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An essential role for *hfq* involved in biofilm formation and virulence in serotype 4b *Listeria monocytogenes*

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ABSTRACT

Regulator factor Hfq has been widely detected among both Gram-positive and Gram-negative bacteria; however, its role in Gram-positive bacteria is less well established and varies among species. In *Listeria monocytogenes* (*Lm*), an organism able to adapt to a range of environments and live both saprobiotic and parasitic lifestyles, the role of Hfq is not fully understood. Serotype 4b *Listeria monocytogenes* strains associated with the majority of listeriosis outbreak, while the function of *hfq* in serotype 4b strains still not referenced. Here, we constructed *hfq* deletion and reversion mutants of serotype 4b *Lm* NTSN and analysed the biological characteristics both *in vitro* and *in vivo*. The deletion of *hfq* resulted in a growth deficiency in medium containing 4.5% ethanol or 1% Triton X-100, and the growth of the mutant was significantly reduced at 4 °C. Furthermore, the *hfq* deletion dramatically decreased biofilm formation in BHI medium and gastric fluid medium, and reduced the invasion and replication rate into the Caco-2BBe cells and RAW264.7 cells. However, complementation restored the wild-type phenotype. Importantly, mouse infection experiments demonstrated that *hfq* played a more important role in the colonisation and virulence in serotype 4b strain *Lm* NTSN than in the serotype 1/2a strain *Lm* EGDe. Taken together, these results demonstrated that *hfq* is a novel factor associated with biofilm formation, and plays an essential role in the stress response and pathogenisis in serotype 4b strain *Lm* NTSN. Our data provide the basis for further research into the function of Hfq in serotype 4b *Listeria monocytogenes*.

1. Introduction

Listeria monocytogenes (*Lm*) can cause a serious life-threatening infection, known as listeriosis, that can result in meningoencephalitis and sepsis with a mortality rate of close to 30%. Molecular epidemiology data show that 98% of listeriosis cases are caused by strains 4b, 1/2a and 1/2b (Swaminathan and Gernersmidt, 2007; Laksanalamai et al., 2014), whereas the majority of listeriosis cases, more than 80% of animal listeriosis cases and ~50% of human listeriosis cases are caused by serotype 4b strains. Chen et al. reported that the risk of 4b strains of lineage I causing listeriosis was 100-fold higher than that of 1/2a and 1/2c strains (Chen et al., 2007); thus 4b strains are the major serotype strains resulting in losses to the livestock industry and threatening human life.

The response of *Lm* to different living environments *in vivo* and *in vitro* is regulated by a variety of factors, such as PrfA (Chaturongakul et al., 2011), SigB (Severino et al., 2007; Giotis et al., 2008; Hain et al., 2008; McGann et al., 2008), VirR (Mandin et al., 2005), HrcA, CtsR and Hfq (Christiansen et al., 2004; Vera et al., 2013). Such multifarious regulation helps *Lm* to adapt to and survive in hostile environments. Increasing attention has been paid to the Hfq protein that plays an integral role in regulating a series of processes relating to adaptations to environmental conditions. Hfq is found widely in both Gram-positive and Gram-negative bacteria. The function of the *hfq* gene, which has been investigated in depth in a range of bacteria including *Escherichia coli*, *Haemophilus* species, *Bordetella pertussis* and *Staphylococcus aureus* (Tsui et al., 1994; Muffler et al., 1997; Bohn et al., 2007; Simonsen et al., 2011; Argaman et al., 2012; Hempel et al., 2013), has been reported to relate to environmental tolerance and virulence in Gram-negative pathogens (Robertson and Roop, 1999; McNealy et al., 2005). The role of Hfq in Gram-positive bacteria is less well established and varies among species (Zheng et al., 2016). Hfq does not contribute to

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the adaptation of stress responses in *S. aureus* (Bohn et al., 2007); however, it plays an important role in the resistance to hypertonic stress and pathogenicity in serovar 1/2a *Lm* EGDe from lineage II (Christiansen et al., 2004).

Here we report the study of *hfq* in the serotype 4b *Lm* NTSN isolated from an ovine outbreak with a high rate of mortality, and compared the biological characteristics of the *hfq* gene deletion and reversion mutants with that of the wild-type. The role of the Hfq protein in environmental adaption and pathogenicity in *Lm* was investigated to provide insight into the functional properties of this protein.

2. Materials and methods

2.1. Bacterial strains and plasmids

Virulent *Lm* NTSN was isolated from a case of ovine listeriosis. Mutant *Lm* EGDeΔ*hfq* was constructed in a previous study (Kang et al., 2015) and preserved at the Jiangsu Key Laboratory of Zoonosis. pMD20-T was purchased from TaKaRa Biotechnology Co., Ltd. (Dalian, China). Strain *Lm* EGDe and plasmid pERL3was gifted by Prof. Chakraborty (Justurs Liebig University, Giessen, Germany). Shuttle vector pKSV7 was kindly donated by Prof. Zhu Guowiang (Yangzhou University, China). All *Lm* strains were cultured in brain heart infusion (BHI) broth (Becton Dickinson Co., Franklin Lakes, NJ, USA). Detailed information regarding the bacteria strains and plasmids used in this study is provided in Supplementary Table 1.

2.2. Experimental animals

Six-week-old female BALB/c mice were purchased from Vital Riverc Co. (Beijing, China). The mice were housed, handled and immunized at an animal biosafety facility and all procedures were approved by the institutional animal experimental committee of Yangzhou University.

2.3. Construction of the hfq deletion and reversion mutants

To achieve homologous recombination, recombinant plasmid pKSV7-*N-hfqab* was constructed with *hfq* gene flanking regions, the oligonucleotide sequences used are provided in Supplementary Table 2. The recombinant plasmid pKSV7-*N-hfqab* was identified and introduced into a competent strain *Lm* NTSN by electroporation according to a previously described protocol (Park and Stewart, 1990). Recombinant strain NTSNΔ*hfq* was screened by PCR with the primer pair NhfqaF and NhfqbR. The amplified fragments were ~600-bp in the deletion mutants, compared with 600-bp plus the size of the deleted gene in the wild-type. The recombinant shuttle plasmid pERL3-hfqAB was constructed with primers hfqaF2/hfqbR2 and verified, then electrotransformed into NTSNΔ*hfq* competent cells and cultured in an incubator at 30 °C. The positive clones were screened on a BHI plate containing 5 mg/L erythromycin (Sigma) at 37 °C. The recombinant strains of NTSNΔ*hfq-hfq* were screened by PCR. RNA was extracted using an EZ-10 spin column RNA purification kit in accordance with the manufacturer's instructions (Bio Basic Inc). Single-stranded cDNA was synthesized using Moloney murine leukaemia virus reverse transcriptase (Promega) with the reverse primer followed by PCR amplification using the primer pair Hfq-s1/Hfq-as. To exclude the possibility of DNA contamination in the extracted RNA pool, PCR was performed using the total RNA template as the negative control.

2.4. Growth curve analysis of Lm

Bacterial cells of exponentially growing cultures of NTSN, NTSNΔ*hfq* and NTSNΔ*hfq-hfq* were harvested and centrifuged at 6010*g* for 2 min. The cell pellets were resuspended in phosphate-buffered saline (PBS), and the optical density at 600 nm (OD_{600}) was measured. The cultures were then transferred into small conical flasks containing 10 mL of BHI medium. Three parallel groups were set for each strain and adjusted to an initial OD_{600} value of 0.05. The bacterial cultures were incubated at 37 °C or 4 °C, and the OD_{600} value of each flask was measured every 1.5 h (37 °C) and 24 h (4 °C).

2.5. Biochemical characteristics of Lm

Strains NTSN, NTSNΔ*hfq* and NTSNΔ*hfq-hfq* were inoculated onto fresh BHI plates and cultured overnight. Bacterial cells were scraped from the plate using an inoculating loop and transferred into 5 mL of 0.45% normal saline. The bacterial turbidity was controlled at ~1.0 with a nephelometer. The bacterial biochemical characteristics were identified using a VITEK® 2 GN card (Biomerieu, France).

2.6. Effect of Triton X-100 and ethanol on Lm growth

The growth of strains NTSN, NTSNΔ*hfq* and NTSNΔ*hfq-hfq* under stress conditions induced by the addition of ethanol (4.5%) or Triton X-100 (1%) was measured. Overnight cultures were washed twice in PBS, then transferred into 10 mL of BHI medium containing different sources of stress (*i.e.*, ethanol or Triton X-100), and adjusted to an initial OD_{600} of 0.05. Three parallel groups were set for each strain, and the cultures were incubated at 37 °C on a shaker. The OD_{600} value in each flask was measured at set time points.

2.7. Determination of biofilm formation by Lm

Overnight cultures of *Lm* strains were collected and adjusted to an OD_{600} value of 1.0 with BHI medium or bile salt medium (pH = 2.5, containing 1.3 mg/mL pepsin, 10 mg/mL lysozyme and 5 mg/mL bile). After 10 serial dilutions (OD_{600} ~0.1), 200 μL of bacterial suspension was added to 96-well U-shaped cell culture plates, and the plates were incubated at 37 °C for 72 h. The plates were washed three times with sterile saline, stained with crystal violet, washed again. After drying, 225 μL of 96% ethanol was added to each well for 15 min for elution, and the absorbance at 595 nm was measured.

2.8. Determination of the invasiveness of Lm

Stabilized human colon carcinoma Caco-2BBE cells and RAW264.7 were respectively seeded into 24-well cell culture plates. Freshly cultured cells of the strains *Lm* NTSN and NTSNΔ*hfq* were then added at a bacterium/cell ratio (multiplicity of infection, MOI) of 20. After 1 h of incubation, the medium was discarded and Dulbecco's modified Eagle's medium (DMEM; containing 100-μg/mL gentamicin sulphate) was added to the wells for another 15 min. or 2 h. Then the monolayer was washed twice with sterile PBS, then overlaid with 0.1% Triton X-100 for 8 min to release the bacteria. The lysate was diluted with sterile PBS and spread onto BHI plates to obtain the CFU of each strain.

2.9. Mouse infection model

Six-week-old BALB/c female mice were used for an *in vivo* infection model, strains of NTSN, NTSNΔ*hfq*, EGDe and EGDeΔ*hfq* were infected *via* the intragastric route at a dose of 1×10^9 CFU, and bacteria in the ileum, lymphoglandulae mesentericae (MLN), spleen and liver were enumerated 24-h post-inoculation by tissue homogenization and serial plating, and ileum samples were spread onto CHROMagar selective plates. The above four strains were also used for intraperitoneal infection at a dose of 3×10^4 CFU, and bacteria in the liver and spleen were enumerated 72-h post-inoculation.

2.10. Determination of the 50% lethal dose (LD_{50}) for Lm in BALB/c mice

Overnight cultures of *Lm* strains were transferred to fresh BHI broth at the ratio of 1:20 and harvested at mid-log phase. The bacteria were