. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Acknowledgments

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