

RESEARCH ARTICLE

A sRNA Subset Present in *Brucella melitensis* 16M OMVs Regulates Intracellular Survival of *Brucella melitensis*

Pan CHEN¹ , Shengnan SONG¹ , Jing ZHANG¹ , Wendong FU² , Ce WANG³ , Yu YANG¹ , Zhixiang SHEN¹ , Xiaochun LIAN³ , Jia GUO^{1,4(*)} , Hui ZHANG^{1(*)} , Yu ZHANG^{1(*)} 

¹ Shihezi University, Faculty of Animal Science and Technology, Veterinary Medicine Department, 832000, CHINA

² Changji Hui Autonomous Prefecture Animal Disease Prevention and Control Center, 831100, CHINA

³ Animal Husbandry and Veterinary General Station of Xinjiang Production and Construction Corps, 830063, CHINA

⁴ Aheqi Animal Husbandry and Agriculture Development Co., Ltd, 843500, CHINA



(*) Corresponding author:

Yu Zhang

Phone: +86 18728773352

Cellular phone: +86 18728773352

E-mail: 362475905@qq.com

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Abstract

Intercellular communication mediated by extracellular vesicles (EVs) is a key component of host-pathogen interactions. Emerging evidence suggests that Outer Membrane Vesicles (OMVs) enter host cells via multiple pathways and release cargo, including proteins and small RNAs (sRNAs), that regulate gene expression and function in recipient cells. In this study, we used *Brucella melitensis* 16M and its OMVs to construct an OMV-specific sRNA dataset and investigate their unique role in *B. melitensis* 16M intracellular survival. A total of 1,783,364 unique sRNA sequences were identified, and the ten most abundant sequences were determined by sequencing sRNAs extracted and purified from *B. melitensis* 16M and its OMVs. Bioinformatic analysis showed that eight sRNAs within OMVs exhibited stable secondary structures, and six were predicted to interact with mouse mRNAs. Notably, sRNA t0000835 was predicted to target the G protein-coupled receptor 45 (GPR45) gene and suppress its expression by binding to Argonaute 2 (AGO2). Transfection of sRNA t0000835 markedly enhanced intracellular survival of *B. melitensis* 16M. Collectively, these findings support the hypothesis that sRNAs t0000835 of *B. melitensis* 16M OMVs modulates host gene expression to promote the intracellular viability of *B. melitensis* 16M.

Keywords: *B. melitensis* 16M, OMVs, Regulatory mechanisms, sRNA

INTRODUCTION

Brucella is a Gram-negative, facultative intracellular bacterium with a short-rod-shaped morphology^[1]. It belongs to the α -Proteobacteria class^[2] and comprises multiple species that infect a wide range of mammalian hosts^[3,4]. Traditionally, the genus has been divided into six distinct species based on host preference: *B. suis*, *B. melitensis*, *B. abortus*, *B. ovis*, *B. neotomae*, and *B. canis*^[5]. More recently, additional species from humans (*B. inopinata*), aquatic mammals (*B. pinnipedialis* and *B. ceti*), and a common vole (*B. microti*) have been identified, bringing the total number to ten recognized species in the genus^[6].

In animal, *Brucella* infection typically causes abortion, infertility, and lameness. As a facultative intracellular pathogen, it survives and replicates within macrophages^[7,8]. This capacity depends on the specialized secretion system of the bacterium, particularly the Type IV Secretion System (T4SS)^[9], which translocates a diverse repertoire

of effector proteins into host cells. These effector proteins alter host signaling pathways, cytoskeletal organization, and immune response mechanisms^[10], thereby creating an intracellular microenvironment that facilitates *Brucella*'s survival and replication.

Extracellular vesicles (EVs) have attracted significant interest in recent years as important mediators of secretory functions and intercellular communication. Specifically, they function in intraspecies cellular information transduction and also act as a crucial nexus in host-pathogen interactions^[11, 12], which are fundamentally shaped by their distinctive biological and structural properties. EVs are nanoscale, membrane-bound particles with a spherical morphology, typically 10-1000 nm in diameter^[13]. These vesicles are delimited by a lipid bilayer and encapsulate a diverse array of cellular constituents, including proteins, lipids, nucleic acids, and other biomolecules^[11,14]. The lipid bilayer protects intravesicular RNA and proteins from extracellular



degradation, thereby significantly enhancing cargo stability. EVs can enter target cells through multiple pathways and molecular components, including proteins, small RNAs (sRNAs), tRNA fragments, and microRNAs (miRNAs), that regulate host gene expression and cellular functions [14].

OMVs (Outer Membrane Vesicles) are spherical structures (approximately 10 to 300 nm in diameter) derived from the outer membrane of Gram-negative bacteria [15,16]. They encapsulate periplasmic and cytoplasmic components, such as sRNAs, tRNA fragments, DNA, lipids, and proteins [14]. OMVs mediate the delivery of sRNAs and tRNA fragments, typically 18-50 nucleotides in length, into mammalian host cells [12]. These genetic elements, derived from transcriptional processes, contain complementary binding sites for specific host miRNAs. They regulate gene expression through two principal mechanisms: 1) base-pairing with either the translation initiation region or coding sequences of target mRNAs and 2) functioning as competitive endogenous RNAs that sequester host miRNAs, thereby acting as sRNA sponges [17-19]. tRNAs (70-100 nucleotides) adopt evolutionarily conserved sequences that assemble into distinctive L-shaped tertiary structures with characteristic cloverleaf secondary configurations [20]. Although systematic investigations remain limited, accumulating evidence indicates that tRNA-derived fragments exert bidirectional regulatory effects on protein translation [21], including mediating gene silencing in eukaryotic cells through an Argonaute 2 (AGO2)-dependent, miRNA-like mechanisms [22]. However, few studies have described the role of OMVs in the delivery of sRNA, tRNA fragments, and miRNAs to target cells or have elucidated the mechanisms by which they mediate intercellular signaling [23].

Current evidence supports the working hypothesis that *Brucella* employs OMV biogenesis as a T4SS-independent non-canonical secretory mechanism for intracellular adaptation. This vesicle-mediated pathway potentially constitutes an evolutionary innovation that enables bacterial persistence under periplasmic stress, particularly during nutrient deprivation, phagosomal acidification, and exposure to host-derived reactive oxygen/nitrogen species (RONS). These microenvironmental pressures are key modulators governing *Brucella*'s parasitic strategy within host cells.

The experimental foundation for this hypothesis was derived from established protocols for the isolation/purification of OMVs from *B. melitensis* 16M strains, coupled with preliminary characterization of their stress-responsive release dynamics and functional proteomic profiles. To mechanistically validate this paradigm, this study employed a dual approach. First, we systematically constructed a comprehensive OMV-associated sRNA dataset using high-throughput sequencing to characterize

vesicular RNA cargo. Second, we evaluated the functional roles of selected sRNA effectors in modulating intracellular survival. This integrated strategy aimed to decode the novel regulatory mechanisms by which *B. melitensis* 16M employs vesicles to mediate host-pathogen co-adaptation.

MATERIAL AND METHODS

Ethical Statement

This study did not involve any procedures requiring ethical approval.

Isolation and Purification of OMVs from *B. melitensis* 16M

Approximately 1×10^6 *Brucella* cells were inoculated onto *Brucella* agar (BD Difco) and statically incubated at 37°C with 5% CO₂ (vol/vol) for 4 days. Single-colony strains were transferred to *Brucella* broth (BD Difco) and cultured at 37°C with shaking (180 × g) until the logarithmic growth phase (OD₆₀₀ nm = 0.4). OMVs were purified as described previously by Gamazo et al. [24]. Briefly, bacterial cells were harvested using a rubber policeman and suspended in 250 mL sterile phosphate-buffered saline (PBS; 1M, pH7.3). The suspension was centrifuged at 10 000 × g for 30 min, and the supernatant was filtered through a 0.22 µm membrane (Millipore Corp.). Sterility test was performed by culturing an aliquot onto a Tryptic Soy Agar (TSA) plate followed by incubation for 72 h at 37°C; the filtrate was stored at 4°C during the viability check. The filtered supernatant was ultracentrifuged at 100.000 × g for 2 h at 4°C. The resulting pellet was washed twice with 25 mL sterile PBS, and the OMVs were resuspended in 1 mL sterile PBS. Total protein concentration was determined using the PIERCE-BCA (PIERCE) reagent, according to the manufacturer's recommendations. The OMVs samples were divided into 0.5 mL aliquots and stored at -20°C until use. All procedures involving live *Brucella* were performed in Biosafety Level 3 (BSL3) facility.

RNA Isolation and Purification from *B. melitensis* 16M

Approximately 5×10^9 CFU of *Brucella* were resuspended in 1 mL of TRIzol (Invitrogen) and RNA purification was conducted using the MiniBEST Universal RNA Extraction kit (Takara, Beijing, China). RNA quantification and purity were determined using an OPTIZEN NanoQ microvolume spectrophotometer (KLAB CO, LTD). Samples consistently showed A260/280 ratios higher than 2.0, and A260/230 ratios higher than 1.8.

RNA Isolation from *Brucella melitensis* 16M OMVs

Enrichment, purification and identification of OMV RNA was performed using a modified lysis and organic extraction protocol. Briefly, 200 µL of OMV suspension was mixed with an equal volume of 7% sodium dodecyl sulfate (SDS) lysis buffer and 10 µL protein digestion solution, vortexed

for 10 s, and incubated at 65°C for 10 min. The sample was transferred to the adsorbent column, mixed with 0.5 mL anhydrous ethanol and centrifuged at $12\,000 \times g$ for 10 min at 4°C. The supernatant was collected and transferred to an 1.5 mL RNase-free centrifuge tube, added chloroform and vortexed for 10 s, and incubated at room temperature for 8 min. After centrifugation ($12\,000 \times g$, 4°C for 10 min), the supernatant was collected and mixed with pre-cooled isopropanol to precipitate RNA. The pellet was washed with 1 mL pre-cooled 75% ethanol, air-dried at room temperature for 5 min and resuspended in 30 µL RNase-free water. The extracted RNA was stored at -80°C for subsequent analysis.

B. melitensis 16M and sRNA Sequencing of Its OMVs

RNA samples isolated and purified from *B. melitensis* 16M and OMVs, were treated with DNase and quality was assessed using a bioanalyzer. For each sample, 1 µg of DNase-treated total RNA was used to prepare cDNA libraries using the TruSeq Small RNA Library Preparation Kit (Illumina). Libraries were sequenced as 50 bp single-end reads using an Illumina Genome Analyzer. Reads were trimmed and aligned to the reference genome (NC_003317.1, NC_003318.1) using the CLC Genomics Workbench v24.0.2. The sRNA-Seq analysis was performed with the following modifications to standard parameters. First, an additional 50 bases upstream and downstream of annotated genes were included to capture sRNAs aligned to intergenic regions. To minimize nonspecific alignment of yeast sequences in the culture medium, the maximum number of mismatches was set to zero, and read mapping was limited to four locations. Uniquely mapped reads were compiled, and sequence frequencies were generated using the CLC small RNA analysis tool for normalization, statistical calculations, and visualization in the R version 4.2.1 (R Core Team, Vienna, Austria; <https://www.r-project.org/>) [25]. Quality control included trimming low-quality bases from read ends, removing adapter sequences, discarding reads shorter than 18 bp, and retaining sequences between 18–30 bp in length based on known sRNA characteristics.

Bioinformatic Prediction of sRNA Target

Secondary structures of the ten most abundant OMV-associated sRNAs were predicted using the RNAfold web server [26]. Complementary sequences of the ten most abundant OMVs sRNAs were aligned with the NCBI mouse reference genome using BLASTN 2.2.31 [27] to identify perfect matches with the mouse mRNA. Target prediction scans were performed using the miRanda algorithm for subsequent analyses.

RNA-AGO2 Molecular Docking

The 3D structures of the AGO2 protein and OMV sRNA were predicted using the Phyre2 server and RNAComposer

software, respectively. Structural optimization was performed using ChemBio3D Ultra 7.0 and AutoDock Tools 1.5.6, including energy minimization, water removal, and addition of nonpolar hydrogens. Protein–RNA docking was performed using the HDock server [28] with a hybrid docking strategy. Protein and RNA molecules were set as rigid bodies, and 100 conformations were generated and scored using the ITScorePP function. A more negative docking score indicated stronger binding affinity. The top-ranked conformation was further optimized using the minimization module using MOE 2019.1 with the Amber10: ETH force field and the water solvation model with the steepest descent and conjugate gradient methods. The final optimized structure was visualized using PyMOL version 2.1 (Schrödinger, New York, NY, USA; <https://pymol.org/>).

RNA Pull-down

Cells (6×10^6) from a 100 mm dish were washed with PBS, trypsinized, and pelleted at $3000 \times g$ for 2 min. The pellet was lysed in buffer A for 15 min on ice, mixed with buffer A containing 2.5% NP40, and centrifuged at $4000 \times g$ for 5 min at 4°C to collect the cytoplasmic supernatant. Biotin-labeled RNA probes were synthesized by in vitro transcription using a Promega kit and incubated with cytoplasmic extracts to form RNA-protein complexes. Complexes were captured using streptavidin magnetic beads, eluted, and analyzed by western blotting to identify specific RNA-binding proteins.

Cellular Infection Assay

RAW264.7 cells (Cell Resource Center, Beijing, China) were seeded in 24-well plates at 4×10^6 cells per well and cultured for 24 h at 37°C with 5% CO₂. Cell infection was performed as previously described [29]. Cells were infected with *B. melitensis* 16M at a multiplicity of infection of 100:1 with gentle mixing, and a mock-infected control was included. After 4 h of incubation, cells were washed three times with sterile PBS and incubated for 1 h in DMEM containing 50 µg/mL gentamicin to eliminate extracellular bacteria. The medium was then replaced with DMEM supplemented with 25 µg/mL gentamicin and 10% fetal bovine serum, and cells were cultured for another 12 h.

CFU Assay

Intracellular bacterial counts were determined at 0, 4, 8, 12, and 24 h post-infection. Cells were washed three times with PBS and lysed with 200 µL 0.1% Triton X-100 for 10 min at room temperature to release intracellular bacteria. Serial 10-fold dilutions of the lysate were prepared, and 100 µL of each dilution was plated onto TSA agar in triplicate. Plates were incubated at 37°C for 72 h and colonies were counted. Only plates containing 30–300 colonies were included in the analysis. Results were expressed as log

CFU per well.

Statistical Analysis

All experiments were independently repeated at least three times. Statistical analyses were performed using GraphPad Prism version 9.00 (GraphPad Software, San Diego, CA, USA; <https://www.graphpad.com/scientific-software/prism/>). Data from multiple groups were analyzed using one-way analysis of variance (ANOVA) or Dunnett's multiple comparison test. The results are presented as mean $\pm$ standard deviation ($\bar{x} \pm SD$), where “*” indicates a significant difference ($P < 0.05$), “**” indicates a highly significant difference ($P < 0.01$), and “ns” indicates no significant difference.

RESULTS

OMVs Contain Differentially Packaged sRNAs

From RNA-Seq analysis of the *B. melitensis* 16M and OMVs conducted to determine whether sRNAs were packaged in OMVs, sRNAs spanning a length range of 18-30 nucleotides were selected for analysis (Fig. 1). Sequences with identical nucleotides were collapsed into unique entries (Table 1), which were then used to evaluate the distribution and relative abundance of shared and specific sRNA sequences in the samples (Fig. 1-A, B). Reads were aligned to the reference genome using the Bowtie algorithm to profile the genomic distribution (Fig. 1-C, D). In total, 1,783,364 unique sRNA sequences were identified in the OMVs of *B. melitensis* 16M (Fig. 2-A). The ten most abundant OMV-associated RNAs mapped to the *B. melitensis* 16M genome. Notably, eight of these ten sRNAs were significantly enriched in OMVs compared to whole-cell samples from *B. melitensis* 16M (Fig. 2-B).

sRNAs in OMVs of Target Mouse mRNAs

When we assessed whether the ten most abundant sRNAs in OMVs form stable secondary structures and interact with host mRNAs, eight sRNAs were predicted to form stable stem-loop structures (Fig. 3). Sequence alignment against of the eight most abundant OMVs sRNAs against the mouse reference mRNA sequences using BLASTN 2.2.31, followed by miRanda analysis identified six sRNAs with perfect matches to the mouse mRNA (Table 2).

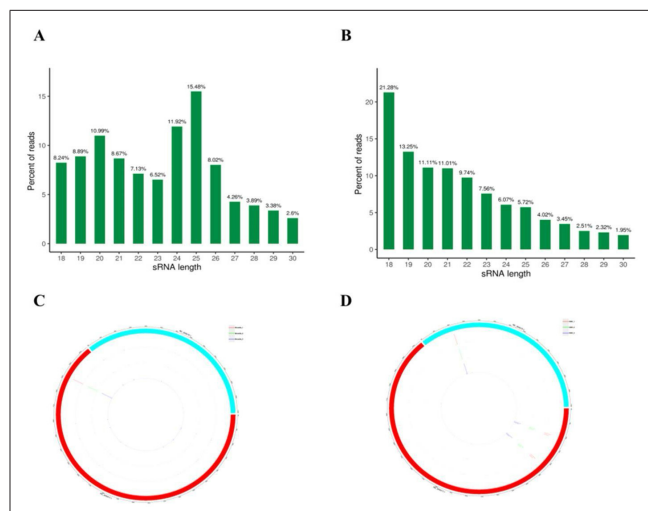


Fig 1. Analysis of repeat merging and shared sRNA sequences across samples. **A-** Read length distribution of *B. melitensis* 16M whole-cell sRNAs; **B-** Read length distribution of *B. melitensis* 16M OMV-derived sRNAs; **C-** Chromosomal distribution density of *B. melitensis* 16M genomic sequences; **D-** Chromosomal distribution density of *B. melitensis* 16M OMV-associated sequences

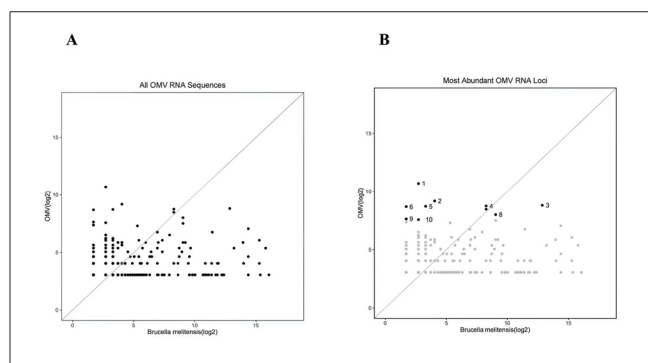


Fig 2. OMVs contain differentially packaged sRNAs. **A-** Relative abundance of 1,783,364 unique OMV sRNA reads versus whole-cell sRNA reads in *B. melitensis* 16M. Sequences to the left of the solid diagonal line are more abundant in OMVs than in whole cells, whereas those to the right are less abundant in OMVs. The sRNA sequence lengths range from 18 to 30 nt. **B-** Relative abundance of OMV-associated sRNAs mapped to genomic loci in *B. melitensis* 16M. Loci to the left of the solid diagonal line are more abundant in OMVs than in whole bacteria, while those to the right are less abundant in OMVs. Overall, the high-abundance genomic loci are enriched in OMVs compared to *B. melitensis* 16M whole cells. Results were presented as mean $\pm$ SD of three individual experiments

OMV sRNA Interact with AGO2

Molecular docking simulations demonstrated that all six high-abundance sRNAs from *B. melitensis* 16M OMVs could

Table 1. Statistical result of sequence profiles and shared sequences across samples

Type	Unique sRNA	Percent of Unique	Total sRNA	Percent of Total
Total_sRNAs	5.775.639	100%	42.548.889	100%
OMVs unique	1.783.364	30.88%	9.107.598	21.41%
Brucella 16M unique	3.977.013	68.86%	16.775.066	39.42%
OMVs & <i>B. melitensis</i> 16 M common	15.262	0.26%	16.666.225	39.17%

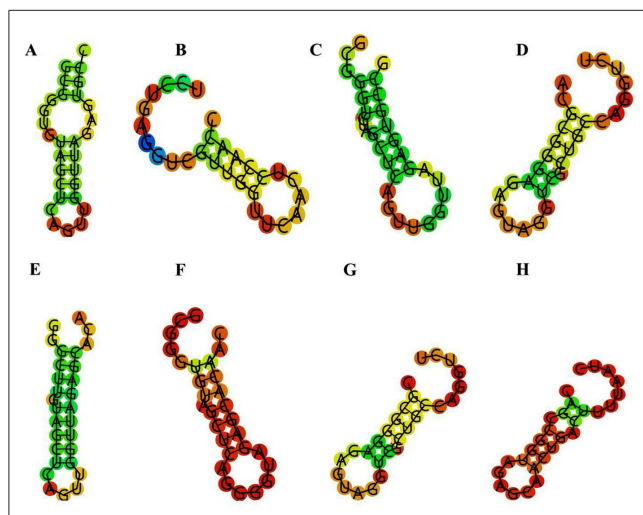


Fig 3. Secondary structure prediction of the 8 most abundant sRNAs in *B. melitensis* 16M OMVs. The secondary structures were predicted using the RNAfold WebServer with default parameters. The figures show the minimum free energy structures of each sRNA. The negative free energy values (ranging from -23.5 kcal/mol to -11.2 kcal/mol) indicate that all these sRNAs can form stable secondary structures

bind to AGO2 (Fig. 4). Among this, sRNA t0000835 exhibited the strongest binding affinity and the most stable interaction with AGO2. Consistent with this, RT-qPCR analysis revealed that sRNA t0000835 significantly suppressed transcription of the G protein-coupled receptor 45 gene (GPR45) (Fig. 5-A). The binding mode of the sRNA-AGO2 complex was visualized using PyMOL version 2.1 (Fig. 5-B).

Molecular docking analysis further revealed that the sRNA t0000835-AGO2 complex was stabilized through multiple interactions between key amino acid residues in AGO2 and specific nucleotides in sRNA t0000835 (Fig. 4), mediated by various intermolecular interactions, including electrostatic and hydrogen bonds, which significantly enhance the stability of the sgRNA-protein complex. Contact mapping between Ca atoms of AGO2 and O5' atoms of the RNA strand further supported these interactions (Fig. 5-C). RNA pull-down assays confirmed that biotinylated sRNA t0000835 specifically binds to AGO2 in vitro (Fig. 5-D). These complementary

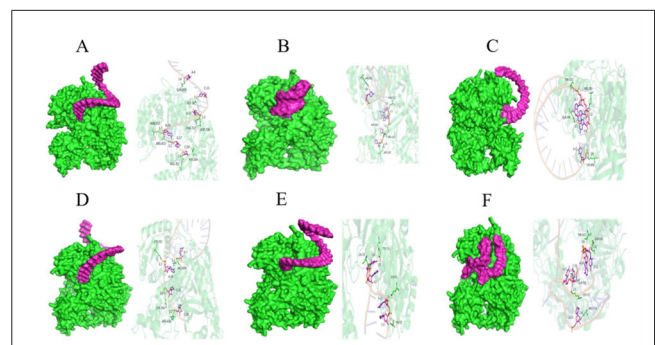


Fig 4. Analysis of sRNA-AGO2 binding in *B. melitensis* 16M. The 3D structures of AGO2, predicted using Phyre2 .0 (<https://www.sbg.bio.ic.ac.uk/phyre2/>), and sRNA, modeled using RNAComposer1.0 (<https://rnacomposer.cs.put.poznan.pl/>), were optimized by energy minimization and non-polar hydrogen addition. Rigid-body molecular docking was performed with HDock server 2017 (<http://hdock.phys.hust.edu.cn/>) under full-surface contact mode with 100 conformations generated and ITCPP scoring applied. The most stable docking conformation was re-optimized using MOE 2019.1 (<https://www.chemcomp.com>) with the Amber10:ETH force field, and finally visualized with PyMOL 2.1 (<https://pymol.org/>)

approaches provided robust evidence for the formation of a stable sRNA t0000835-AGO2 complex.

OMVs and sRNA t0000835 Promote Intracellular Replication of *B. melitensis* 16M

Functional assessment of OMVs during infection showed that OMV treatment significantly enhanced intracellular bacterial proliferation, particularly during the early stages (0.5-8 h) (Fig. 6). This suggests that OMVs may facilitate initial intracellular colonization and replication prior to full activation of the type IV secretion system (T4SS). Notably, intracellular survival assays using RAW264.7 revealed that cells expressing sRNA t0000835 exhibited significantly enhanced intracellular *Brucella* survival compared to the controls (Fig. 7), supporting the hypothesis that sRNA modulates host pathways to create a favorable intracellular niche for bacterial survival.

DISCUSSION

In the present study, we described an example of trans-kingdom regulatory activities of OMV-derived sRNAs

Table 2. sRNAs in OMVs predicted to target mouse mRNAs

OMVs sRNA	OMVs Counts ^a	Log2 Ratio ^b	Stable Secondary Structure	Targeted mRNA	Function
t0000206	1657	7.99	YES	Lanc1	lanC-like protein 1
t0000788	447	-4.05	YES	Lanc1	lanC-like protein 1
t0000833	430	0.49	YES	Mdfic2	Uncharacterized protein
t0000835	422	5.43	YES	Gpr45	G-protein coupled receptor 45
t0000964	356	0.21	YES	Mdfic2	Uncharacterized protein
t0001585	198	5.93	YES	Sil1	Nucleotide exchange factor SIL1

^a OMV counts show the total number of reads for each locus in OMV-derived sRNAs; ^b The log2 ratio is the number of total reads in OMVs divided by the number of total reads in *Brucella* 16M whole cells for each locus

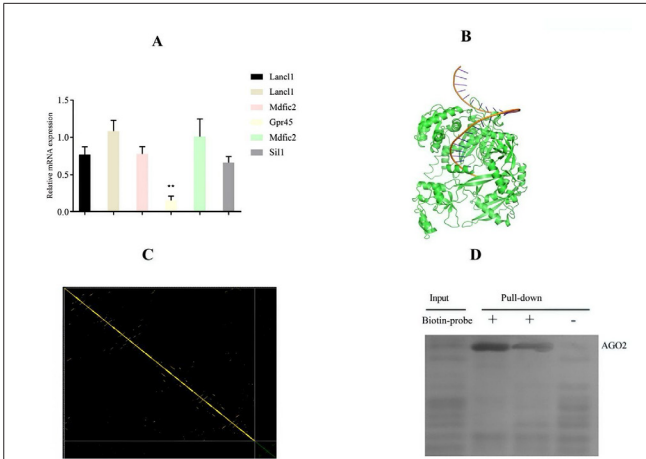


Fig 5. *B. melitensis* 16M sRNA binding AGO2 in vitro. **A-** Real-time fluorescence quantitative PCR detection of the effect of *B. melitensis* 16M OMVs sRNA on the mRNA of host target cells (* $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$); **B-** sRNA t0000835-AGO2 protein molecular docking complex; **C-** Residue-level resolution contact map of sRNA t0000835-AGO2 protein docking complex; **D-** RNA pull-down. Lane 1: Input group, representing the total protein sample of cells. Lane 2 and Lane 3: After adding the biotin-labeled RNA probe, the AGO2 protein forms an RNA-AGO2 protein complex with the biotin-labeled RNA. Lane 4: No biotin-labeled RNA probe was added, serving as a negative control. Results were presented as mean $\pm$ SD of three individual experiments

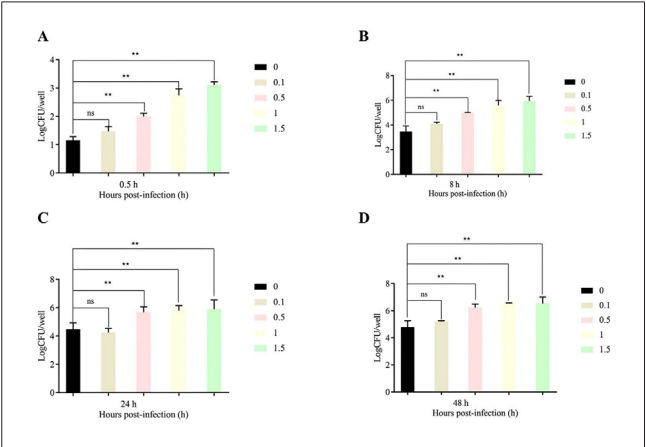


Fig 6. OMVs enhance the intracellular survival of *Brucella*. RAW264.7 cells were pre-incubated with 0, 0.1, 0.5, 1, or 1.5 μ g of OMVs purified from *B. melitensis* 16M for 1 h before infection with *Brucella* at MOI=100:1. At 0.5, 8, 24 and 48 h post-infection, cells were lysed with 0.1% Triton-100, serially diluted, and plated to enumerate intracellular *Brucella*. Statistical analysis was performed using GraphPad Prism version 9.00 (* $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$). Results were presented as mean $\pm$ SD of three individual experiments

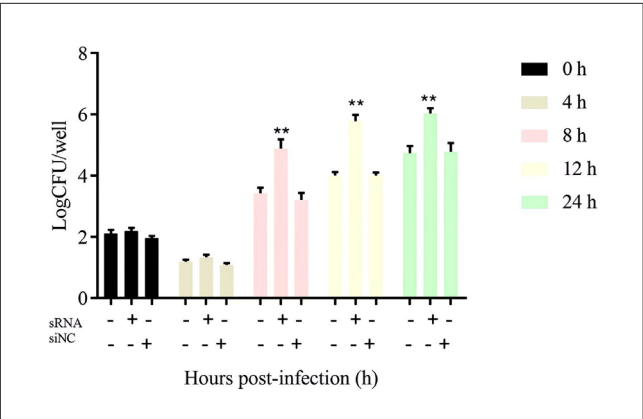


Fig 7. Transfection with sRNA t0000835 can increase the intracellular survival count of *Brucella*. Compared with cells transfected with negative control small interfering RNA (siNC), the intracellular survival count of *Brucella* in Raw264.7 cells transfected with sRNA t0000835 is significantly increased (* $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$). Results were presented as mean $\pm$ SD of three individual experiments

in *Brucella* infection. sRNA t0000835, encapsulated in *B. melitensis* 16M OMVs, represents a novel mechanism of pathogen–host communication, which modulates host gene expression to promote intracellular survival of bacteria. This finding reveals a T4SS-independent regulatory strategy used by *Brucella* during early stages of infection.

We demonstrated that OMVs produced by *Brucella* delivered functional sRNAs to host macrophages. However, the relative abundance of sRNAs in OMVs did not fully mirror their predicted targeting efficiency, probably owing to variable sRNA stability or AGO2-binding affinity among sequences, which led to differential functional availability in host cells. sRNAs in bacterial OMVs have been previously characterized [12,30]; however, a direct role of OMV-derived sRNAs in promoting intracellular survival of bacteria by targeting GPCR signaling pathways has not been demonstrated.

The presence of sRNAs in OMVs has been recently reported in various gram-negative bacteria including *Pseudomonas aeruginosa* [12], *Helicobacter pylori* [31], and *Escherichia coli* [32]. However, the functional significance of these sRNAs in modulating host cell biology to benefit intracellular survival of bacteria has remained largely unexplored. For example, sRNA52320 has been reported

Table 3. All primers were listed for qPCR

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Length (bp)
Lanc1	TAAACGCGCCGCATCTGTT	GAGCACACGGAGATGATGGA	87
Mdfic2	CACAATCGCATGTCTCGCTG	GCCACAATGCAGCTACCAAG	156
Gpr45	GTGCCAACTCAGGGTCTCCA	TGTCTCTGTTTCTGTGGTAGCC	86
Sil1	TCCCACCCACTCAGGTTTCT	CCTGCTCTTGTTCACCT	106
GAPDH	GATGCCCCCATGTTTGAT	GGCATGGACTGTGGTCATGAG	162

to reduce IL-8 secretion [12]; however, this study has focused on an extracellular pathogen in airway epithelial cells, without addressing the query whether intracellular pathogens, like *Brucella*, use analogous sRNA-based mechanisms to establish a replicative niche. Differential packaging of tRNA fragments into *E. coli* OMVs has been noticed [32]; however, the biological consequences of this selective packaging during infection have not been reported. Our study fills this gap by demonstrating that sRNA t0000835, a tRNA-derived fragment, directly suppresses GPR45 expression via AGO2 binding, thereby enhancing intracellular survival of *Brucella*.

Notably, sRNA t0000835 targeted GPR45, a GPCR family member. GPCRs are the largest family of cell surface receptors and play central roles in regulating immune responses including phagocytosis and cytokine secretion [33]. Although bacterial effectors modulate GPCR signaling [34], our study provides the first evidence that a bacterial OMV-derived sRNA directly suppresses GPCR expression at the transcriptional level by recruiting AGO2. Given that GPR45 has been implicated in intracellular calcium homeostasis and MAPK signaling pathways [35], its down regulation may create a relatively highly permissive intracellular environment for *Brucella* by attenuating bactericidal responses.

Remarkably, sRNA t0000835 appeared to specifically target GPR45, rather than globally suppressing host gene expression, probably because the secondary structure of sRNA t0000835 enabled sequence-specific binding to the 3'UTR of *GPR45* mRNA via AGO2-mediated base pairing. This specificity may be biologically advantageous for *Brucella* as global suppression of host gene expression may trigger stress responses or be metabolically costly. Consistent with this hypothesis, bacterial sRNAs often target key nodes in host signaling networks to fine-tune immune responses without causing catastrophic cellular damage [36,37].

The ability of *Brucella* to use OMV-delivered sRNAs to enhance intracellular survival has important implications for understanding its pathogenesis. Brucellosis is characterized by chronic, persistent infections that are difficult to eradicate. Our findings suggest that OMV sRNAs may contribute to this persistence by subverting host defense mechanisms from the earliest stage of infection, even before T4SS-mediated effector delivery becomes fully operational. Therefore, targeting OMV biogenesis or sRNA packaging could represent a novel therapeutic strategy to attenuate *Brucella* virulence, particularly given the increasing concerns regarding antibiotic resistance in chronic brucellosis.

OMV-mediated delivery of functional sRNAs to host cells may be a common strategy among intracellular

pathogens that need to establish a replicative niche. Other clinically relevant intracellular bacteria, such as *Salmonella*, *Legionella*, and *Mycobacterium*, may also use OMV-based sRNA-related mechanisms during the early “stealth” phase of infection before their dedicated secretion systems are fully activated. Beyond the host-pathogen interface, *Brucella* OMV sRNAs may play roles in microbe-microbe competition within the host or environment.

This study has some limitations. First, the precise mechanism, by which sRNA t0000835 is selectively packaged into *Brucella* OMVs has not been elucidated. Second, whether sRNA t0000835 targets additional host mRNAs besides GPR45 awaits further investigation. Third, the temporal dynamics of sRNA delivery relative to T4SS activation during infection need to be characterized. Future studies considering genetic deletion of sRNA t0000835 in *B. melitensis* 16M will be essential to confirm its role in virulence *in vivo*. An in-depth understanding of OMV sRNA biology may open new avenues for therapeutic intervention against chronic brucellosis and other intracellular bacterial infections.

In summary, sRNA t0000835 significantly enhances intracellular survival of *B. melitensis* 16M through an AGO2-dependent mechanism that represses GPR45 expression. This study provides new experimental evidence for understanding how bacterial sRNAs promote their own survival by modulating host gene expression.

DECLARATIONS

Availability of Data and Materials: The raw sRNA sequencing data generated in this study have been deposited in the NCBI Sequence Read Archive (SRA) under the BioProject accession number PRJNA1452185 (BioSample: SAMN57209791, SAMN57209792). Since the data are currently private and not yet published, a reviewer link is provided for temporary access: <https://dataview.ncbi.nlm.nih.gov/object/PRJNA1452185?reviewer=75hfrmg97ccubv73jk4dkfcdt>.

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