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Hfq mutation confers increased cephalosporin resistance in *Klebsiella pneumoniae*

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Abstract: *Klebsiella pneumoniae* (*K. pneumoniae*), is an opportunistic pathogen raising significant public health concerns owing to its multi-drug resistance. Hfq, one of the main RNA-binding proteins, is a key post-transcriptional regulator. This protein is closely related to virulence and resistance in various pathogenic bacteria. Although the role of *hfq* in *K. pneumoniae* virulence has been explored, its influence on resistance remains largely unknown. The aim of this study was to investigate the role of *hfq* in the resistance of *K. pneumoniae* to cephalosporins. An *hfq* mutant was constructed, and its resistance to cephalosporins was investigated. The *hfq* mutant exhibited over 16-fold higher cephalosporin resistance than that exhibited by the wild type. Time-kill curve analysis showed that the *hfq* mutant could survive under higher concentrations of cephalosporins than the wild-type strain could. Quantitative RT-PCR showed that expression levels for 8 out of the 9 penicillin-binding proteins, which are the targets of cephalosporins, were downregulated in the *hfq* mutant. Taken together, contrary to its role in many other bacteria, *hfq* is involved in a negative regulation of *K. pneumoniae* resistance to cephalosporins by downregulating the expression of penicillin-binding proteins

Key words: Cephalosporins; *hfq*; *Klebsiella pneumoniae*; penicillin-binding proteins; resistance

INTRODUCTION

Klebsiella pneumoniae, a gram-negative bacterium, is a major opportunistic pathogen, causing a variety of human diseases such as pneumonia [1], urinary tract infection [2], bacterial meningitis [3], septicemia [4], and even liver abscess [5]. Infections range from local to life-threatening disseminated diseases, especially at the hospital and community levels. Even with appropriate antibiotic treatment, in vulnerable patients the mortality rate of hospital-acquired pneumonia due to *K. pneumoniae* exceeds 50% [6]. *K. pneumoniae* is one of the main causes of carbapenem-resistant bacterial infections, which have no effective treatment. This pathogen can resist third-generation cephalosporins and carbapenems, which are the last line of defense against severe infections [6]. *K. pneumoniae* poses grave public health risks owing to its multidrug-resistance and global reach. In light of the high pathogenicity and dangerous resistance of *K. pneumoniae*, it is necessary to understand more about the resistance mechanisms of this pathogen.

Hfq, an RNA-binding protein, is an important post-transcriptional regulator in various pathogens, including *Pseudomonas aeruginosa* [7], *Listeria monocytogenes* [8], *Vibrio cholerae* [9], *Shigella flexneri* [10], *Salmonella* [11,12], *Neisseria gonorrhoeae* [13], *Neisseria meningitidis* [14], *Yersinia* [15,16], *Francisella tularensis* [17], *Erwinia amylovora* [18] and *Escherichia*

coli [19,20]. There have been many reports of the effects of Hfq on bacterial growth, virulence and biofilm formation; this protein is a key virulence- and stress-response factor [21]. Hfq facilitates the interaction between small non-coding RNAs (sRNAs) and mRNA, which can impact the stability and translation of mRNA. Through its influence on gene expression, Hfq affects multiple cellular processes and physiological reactions, particularly virulence and resistance [21-24]. This vital role in regulating virulence has been described in a variety of bacteria. For *K. pneumoniae*, Chiang et al. [25] reported that the Hfq protein is a key factor in the regulation of gene expression, being critical for virulence, and is involved in many stress conditions. However, the relationship between *hfq* and drug resistance in *K. pneumoniae* remains largely unknown.

Cephalosporins are one of the most commonly used antibiotics, and *K. pneumoniae* resistance to this type of antibiotic is becoming a public health problem. Several explanations for cephalosporin resistance have been developed over the years. The expression of chromosome- or plasmid-mediated β-lactamases, which would bind and hydrolyze β-lactam antibiotics, including cephalosporins, could provide protection to penicillin-binding proteins (PBPs), the targets of cephalosporin, thereby conferring cephalosporin resistance [26]. Alternatively, modifications to cephalosporin targets may influence resistance. The modification of PBPs was mediated by the expression of low-affinity PBPs or acquisition of supplementary cephalosporin-insensitive PBPs [27]. Moreover, a number of recent studies have demonstrated that changes in PBP expression levels can impact cephalosporin resistance [28-30].

In the present study, we investigated the role of *hfq* in *K. pneumoniae* resistance to cephalosporins. We also examined PBP expression levels for indications of regulatory processes, since these proteins can directly influence cephalosporin resistance.

MATERIALS AND METHODS

Strains, plasmids and growth conditions

The *K. pneumoniae* strains and plasmids used in this study are listed in Table 1. We used *K. pneumoniae* from the wild-type (WT) isolate *K. pneumoniae* WJ101, which was a kind gift from Y. F. Wang. All strains were maintained in Luria-Bertani agar (LB agar: 1% tryptone, 0.5% yeast extract, 1% NaCl and 3% agar) plates and stored at 4°C during the experimental period. Mueller-Hinton broth (MHB) was used as the medium for susceptibility testing.

Antibacterial agents

Antibiotics for plasmid maintenance were added into the medium at the following concentrations: kanamycin 60 μg/mL and tetracycline 40 μg/mL. All antimicrobial agent stock solutions were maintained at -20°C.

Construction of *K. pneumoniae* WJ101 *hfq*-mutant and complemented strain

The genome sequence of *K. pneumoniae* NTUH-K2044 (GenBank accession number NC_012731.1) was retrieved from the NCBI GenBank database and used for primer design. All primers used in this study were synthesized commercially (Sangon Biotech Co., Ltd., Shanghai, China). These primers are listed in Table 2.

The deletion of the *hfq* gene from *K. pneumoniae* WJ101 chromosomal DNA was performed using the Red recombinase system [31]. Since the ampicillin resistance marker was invalid for *K. pneumoniae*, a new recombinant plasmid, pKD46T, was constructed. The tetracycline resistance gene fragment was cut from pBR322 and inserted at the *Xmn*I site of pKD46 [32]. Thus, ampicillin resistance was replaced with tetra-

Table1. Strains and plasmids used in this study.

Strains or plasmids	Description[a]	Reference or source
Strains		
WJ101	*K. pneumoniae*WJ101 wild-type	This study
WJ101ΔH	WJ101 derivative; *hfq::Km*^{R}	This study
WJ101ΔHCH	WJ101 derivative; harboring pHSGCH	This study
Plasmids		
pKD46	λ recombination plasmids; Ap^R	[32]
pKD46T	modified pKD46 plasmids; T^R	This study
pBRR1mcs2	cloning vector;	This study
pHSG298	cloning vector;	This study
pHSGCH	pHSG298 derivative; containing *hfq* fragments	This study
pBR332	cloning vector;	This study

Ap^R – resistance to ampicillin; Km^R – resistance to kanamycin; T^R – resistance to tetracycline.

Table 2. Oligonucleotides used for PCR in this study.

Primers name	Forward primer (5'-3')	Reverse primer (5'-3')	Reference
PTF/R [a]	AA*CTGCAGG*TTTGACAGCTTATCATCG	AA*CTGCAGG*AGTGGTGAATCCGTTAGCG	This study
KPhfq-N-F/R	ACTGACGCAAACGTCAGGAG	GCTTCTCAGTGCGTTACATAGCGTTCAG-GAACGGATCTTG	This study
KPhfq-C-F/R	GCCTACACAATCGCTCAAGTCTTCTGC-GCCGCAACAAGAC	AGTCGCCATATGGCGCAGC	This study
KANA-F/R	GCCACCTGGGATGAATGTC	CGGTCATTTCGAACCCCAGA	This study
KPhfq-I-F/R	TCAGCAATTACAGGAGATCG	ATCTGCATGATACGGTTACG	This study
Kana-I-F/R	ATCAGGACATAGCGTTGGC	GGCAAGAAAGCCATCCAGT	This study
KPhfqF/R	G*CTCTAGA*GCAAACGTCAGGAG	GG*AATTCC*AGTCGCCATATGGCGCAGC	This study
rpoB-F/R	GCGGTTGGTCGTATGAAGTT	TGGCGTTGATCATATCCTGA	[34]
PBP1a-F/R	CCCTTGTCGGCGGGTTTGA	TCGGCACATCGTTGAGCAT	This study
PBP1b- F/R	GTTTCATGGCCCGGTGAGC	CGCCGAGGATTAGCGACAGA	This study
PBP2- F/R	ACGGCCATCTGAACGTCAC	TAGCCAAACTTGCTCATCCAC	This study
PBP3- F/R	AAACAACGGTGGTCTGACATAG	CGACGGTACGAACGAGTGA	This study
PBP4- F/R	ATCGACTATCACAGCCAGCAAA	TTTCAGCGTCGCCACCATA	This study
PBP5- F/R	CTCTTTCACCGCTCGTTTACT	TCCAGGATTCGGCGTCTAT	This study
PBP6a- F/R	TCGGTGATGTTCCTCAAGCC	GGACGGTCATAAAGGTGGTGT	This study
PBP6b-F/R	GCTGTATCTTTGCTCGCTTCG	GGTCAGACTGGCAGGATTGC	This study
PBP7- F/R	GTTCCGCAACACCAACCATC	AAGGCATCCATCACCACCAG	This study

Italicized residues are the cleavage sites.

cycline resistance. The preparation of *K. pneumoniae* electrocompetent cells was conducted following the procedure described by Wei et al. [31], with slight modification. Briefly, *K. pneumoniae* WJ101 was inoculated into a flask containing 100 mL of LB, and incubated on ice for 30 min after the cell density value (OD_{600}) was approximately 0.7. The *K. pneumoniae* WJ101 cells were collected by centrifugation at 5000×g for 10 min at 4°C, and washed twice using ice-cold sterile ddH_2O. Next, the cells were resuspended in ice-cold sterile ddH_2O to a final volume of 0.5 mL. To construct the Δ*hfq* mutant, a disruption cassette consisting of a kanamycin-resistance marker flanked by *hup* and *hdown* (the upstream and downstream fragments of *hfq*) was constructed by fusion PCR using the primer set KPhfq-N-F/R and KPhfq-C-F/R. Purified pKD46T was electroporated into competent *K. pneumoniae* cells using a Bio-Rad MicroPulser, followed by transformation of the disruption cassette. The recombinant *K. pneumoniae* were screened on LB plates supplemented with kanamycin. The pKD46T plasmid was eliminated from *K. pneumoniae* by culturing this recombinant strain at 42°C. The *hfq*-complemented strain was constructed according to the method reported by Jayol et al. [33], to demonstrate the specificity of deletion. Amplification of the *hfq* fragment from the genomic DNA of *K. pneumoniae* WJ101 was performed using primers hfqF and hfqR, which contained the *Eco*RI and *Xba*I cleavage sites, respectively. Next, this *hfq* fragment was digested with *Eco*RI and *Xba*I, and cloned into the pHSG298 plasmid, which contains a kanamycin-resistance gene. The recombinant plasmid pHSG298CH was transferred into *K. pneumoniae* WJ101 *hfq* mutant strains, yielding the complemented strains. The recombinant *K. pneumoniae* WJ101ΔHCH were screened on LB plates supplemented with kanamycin in higher concentrations (125 μg/mL) than was once used. All constructed strains were identified by PCR and further verified by DNA sequencing (Supplementary Figs. 1S and 2S).

Antimicrobial susceptibility

Cefazolin sodium (CFZN), cefotaxime (CTX), cefuroxime sodium (CXMN) and ceftazidime (CAZ) were used in this study. These antimicrobial agents (all ≥98% pure) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China. Sterile water was used to prepare stock solutions of these drugs (1024 μg/mL), which were maintained at -20°C.

The minimum inhibitory concentrations (MICs) of cephalosporins against the *K. pneumoniae* strains

were determined using the standard broth microdilution method, as described by the Clinical and Laboratory Standards Institute (CLSI, formerly the National Committee for Clinical Laboratory Standards). All test isolates were diluted with NaCl 0.85% medium (BIOMERIEUX) to a turbidity of 0.5 McFarland. These dilute samples were further diluted (1:1000) into fresh Mueller-Hinton (MH) medium in the plates, and incubated for 18h at 37°C. The MIC was defined as the lowest concentration of antibacterial agent that showed no visible growth compared to that of the drug-free control. Meanwhile, the minimum bactericidal concentrations (MBCs) were determined using the colony-counting method. The complete content of every dilution was spread on an LB agar plates and incubated at 37°C for 24 h. We counted the number of colonies on each plate. The MBC was defined as the lowest concentration of bactericide with which no colonies appear on the LB agar plate. Each assay was performed in triplicate.

Time-kill curves

Time-kill curves for CFZN and CXMN against WT *K. pneumoniae* WJ101 and the *hfq* mutant, respectively, were analyzed**.** The test isolates were diluted to 0.5 McFarland turbidity using NaCl 0.85% medium (BIOMERIEUX), and then inoculated (1:500) into fresh MH medium in 5-mL test tubes. The tubes contained 0.5×drug concentrations obtained from the previous experiment. We incubated these tubes at 37°C. At the 0, 4, 8, 12, and 24 h time points, a 100-μL aliquot was removed from each test tube and serially diluted 10-fold in sterile water. One hundred mL of each dilution was spread on the LB agar plates and incubated at 37°C for 18 h. Subsequently, we counted the colonies on each plate. Each assay was performed in triplicate.

PCR

We used PCR to confirm the presence of PBP genes in *K. pneumoniae* WJ101. Genomic DNA from WJ101 was extracted using the TIANamp bacteria DNA kit (TianGen), and this DNA was the PCR template. The reaction system consisted of 12.5 μL 2×Taq MasterMix, 1 μL of each primer (PBPs-F/R primers, Table 2), 1 μL genomic DNA as template, and ddH_2O, for a final volume of 25 μL. The PCR was performed using the GeneAmp PCR system 9700 (Applied Biosystem) according to following program: 5 min of predenaturation at 94°C, followed by 35 cycles of denaturation for 30 s at 94°C, primer annealing for 30 s at 55°C and extension for 30s at 72°C. The final step was extension for 5 min at 72°C. Five mL of each reaction mixture was electrophoresed in 1% agarose gel containing ethidium bromide (EB) at 125 V for 35 min. The DNA bands were imaged using Gel Doc™ XR System (Bio-Rad Laboratories).

Quantitative reverse transcription PCR

RNA was isolated from overnight bacterial cultures using TRIzol reagent (Invitrogen). The RNA yield and purity were determined using a NanoDrop ND-1000 (Thermo Scientific). The purified RNA was reverse transcribed to cDNA using an ImProm-II™ Reverse Transcription System (Promega). Quantitative reverse transcription PCR (qRT-PCR) was carried out to detect PBP gene expression levels in WJ101 and the *hfq* mutant. The primers and their target genes are listed in Table 2. RT-PCR was performed on an IQ5 real-time PCR detection system (Bio-Rad) using the SYBR Premix Ex Taq™ (TliRNaseH Plus) (Takara). Amplification was performed in a 25-μL reaction system consisting of 12.5 μL Ex Taq mixture, 0.6 μL of each primer, 1μL cDNA and ddH_2O. We used the following program: predenaturation for 30 s at 95°C, followed by 40 cycles of denaturation for 30 s at 95°C and primer annealing for 30 s at 60°C. To acquire melting curves, we performed 71 denaturation-annealing cycles to confirm that this amplification was specific. The analyses of the expression levels were conducted on the following targets: PBP1a, PBP1b, PBP2, PBP3, PBP4, PBP5, PBP6a and PBP7. We normalized measurements to those for the housekeeping gene *rpoB* [34] and used the $2^{-\Delta\Delta t}$ method. Each assay was performed in triplicate.

Statistical analysis

Statistical analyses of qRT-PCR data were performed using the Student's t-test for paired samples. A *p* value of less than 0.05 was considered significant. Plotting and *p* value calculation were facilitated by GraphPad Prism 5 (GraphPad Software, Incorporated, CA).

RESULTS

Hfq mutant of *K. pneumoniae* exhibits increased MIC for cephalosporin

To test the possible roles of the Hfq protein in *K. pneumoniae* resistance to cephalosporins, a deletion mutant of *hfq* was constructed. The *hfq* open reading frame (ORF) was deleted using a lambda red-based replacement system. PCR and DNA sequencing confirmed that the *hfq* deletion was successful (Supplementary Figs. 1S and 2S). Next, the antibacterial activities of cephalosporins against *K. pneumoniae* and the *hfq* mutant were assessed in a microtiter plate model. The MIC values of the two strains against four cephalosporin antibiotics are shown in Table 3. MIC values for the WT strain ranged from 0.0625 of CAZ to 16 of CTX. For the *hfq* mutant, the MIC ranged from 1 of CAZ to 1024 of CFZN. The cephalosporin MIC against the *hfq* mutant was significantly higher than against WT *K. pneumoniae*. With respect to the WT MICs, the CTX, CFZN, CXMN and CAZ MICs for the *hfq* mutant increased by 32-, 1024-, 128- and 16-fold, respectively (Table 3). This effect was impaired by an *hfq* complementary strain (Table 3).

Table 3. MIC fold increases for *hfq* mutant over wild-type *K. pneumoniae*

Antibioticsa	MIC (μg/ml)			Increased folds
	WJ101	WJ101ΔH	WJ101ΔHCH	
CTX	16	512	—	32
CFZN	1	1024	1	1024
CXMN	0.5	64	0.5	128
CAZ	0.0625	1	0.625	16

CTX – cefotaxime; CFZN – cefazolin sodium; CXMN – cefuroxime sodium; CAZ – ceftazidime.

Table 4. MBC fold increases for *hfq* mutant over wild-type *K. pneumoniae*.

Antibioticsa	MBC(μg/mL)		Fold increase
	WJ101	WJ101ΔH	
CTX	32	512	16
CFZN	16	1024	64
CXMN	4	64	16
CAZ	0.25	1	4

CTX – cefotaxime; CFZN – cefazolin sodium; CXMN – cefuroxime sodium; CAZ – ceftazidime.

Increased MBC of the *hfq*-mutant of *K. pneumoniae* to cephalosporin

To further confirm the increased resistance of the *hfq* mutant to cephalosporin antibiotics, the MBCs for the *hfq* mutant and WT strains were tested. MBCs for CTX, CFZN, CXMN and CAZ against the WT strain were 32, 16, 4 and 0.25 μg/mL, respectively. MBCs for the *hfq* mutant were 512, 1024, 64 and 1 μg/mL, respectively (Table 4). Compared with those of the WT strain, the MBCs for these antibiotics in the *hfq* mutant increased between 4-fold (CAZ) and 64-fold (CFZN). These results demonstrated that the deletion of *hfq* increased the MBCs for *K. pneumoniae*.

Time-kill curves

To assess the role of *hfq* in *K. pneumoniae* cephalosporin insensitivity, the antimicrobial activities of CFZN and CXMN against WJ101 and its *hfq* mutant, respectively, were determined using the time-kill approach. The results are shown in Fig. 1. As presented in the figure, although the curves of WJ101and the *hfq* mutant show a similar trend, the *hfq* mutant exhibited a slow-growing state when compared with WT WJ101. While CFZN exhibited excellent bactericidal activity against WJ101 at 1/2×*hfq*-mutant MIC during 12 h, the mutant exhibited only a minor growth inhibition during the same period. There was no apparent impact on the mutant when we treated it with CFZN at 1/2×WT MIC concentration, whereas WT WJ101 exhibited significant growth inhibition. As Fig. 1 illustrates, there was a parallel trend for CXMN against WJ101 and the *hfq*-mutant at 1/2×*hfq*-mutant MIC and 1/2×WT MIC. These time-kill curves confirmed that *hfq* plays an important role in resistance of *K. pneumoniae* to cephalosporins.

Decreased expression level of PBP genes in the *hfq* mutant

Bacterial resistance to cephalosporins is usually mediated by PBPs. Decreased expression of PBPs results in increased resistance to the antibiotics. To test whether the *hfq* deletion influenced the expression of PBPs, the distribution of PBPs and their expression were determined. Firstly, *K. pneumoniae* WJ101 was detected by PCR to confirm the presence of genes that encode PBPs. As shown in Fig. 2(a), all 9 genes encoding PBPs

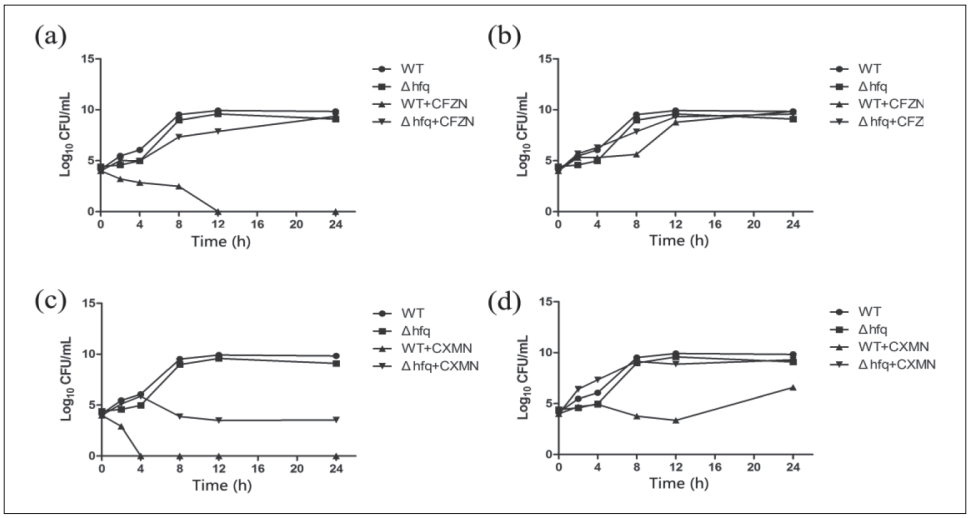

Fig. 1. Survival curves of WJ101 and *hfq* mutant treated with CFZN and CXMN, respectively. **a** – 512 μg/mL (1/2×*hfq* mutant MIC of CFZN); **b** – 0.5 μg/mL (1/2×WT MIC of CFZN); **c** – 32 μg/mL (1/2×*hfq* mutant MIC of CXMN); **d** – 0.25 μg/mL (1/2×WT MIC of CXMN).

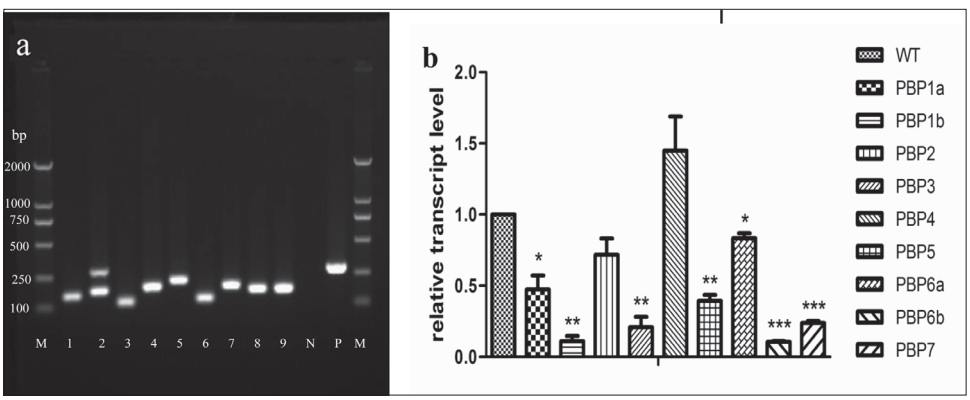

Fig. 2. Expression levels of PBPs were changed in the *hfq* mutant of *K. pneumoniae*. a – all major PBPs are present in *K. pneumoniae* WJ101. M – marker; 1 – PBP1a; 2 – PBP1b; 3 – PBP2; 4 – PBP3; 5 – PBP4; 6 – PBP5; 7 – PBP6a; 8 – PBP6b; 9 – PBP7; N – negative control; P – positive control (*rpoB*); **b** – relative mRNA expression levels of PBPs (1a, 1b, 2, 3, 4, 5, 6a, 6b, 7) were compared between the wild-type strain and the *hfq* mutant. The mutant expression levels are presented as values relative to the WT levels. All values were normalized to the housekeeping gene *rpoB*. Error bars represent standard deviations. Significant differences are indicated with asterisks (* indicates $P<0.05$; ** indicates $P<0.01$;*** indicates $P<0.001$).

were successfully detected in the WJ101 strain, indicating that these genes do exist in the strain and it was feasible to analyze their expression. To test the influence of *hfq* on PBPs, gene expression of was analyzed by qRT-PCR in WT and the *hfq*-mutant strain as described in the Materials and Methods. As shown in Fig. 2(b), compared with that in the WT strain, the expressions of most of these genes, including 1a, 1b, 2, 3, 5, 6a, 6b and 7, were decreased. The expression levels of these genes were inhibited from 0.3- to 5-fold. Among these, PBP1b, PBP3, PBP6b and PBP7 showed the greatest decrease. The only exception was PBP4, which showed increased expression.

DISCUSSION

Cephalosporins, which have a broad spectrum of antimicrobial activity against a large variety of pathogens, are widely used as first-line drugs for both the preven-

tion and the treatment of bacterial infection. However, recent studies have shown that they have a reduced effect against many drug-resistant pathogens, such as *K. pneumoniae*. In the present work, we have investigated the role of *hfq* in the regulation of *K. pneumoniae* resistance to cephalosporins. One interesting finding here was that an *hfq* mutant exhibited increased resistance to cephalosporins in *K. pneumoniae*.

There have been many reports describing the specific mechanism of the antimicrobial effect of cephalosporins. In brief, this kind of antibiotic can inhibit the activity of the penicillin-binding proteins, which is necessary for the biosynthesis of peptidoglycan, the main component in the negative-bacteria cell wall. Hence, cephalosporins can influence cell-wall integrity, resulting in damage to the pathogen cell. As an important factor contributing to the multifactorial mechanism of cephalosporin resistance, the changes in PBP level have been found to be involved in drug susceptibility in some bacteria. Another interesting finding here is that the inactivation of *hfq* leads to altered expression levels of PBPs, the targets of cephalosporins in *K. pneumoniae*. This finding implies that the increased resistance might be mediated by an altered expression of PBPs, the main mediators of resistance. The reduction or loss of one or more PBPs has been identified as a reason for the increased resistance to β-lactam in many reports. Examples include PBP3 for clinical ceftazidime-resistance in *Burkholderia pseudomallei* [30] and cefotaxime-resistance in *Streptococcus pneumoniae* [29], and PBP4 for triggering overproduction of AmpC in *Enterococcus faecium* [35]. In addition, the similar phenomenon where increased resistance accompanies a decreased expression level of most PBPs was also observed by Vashist et al. [36] in *Acinetobacter baumannii*. Contrary to the above results, Mottl et al. [37] and Sanders et al. [38] reported respectively that the loss of PBP4 would result in diminishing the induction of AmpC in *Escherichia coli*, which is in line with the increased expression of PBP4 in the cephalosporin-resistant *hfq* mutant.

In conclusion, one key finding in this investigation is that *hfq* negatively regulates the sensitivity of *K. pneumoniae* to cephalosporins. Inactivation of *hfq* leads to reduced expression levels of most of the PBPs, which is the main mechanism of resistance to cephalosporin. Therefore, *hfq* mediated the sensitivity of *K. pneumoniae* to cephalosporins probably by decreasing the expression of PBPs. Because the roles of the Hfq protein are mainly mediated by sRNA, our future work is to identify sRNA involved in expression regulation of PBPs.

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Supplementary Figures

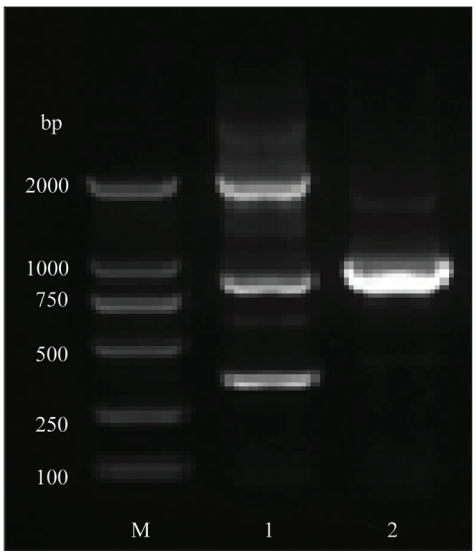

Fig. 1S. *hfq* mutant strain was identified by PCR. M – marker; 1 – upstream of the identification fragment; 2 – downstream of the identification fragment.

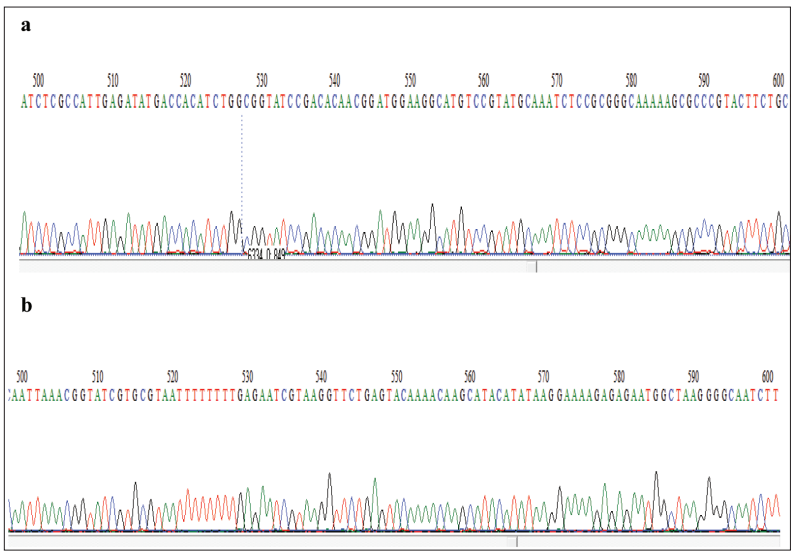

Fig. 2S. *hfq*-Mutant strain was identified by sequencing. The figure shows part of the fragment (500 bp-600 bp) which was excised from the whole sequence: **a** – upstream of the identification fragment and **b** – downstream of the identification fragment.