International Journal of Medical Microbiology 309 (2019) 225–231

Contents lists available at ScienceDirect

International Journal of Medical Microbiology

journal homepage: www.elsevier.com/locate/ijmm

A small non-coding RNA facilitates *Brucella melitensis* intracellular survival by regulating the expression of virulence factor

Yufei Wang[a,1], Yuehua Ke[b,1], Cuijuan Duan[a,1], Xueping Ma[a], Qinfang Hao[a], Lijie Song[a], Xiaojin Guo[a], Tao Sun[a], Wei Zhang[a], Jing Zhang[a], Yiwen Zhao[a], Zhijun Zhong[c,***], Xiaoli Yang[a,**], Zeliang Chen[b,d,*]

[a] Department of laboratory medicine, The Third Medical Center, General Hospital of the Chinese People's Liberation Army, Beijing 100039, China
[b] Department of Infectious Disease Control, Center of Disease Control and Prevention, Beijing 100071, China
[c] Key Laboratory of Animal Disease and Human Health of Sichuan Province, College of Veterinary Medicine, Sichuan Agricultural University, Sichuan 611130, China
[d] Key Laboratory of Livestock Infectious Diseases in Northeast China, Ministry of Education, Key Laboratory of Zoonosis of Liaoning Province, College of Aninal Science and Veterinary Medicine, Shenyang Agricultural University, Liaoning, 110866, China

ARTICLE INFO

Keywords:
Brucella
sRNA
Intracellular survival
Stress response
Virulence
Chronic infection

ABSTRACT

Brucella species are the causative agents of brucellosis, a worldwide zoonotic disease that affects a broad range of mammals and causes great economic losses. Small regulatory RNAs (sRNAs) are post-transcriptional regulatory molecules that participate in the stress adaptation and pathogenesis of *Brucella*. In this study, we characterized the role of a novel sRNA, BSR1141, in the intracellular survival and virulence of *Brucella melitensis*. The results show that BSR1141 was highly induced during host infections and under in vitro stress situations that simulated the conditions encountered within host phagocytes. In addition, a BSR1141 mutant showed reduced survival both under in vitro stress conditions and in mice, confirming the role of BSR1141 in *Brucella* intracellular survival. Bioinformatic and experimental approaches revealed that BSR1141 affects the expression of many target genes, including the *Brucella* virulence component *virB2*. These data indicate that BSR1141 could influence the expression of *virB2*, which is important for *B. melitensis* pathogenesis and intracellular survival. This work provides new insight into the mechanism of adaptation to environmental stress and into the pathogenesis of intracellular pathogens.

1. Introduction

Brucella species are facultative intracellular pathogens that cause abortion in domestic animals and undulant fever in humans. Human brucellosis is a debilitating febrile illness that can develop into a long-lasting disease with the appearance of severe complications (Atluri et al., 2011; de Figueiredo et al., 2015). *Brucella* infects hosts primarily by penetrating the host mucosa and then residing within macrophages, where they can replicate and escape host immunity. This process is essential for the ability of these pathogens to establish chronic infections and for their virulence (Celli and Gorvel, 2004). Successful in vivo infection is a complex procedure in which the pathogenic bacteria need to cope with diverse stresses imposed by host defenses. Thus, this process largely relies on the coordinated and sequential expression of metabolic and stress-related genes. Transcription factors have been found to play important roles in linking metabolic pathways and virulence gene expression (Papenfort and Vogel, 2014). Over the last decade, multiple *Brucella* metabolic and stress-related genes required for intracellular survival have been identified, and many of these genes are regulated by different transcription factors.

Small regulatory RNAs (sRNAs) are post-transcriptional regulatory molecules that participate in adaptive responses by cells against environmental change, such as temperature or pH shift, nutrient stress, osmotic shock, iron limitation, envelope stress, and metabolic imbalance. It is becoming evident that sRNAs also regulate the expression of virulence genes in pathogenic bacteria and affect bacterial survival in hosts (Michaux et al., 2014). Bacterial sRNAs are generally 50–300 nucleotides (nt) in length, and this groups includes cis-encoded

* Corresponding author at: No. 120, Dongling Road, Shenhe District, Shenyang 110866, China.
** Corresponding author at: No. 69, Yongding Road, Haidian District, Beijing 100039, China.
*** Corresponding author at: No. 211, Huimin Road, Wenjiang District, Chengdu 611130, China.
E-mail addresses: zhongzhijun488@126.com (Z. Zhong), yangxiaolitwins@163.com (X. Yang), zeliangchen@yahoo.com (Z. Chen).
[1] The authors contributed equally to this work.

https://doi.org/10.1016/j.ijmm.2019.04.002
Received 16 December 2018; Received in revised form 10 March 2019; Accepted 18 April 2019

antisense RNAs, trans-encoded base-pairing sRNAs, and cis-acting regulatory regions of mRNAs (i.e., riboswitches). The trans-encoded sRNAs, which are the most common type of sRNA, are usually encoded within intergenic regions and regulate gene expression through imperfect base pairing within the 5′ untranslated region (UTR) of target mRNAs, thus modulating mRNA stability and/or altering its accessibility to the translational machinery (Storz, 2002; Waters and Storz, 2009).

The interaction between trans-encoded sRNAs and their targets usually requires the RNA chaperone Hfq. Previous work reported that a *Brucella* Hfq deletion mutant showed reduced stress tolerance and intracellular survival in macrophages and mice, suggesting that sRNAs may play regulatory roles in *Brucella* intracellular survival (Robertson and Roop, 1999). Recently, a large number of sRNAs have been identified in different *Brucella* spp. (Dong et al., 2014; Saadeh et al., 2015; Zhong et al., 2016); however, the associated functions of these sRNAs have been determined for only a small subset. Thus, clarifying the functions of these sRNAs and identifying virulence-associated sRNAs may provide new insights into the pathogenesis of *Brucella*. In previous work, we identified a group of trans-encoded sRNAs in *B. melitensis* and found several sRNAs that may play roles in the bacteria–host interactions during *Brucella* infection (Wang et al., 2015). Here, we elucidated the function and associated mechanism of one of these sRNAs.

2. Materials and methods

2.1. Animal use

Female BALB/c mice of six-week-old were acquired from the Animal Center of the Academy of Military Medical Sciences. All animals were handled in strict accordance with Experimental Animal Regulation Ordinances defined by China National Science and Technology Commission, and the animal work was preapproved by Beijing Institute of Disease Control and Prevention animal ethics committee (Ethical Approval BIDCP003-2014). The mice were provided with humane care and healthful conditions during their stay in the facility. All individuals who use animals receive instruction in experimental methods and in the care, maintenance and handling of mice, and are under supervision of the committee.

2.2. Bacterial strains, plasmid, and growth conditions

B. melitensis 16 M and its derivative strains were routinely cultured on Tryptic Soy Agar (TSA) or in Tryptic Soy Broth (TSB). *Escherichia coli* strains were routinely grown on Luria–Bertani (LB) medium. When required, antibiotics were added to media at final concentrations of 100 μg/ml of ampicillin or 50 μg/ml of kanamycin. Plasmid pBBR1 MCS-4 is a broad-host-range plasmid capable of replicating in *Brucella* (Elzer et al., 1995). All experiments with live *Brucella* cells, including animal experiments, were performed in biosafety level 3 facilities.

2.3. Construction of sRNA mutant and overexpression B. melitensis strains

The previously described resistance gene replacement method was used to construct the BSR1141 deletion mutant (Cui et al., 2013). Firstly the upstream homologous arm of the BSR1141 coding region was amplified with primers BSR1141-N-F and BSR1141-N-R, and the downstream homologous arm was amplified with BSR1141-C-F and BSR1141-C-R. Then, both the PCR products were digested and ligated respectively to the upstream and downstream of the kanamycin gene of pUC19 K as described previously (Wang et al., 2011). The resulting suicide plasmid pUC19K-BSR1141 was electroporated into the *B. melitensis* 16 M recipient strain, and the resulting potential deletion mutants were screened on TSA plates based on their amp^S kan^R phenotype. Deletion mutant was confirmed by PCR with primers located in kanamycin resistance gene and outside of the homologous arms. PCR products were sequenced to confirm the sequence. The final selected mutant strain was named 16 MΔBSR1141. To construct the BSR1141-overexpression strain, a DNA fragment containing the BSR1141 locus was amplified from the wildtype 16 M strain by PCR using primers BSR1141-N-F and BSR1141-C-R. The resulting DNA fragment was then ligated into the *KpnI-PstI* sites of pBBR1 MCS-4 to create pBBR-BSR1141. Finally, pBBR-BSR1141 was introduced into *B. melitensis* strain 16 M by electroporation, and resulting overexpressing strain, named 16 M-BSR1141, was selected based on its resistance to ampicillin.

2.4. RNA preparation

Total RNA from different *B. melitensis* strains was prepared using Trizol reagent (Invitrogen) according to the manufacturer's recommendations. The total RNA concentration and purity of each sample were assessed using a ND-1000 Spectrophotometer Nanodrop (Technologies) and agarose gel electrophoresis, respectively.

2.5. Northern blot

Northern blot analyses were performed using a DIG Northern Starter Kit (Roche) as previously described by Beckmann et al (Beckmann et al., 2010). Total RNA (15–20 μg) was separated on a 10% polyacrylamide–7 M urea gel and transferred to Hybond N + membranes (GE) by electroblotting. The membranes were UV-cross-linked and prehybridized in ULTRAhyb® Ultrasensitive Hybridization Buffer (Ambion). The membranes were then hybridized with gene-specific 3′-end digoxigenin-labeled RNA probes, and the resulting hybridization signals were detected according to the manufacturer's instructions.

2.6. 5′ and 3′ RACE

To determine the sequences of the BSR1141 termini, RACE was performed as previously described by Wang et al (Wang et al., 2015). Both 5′ and 3′ RACE were carried out using a Full RACE Core set (Takara Biochemicals) according to the manufacturer's instructions. For each RACE analysis, six to ten clones were sequenced, and the farthest end was regarded as the end of the sRNA.

2.7. qRT-PCR

For transcription analyses of BSR1141 under in vitro stress conditions, total RNA was isolated from cultures of *B. melitensis* 16 M grown under different stress conditions. To induce acid stress, cells were re-suspended in TSB broth adjusted to pH of 4.0 with 1 M HCl at 37℃. To starve bacteria of nutrients, cells were re-suspended in GEM medium ($MgSO_4.7H_2O$ 0.2 g/L, Citric acid $\cdot H_2O$ 2.0 g/L, K_2HPO_4 10.0 g/L, $NaNH_4HPO_4.4H_2O$ 3.5 g/L, Glucose 20 g/L, pH 7.0) at 37℃. To induce oxidative stress, H_2O_2 was added to the broth at a final concentration of 1.5 mM at 37℃. To induce heat shock, cells were re-suspended in TSB broth at 42℃. TSB 7.0 is the standard in vitro growth condition for *B. melitensis*. The resulting RNA samples were treated with DNaseI (Promega) and reverse-transcribed (RT) into cDNA using a reverse transcription kit (Promega) according to the manufacturer's instructions. qRT-PCR was performed with the method previously described by Wang et al (Wang et al., 2015). The average expression levels and SD were calculated using data from three technical replicates of three independent experiments. The primers used for qRT-PCR are listed in Table S1.

For transcription analysis of BSR1141 during mouse infection, BALB/c female mice (6–8 weeks old) were challenged with 2×10^6 colony-forming units (CFU) of *B. melitensis* strain 16 M in sterile PBS. At 3, 7, 14, and 42 days post-inoculation, five infected mice were anesthetized by cervical dislocation. Their spleens were collected

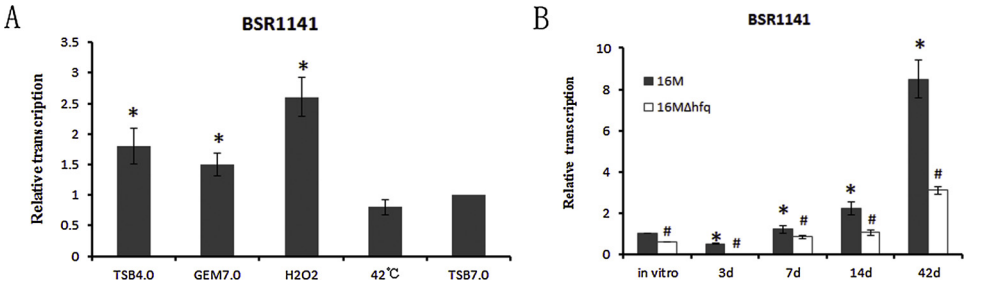

Fig. 1. BSR1141 transcription levels under in vitro stress conditions and during in vivo *B. melitensis* infection. (A) The relative transcription levels of BSR1141 under various stress conditions. Asterisks (*) denote values that are significantly different ($P < 0.05$) from that for *B. melitensis* cultured under TSB7.0. (B) The BSR1141 expression in *B. melitensis* 16 M and 16 MΔhfq strains during mouse infection. The asterisks above the gray boxes represent significant differences ($P < 0.05$) compared with those of the in vitro condition, and the asterisks above the white boxes represent significant differences ($P < 0.05$) between strains 16 M and 16 MΔhfq.

aseptically, and the total RNA was extracted from each spleen. Reverse transcription and qRT-PCR analysis were then performed as described above. The expression levels of BSR1141 are expressed here as n-fold differences relative to the calibrator.

2.8. Growth curve, in vitro environmental stress, and in vivo mouse virulence assays

For growth curve experiments, *B. melitensis* cultures grown in TSB medium, pH 7 (TSB7.0) were diluted to an optical density at 600 nm (OD_{600}) of 0.05 and incubated in 125-ml Erlenmeyer flasks at 37 °C with shaking at 250 rpm. The resulting cell density at each timepoint was assessed at OD_{600}.

For in vitro environmental stress assays, all the tested *B. melitensis* strains were cultured at 37 °C in TSB7.0 to the stationary growth phase (OD_{600} = 2.5) and then exposed to different stress conditions resembling those that *Brucella* probably encounter during infection: osmotic stress (incubated in the presence of 1.5 M NaCl for 20 min), oxidative stress (incubated in the presence of 440 mM H_2O_2 for 40 min), and acid stress (incubated in TSB medium at pH 4.0 [TSB4.0] for 15 min). The cultures were then diluted and spread on TSA plates to measure their CFU. Results shown represent the mean of at least three technical replicates of three independent experiments.

For virulence assays, six-week-old BALB/c female mice (n = 15 per group) were infected intraperitoneally with 2×10^6 CFU of *B. melitensis* strain 16 M, 16 MΔBSR1141, or 16 M-BSR1141 in sterile PBS. Five infected mice from each infected group were randomly selected and euthanized by cervical dislocation at 1 and 6 weeks post-inoculation. Their spleens were collected and homogenized with PBS containing 0.1% Triton X-100. The homogenized spleen samples were serially diluted, plated on TSA with or without kanamycin or ampicillin, and then incubated for 3–5 days at 37 °C, after which the bacteria were counted to determine the number of CFU. The experimental limit of detection was determined to be 10 CFU per spleen and the results described represent the accumulated data from 3 independent experiments.

2.9. BSR1141 target identification

The TargetRNA and RNApredator algorithms were used against the entire genome of wildtype *B. melitensis* strain 16 M to predict the target mRNAs of BSR1141 (Tjaden et al., 2006). The predicted mRNA targets of BSR1141 were then further validated by semi-quantitative RT-PCR. 16S rRNA was chosen as an internal control because its transcription is relatively constant in bacteria. The primers used for RT-PCR are listed in Table S1. The PCR products were separated electrophoretically in a 1.2% agarose gel containing ethidium bromide and detected using the Gel Doc™ XR System (Bio-Rad Laboratories).

2.10. Two-plasmid system for assessing target regulation by BSR1141

The *E. coli*-based system for studying sRNA gene regulation developed by Urban and Vogel (Urban and Vogel, 2007) was used here to assess BSR1141 target mRNAs regulation as previously described by Wang et al (Wang et al., 2015).

2.11. Statistical analysis

The bacterial survival under in vitro stress conditions and during in vivo infections are expressed as the mean percentage of survival compared with that of untreated controls ± SD. Statistical analyses were performed using Student's unpaired *t*-tests. For the competitive index assays, the data were analyzed by Student's *t*-tests. For qRT-PCR experiments, the significance of differences was assessed by the Wilcoxon signed-rank test. In all cases, a *p*-value of less than 0.05 was considered significant.

3. Results

3.1. BSR1141 is activated under in vitro stress conditions and during in vivo mouse infection

In our previous analysis of *B. melitensis* 16 M sRNAs, we identified a trans-encoded highly abundant sRNA, BSR1141 (Wang et al., 2015). The BSR1141 sequence is highly conserved among different *Brucella* species, and it showed an Hfq-dependent expression profile in vitro. 5′ and 3′ RACE were used to precisely determine the start and termination sites of the BSR1141 transcript. The transcript was found to be 75 nt in length and located in a clockwise orientation at bps 1187596–1187668 of *B. melitensis* chromosome I.

Previous work demonstrated that *Brucella* could withstand different environmental conditions during their long-term residence in host macrophages, including exposure to acidic pH, reactive oxygen intermediates, and nutrient deprivation (Caldelari et al., 2013). To understand the role of BSR1141 in *Brucella* intracellular survival, we monitored the BSR1141 expression under stress conditions mimicking those that the bacteria encounter during infection. As shown in Fig. 1A, the expression of BSR1141 was upregulated 2.6-fold, 1.8-fold, and 1.5-fold, respectively, under oxidative stress (induced by H_2O_2), acidic stress (TSB4.0), and nutrient limitation (GEM 7.0) conditions compared with that in the standard laboratory growth condition (TSB7.0). The results suggest that BSR1141 may play a role during *B. melitensis* infection. To test this possibility, we analyzed the BSR1141 expression during intracellular growth in vivo. Mice were injected with *B. melitensis* 16 M, and the expression of *B. melitensis* RNA recovered from their spleens was measured by qRT-PCR. At 3 days post-infection, BSR1141 was detected at ˜2-fold lower levels compared with cells infected with *B. melitensis* in vitro (Fig. 1B). In contrast, as the infection progressed, the transcript of BSR1141 became upregulated in vivo compared with the in vitro growth condition. At 42 days post-infection, the level of BSR1141 sRNA transcription in vivo was 8.5-fold higher than the in vitro levels (Fig. 1B), suggesting that BSR1141 may play a role in the chronic stage of *B. melitensis* infection. Moreover, at all post-infection

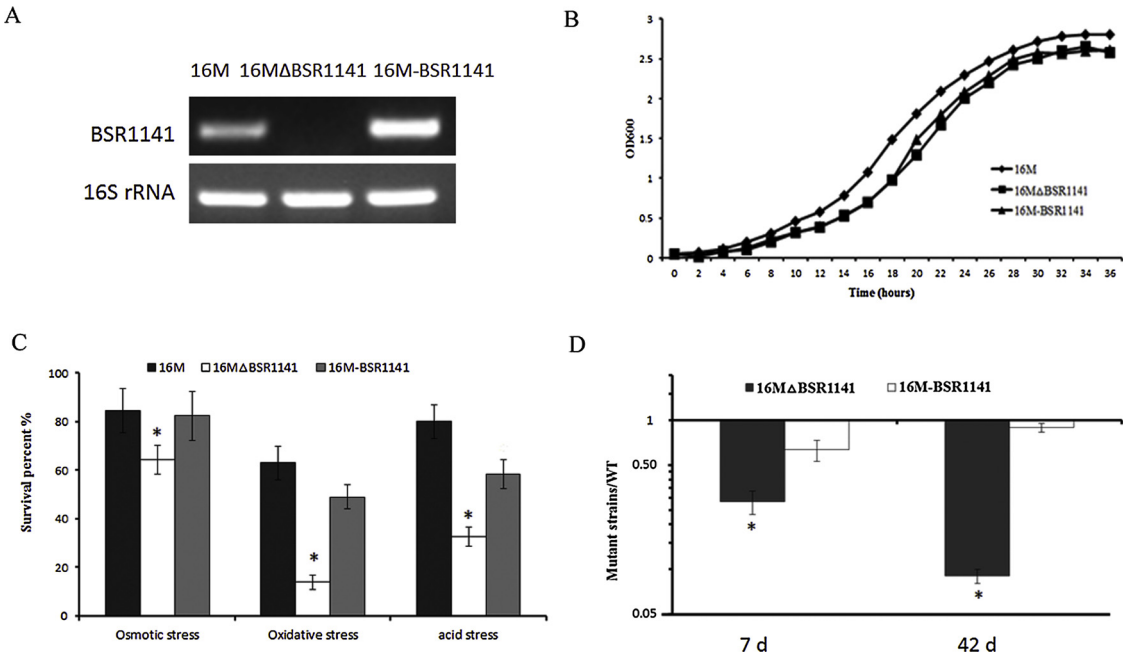

Fig. 2. Assessment of BSR1141 necessity for *B. melitensis* 16 M intracellular survival. (A) RT-PCR verification of BSR1141 transcription in *B. melitensis* strains 16 M, 16 MΔBSR1141, and 16 M-BSR1141. (B) Growth characteristics of strains 16 M, 16 MΔBSR1141, and 16 M-BSR1141 in TSB7.0. (C) Survival of *B. melitensis* strains under stress conditions. Strains 16 M, 16 MΔBSR1141, and 16 M-BSR1141 were grown in TSB7.0 to the logarithmic phase and then subjected to high osmotic, oxidative, or acid stress. The recovered CFU of each strain/condition were determined by counting plated serial dilutions. Bars represent the mean percentage survival compared with untreated strains. Each assay was conducted with three replicates. Asterisks (*) represent significant differences ($P < 0.05$) compared with strain 16 M. (D) Survival of *B. melitensis* strains in mice. Mice were infected with strains 16 M, 16 MΔBSR1141, or 16 M-BSR1141, and the resulting levels of spleen colonization were measured. Data are presented as the ratios of CFU from mutant strains to those of wildtype. Asterisks (*) represent significant differences ($P < 0.05$).

timepoints, the BSR1141 transcription level in the *B. melitensis* hfq deletion mutant (strain 16 MΔhfq) was significantly lower compared with that in the wildtype *B. melitensis* strain 16 M (Fig. 1B), demonstrating that BSR1141 is also Hfq-dependent in vivo. In summary, the above results indicate that BSR1141, an abundant Hfq-dependent sRNA, was highly activated both under in vitro stress conditions and during in vivo infection.

3.2. BSR1141 is required for B. melitensis 16 M intracellular survival

To further elucidate the functions of BSR1141 in *Brucella* intracellular survival, we next aimed to analyze the phenotypes of *B. melitensis* strains with different sRNA expression levels. BSR1141 deletion (16 MΔBSR1141) and overexpression (16 M-BSR1141) mutant strains were constructed for these experiments (Fig. 2A). First, the role of BSR1141 in *B. melitensis* growth under the standard laboratory growth condition (TSB7.0) was examined. Compared with the wildtype *B. melitensis* 16 M strain, the 16 MΔBSR1141 and the 16 M-BSR1141 mutant strains each exhibited a longer lag phase and reached the stationary phase at a lower optical density (Fig. 2B). These results indicate that both the deletion and overexpression of BSR1141 had slight but clearly detectable effects on *B. melitensis* growth.

We next compared the intracellular survival phenotypes of the BSR1141 deletion and overexpression mutant strains with that of the wildtype 16 M strain by subjecting them to stress conditions that mimic bacterial intracellular environments, specifically high osmotic, oxidative, and low pH conditions, and calculating their percentages of survival. The 16 MΔBSR1141 mutant was significantly more sensitive to all stress conditions than its parental strain (Fig. 2C). Compared with strain 16 M, the survival percentage of strain 16 MΔBSR1141 decreased approximately 77% and 59% under oxidative and acid stress, respectively. The overexpression strain 16 M-BSR1141 had high osmotic-resistance levels that were similar to those of the wildtype 16 M; however, this strain had lower resistance against oxidative and acid stress compared with strain 16 M. Thus, the overexpression of BSR1141 also has deleterious effects for the stress adaptation ability of *Brucella*.

Give the apparent role of BSR1141 in *B. melitensis* resistance to environmental stresses that are likely encountered in host macrophages, we further investigated if BSR1141 is essential for bacterial survival in vivo. BALB/c mice were infected with the *B. melitensis* 16 M, 16 MΔBSR1141, or 16 M-BSR1141 strains, and the resulting spleen colonization was measured at different timepoints post-infection. At 7 and 42 days post-infection, the amount of strain 16 MΔBSR1141 was significantly lower than that of the wildtype strain (Fig. 2D). In contrast, the level of colonization by strain 16 M-BSR1141 was similar to that of strain 16 M (Fig. 2D). Especially at 42 days post-infection, sharp reductions of the spleen bacterial load were observed in mice infected with 16 MΔBSR1141, and the ratio of 16 MΔBSR1141/16 M was only 0.09. While approximately log 4.5 CFU of *Brucella* remained in the spleens of mice infected with *B. melitensis* 16 M-BSR1141, with the ratio of 16 M-BSR1141/16 M 0.89. These data show that BSR1141 sRNA plays an important role in *Brucella* survival in mice. Thus, the results from the in vitro stress and in vivo infection assays confirmed that BSR1141 sRNA is required for *B. melitensis* intracellular survival.

3.3. BSR1141 post-transcriptionally regulates multiple target mRNAs

As an Hfq-dependent, trans-encoded sRNA, BSR1141 may act via imperfect base pairing with the 5′ UTR of target mRNAs. Thus, we next aimed to link BSR1141 to mRNA targets to reveal a BSR1141-dependent regulatory circuit in *B. melitensis* intracellular survival. Using TargetRNA and RNApredator, we searched the potential intermolecular base pairings between the BSR1141 conserved unpaired region and mRNA ribosome-binding sites (20 nt upstream and 30 nt downstream of the AUG codon). In total, 43 putative target mRNA genes were identified (Table S2). Almost half of these genes were related to transport and

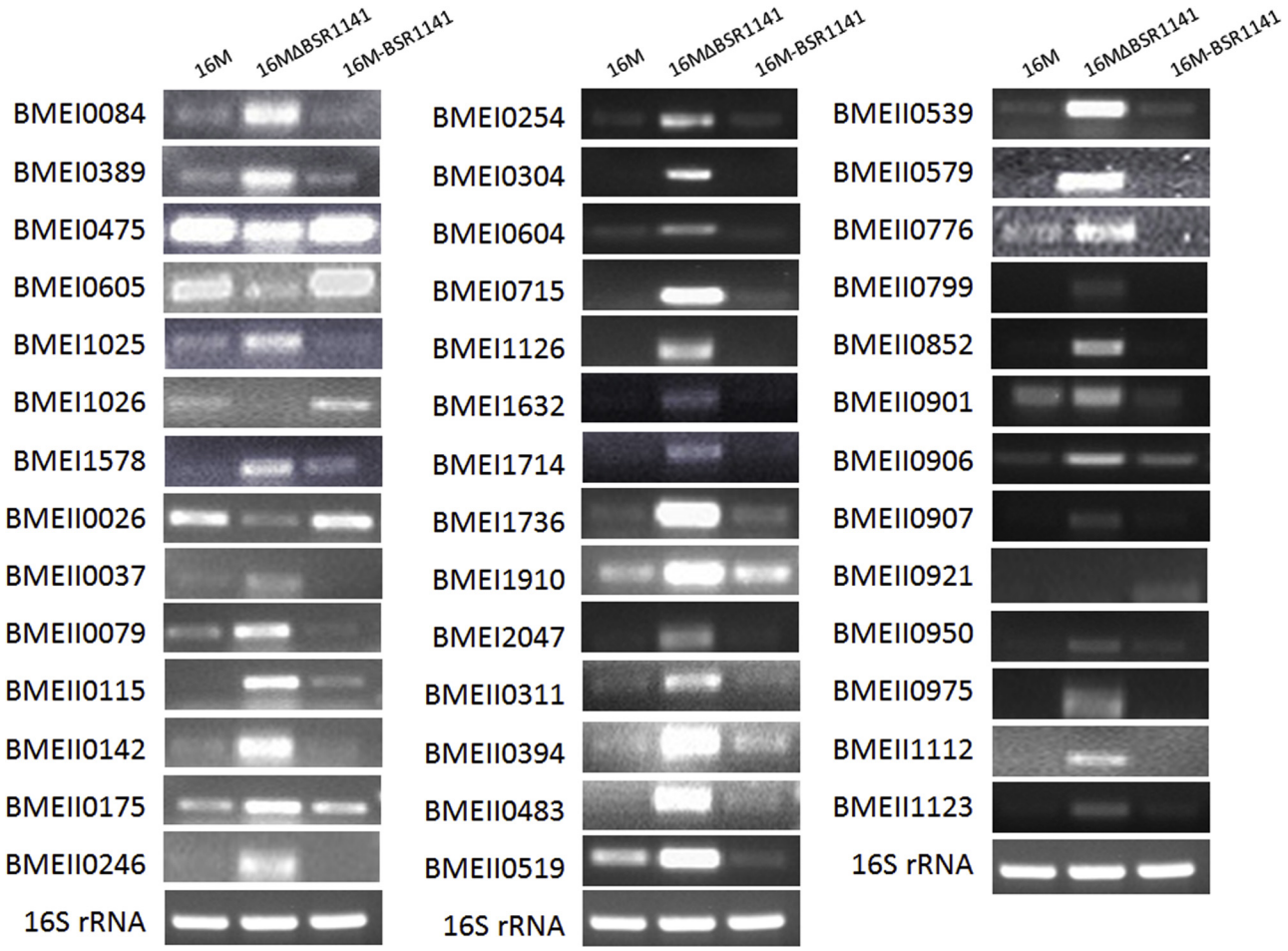

Fig. 3. RT-PCR verification of predicted BSR1141 mRNA transcription targets. RNA was isolated from *B. melitensis* strains 16 M, 16 MΔBSR1141, and 16 M-BSR1141, and the relative transcription of predicted target mRNA genes was quantified and normalized with 16S rRNA.

metabolism (21/43), which further supports the important role of sRNA in bacterial metabolism. To verify these targets, qPCR was used to examine the expression of each target gene in the wildtype 16 M, ΔBSR1141 mutant, and BSR1141 overexpression strains. For most of these candidate target genes, their expression level was increased when BSR1141 was inactivated, indicating that these genes were negatively regulated by BSR1141 (Fig. 3). Of the 43 putative target genes, only four genes (BMEI0475, BMEI0605, BMEI1026, and BMEII0026) were positively regulated by BSR1141. Compared with *B. melitensis* strain 16 M, the transcript levels of these four target genes were lower in the ΔBSR1141 mutant strain and not different in the 16 M-BSR1141 overexpression strain.

3.4. BSR1141 facilitates B. melitensis intracellular survival via target mRNA

As our ultimate aim is to understand how BSR1141 influences *B. melitensis* intracellular survival, we were intrigued by our identification of *virB2* (encoded by BMEII0026) as a BSR1141 target. *virB2* is one of the 12 genes (*virB1–12*) of the VirB type IV secretion (T4SS) of *Brucella*. T4SS is a key virulence factor in *Brucella* that plays important roles in mediating intracellular survival and manipulating the host immune response to infection. Both RT-PCR and northern blot results show that *virB2* was positively regulated by BSR1141 (Figs. 3 and 4A). BMEI0254 (GntR family) and BMEI0604 (TetR family) both belong to transcriptional regulator families whose members are known to be involved in host–bacteria interactions (Haine et al., 2005). To further determine if BSR1141 directly regulates these three target mRNAs, a two-plasmid GFP system (Corcoran et al., 2012) was employed to exam the functional relevance of these predicted RNA duplexes in vivo. Of the three target fusions, *tetR* (BMEI0604) and *gntR* (BMEI0254) were repressed in cells co-expressing BSR1141 (Fig. 4B), suggesting that BSR1141 could repress *tetR* and *gntR* mRNAs at the post-transcriptional level. As shown in Fig. 4B, the amounts of GFP were not significantly different between the strain carrying both the *gfp* fusion and the nonsense sRNA and the "fusion-only" strain, whereas the *virB2-gfp* fusion strain showed increased GFP expression in cells co-expressing BSR1141. These data indicate that BSR1141 directly positively regulates *virB2* expression. Thus, we speculate that the decreased intracellular survival of the 16 MΔBSR1141 mutant strain may be caused by the decreased expression of *virB2* and the increased expression of *tetR* and *gntR*.

As mentioned above, BSR1141 is a trans-encoded sRNA that is Hfq-dependent both in vitro and during in vivo infection. To test whether Hfq plays a role in the interaction between BSR1141 and its mRNA targets, the plasmid pBBR1-BSR1141 was electroporated into *B. melitensis* strains 16 M, the *hfq* mutant strain 16 MΔhfq, and its complementary strain 16 MΔhfq-C, resulting in 16 M-pBBR1141, 16 MΔhfq-pBBR1141, and 16 MΔhfq-pBBR1141-C, respectively. The expressions of three mRNA targets (BMEI0254, BMEI0605, and BMEII0026) were then compared among these strains (Fig. 4C). The *hfq* mutant strain (16 MΔhfq-pBBR1141) had significantly lower *virB2* (BMEII0026) levels and higher expression levels of *tetR* (BMEI0604) and *gntR* (BMEI0254) compared with strain 16 M-pBBR1141; these results are similar to those of the BSR1141 mutant strain (16 MΔBSR1141). Thus, when *hfq* was deleted, the regulation role of BSR1141 disappeared. However, the expression levels of the three target genes in strain

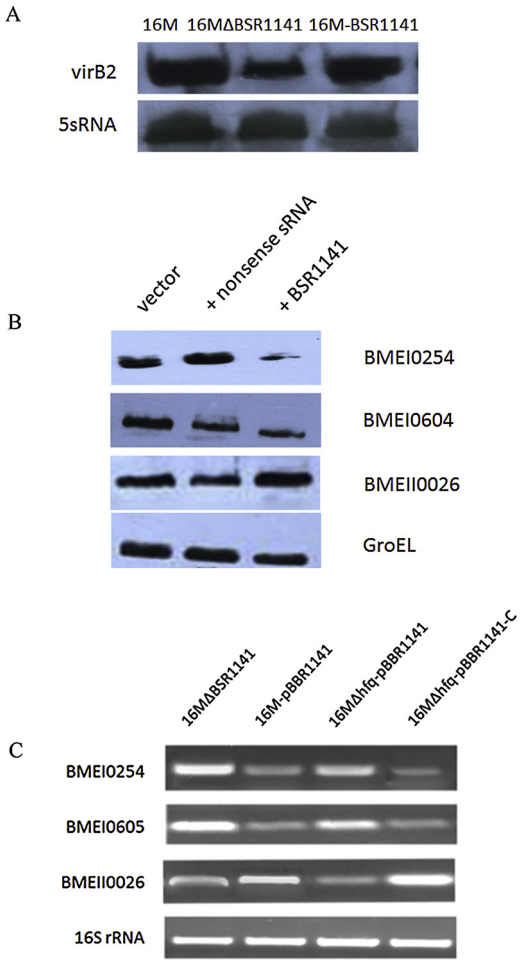

Fig. 4. Assessment of direct regulation by BSR1141 on target mRNAs *virB2*, *gntR*, and *tetR*. (A) Regulation of GFP-target mRNA reporter fusions by BSR1141. *E. coli* strains carrying only a *gfp* fusion plasmid or a combination of both the *gfp* fusion plasmid and a sRNA-encoding plasmid were grown in LB broth, and immunoblot analyses were performed on total protein lysates of these strains to detect the levels of GFP or GroEL. (B) RT-PCR detection of the *virB2* (BMEII0026), *gntR* (BMEI0254), and *tetR* (BMEI0604) expression in *B. melitensis* strains 16 M-pBBR1141, 16 MΔhfq-pBBR1141, and 16 MΔhfq-pBBR1141-C.

16 MΔhfq-pBBR1141-C were close to their levels in strain 16 M-pBBR1141, suggesting that the regulation role of BSR1141 was restored in the *hfq* complementary strain. Together, these results suggest that Hfq is involved in the interaction between BSR1141 and its mRNA targets.

4. Discussion

Successful host infection by *Brucella* largely depends on the ability of the bacteria to adapt to changing environments within the host macrophages, and this requires the coordinated expression of a multitude of stress-related and metabolic genes (Martirosyan et al., 2011; Ahmed et al., 2016). As important post-transcriptional regulatory molecules, sRNAs can enable precise gene regulation and rapid adaptation of cellular physiology in response to environmental changes (Harris et al., 2013). Thus, the identification of sRNAs associated with *B. melitensis* intracellular survival could offer new insights into its virulence. In the present study, we found that BSR11141 could influence the expression of *virB2*, which is important for *B. melitensis* pathogenesis and intracellular survival.

As gene induction is closely related to the function of the encoded protein, we first manipulated the culture conditions of *B. melitensis* and measured the resulting level of BSR1141 sRNA expression. This sRNA was induced upon exposure to H_2O_2, low pH, and nutrient-deprivation (Fig. 1A), suggesting that BSR1141 may play a role during stress conditions. Moreover, the 16 MΔBSR1141 mutant was more sensitive than the wildtype 16 M strain to environmental stresses that mimic those encountered in a host cell (Fig. 2C), indicating that BSR1141 is associated with the adaptation of the bacterium to its host. Therefore, the expression profile of BSR1141 is closely related to the survival capacity of *B. melitensis* under stress. Transcription analysis of BSR1141 in infected mice revealed that BSR1141 accumulates at high levels during *B. melitensis* infection, especially the late stage of infection (Fig. 1B). Consistent with its expression profile, the survival capacity of the 16 MΔBSR1141 mutant was also significantly lower than that of the wildtype 16 M strain in the late stage of mouse infection. The BSR1141 deletion mutation significantly reduced the number of *B. melitensis* bacteria in the spleens from infected mice at 42 days post-infection, but this number was only slightly reduced at 7 days post-infection (Fig. 2D). Together, these findings imply that BSR1141 may function during the chronic stage of infection. Generally, *Brucella* infection in mice can be divided into three steps: 1) bacteria invade mouse tissues and organs within two days of infection; 2) bacteria replicate in different organs of the reticuloendothelial and reproductive systems from 2 days to 3 weeks, which is known as the acute phase of infection; and 3) bacteria persist for 6 months or more, which is known as the chronic phase (Martirosyan et al., 2011; Grillo et al., 2012; Ahmed et al., 2016). *Brucella* may develop multiple strategies to defeat host defense mechanisms and successfully establish chronic infection (Monack et al., 2004). Our results demonstrate that BSR1141 may play a role in *Brucella* pathogenicity and chronic infection.

To fully understand the function of BSR1141 in *Brucella* chronic infection, the regulatory pathway of BSR1141 must be identified. Thus, the target mRNAs of BSR1141 were searched computationally using two different software programs, TargetRNA and RNApredator. Our data show that almost half of the putative target mRNA genes are associated with bacterial transport and metabolism, suggesting that BSR1141 plays a role in metabolism; this may be the cause of the growth deficiency observed in the BSR1141 mutant (Fig. 2B). Interestingly, the results of both qRT-PCR and northern blot assays indicate that the *virB2* gene of *Brucella* T4SS was upregulated by BSR1141. Furthermore, a GFP reporter system analysis confirmed that BSR1141 directly positively regulates *virB2* expression. As an intracellular pathogen, to survive and replicate in host cells, *B. melitensis* must interfere with host function and adapt to the intracellular environment. It has been proven that the T4SS is essential for *Brucella* intracellular survival and replication (O'Callaghan et al., 1999). Thus, *Brucella* may use a variety of strategies to establish and maintain chronic infection, such as the fusion of T4SS-dependent BCV (*Brucella* containing vacuole) with host lysosomes to evade intracellular destruction. VirB mutant strains are unable to persist within host cells and are therefore unable to cause chronic infection in mice (Zhong et al., 2009; Ke et al., 2015). Thus, it seems likely that BSR1141 favors *Brucella* chronic infection via the modulation of VirB activity. Similarly, the sRNA RyhB was also found to regulate *virB* expression at the level of *virB* transcription directly or indirectly in Shigella species, and this regulation is facilitated by specific nucleic acid sequences within *virB* (Prosseda et al., 1998; Murphy and Payne, 2007; Broach et al., 2012).

Hfq is a bacterial Sm-like protein that acts as an important post-transcriptional regulator of gene expression by mediating the interactions between sRNAs and their target mRNA molecules (Aiba, 2007). This protein is required for the intracellular survival and pathogenicity of *B. melitensis* (Robertson and Roop, 1999; Cui et al., 2013). Our results show that BSR1141 is a trans-encoded sRNA that is Hfq-dependent both in vitro and during in vivo infection. Additionally, Hfq was also required for the interaction between BSR1141 and its mRNA targets *virB2*, *tetR*, and *gntR*. The BSR1141-mediated regulation of its mRNA

targets was disappeared when Hfq was inactivated, and it was restored in the complementary strain. Together, these data demonstrate the role of Hfq in BSR1141–*virB2* interactions. In many cases, the sRNA–mRNA interactions facilitated by Hfq inhibit gene expression, usually by occluding the ribosome-binding site (RBS) and/or decreasing the mRNA stability, thus leading to transcript degradation (Arocena et al., 2010; Caswell et al., 2012). However, sRNAs and Hfq can also act together to activate gene expression by alleviating the secondary structure in the mRNA RBS region, thus allowing translation to proceed, or by binding to the mRNA 3′ end, which stabilizes the transcript (Frohlich and Vogel, 2009). Here, our results show that *virB2* was positively regulated by BSR1141 and Hfq in *B. melitensis*. Studies by Caswell and colleagues demonstrated that Hfq has a positive influence on VirB production in the *Brucella abortus* 2308 strain (Caswell et al., 2012). They reported that Hfq coordinates *virB* expression through both BabR-dependent and BabR-independent mechanisms in *B. abortus* 2308. Future work will characterize how Hfq and BSR1141 regulate *virB* expression in *B. melitensis*. In conclusion, we identified an Hfq-dependent sRNA, BSR1141, which facilitates *B. melitensis* intracellular survival and chronic infection by modulating its mRNA targets.

Funding

This study was supported by the National Key Research and Development Program of China (2017YFD0500305, 2017YFD0500901, 2018YFD0500900), National Natural Science Foundation of China (81171530, U1808202) and Beijing Novo Program (Z131102000413062). The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Author contributions

YW, YK, ZZ, XY, and ZC designed the research, assessed and interpreted the results, and prepared the manuscript. YW, YK, CD, XM, QH, LS, XG, TS, WZ, and ZZ executed the experiments, the other authors helped to revising it for important intellectual content. ZZ, ZC, and XY have decided the final approval of the version to be published. All authors have contributed to this review from their complementing areas of expertise. All authors read and approved the final manuscript.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijmm.2019.04.002.

References

Ahmed, W., Zheng, K., Liu, Z.F., 2016. Establishment of chronic infection: brucella's stealth strategy. Front. Cell. Infect. Microbiol. 6, 30.
Aiba, H., 2007. Mechanism of RNA silencing by hfq-binding small RNAs. Curr. Opin. Microbiol. 10, 134–139.
Arocena, G.M., Sieira, R., Comerci, D.J., Ugalde, R.A., 2010. Identification of the quorum-sensing target DNA sequence and N-Acyl homoserine lactone responsiveness of the Brucella abortus virB promoter. J. Bacteriol. 192, 3434–3440.
Atluri, V.L., Xavier, M.N., de Jong, M.F., den Hartigh, A.B., Tsolis, R.M., 2011. Interactions of the human pathogenic Brucella species with their hosts. Annu. Rev. Microbiol. 65, 523–541.
Beckmann, B.M., Grunweller, A., Weber, M.H., Hartmann, R.K., 2010. Northern blot detection of endogenous small RNAs (approximately 14 nt) in bacterial total RNA extracts. Nucleic Acids Res. 38, e147.
Broach, W.H., Egan, N., Wing, H.J., Payne, S.M., Murphy, E.R., 2012. VirF-independent regulation of Shigella virB transcription is mediated by the small RNA RyhB. PLoS One 7, e38592.
Caldelari, I., Chao, Y., Romby, P., Vogel, J., 2013. RNA-mediated regulation in pathogenic bacteria. Cold Spring Harb. Perspect. Med. 3, a010298.
Caswell, C.C., Gaines, J.M., Roop 2nd, R.M., 2012. The RNA chaperone Hfq independently coordinates expression of the VirB type IV secretion system and the LuxR-type regulator BabR in Brucella abortus 2308. J. Bacteriol. 194, 3–14.
Celli, J., Gorvel, J.P., 2004. Organelle robbery: brucella interactions with the endoplasmic reticulum. Curr. Opin. Microbiol. 7, 93–97.
Corcoran, C.P., Podkaminski, D., Papenfort, K., Urban, J.H., Hinton, J.C., Vogel, J., 2012. Superfolder GFP reporters validate diverse new mRNA targets of the classic porin regulator, MicF RNA. Mol. Microbiol. 84, 428–445.
Cui, M., Wang, T., Xu, J., Ke, Y., Du, X., Yuan, X., Wang, Z., Gong, C., Zhuang, Y., Lei, S., 2013. Impact of Hfq on global gene expression and intracellular survival in Brucella melitensis. PLoS One 8, e71933.
de Figueiredo, P., Ficht, T.A., Rice-Ficht, A., Rossetti, C.A., Adams, L.G., 2015. Pathogenesis and immunobiology of brucellosis: review of Brucella-host interactions. Am. J. Pathol. 185, 1505–1517.
Dong, H., Peng, X., Wang, N., Wu, Q., 2014. Identification of novel sRNAs in Brucella abortus 2308. FEMS Microbiol. Lett. 354, 119–125.
Elzer, P.H., Kovach, M.E., Phillips, R.W., Robertson, G.T., Peterson, K.M., Roop 2nd, R.M., 1995. In vivo and in vitro stability of the broad-host-range cloning vector pBBR1MCS in six Brucella species. Plasmid 33, 51–57.
Frohlich, K.S., Vogel, J., 2009. Activation of gene expression by small RNA. Curr. Opin. Microbiol. 12, 674–682.
Grillo, M.J., Blasco, J.M., Gorvel, J.P., Moriyon, I., Moreno, E., 2012. What have we learned from brucellosis in the mouse model? Vet. Res. 43, 29.
Haine, V., Sinon, A., Van Steen, F., Rousseau, S., Dozot, M., Lestrate, P., Lambert, C., Letesson, J.J., De Bolle, X., 2005. Systematic targeted mutagenesis of Brucella melitensis 16M reveals a major role for GntR regulators in the control of virulence. Infect. Immun. 73, 5578–5586.
Harris, J.F., Micheva-Viteva, S., Li, N., Hong-Geller, E., 2013. Small RNA-mediated regulation of host-pathogen interactions. Virulence 4, 785–795.
Ke, Y., Wang, Y., Li, W., Chen, Z., 2015. Type IV secretion system of Brucella spp. And its effectors. Front. Cell. Infect. Microbiol. 5, 72.
Martirosyan, A., Moreno, E., Gorvel, J.P., 2011. An evolutionary strategy for a stealthy intracellular Brucella pathogen. Immunol. Rev. 240, 211–234.
Michaux, C., Verneuil, N., Hartke, A., Giard, J.C., 2014. Physiological roles of small RNA molecules. Microbiology 160, 1007–1019.
Monack, D.M., Mueller, A., Falkow, S., 2004. Persistent bacterial infections: the interface of the pathogen and the host immune system. Nat. Rev. Microbiol. 2, 747–765.
Murphy, E.R., Payne, S.M., 2007. RyhB, an iron-responsive small RNA molecule, regulates Shigella dysenteriae virulence. Infect. Immun. 75, 3470–3477.
O'Callaghan, D., Cazevieille, C., Allardet-Servent, A., Boschiroli, M.L., Bourg, G., Foulongne, V., Frutos, P., Kulakov, Y., Ramuz, M., 1999. A homologue of the Agrobacterium tumefaciens VirB and Bordetella pertussis Ptl type IV secretion systems is essential for intracellular survival of Brucella suis. Mol. Microbiol. 33, 1210–1220.
Papenfort, K., Vogel, J., 2014. Small RNA functions in carbon metabolism and virulence of enteric pathogens. Front. Cell. Infect. Microbiol. 4, 91.
Prosseda, G., Fradiani, P.A., Di Lorenzo, M., Falconi, M., Micheli, G., Casalino, M., Nicoletti, M., Colonna, B., 1998. A role for H-NS in the regulation of the virF gene of Shigella and enteroinvasive Escherichia coli. Res. Microbiol. 149, 15–25.
Robertson, G.T., Roop Jr., R.M., 1999. The Brucella abortus host factor I (HF-I) protein contributes to stress resistance during stationary phase and is a major determinant of virulence in mice. Mol. Microbiol. 34, 690–700.
Saadeh, B., Caswell, C.C., Chao, Y., Berta, P., Wattam, A.R., Roop 2nd, R.M., O'Callaghan, D., 2015. Transcriptome-wide identification of hfq-associated RNAs in Brucella suis by deep sequencing. J. Bacteriol. 198, 427–435.
Storz, G., 2002. An expanding universe of noncoding RNAs. Science 296, 1260–1263.
Tjaden, B., Goodwin, S.S., Opdyke, J.A., Guillier, M., Fu, D.X., Gottesman, S., Storz, G., 2006. Target prediction for small, noncoding RNAs in bacteria. Nucleic Acids Res. 34, 2791–2802.
Urban, J.H., Vogel, J., 2007. Translational control and target recognition by Escherichia coli small RNAs in vivo. Nucleic Acids Res. 35, 1018–1037.
Wang, Y., Bai, Y., Qu, Q., Xu, J., Chen, Y., Zhong, Z., Qiu, Y., Wang, T., Du, X., Wang, Z., 2011. The 16MDeltavjbR as an ideal live attenuated vaccine candidate for differentiation between Brucella vaccination and infection. Vet. Microbiol. 151, 354–362.
Wang, Y., Ke, Y., Xu, J., Wang, L., Wang, T., Liang, H., Zhang, W., Gong, C., Yuan, J., Zhuang, Y., An, C., Lei, S., Du, X., Wang, Z., Li, W., Yuan, X., Huang, L., Yang, X., Chen, Z., 2015. Identification of a novel small non-coding RNA modulating the intracellular survival of Brucella melitensis. Front. Microbiol. 6, 164.
Waters, L.S., Storz, G., 2009. Regulatory RNAs in bacteria. Cell 136, 615–628.
Zhong, Z., Wang, Y., Qiao, F., Wang, Z., Du, X., Xu, J., Zhao, J., Qu, Q., Dong, S., Sun, Y., Huang, L., Huang, K., Chen, Z., 2009. Cytotoxicity of Brucella smooth strains for macrophages is mediated by increased secretion of the type IV secretion system. Microbiology 155, 3392–3402.
Zhong, Z., Xu, X., Li, X., Liu, S., Lei, S., Yang, M., Yu, J., Yuan, J., Ke, Y., Du, X., Wang, Z., Ren, Z., Peng, G., Wang, Y., Chen, Z., 2016. Large-scale identification of small non-coding RNA with strand-specific deep sequencing and characterization of a novel virulence-related sRNA in Brucella melitensis. Sci. Rep. 6, 25123.