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A novel small RNA Bmsr1 enhances virulence in *Brucella melitensis* M28

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ABSTRACT

Brucellosis, caused by *Brucella* spp., is one of the most serious zoonotic bacterial diseases. Small RNAs (sRNAs) are recognized as a key player in bacterial post-transcription regulation, since they participate in many biological processes with high efficiency and may govern the intracellular biochemistry and virulence of some pathogenic bacteria. Here, a novel small regulatory RNA, Bmsr1 (*Brucella melitensis* M28 small RNA 1), was identified in a virulent *Brucella melitensis* M28 strain based on bioinformatic analysis, reverse transcription PCR (RT-PCR), and Northern blot. The Bmsr1 expression level was highly induced after infection of macrophage cells RAW264.7 at 48 h, suggesting a role for Bmsr1 during *in vitro* infection. Indeed, *bmsr1* deletion mutant of M28 attenuated its intracellular survival in RAW264.7 at 24 h and 48 h post-infection. In a mouse model of chronic infection, *bmsr1* deletion strain displayed decreased colonization in the spleen while Bmsr1-overexpressed strain showed higher colonization levels than wild type pathogen. Isobaric tags for relative and absolute quantification (iTRAQ) revealed that 314 proteins were differentially expressed in M28Δ*bmsr1* compared with wild type. Functional annotation analysis demonstrated that most of those proteins are involved in biological processes and those proteins in the ribosome and nitrogen metabolism pathways were enriched. iTRAQ results combined with target prediction identified several potential target genes related to virulence, including *virB2*, *virB9*, *virB10*, *virB11*, and *vjbR* and many metabolism genes. Taken together, this study revealed the contribution of a novel sRNA Bmsr1 to virulence of *B. melitensis* M28, probably by influencing genes involved in T4SS, virulence regulator *VjbR* and other metabolism genes.

1. Introduction

Brucellosis, caused by *Brucella* spp., is one of the most serious zoonotic bacterial diseases. This chronic disease mainly affects the reproductive tract, and can lead to abortion and infertility in natural hosts, therefore incurring substantial economic losses (D'Anastasio et al., 2011). At a conservative estimate, more than 300 million cattle are infected with this pathogen and every year more than half a million incident human infections occur (De et al., 2015). The alphaproteobacterium *Brucella* spp. are small Gram-negative coccobacilli, which are facultatively anaerobic, and intracellular (D'Anastasio et al., 2011). These gram-negative bacteria infect a variety of mammals. Historically, according to host specificity, they are classified into 6 species. These include *B. melitensis* (goat and sheep), *B. abortus* (cattle), *B. suis* (pigs), *B. ovis* (sheep), *B. canis* (dogs) and *B. neotomae* (desert mice). Recently, new species were isolated from humans, aquatic mammals, and voles (Audic et al., 2009). Among these species, *B. melitensis* is one of the most frequent causes of human brucellosis (Corbel, 1997; Young, 1995).

Brucella virulence relies on its ability to evade or modulate the host immune response and these organisms can infect and persist inside mammalian host cells. Macrophages, placental trophoblasts, and dendritic cells are the main cells in which the organism survives and replicate (Martirosyan et al., 2011). After internalization, several key factors are related to the intracellular life of *Brucella* such as the type IV secretion system (T4SS), two-component regulatory system, the lipopolysaccharide (LPS) and flagellum-like structures (Ii et al., 2009; Ke et al., 2015; Weeks et al., 2010). Those factors can facilitate *Brucella* to invade and adapt to host cells.

Small non-coding RNAs in bacteria, known as small RNAs (sRNAs),

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have attracted considerable attention due to their abundance and significant regulatory roles in many cellular processes, such as stress response, metabolism, and virulence (Hoe et al., 2013; Jørgensen et al., 2013; Oliva et al., 2015). Recently, two bioinformatics analysis programs SIPHT and NAPP were used to predict novel sRNAs in *B. abortus* 2308 and 112 novel sRNAs were identified. In addition, strategies of immunoprecipitation with Hfq and RNA-seq deep sequencing were also successfully applied to identify novel sRNAs in *Brucella suis* (Saadeh et al., 2015) and *melitensis* (Zhong et al., 2016). Several sRNA have been characterized and confirmed to be involved in the virulence of *Brucella* (Caswell et al., 2012). In *B. abortus,* abcR1 and abcR2 were shown to be important for chronic infection and a double deletion (abcR1/2) resulted in significant attenuation of virulence (Caswell et al., 2012). Further study indicates that the M2 motif of AbcR sRNA and its target BAB2_0612 are critical for the virulence of B. *abortus* (Sheehan and Caswell, 2017). Although several putative sRNAs have been predicted, few have been experimentally verified and characterized for their biological functions in *Brucella* spp. Here we identified a novel sRNA Bmsr1 in virulent *B. melitensis* M28, and demonstrated that the Bmsr1 contributed to virulence of *B. melitensis* M28, probably by influencing genes involved in T4SS, virulence regulator *VjbR* and other metabolism genes.

2. Materials and methods

2.1. Bacterial growth and cell culture

B. melitensis M28 is a hypervirulent strain. All studies involving live *B. melitensis* M28 and its derivatives were performed under biosafety level 3 (BSL3) conditions. Smooth *B. melitensis* M28 and its derivatives were grown in tryptic soy broth (TSB) or on TSA (TSB with agar) plates containing 1% agar at 37 °C. *E. coli* DH5α strains were grown in lysogeny broth (LB). When needed, appropriate antibiotics were added to media at the following concentrations: kanamycin, 50 μg/mL; ampicillin, 100 μg/mL. RAW264.7 (obtained from the ATCC) murine macrophage cells were cultured in Dulbecco's Minimal Essential Medium (DMEM) basic (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) without antibiotics, and incubated at 37 °C in 5% CO_2.

2.2. RNA isolation

For RT-PCR and Northern blot analyzes, total RNA was extracted as follows. *B. melitensis* M28 was cultured at 37 °C in TSB medium to mid-exponential phase or appropriate time (OD_{600} = 0.5, 1.2, 2.0). Cells were stabilized with the RNA Protect Bacteria Reagent (Qiagen, GER), and then treated with lysozyme and RNase inhibitor RNasin for 15 min. Total RNA was extracted using TRIzol RNA Isolation Reagent (Invitrogen, USA) according to the instructions. Genomic DNA was removed by using RQ1 RNase-free DNase (Promega, USA) at 37 °C for 1 h. Treated RNA was purified through phenol/chloroform extraction and ethanol precipitation, and stored at −80 °C until use. RNA quality was examined by formaldehyde gel electrophoresis and RNA concentrations were determined using a nanodrop spectrophotometer (Thermo Scientific, USA).

After cell infection at 8 h, 24 h, and 48 h, cells were washed three times with PBS (DEPC-treated). TRIzol reagent (800 μL/well) was used to collect cells and bacteria. When precipitating RNA, 5 μg of RNase-free glycogen was added and incubated at −20 °C. After 4 h incubation, the RNA pellet was collected and washed. The total prokaryotic and eukaryotic RNA isolated from infected RAW264.7 cells was obtained and used for real-time qPCR analyzes. The RNA was treated according to the instructions of the PrimeScript™ RT reagent kit with gDNA Eraser (Perfect Real Time) (Takara, JPN).

2.3. Bioinformatic prediction of sRNAs and targets

To extract candidate sRNAs, intergenic regions were selected and regions of length less than 100 bp or exceeding 300 bp were discarded. Promoters and Rho-independent terminators were predicted using bioinformatics software Softberry (BProm and TermFind) and ARnold (http://rna.igmors.u-psud.fr/toolbox/arnold/) with default settings.

The TargetRNA2 program (version 2.01) was used to predict sRNA target genes in order to indicate their potential function (Kery et al., 2014). Parameter settings were as below: "NTs before start codon: 80; NTs after start codon: 20 and seed length: 7. "*B. melitensis* M28 chromosome I (CP002459.1) and chromosome II (CP002460.1) were selected for target prediction. This program is available at http://cs.wellesley.edu/~btjaden/TargetRNA2/.

2.4. Expression analysis by RT-PCR and real-time qPCR

RT-PCR was performed to confirm the expression of candidate sRNAs. RNA was prepared as previously mentioned. No more than 5 μg of total RNA was reverse transcribed to cDNA with EasyScript® First-Strand cDNA Synthesis SuperMix (TransGen, CHN). All primers are listed in Table S1.

The relative expression levels of candidate sRNA during infection of macrophages were examined by real-time qPCR in a MX3005P qPCR system (Agilent). Real-time qPCR was performed with SYBR Premix Ex Taq™ (Tli RNaseH Plus) (Takara, JPN). Each 20 μL PCR mix contained 10 μL SYBR Premix Ex Taq™, 0.4 μL of each primer (10 μM), 2 μL diluted cDNA template and 6.4 μL RNase-free H_2O. Thermal cycles used a program at 95 °C for 30 s, followed by 40 cycles of amplification at 95 °C for 5 s, and 60 °C for 30 s. The fold change of transcript levels was calculated using the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001), and normalized according to 16S rRNA expression levels.

2.5. Northern blot analysis

A DIG-labelled RNA probe was generated by T7 RNA polymerase-mediated transcription according to the DIG Northern Start Kit (Roche, Switzerland) (Table S1). Northern blot analysis was performed by using the Northernmax kit (Ambion, USA) according to the manufacturer's protocol. Briefly, 1–15 μg of total RNA was denatured for 15 min at 65 °C, followed by separation on a formaldehyde denaturing 1% agarose gel. RNA samples were then transferred to a BrightStar®-Plus Membrane (Ambion) and cross-linked by UV. After a pre-hybridization step for 45 min at 68 °C, DIG-labelled RNA probes were added to pre-hybridized membranes and incubated. For maximum sensitivity, an overnight hybridization step was performed at 68 °C. Membranes were then washed and samples were detected by a DIG Northern Start Kit (Roche).

2.6. 5'-rapid amplification of cDNA ends (5' RACE)

For generation of full-length sRNA, rapid amplification of complementary DNA ends (RACE) were performed using a SMARTer® RACE kit (Clontech, JPN) as recommended by the manufacturer's instructions. The gel-purified 5' RACE products were cloned into linearized pRACE vector (a pUC19-based vector provided with the SMARTer® RACE kit) and then the largest gene-specific inserts were obtained and sequenced.

2.7. Construction of deletion and complementation strains

Deletion of genes encoding sRNAs was achieved by homologous recombination (Wang et al., 2011b). Briefly, 500 bp upstream and downstream regions of the target genes were PCR-amplified from *B. melitensis* M28, respectively, and cloned to flank a kanamycin resistance gene cassette on plasmid vector pSP72 (Amp^R). This was performed