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Identification of OtpR regulated sRNAs in *Brucella melitensis* expressed under acidic stress and their roles in pathogenesis and metabolism

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ABSTRACT

Small RNAs (sRNAs) are the small regulatory molecules that regulate various biological processes in bacteria. Though the functions of sRNAs are well documented, very little information is available on the sRNAs of *Brucella* spp. The *otpR* is the response regulator of a two-component regulatory system, which plays a significant role in *Brucella* virulence. In this study, we identified the sRNAs expressed in *B. melitensis* 16M and its *otpR* mutant under acidic stress from the RNAseq dataset. We identified 94 *trans*-encoded and 948 *cis*-encoded sRNAs in *B. melitensis* 16M. In *B. melitensis* 16M Δ*otpR* under acidic stress 99 *trans*-encoded and 877 *cis*-encoded sRNAs were identified. Among these, 12 *trans*-encoded and 43 *cis*-encoded sRNAs were upregulated in *B. melitensis* 16M Δ*otpR*, with an adjusted P-value ≤ 0.05. The mRNA targets of these sRNAs were predicted. Further, the levels of mRNA targets were examined, and the sRNA-mediated regulatory network was predicted. Functional classification and pathway analysis of mRNA targets provided evidence that sRNAs are involved in different metabolic pathways including carbohydrates, amino acids, lipids, nucleotides transport and metabolism, cell membrane biogenesis and intracellular trafficking of *Brucella*. We also found that eight transcriptional regulators including a quorum sensing regulator, *vjbR* are down-regulated by sRNAs. These transcriptional regulators might be responsible for the regulation of several other genes in the *otpR* mutant. The *trans*-encoded BsnR88 and *cis*-encoded BsnR980, BsnR998, BsnR881, BsnR1001, BsnR891, BsnR883, BsnR905 are regulating *virB* operon genes coding for type IV secretion system (T4SS), which is the major virulence factor of *Brucella*.

1. Introduction

Brucella is a Gram-negative, facultative intracellular pathogen that causes the zoonotic disease, brucellosis. Brucellosis is responsible for high economic losses with an annual incidence of approximately 500,000 cases of human brucellosis worldwide [1]. *Brucella* infects a wide array of aquatic and terrestrial mammals including humans, cattle, goat, sheep, swine, dogs, dolphins, whales, seals, and desert wood rats. Humans are incidental hosts of *Brucella.* Most of the human cases occur due to the direct contact with the infected animals, and its secretions or consumption of unpasteurized milk and other animal products [2]. Brucellosis is characterised by abortion and infertility in animals and undulant fever in humans. Chronic brucellosis can cause arthritis, encephalomyelitis, and endocarditis [3]. Currently, the genus *Brucella* is classified into ten species based on host preferences, antigenic and biochemical characteristics. Of these, the dominant species causing human brucellosis are *B. melitensis, B. abortus,* and *B. suis* [4]. Comparative genomic analysis of *Brucella* genomes has revealed that *Brucella* species are highly similar at the nucleotide level, and the organism evolved clonally [5,6]. *Brucella* genome is approximately 3.2–3.3 Mb in size, which is divided into two chromosomes with a larger chromosome of ~2.1 Mb and a smaller chromosome of ~1.2 Mb size. *Brucella* lacks classical bacterial virulence factors such as exotoxins, capsules, plasmids, fimbriae, etc. *Brucella* virulence is based on its ability to enter, survive and replicate within the phagocytic and non-phagocytic cells. *Brucella* evades the host immune system and survives intracellularly. Type IV secretion system (T4SS), LPS and BvrR/BvrS two component system have been shown to have significant roles in intracellular survival and replication [7].

Small RNAs (sRNAs) are ubiquitous in all life forms. Bacterial sRNAs are typically short transcripts of ~50–500 nucleotides in length, which are analogous to the eukaryotic miRNAs. The sRNAs

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regulate (positively/negatively) diverse biological process such as quorum sensing, virulence, stress response, secretion, outer membrane and iron homeostasis, host – cell contact, etc. The sRNAs modulate gene expression by base pairing with the mRNA targets. The majority of the sRNAs downregulate the gene expression by an imperfect base pairing at the 5′-UTR region of the mRNAs and thereby inhibiting the ribosomal binding or by inducing mRNA degradation [8]. Very few sRNAs have been shown to regulate the gene expression positively by exposing the ribosome binding site or stabilizing the mRNA [9] The sRNAs can be either *trans*-encoded or *cis*-coded. The *trans*-coded sRNAs are located in the intergenic regions and transcribed from their promoters, which often are induced by specific stress conditions or environmental and morphological changes. The mRNA targets of these sRNAs may not be located in the proximity of the sRNA gene, and their hybridization occurs through short imperfect base pairing [10]. The *cis*-encoded or the antisense sRNAs (asRNAs) are expressed from the complementary strand of the protein-coding genes. These sRNAs have an impact on gene expression and/or mRNA stability of the fully complementary sense gene [11]. The roles of several sRNAs have been elucidated in different bacterial pathogens [12–16]. However, very few reports are available on the sRNAs of *Brucella* and their roles in pathogenicity [17,18]. Recently, Liu et al. [19] have reported the role of *otpR* and genes regulated by *otpR* in *B. melitensis* through transcriptome analysis. OtpR is a two-component response regulator (BMEI0066), whose function is not completely studied. But the recent studies identified the role of *otpR* in withstanding various sensitive conditions bacteria come across during the infection. In this study, we have identified sRNAs expressed in the *B. melitensis* 16M and its *ΔotpR* mutant. Further, we have identified the *OtpR* regulated *cis*- and *trans*-encoded sRNAs in *B. melitensis* expressed under acidic stress. Also, the mRNA targets of these sRNAs and the possible network of sRNA-mRNA interactions were predicted.

2. Methodology

The RNA-seq dataset was obtained from the NCBI Gene Expression Omnibus (GEO) (Accession No: GSE48165) [19]. The raw reads of *B. melitensis* 16M and its *ΔotpR* mutant were downloaded from the sequence read archive (SRA) database (Accession No: SRP026210).

2.1. Overview of the dataset

Bacterial growth conditions, RNA extraction, and the details of sequencing are described by Liu et al. [19]. Briefly, *B. melitensis* 16M and its *ΔotpR* mutant were grown in 100 ml Tryptic Soy Broth (TSB) at 37 °C until early-log phase. Subsequently, acid stress was given, RNA was extracted, and the library was prepared for sequencing. The RNA-seq was done on the Illumina HiSeq 2000 platform. The fastq format files containing the raw reads were downloaded from the SRA database (SRA accession: SRP026210).

2.2. Prediction of sRNAs

The raw reads obtained from the above mentioned RNA-seq dataset were processed and analysed using the Rockhopper [20,21]. First, the reads were mapped to the two chromosomes of *B. melitensis* 16M individually. After the mapping, the reads were normalised by upper quartile normalization. Subsequently, the transcripts were assembled, and the transcript boundaries were identified. The relative levels of transcripts were quantified based on the "reads per kilobase per million mapped reads" (RPKM) measure. Initially, we have identified the sRNAs expressed in the *B. melitensis* 16M wild type under acidic stress. Further, we have identified the sRNAs differentially expressed in *B. melitensis* 16M Δ*otpR*.

2.3. Target prediction

The mRNA targets of the *trans*-encoded sRNAs were predicted using TargetRNA2 (http://cs.wellesley.edu/~btjaden/TargetRNA2) with default parameters (P-value: 0.05; NTs downstream of the mRNA translation start site: 20; NTs upstream of the mRNA translation start site: 80; NTs in interaction region: 20) [22]. The targets of the sRNAs expressed in *B. melitensis* 16M and its Δ*otpR* mutant were identified. Among the predicted targets of each differentially expressed sRNAs, those mRNAs showed significant differential expression or regulated under the same condition were considered as the targets of the sRNA and considered for downstream analysis. The sRNA-mRNA interactions were predicted using IntaRNA [23].

2.4. Functional enrichment analysis

Functional categorization of the predicted target mRNAs was done by clusters of orthologous group (COG) analysis, and gene ontology (GO) annotations using the COG database [24] and the comparative GO [25], respectively. Pathway annotations were done using KEGG database with default parameters. The network was visualised using Cytoscape [26].

3. Results

3.1. Identification of sRNAs in B. melitensis 16M

The raw RNASeq reads of *B. melitensis* 16M and *B. melitensis* 16M *ΔotpR* under acidic stress were filtered for the poor quality and ambiguous sequences using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). High quality reads were mapped to the two chromosomes of *B. melitensis* 16M (NC_003317 and NC_003318) using Rockhopper. The alignment statistics are given in Table 1.

After aligning the reads to the genome, transcripts from the intergenic regions and the antisense regions from the complementary strand of the protein coding genes were identified. The transcripts having a length of >500 nucleotides and <40 nucleotides were removed. We identified 94 *trans*-encoded sRNAs and 948 *cis*-encoded sRNAs expressed in the *B. melitensis* 16M under acidic stress. These sRNAs were named as BsnR (*Brucella* small non-coding RNA) followed by a number. Identified sRNAs were searched against Rfam database to compare with existing orthologs if any. Five *trans*-encoded sRNAs showed homology with existing sRNAs in the Rfam database. BsnR28 and BsnR29 showed homology with Atu_c9 (Rfam ID: RF02503) and TPP (Rfam ID: RF00059), respectively. TPP is a riboswitch, which is also known as the

Table 1
Summary of reads alignment to the *B. melitensis* 16M genome.

Attributes	*B. melitensis* 16M wild type	*B. melitensis* 16M ΔotpR
Total reads	45,04,212	45,15,172
Reads aligned (antisense) to protein-coding genes in chromosome I	41%	39%
Reads aligned (antisense) to protein-coding genes in chromosome II	41%	41%
Reads aligned to the intergenic regions in chromosome I	20%	20%
Reads aligned to the intergenic regions in chromosome II	14%	18%