



(P)ppGpp synthase Rsh promotes persister cells formation by regulating sulfur metabolism mediated by sulfate reductase CysI in *Brucella abortus*

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ABSTRACT

Persister cells constitute a subpopulation of dormant cells that are transiently tolerant to antibiotics and associated with chronic infection. We have known (p)ppGpp synthetase Rsh promoted persister cells formation during rifampicin exposure in *Brucella*, but the metabolic regulation mechanism by which Rsh promotes persister cells formation is unknown. In this study, we firstly characterized the proteome and metabolome of persister cells formation during rifampicin exposure between *B. abortus* A19 (WT) and Δrsh strains. We found that sulfur metabolism-related protein (NAD(P)/FAD-dependent sulfite reductase, *cysI*) was significantly down-regulated in Δrsh compared with WT strain during persister cells formation ($P < 0.01$). The Rsh significantly affected hydrogen sulfide consumption by hydrogen sulfide measurement ($P < 0.01$). And then we found Rsh positively regulated CysI that its promoter region was 122 bp of upstream of *cysI* gene. In addition, the *cysI* knockout strain ($\Delta cysI$) was constructed which significantly reduced persister cells formation ($P < 0.01$) and also reduced consumption of hydrogen sulfide compared with WT strain. Taken together, we firstly reported that (p)ppGpp synthetase Rsh promotes persister cells formation through regulating sulfur metabolism mediated by sulfite reductase CysI in *B. abortus*. Our results provide a potential target and new strategy for clearing persister cells as well as prevention of *Brucella* infection.

1. Introduction

Brucella spp., a gram-negative facultative intracellular bacteria, cause brucellosis that is a widely prevalent zoonotic infectious disease in many countries and regions which bring the extreme economic losses to animal husbandry industry, bio-security and human health (Roop et al., 2021). *Brucella* can invade host cells and resides within *Brucella*-containing vacuoles (BCVs), which interact with components of endocytic and secretory pathways to ensure bacterial survival in host cells (Yu et al., 2024). *Brucella* undergoes an intracellular cycle within host cells and persists, ultimately leading to chronic infection (Alakavuklar et al., 2023). *Brucella*-induced persistent infections in hosts pose significant challenges to the eradication and control of brucellosis (Myeni et al., 2013). In addition to host-related factors, the underlying mechanisms of

persistent infection caused by *Brucella* that may also be associated with antibiotic resistance and persister cells.

Persister cells are a phenotypic switched subpopulation that survive in high dose of antibiotics exposure without genetic changes which are widely present almost all pathogens and likely connected to persistent infections caused by some of these pathogens (Niu et al., 2024). This phenomenon have not only been extensively studied in bacteria but have also been identified in fungi (Ke et al., 2024) and parasites (Jorge et al., 2020) which is difficult to eradicating them with conventional antibiotics. Currently, the molecular mechanisms underlying bacterial persister cells remain poorly understood. Extensive research on molecular mechanisms, primarily conducted in *Escherichia coli* and other model bacteria, have been established that are clearly associated with various factors (Roy et al., 2024). Studies indicate that toxin-antitoxin

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systems (Xiang et al., 2024), metabolism (Li et al., 2023), nutrient limitation oxidative stress (Zhang et al., 2023), general stress responses (Ronneau et al., 2023) and the second messenger molecule (p)ppGpp are all linked to bacterial persister cells formation (Tkachenko et al., 2021). However, the mechanisms driving persister cells formation are not attributable to a single factor but rather involve a complex regulatory network that control the expression of multiple genes to promote persister cells formation in bacteria.

The second messenger (p)ppGpp triggers the stringent response under nutrient starvation and other stress conditions for regulating bacterial physiological and metabolic processes to sustain bacterial survival (Winther et al., 2018). Related research indicates that the bacterial (p)ppGpp plays a significant regulatory role in persister cells formation such as *E. coli* and *Pseudomonas aeruginosa* (Svenningsen et al., 2019). The (p)ppGpp is synthesized by RelA/SpoT homologs (Rsh) in *Brucella* (Das and Bhadra, 2020). In our previous study, it was shown that the *B. abortus* is capable of forming persister cells and the (p)ppGpp synthase Rsh facilitates persister cells formation in *B. abortus* in the presence of rifampicin (Liu et al., 2023). And we have demonstrated that the partial regulatory mechanism of the (p)ppGpp synthase Rsh promoting persister cells formation that is associated with ATP level in *Brucella* (Liu et al., 2024).

Sulfur metabolism refers to various biochemical processes related to sulfur in organisms including sulfur acquisition, transformation, utilization and sulfur-related metabolic pathways (Tang et al., 2025). The key metabolites in the sulfur metabolic pathway include hydrogen sulfide (H₂S), sulfate (SO₄²⁻), and cysteine. These metabolites play a significant role in regulating bacterial energy metabolism, DNA repair and stress response (Miller and Schmidt, 2020). In this study, we sought to investigate the underlying metabolic regulatory mechanism of Rsh influencing persister cells in *B. abortus*. We integrated and analyzed the proteomic and metabolomic libraries of persister cells during rifampicin treatment between wild-type (WT) and Δ rsh strains. We identified that Rsh influences many differentially expressed proteins and enriched metabolic pathways during persister cells formation when compared with WT strain. Specifically, we reveal that (p)ppGpp synthase Rsh promotes persister cells formation by regulating the sulfur metabolism involving sulfite reductase CysI in *B. abortus*.

2. Material and methods

2.1. Bacteria, culture conditions

The *B. abortus* A19 strain was provided by Shaanxi Veterinary Drug Supervision Institute. All experiments about *B. abortus* strains were performed in a biosafety level 2 facility. All deletion strains were derived from wild type *B. abortus* A19 (WT). All *Brucella* strains were cultured at 37°C in Tryptic Soy Broth (TSB) liquid medium with 180 rpm shaking or on solid TSA medium. The *E. coli* (DH5 α and BL21) were cultured at 37°C in Luria-Bertani (LB) liquid medium with 180 rpm or on solid LA medium. All plasmids and strains were shown in Table 1 in this study.

2.2. Protein sample preparation and proteomics analysis

The Bacterial cultures (WT and Δ rsh) were grown to stationary phase for 72 h in TSB liquid medium. And then the bacterial samples were treated with rifampicin for 24 h to induce persister cells formation (Liu et al., 2024). The treated samples were concentrated at 8000 rpm for 30 min at 4°C. The pellets were washed and suspended in PBS and again concentrated after discarding the supernatant. And then total proteins were extracted and quantified with SDT lysis buffer, a BCA kit (Thermo Fisher Scientific, Waltham, MA, USA), respectively. And then the total proteins were digested with trypsin prior to mass spectroscopy (MS) and the trypsin extracts were subjected to LC-MS/MS-DIA as previously described (Cranney and Meyer, 2021). The raw data was analyzed as our previous described (Liu et al., 2025).

Table 1

Strains and plasmids used in this study.

Plasmids/strains	Description	Reference/ source
<i>E. coli</i> DH5 α	Host strain	Lab stock
<i>E. coli</i> BL21 (DE3)	Expression strain	Lab stock
pet32a	plasmid	Lab stock
pet32a-cysI	plasmid	This study
<i>Brucella abortus</i> A19	Wild type strain	Lab stock
Δ Rsh	Rsh: KanR derivative of <i>B. abortus</i> A19	Lab stock
Δ cysI	cysI: KanR derivative of <i>B. abortus</i> A19	This study
pBBR1MCS-5	Gentamicin resistance gene, mod, broad host range cloning vector	Lab stock
pBB-Amp-lacZ	Ampicillin(Amp) resistance gene, Reporter vector	Lab stock
pBB-cysI (500)-lacZ, (cysI-500)	Amp resistance gene, Reporter vector	This study
pBB-cysI (300)-lacZ, (cysI-300)	Amp resistance gene, Reporter vector	This study
pBB-cysI (200)-lacZ, (cysI-200)	Amp resistance gene, Reporter vector	This study
pBB-cysI (100)-lacZ, (cysI-100)	Amp resistance gene, Reporter vector	This study
Δ Rsh-cysI-500p	Amp resistance gene, Δ Rsh carries a pBB-cysI (500)-lacZ;	This study
WT-cysI-500p	Amp resistance gene, <i>B. abortus</i> carries a pBB-cysI (500)-lacZ;	This study
Δ Rsh-cysI-300p	Amp resistance gene, Δ Rsh carries a pBB-cysI (300)-lacZ;	This study
WT-cysI-300p	Amp resistance gene, <i>B. abortus</i> carries a pBB-cysI (300)-lacZ;	This study
Δ Rsh-cysI-200p	Amp resistance gene, Δ Rsh carries a pBB-cysI (200)-lacZ;	This study
WT-cysI-200p	Amp resistance gene, <i>B. abortus</i> carries a pBB-cysI (200)-lacZ;	This study
Δ Rsh-cysI-100p	Amp resistance gene, Δ Rsh carries a pBB-cysI (100)-lacZ;	This study
WT-cysI-100p	Amp resistance gene, <i>B. abortus</i> carries a pBB-cysI (100)-lacZ;	This study

2.3. Metabolite sample preparation and metabolomics analysis

In briefly, the metabolite samples preparation of *Brucella* persister cells (WT and Δ rsh) were consistent with protein sample preparation. Metabolite samples were placed in EP tubes containing 300 μ L of pre-cooled 80 % methanol and placed on ice for 30 s and sonicated for 6 min and then centrifuged at 5000 rpm for 1 min at 4°C. The supernatant was freeze-dried and dissolved with 10 % methanol. The dissolved matter was loaded onto a Vanquish UHPLC system (Thermo Fisher, Germany) in line with an Orbitrap Q Exactive TMHF mass spectrometer or Orbitrap Q Exactive TMHF- Xmass spectrometer (Thermo) for analysis at Novogene (Beijing, China). Qualitative and relative quantitative results were obtained using MzVault and MassList database. Statistical analyses were carried out using the statistical software R, Python and CentOS. The obtained metabolites were annotated using the KEGG database (<https://www.genome.jp/kegg/pathway.html>), HMDB database (<https://hmdb.ca/metabolites>) and LIPIDMaps database (<http://www.lipidmaps.org/>). Principal components analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were performed at MetaX (Shanghai, China). Statistical significance (P-value) was calculated by using univariate analysis (T-test). The metabolites were considered to be differential metabolites with VIP > 1 and P-value and fold change $\geq$ 2 or FC $\leq$ 0.5. Based on log₂ (Fold Change) and -log₁₀(p-value) of metabolites, volcano plots were constructed using ggplot2 in R.

2.4. Quantitative real-time PCR (qRT-PCR) analysis

Total RNA of persister cells in *B. abortus* (WT and Δ rsh) was extracted using RNAiso Plus (Takara, Dalian, China). The RNA was reverse transcribed to cDNA using PrimeScript RT Master Mix. The cDNA was reverse transcribed from RNA samples using PrimeScript™ RT Master

Mix (Takara, Dalian, china). The primers were described as Table 2. The qRT-PCR was performed using ChamQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China). The 16sRNA of *B. abortus* was served as an internal reference for normalization. The reaction program was set to 95 °C for 30 s; 95 °C for 10 s; 56 °C for 30 s, 40 cycles. The relative gene expression was calculated as $2^{-\Delta\Delta C_t}$. All assays were performed at least three times.

2.5. Detection of hydrogen sulfide (H_2S) content

According to instructions with slight modifications (Nanjing Jiancheng bioengineering insititude, Nanjing, Jiangsu). The WT and knockout strain (Δrsh and $\Delta cysI$) were cultured to stationary phase for 72 h. The bacterial cells were centrifuged at 4 °C for 5 min. And the supernatant was discarded to collect the pellet. The pellet was washed once with PBS and centrifuged again at 4 °C for 5 min. The pellet was resuspended in PBS, followed by addition of 100 μ L lysozyme (final concentration: 1 mg/mL). The suspension was incubated at room temperature for 5 min and then placed on ice for further testing after vortex mixing.

2.6. β -galactosidase assays

The β -galactosidase assays were performed according to our previously describe (Liu et al., 2024). The fragments of *cysI* promoter were amplified using *B. abortus* A19 DNA as the template. The fragments were gel-purified, digested with restriction enzymes and verified by sequencing. The digested promoter fragments were then ligated with the plasmids pBB-Amp-lacZ that were pBB-cysI (500)-lacZ, pBB-cysI (300)-lacZ, pBB-cysI (200)-lacZ and pBB-cysI (100)-lacZ, respectively. And then the reported plasmids were transformed into both wild-type (WT) strain and *rsh* deletion strain, respectively. The *Brucella* strains containing reporter plasmids were cultured at 37 °C in TSB medium and then diluted to $OD_{600} = 0.05$ in the same medium. The Cultures were harvested by centrifugation and washed in PBS (pH=7.2) for β -galactosidase assay.

Table 2
PCR primers used in this study.

Primer name	Primer	Sequence (5'-3')	Amplicon sizes (bp)
<i>Q-cysI</i>	q-cysI-F q-cysI-R	CGCAACCATCATCTGTTC GTCACGAAAAAGATTGCGGAT	99
<i>E-cysI</i>	e-cysI-F e-cysI-R	GGATCCATGGATGCAGCAACAGC CTCGAGCTAGCGAAACTCATCCGACG	1275
<i>cysI</i> (500)	<i>cysI</i> (500)-F <i>cysI</i> (500)-R	GGATCCCAACGCTTTTCGTTTCGTGATAC CTCGAGCTGAAAAATACCTGCTTTTGAATGC	500
<i>cysI</i> (300)	<i>cysI</i> (300)-F <i>cysI</i> (300)-R	GGATCCAACTCTGCGCATGGACCA CTCGAGCTGAAAAATACCTGCTTTTGAATGC	333
<i>cysI</i> (200)	<i>cysI</i> (200)-F <i>cysI</i> (200)-R	GGATCCAAAGGCCGTTCTTCGCT CTCGAGCTGAAAAATACCTGCTTTTGAATGC	203
<i>cysI</i> (100)	<i>cysI</i> (100)-F <i>cysI</i> (100)-R	GGATCCCGATGCACTGCGACATGA CTCGAGCTGAAAAATACCTGCTTTTGAATGC	122
<i>Q-gpt</i>	q-gpt-F q-gpt-R	GGTTTCATTTAGCCGCGT GGATACCTGGATCTATTTCCCAT	84
16srRNA	q-16srRNA -F q-16srRNA -R	AGAGTTTGATCCTGGCTCAG ATTCCGTAGCAATGGTACG	123
<i>Q-hdhA</i>	q-hdhA -F q-hdhA -R	CACCTTCAGGTCTCAGATATAG CGGGCTCTCATAGGACATATA	99
<i>Q-iolG</i>	q-iolG-F q-iolG-R	CGTATCGCAAGGTTTCAT TTTGGCAGCGTCTCATT	90
U- <i>cysI</i>	U- <i>cysI</i> -F U- <i>cysI</i> -R	CGAAACCGTTTCACACTTTTC TTCCGCGCACATTTCCCGAAAGCTGAAAAATACCTGCTTTT	729
D- <i>cysI</i>	D- <i>cysI</i> -F D- <i>cysI</i> -R	GGACTCTGGGGTTTGAATGAAGCCTTTCGAGCCAAAAG ATGACCCAGCCCAAGAATA	808
<i>KanR</i> -F	<i>KanR</i> -F <i>KanR</i> -R	CTCGAGAAGGCCGTTTCAGGATGGA GGATCCTTGATTGCACTTCGACGCGA	1373
I- <i>cysI</i>	I- <i>cysI</i> -F I- <i>cysI</i> -R	GATATGCTCGTGTCTCGCTA ATCATCGCCTTCAACCCAT	627

2.7. Construction of the *cysI* gene knockout strain ($\Delta cysI$) in *B. abortus*

Construction of the *cysI* gene knockout strain ($\Delta cysI$) in *B. abortus* was conducted with reference to our previous study (Liu et al., 2023). Briefly, the upstream, downstream of the *cysI* gene and kanamycin resistance gene (*KanR*) were amplified from *B. abortus* A19 DNA and pET28a, respectively. The primer pairs were designed as U- *cysI* -F/R, D- *cysI* -F/R and *kanR*- F/ R. The three fragments were purified and ligated into the pMD19T-simple vector to generate the suicide plasmid (pMD19T-simple-*cysI*). The recombinant plasmid was identified by PCR (I- *cysI* -F/R) and sequenced. And then the pMD19T-simple-*cysI* plasmid was electroporated into *B. abortus* A19 competent cells with shaking for 24 h at 37 °C immediately and plated on TSA agar with 25 μ g/mL kanamycin for selecting a correct *cysI* deletion strain.

2.8. Determination of growth curve

The *B. abortus* A19 and $\Delta cysI$ strains were retrieved from -80 °C and inoculated into TSB liquid medium at 37 °C with 180 rpm shaking for 24 h. Subsequently, both strains were sub-cultured at 1:50 ratio into 100 mL TSB medium and cultured under identical conditions, separately. Then, the OD_{600} of two stains was continuously monitored for 96 h. The bacterial samples were collected every 12 h for measuring OD_{600} .

2.9. Persister cells assay in *B. abortus* in vivo

The persister cells assay was conducted with reference to our previous study (Liu et al., 2023). In short, the bacterial cultures (*B. abortus* A19 and $\Delta cysI$) were inoculated at 1: 50 into 8 mL fresh TSB medium and cultured to exponential and stationary phase with shaking at 37 °C for 72 h. A 1 mL of bacterial culture was 10-fold serially diluted in PBS as initial value. The remaining culture was treated with rifampicin (40 μ g/mL) for 24 h. The bacterial cells were collected, centrifuged and washed once with sterile PBS and resuspended in PBS for detecting persister cells.

2.10. Statistical analysis

All experiments were performed with three biological replicates. Data visualization was performed using GraphPad Prism 7.0, while statistical analysis was conducted with SPSS 22.0 using one-way or two-way ANOVA. Significance levels were defined as follows: $P > 0.05$ (ns, not significant); $P < 0.05$ (*, significant); $P < 0.01$ (**, highly significant). Data points represent means at least three biological replicates, with error bars indicating standard error of the mean.

3. Result

3.1. Protein qualitative data quality control and reproducibility analysis between WT and *Rsh* mutant

To investigate the impact of *rsh* on the persister cells formation in *Brucella abortus*, we firstly performed proteomic analysis between WT and Δrsh . These results showed that a total of 34,544 peptides and 2424 proteins were identified across three biological replicates between WT and Δrsh strains using Data-Independent Acquisition (DIA) analysis (Fig. 1 A, B). The coefficient of variation (CV) plots and principal component analysis (PCA) plots of the proteomic data demonstrated high reproducibility between biological replicates of both WT and Δrsh strains (Fig. 1 C, D).

3.2. Proteomics analysis of *B. abortus* (p)ppGpp synthetase *Rsh* during persister cells

To better understand the regulatory mechanisms of persister cells formation *Rsh* involving in *B. abortus*, we compared the proteome of persister cells between WT and Δrsh strain during persister cells. Our result showed that 574 differential expressed proteins (DEPs) were identified in strain Δrsh compared with WT which accounted for 23.68 % of proteins. We found that 537 of DEPs (93.56 %) were down-regulated and 37 (6.44 %) were up-regulated in Δrsh mutant contrast to WT strain (Fig. 2 A, B). The Δrsh -dependent down-regulated proteins varied less 2-fold accounted for 17.88 % (96/537) which partially encoded oxidoreductase, flagellar protein, ABC transporters, outer membrane autotransporters, large and small ribosomal subunit proteins, type IV secretion system proteins, sulfite reductase and efflux transporter. The Δrsh -dependent up-regulated proteins varied above than 2-fold accounted for 9.44 % (5/37) which encoded peroxamine synthetase WbkB, 6-phosphogluconate dehydrogenase NAD-binding, carbonic anhydrase, Xylose isomerase domain-containing protein TIM barrel and lytic transglycosylase catalytic.

To further investigate metabolic pathways enriched with differential expressed genes during persister cells formation between WT and Δrsh strain. GO enrichment analysis revealed that the up-regulated proteins in Δrsh strain during persister cells formation were primarily enriched in molecular functions including transition metal ion binding, isocitrate lyase activity, carbonate dehydratase activity, transporter activity

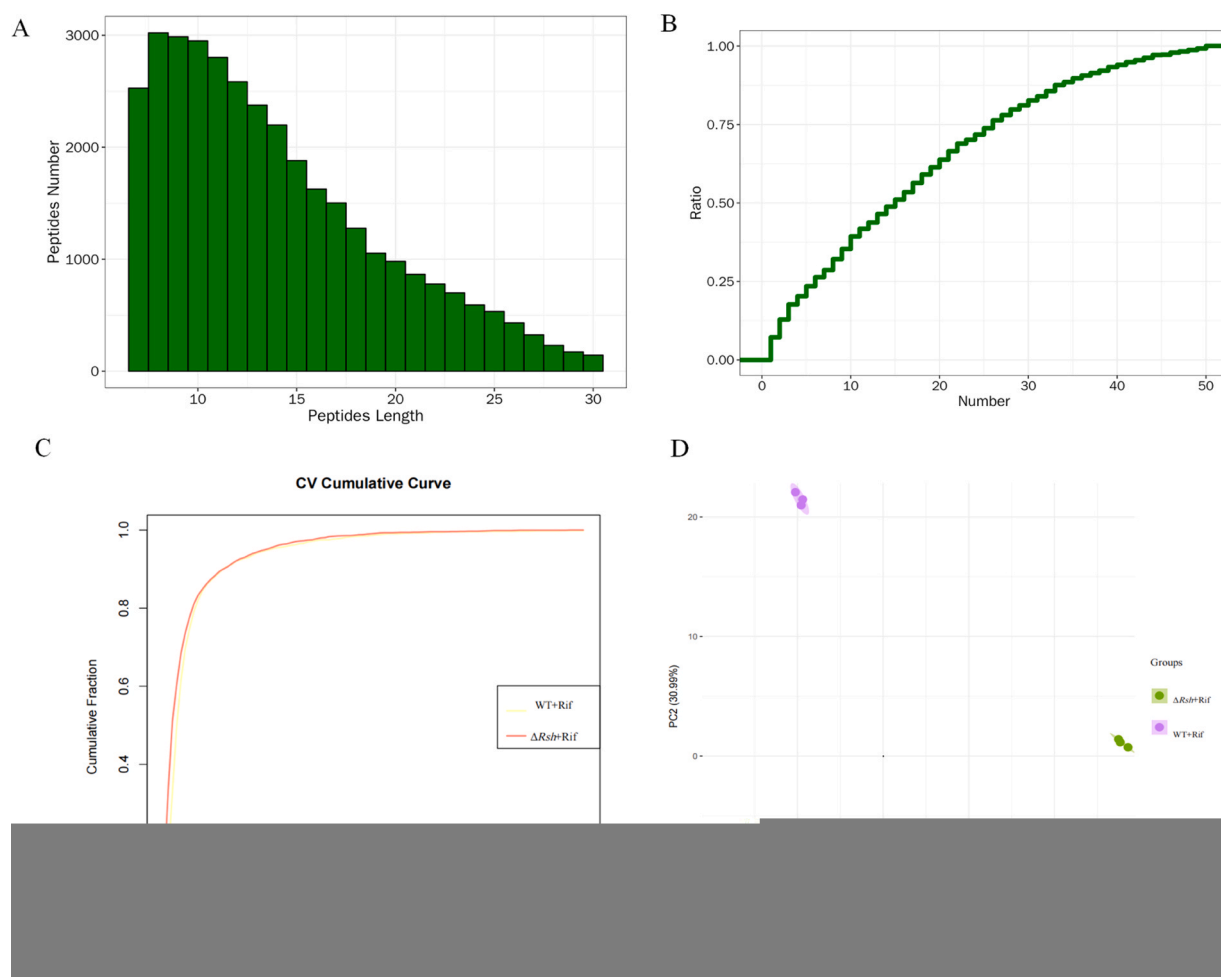


Fig. 1. The PCA analysis of proteomic analysis between WT and ΔRsh during the persister cells formation. (A) Protein qualitative data quality control of WT and ΔRsh during persister cells formation. (B) Data-Independent Acquisition (DIA) analysis of WT and ΔRsh during persister cells formation. (C) The CV analysis between WT and ΔRsh during persister cells formation; (D) The PCA analysis between WT and ΔRsh during persister cells formation.

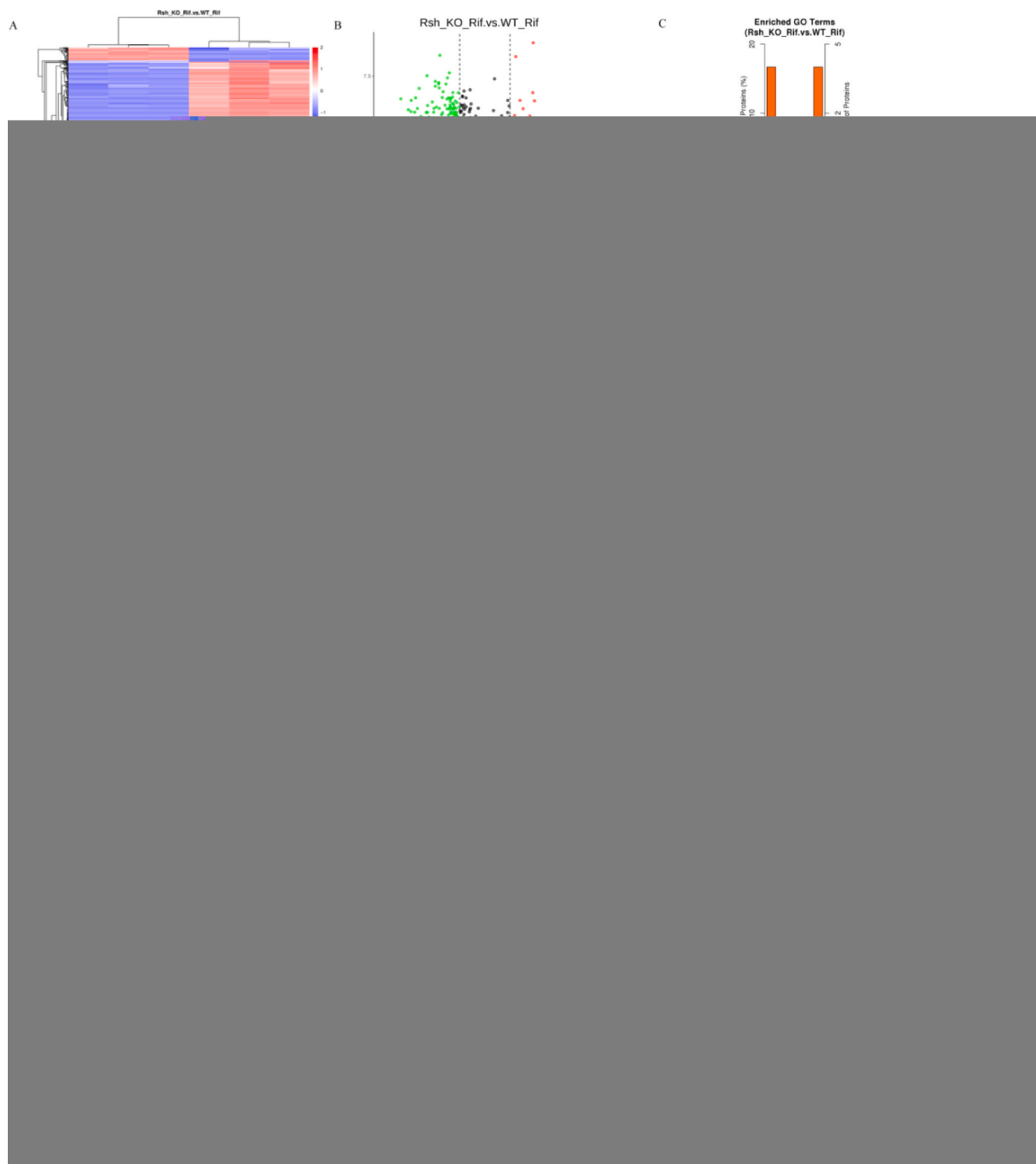


Fig. 2. Proteomic analysis between WT and ΔRsh strain during persister cells formation. (A) The heatmap of DEPs between WT and ΔRsh strains during persister cells formation; (B) The volcano of DEPs between WT and ΔRsh strains during persister cells formation; (C) GO enrichment of upregulated DEPs between WT and ΔRsh during persister cells formation; (D) GO enrichment of upregulated DEPs between WT and ΔRsh during persister cells formation; X-axis, GO term and y-axis, number of enriched proteins. Enriched DEPs are labeled as follows: Crimson, biological process; Purple, cellular components; Orange, molecular functions. (E) KEGG pathway of upregulated DEPs between WT and ΔRsh during persister cells formation. X-axis, enrichment ratio; y-axis, name of enrichment pathway; Dot sizes are proportional to the number of DEPs in the pathway and dot color intensities represent different q-value ranges.

compared to wild-type strain. In contrast, the down-regulated proteins were mainly associated with biological processes including translation and metabolism. Cellular component (CC) analysis indicated enrichment in processes including intracellular ribosome formation, ribosomes, cellular components, cytoplasm. Molecular functions were predominantly related to structural molecule activity, RNA binding, nucleic acid binding, DNA binding, translation initiation factor activity and peptidase activity (Fig. 2 C, D).

To further elucidate the potential metabolic pathways in *Rsh* promoting persister cells formation in *Brucella*, KEGG enrichment analysis were performed on differential expressed proteins identified in WT and ΔRsh strains during persister cells-formation. The results revealed that the enriched KEGG pathways primarily included ribosome, flagellar assembly, bacterial chemotaxis, biosynthesis of unsaturated fatty acids, thiamine metabolism, phosphotransferase system (PTS), drug metabolism-other enzymes, D-Glutamine and D-glutamate metabolism, chlorocyclohexane and chlorobenzene degradation, biofilm formation, β -Lactam resistance, vitamin B6 metabolism, nitrogen metabolism, ABC transporters, ubiquinone and other terpenoid-quinone biosynthesis, inositol phosphate metabolism, purine metabolism and sulfur metabolism (Fig. 2E, F). Our results show that *Rsh* may alter the multiple metabolism pathways during persister cells formation in *B. abortus*.

3.3. Metabolite qualitative data quality control and reproducibility analysis between WT and *Rsh* mutant during persister cells

To investigate the differences in metabolites among *Rsh* on persister cells formation in *Brucella abortus*, we performed metabolomics and detected the principal component analysis (PCA) between WT and ΔRsh . Metabolomic analysis detected a total of 437 metabolites in samples. The PCA model showed good validity ($R^2X = 0.76$) (Fig. 3A), with PC1 contributing 28.39 %, PC2 contributing 22.64 % and PC3 contributing 9.63 % of the variance (Fig. 3B, C). The samples from the two groups were distributed in distinct regions, indicating good intra-group reproducibility and clear metabolic differences among the strains during persister cells formation.

3.4. Metabolomic analysis of (p)ppGpp synthetase *Rsh* during persister cells formation in *B. abortus*

We compared the metabolites of persister cells between WT and ΔRsh strain during persister cells formation. Our result showed that a total of 52 differential metabolites were identified in strain ΔRsh

compared with WT which accounted for 11.90 % of metabolites. We found that 24 of metabolites (5.50 %) were upregulated and 28 (6.40 %) were down-regulated in ΔRsh mutant contrast to WT strain (Fig. 4 A). We found the upregulated metabolites in ΔRsh strain compared to WT strain mainly included phytic acid, N-Desmethylramadol, 1-[2-(1H-imidazol-5-yl)ethyl]pyrrolidine 2,5-dione, N1-[4-[amino(hydroxyimino)methyl]phenyl]acetamide, DMK, methylamino-L-alanine, 2-[2-oxo-2-(2-pyridylamino)ethoxy]acetic acid, 2-Naphthol, Methyl dopa, 12-oxo Phytodienoic Acid, L-Cystathionine, L-iditol, Phe-Phe, Riboflavin, 1,5-diphenyl-1H-1,2,4-triazole-3-thiol, Spermine, N-Methyldiocyrlamine, Cyclohexylsulfamate, 2-Methylpentanedioic acid and Linolenoyl ethanolamide. However, downregulated metabolites in ΔRsh mutant mainly included 6-styryl-3-phenyl-2,5-dihydro-1,2,4-triazin-5-one, CYM-5442, 2-Aminobenzenesulfonic acid, 4-Pyridoxic acid, FOH, N-benzyl-2-(6-methoxy-2-naphthyl)propanamide, Cysteinylglycine, P-Amino-thipuric Acid, S-(Methyl)Glutathione, 2-[2-oxo-2H,8H-9H-1,4-trg[2,3-h]chromen-8-yl]propan-2-yl acetate, L-cysteine, L-Histidinol, ELK, Lithocholic Acid, SLH, 4-oxo-4-[(pyridin-4-ylmethyl)amino]but-2-enoic acid, SLK, 4-methoxy-6-[2-(4-methoxyphenyl)ethyl]-2H-pyran-2-one-Kanosamine and Oxoadipic Acid (Fig. 4 B, C).

To further investigate metabolic pathways enriched with differential metabolites during persister cells formation between WT and ΔRsh strain. To further elucidate the potential metabolic pathways involved in *Brucella* *Rsh* during persister cells formation, KEGG enrichment analysis was performed on differential metabolites identified in WT and ΔRsh strains during persister cells formation. These results revealed that enriched metabolic pathways primarily included microbial metabolism in diverse environments, glutathione metabolism, biosynthesis of amino acids, glycine, serine and threonine metabolism, vitamin B6 metabolism, thiamine metabolism, taurine and hypotaurine metabolism, sulfur relay system, sulfur metabolism, secondary bile acid biosynthesis, riboflavin metabolism, pantothenate and CoA biosynthesis, inositol phosphate metabolism, glycerophospholipid metabolism, cyanoamino acid metabolism, carbon metabolism, carbapenem biosynthesis, caprolactam degradation, beta-Alanine metabolism and Benzoate degradation (Fig. 4D). We classified the metabolites into KEGG pathways and found that global and overview maps accounted for 34.48 %, amino acid metabolism 13.79 %, metabolism of cofactors and vitamins 10.34 %, metabolism of other amino acids 10.34 % (Fig. 4E).

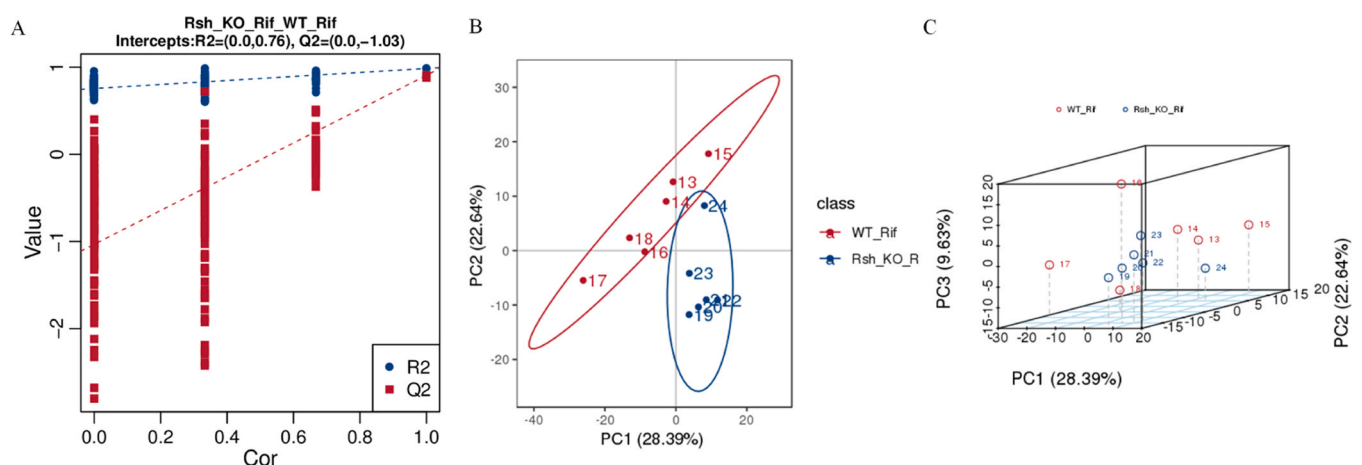


Fig. 3. The PCA analysis of WT and ΔRsh strains during persister cells formation. (A) The PCA analysis of differential metabolites in two group (B) The 2D PCA analysis of differential metabolites WT and ΔRsh during persister cells formation. The red represents six replicates of *Rsh* mutant during persister cells formation. The blue color represents the 6 replicates of WT strain during persister cells formation. (C) The 3D PCA analysis of differential metabolites WT and ΔRsh during persister cells formation. The red represents six replicates of *Rsh* mutant during persister cells formation. The blue color represents the 6 replicates of WT strain during persister cells formation.

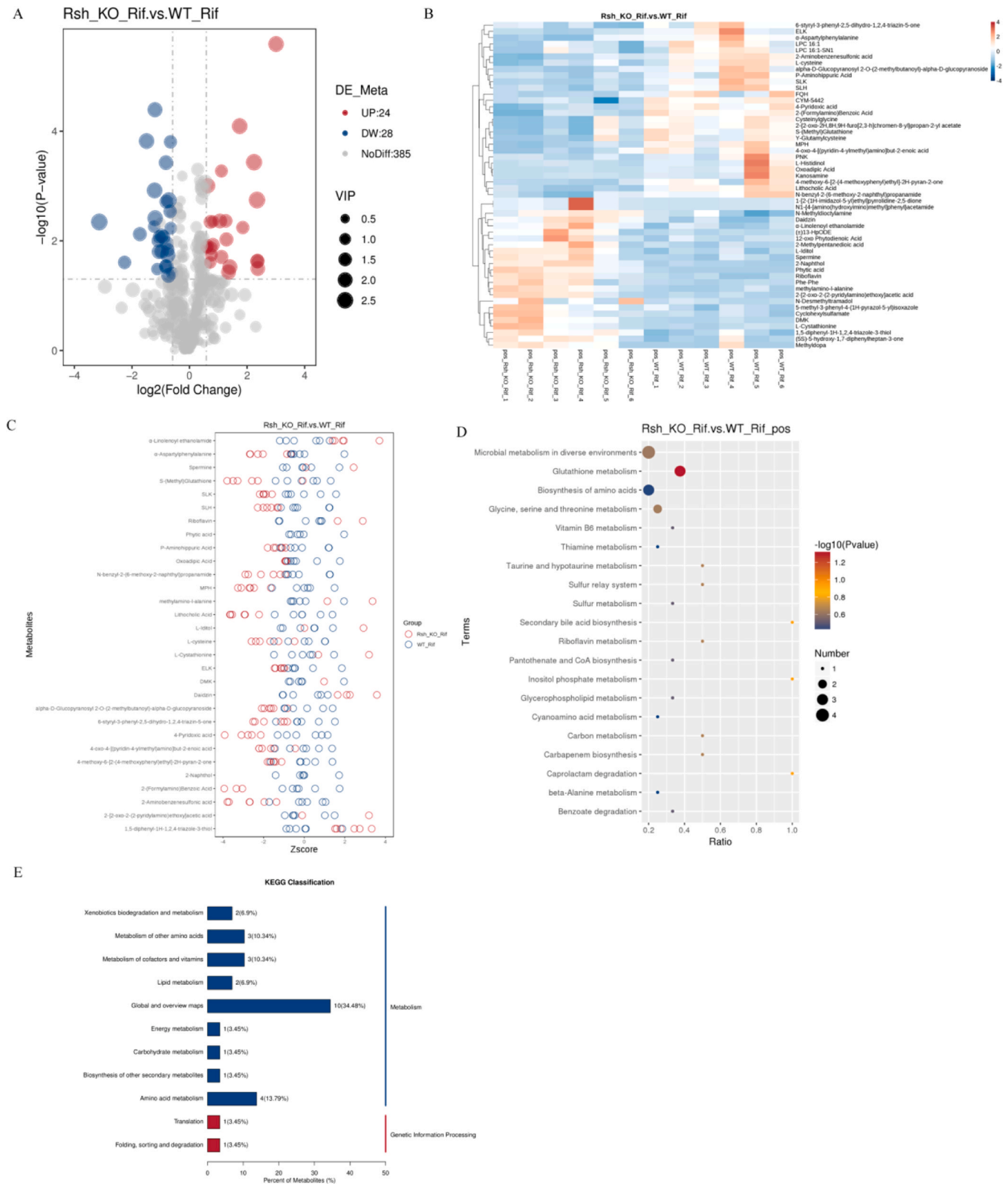


Fig. 4. Metabonomic analysis for between WT and ΔRsh strains during persister cells formation. (A) The volcano plot of analysis between WT and ΔRsh during persister cells formation. Down-regulated and up-regulated metabolites were showed in blue and red, respectively. (B) Heat map analysis of between WT and ΔRsh mutant during persister cells formation. Differential metabolites are showed in ΔRsh compared to WT strain during persister cells formation. (C) KEGG pathway analysis between WT and ΔRsh during persister cells formation. The x-axis and y-axis show enrichment ratio and enrichment pathway. And the size of dots represents the number of differential metabolites in KEGG pathway. (D) Enrichment of KEGG pathway between WT and ΔRsh during persister cells formation. (E) Classifications of KEGG pathway between WT and ΔRsh during persister cells formation.

3.5. Integrated analysis of proteomic and metabolomic of (p)ppGpp synthetase Rsh during persister cells formation in *B. abortus*

We detected the correlation analysis of differential metabolites and proteins between WT and Δ rsh during persister cells formation. We found that these differential expression proteins including A0A7U8JN09 (6-phosphogluconate dehydrogenase NAD-binding), A0A0F6AU88

(bacterial regulatory protein, MerR family), A0A7U8JSQ7(DUF4065 domain-containing protein), A0A0F6ARZ6 (Flagellar export protein FliJ), A0A7U8JDE0(phosphomethylpyrimidine kinase), A0A0F6AQT5 (xanthine-guanine phosphoribosyltransferase, gpt), A0A7U8PNS6 (carbonic anhydrase), A0A0F6ARE6(urease subunit beta, ureB), A0A7U8J779(acetyl-coenzyme A synthetase) and A0A0F6ANT4(Sulfite reductase (NADPH) hemoprotein beta-component, CysI) exhibited

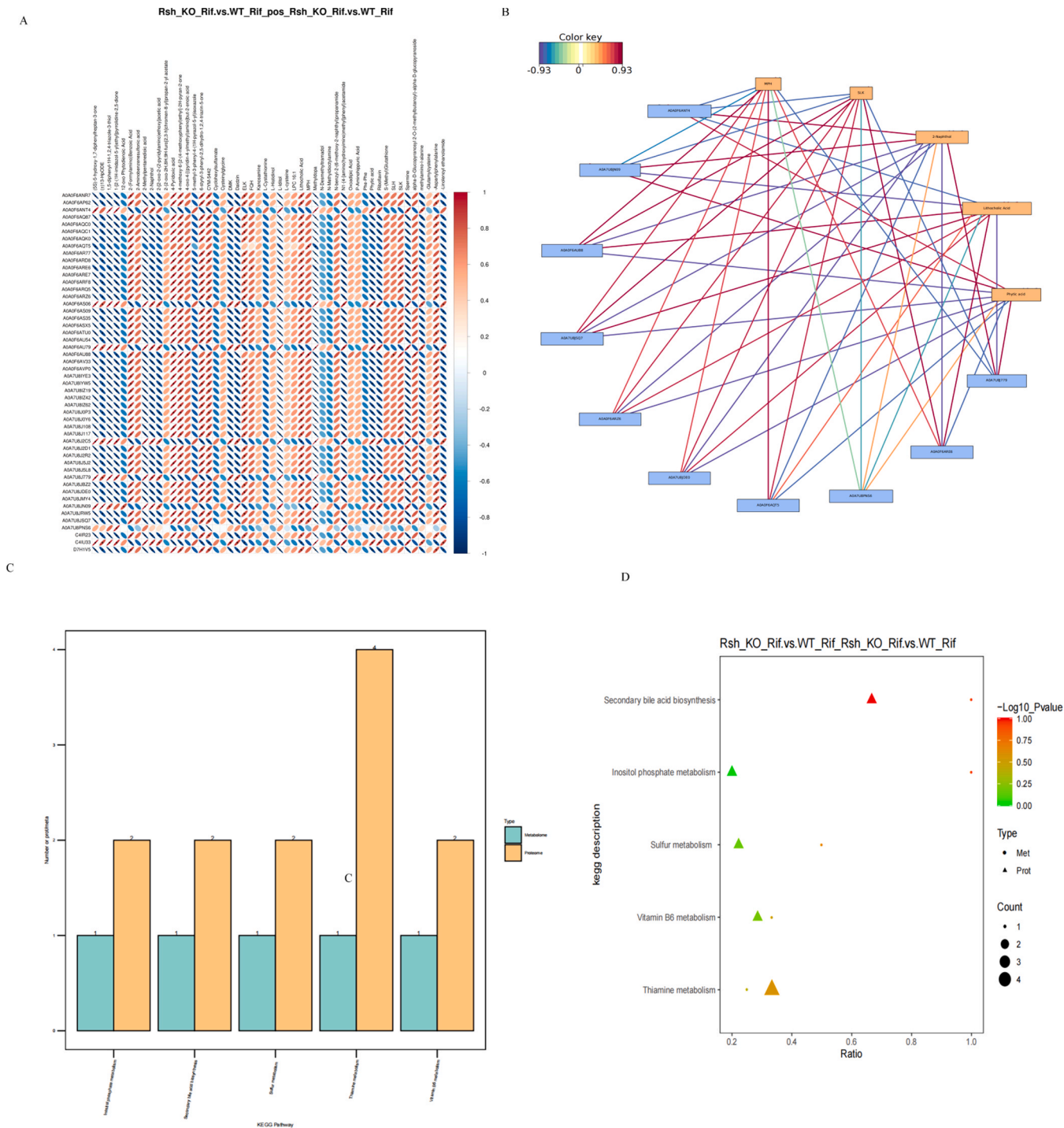


Fig. 5. Integrated analysis of proteomic and metabolomic between *B. abortus* A19 and Δ Rsh during persister cells formation, (A) Correlation analysis for DEPs and metabolites in the deletion of Rsh compared with WT strain during the persister cells formation. Color intensity is positively correlated to numbers of respective groups members; red, positive correlation; blue, negative correlation. The ellipse flatness corresponds to higher absolute values of the correlation. (B) Correlation analysis for DEPs and metabolites during persister cells formation. (C) KEGG pathway of differential proteins and metabolites between WT and Δ Rsh during persister cells formation. The green color represents the metabolome; yellow color represents the proteome. (D) KEGG enrichment analysis of integrated differential metabolites and proteins between WT and Δ Rsh strains during persister cells formation.

significant associations with these metabolites including MPH, SLK, 2-naphthol, lithocholic acid and phytic acid (Fig. 5A, B). Among the up-regulated protein was A0A7U8JN09, other proteins were down-regulated proteins. The other differential proteins (perosamine synthetase WbkB, RNA polymerase sigma factor RpoH and Acetyl-coenzyme A synthetase, Peptidase M23B) not shown in the figure also exhibited significant correlations with differential metabolites (Gibberellin A4, LPE 14:1, gibberellin A4, LPE 12:0). And we observed varying correlations between the differential expressed proteins and differential metabolites.

Next, KEGG revealed that these differential metabolites and proteins were co-enriched in different metabolic pathways including secondary bile acid biosynthesis, inositol phosphate metabolism, sulfur metabolism as well as vitamin B6 metabolism and thiamine metabolism (Fig. 5C, D). We found that differential metabolites of KEGG enrichment included lithocholic acid, phytic acid, L-cysteine and 4-pyridoxic acid. The differential proteins of KEGG enrichment included 7- α -hydroxysteroid dehydrogenase (WP_002964715.1), IolB protein (WP_002965925.1), inositol 2-dehydrogenase (WP_004682008.1), sulfite reductase (WP_006097741.1), serine O-acetyltransferase (WP_006119580.1), pyridoxal kinase (WP_006178234.1), phosphoserine transaminase (WP_002967888.1), thiamine-phosphate synthase (WP_002964797.1), D-amino-acid oxidase (WP_006162851.1), thiazole synthase (WP_010658053.1) and phosphomethylpyrimidine kinase (WP_005970144.1) (Fig. 5C, D). Based on the most significant metabolic pathways were identified from the proteomic and metabolomic data, we thought these metabolic pathways may represent key mechanisms through which Rsh facilitates persister cells formation in *B. abortus*.

4. qRT-PCR validation

Integrated multi-omics analysis was performed on differentially expressed proteins and metabolites identified during persister cells formation in both WT and Δrsh strains in *Brucella*. These results revealed that Rsh is associated with multiple metabolic pathways during persister cells formation in *B. abortus*. We selected a total of 4 differential proteins including linked to these pathways were for quantitative real-time PCR (qRT-PCR) validation. Our result showed that Δrsh strain exhibited significantly reduced transcriptional levels compared to WT strain including DR186_RS05395 (xanthine phosphoribosyltransferase, *gpt*), DR186_RS14785 (NAD(P)/FAD-dependent sulfite reductase, *cysI*), DR186_RS08265 (7- α -hydroxysteroid dehydrogenase, *hdhA*)

($P < 0.01$). However, there was a significant upward trend about DR186_RS13670 (inositol 2-dehydrogenase, *iolG*) in *rsh* deletion strain compared to WT strain ($P < 0.01$) (Fig. 6A). These findings were consistent with the proteomics data (Fig. 6B). Our results further supporting the regulatory role of Rsh in these metabolic pathways during persister cells formation in *Brucella*.

4.1. *Brucella* Rsh affects the enrichment of sulfur metabolism-related proteins during persister cells formation

As shown in Fig. 7A, the enrichment analysis of proteins and metabolites related to sulfur metabolism involving Rsh are presented. The expression levels of sulfur metabolism-related proteins including NAD(P)/FAD-dependent sulfite reductase enriched in ΔRsh strain are lower than compared with WT strain ($P < 0.01$). The levels of sulfur metabolism-related metabolites including L-cysteine in ΔRsh strain are lower than compared with WT strain ($P < 0.01$). In addition, we found the KEGG enrichment map shows that sulfite reductase acts on sulfur donors and can reduce hydrogen sulfide to sulfate with NAD⁺ or NADP⁺ as the acceptor (Fig. 7B). To further confirm whether the (p)ppGpp synthase Rsh promotes sulfur metabolism during the persister cells formation in *Brucella*, sulfur metabolism related indicator was measured. This result showed that the ΔRsh strain exhibited a significant increase in H₂S production compared to WT strain ($P < 0.01$) (Fig. 7C), indicating that the (p)ppGpp synthase Rsh in *Brucella* significantly promotes the consumption of H₂S in sulfur metabolic pathway.

4.2. (P)ppGpp synthetase Rsh regulated *CysI* in *B. abortus* during persister cells formation

To further investigate whether Rsh regulated *CysI* in *B. abortus* during persister cells formation. The β -galactosidase activity assay was performed for assessing promoter activity of *CysI* both WT and ΔRsh strain. We firstly selected the 500-bp fragment of *cysI* upstream for promoter analysis. A promoter fragment of expected size was amplified by PCR using *B. abortus* A19 as template (Fig. 8A). The WT and ΔRsh strain with the *cysI* promoter fragment were designated as WT-*cysIP* and ΔRsh -*cysIP*, respectively. We found a significant reduced activity of *cysI*: LacZ in strain ΔRsh compared with WT strain (Fig. 8B, C) ($P < 0.01$). Our result show that (p)ppGpp synthetase Rsh positively regulates *cysI* promoter in *B. abortus*. And we identified a 500-bp region of upstream of *cysI* initiation codon that is promoter region.

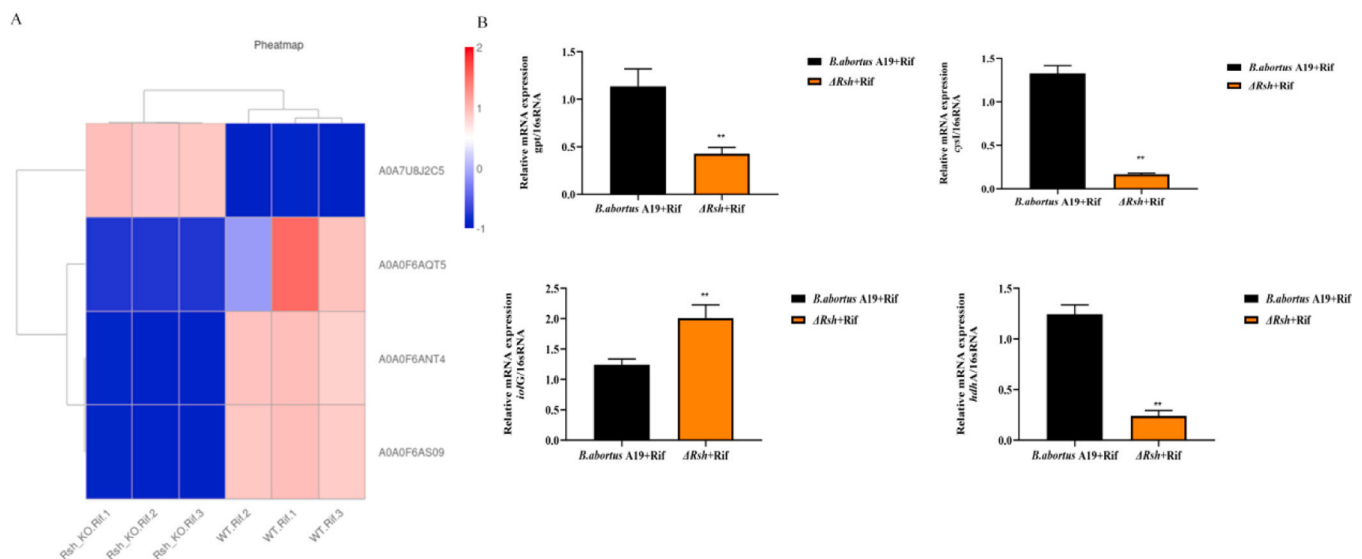


Fig. 6. Validation of integrated omics results by qRT-PCR. (A) The result of qRT-PCR between Δrsh and WT strains during the persister cells formation; (B) The result of integrated omics between Δrsh and WT strains during the persister cells formation; Data are shown as mean $\pm$ SEM. "ns", $P > 0.05$; "**", $P < 0.05$; "***", $P < 0.01$.

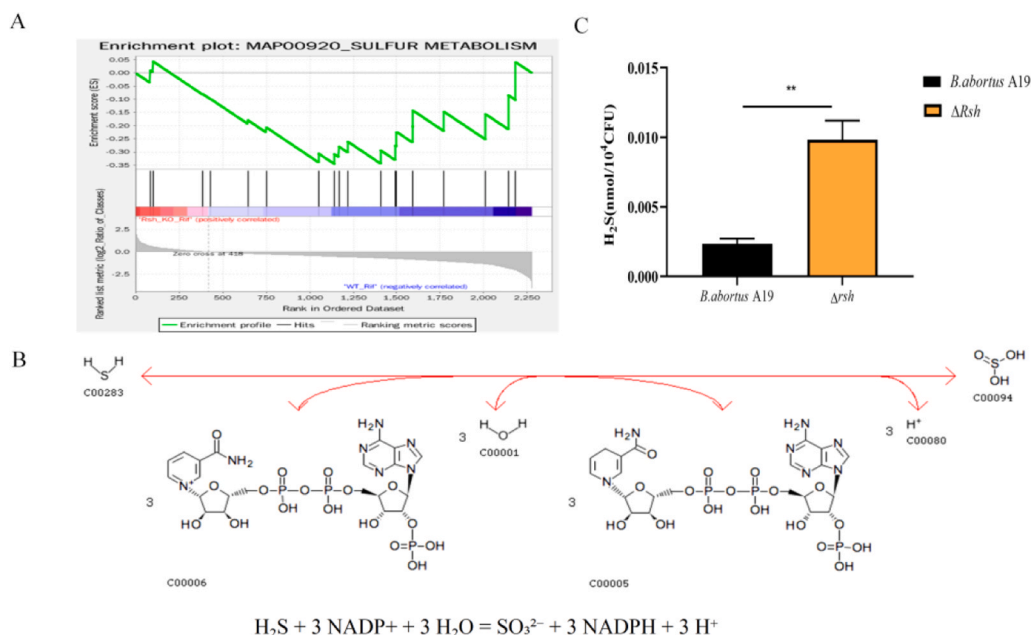


Fig. 7. The Rsh modulates sulfur metabolism and facilitates hydrogen sulfide (H_2S) consumption in *B. abortus*. (A) GSEA enriched sulfur metabolism of all proteins in the comparison pairs; (B) KEGG enrichment pathway results of *cysI* participated in; (C) Consumption of hydrogen sulfide Rsh promoting sulfur metabolism in *B. abortus*. All data provided show the average of three biological replicates. Error bars depict standard deviation. Data are shown as mean $\pm$ SEM. “ns”, $P > 0.05$; “*”, $P < 0.05$; “***”, $P < 0.01$.

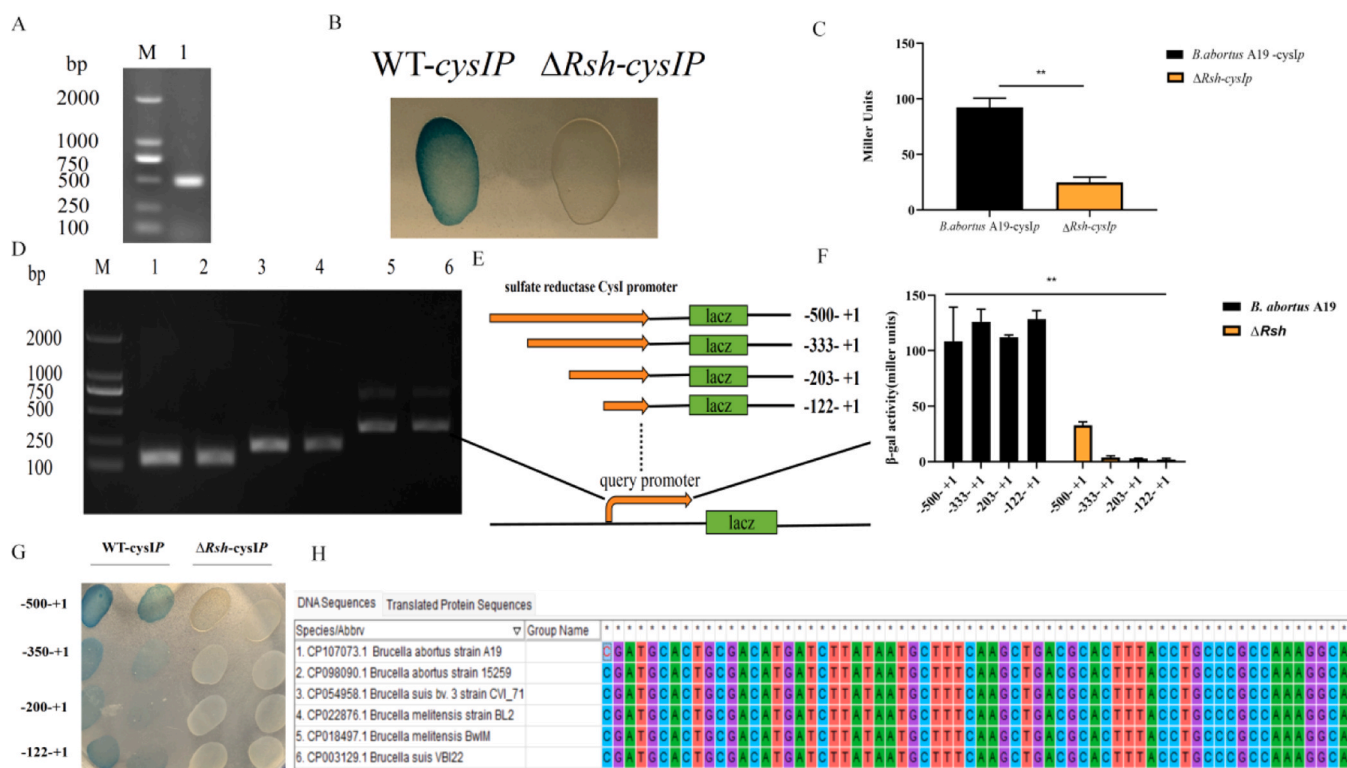


Fig. 8. Rsh positively regulated the *cysI* promoter region in *B. abortus*. (A) PCR amplification of the *cysI* promoter region; (B) Blue-white screening of *cysI* promoter between ΔRsh and WT strains. (C) β -galactosidase activity of *cysI* between ΔRsh and WT strains.

(D) PCR amplification of the *cysI* promoter region; M: DNA marker; 1: p-333; 2: p-333; 3: p-203; 4: p-203; 5: p-122; 6: p-122; (E) Schematic diagram of the *cysI* promoter region; (F) β -galactosidase activity assay; (G) Blue-white screening; (H) Conservation analysis of the *cysI* promoter region. Data are shown as mean $\pm$ SEM. “ns”, $P > 0.05$; “*”, $P < 0.05$; “***”, $P < 0.01$.

To further precisely define the functional promoter region of *cysI*. We generated a series of truncated promoter plasmids of the *cysI* promoter. We amplified the promoter fragment (333 bp, 203 bp, and 122 bp) of

upstream of *cysI* initiation codon by PCR using genomic DNA of *B. abortus* A19 as template (Fig. 8 D). Each truncated fragment was cloned into pBB-Amp-LacZ reporter vector through restriction enzyme-

based ligation, transformation and sequence verification. The successfully constructed plasmids were electroporated into both WT and ΔRsh strains (WT-*cysIP*-500, ΔRsh -*cysIP*-500, WT-*cysIP*-300, ΔRsh -*cysIP*-300, WT-*cysIP*-200, ΔRsh -*cysIP*-200, WT-*cysIP*-100, ΔRsh -*cysIP*-100). The transformants were selected on ampicillin-containing TSB plates. And the promoter activity was assessed through β -galactosidase assays, blue-white screening which is identified the 122 bp fragment as the minimal functional promoter region of *cysI* (Fig. 8 E, F, G). And we found that promoter region of *cysI* is conserved in *Brucella*. (Fig. 8 H). In a word, our result confirmed that promoter region of *cysI* is 122 bp.

4.3. Sulfite reductase *CysI* promotes persister cells formation and sulfur metabolism

Previous studies had demonstrated the (p)ppGpp synthase *Rsh* positively regulates the sulfur metabolism-related *cysI* gene in *B. abortus*. To further investigate whether sulfite reductase *CysI* participate in sulfur metabolism which is associated with persister cells formation *Rsh* involved in *B. abortus*. We constructed a *cysI* deletion strain ($\Delta cysI$) of *B. abortus* A19 by homologous recombination. The upstream (729 bp), downstream (808 bp) homologous arms of *cysI* and kanamycin resistant gene (1373 bp) were amplified by PCR (Fig. 9 A, B and C). The three fragments were then fused to generate the recombinant plasmid 19T-*cysI* (Fig. 9D). This plasmid was electroporated into competent cells of WT strain for constructing *cysI* deletion strain. The *cysI* knockout strain was

screened and identified by PCR amplification (Fig. 9 E). Our result show that we successfully construct the *cysI* deletion strain.

To further confirm the effect of *cysI* on growth of *B. abortus* *in vitro*, growth curves of the *cysI* knockout strain were determined by measuring OD₆₀₀. This result showed no significant difference on growth *in vitro* between $\Delta cysI$ and *B. abortus* A19 in exponential phase, but there was an obvious difference in stationary phase ($P < 0.01$) (Fig. 9 F). In addition, the WT and *cysI* knockout strain were cultured to exponential phase (24 h) and stationary phase (72 h), respectively. The samples were treated with 40 μ g/mL rifampicin for 24 h to assess persister cells formation. The *cysI* deletion strain exhibited a significant reduction in persister cells formation levels either exponential or stationary phases compared to the wild-type strain, ($P < 0.01$) (Fig. 9 G, H). Our result show that sulfite reductase *CysI* also promotes persister cells formation in *B. abortus*.

To further investigate whether *Rsh* promotes sulfur metabolism during persister cells formation, a key sulfur metabolic indicators were measured. The strains (WT and $\Delta cysI$) were cultured to stationary phase (72 h) and then treated with rifampicin for 24 h. Then, the hydrogen sulfide (H_2S) level was measured according to the manufacturer's protocol. The results showed that the H_2S of *cysI* knockout strain exhibited an increased level compared to the wild-type, though the difference was not statistically significant (Fig. 9 I). In a word, these findings suggest that *CysI* facilitates the consumption of hydrogen sulfide in sulfur metabolic pathway in *B. abortus*.

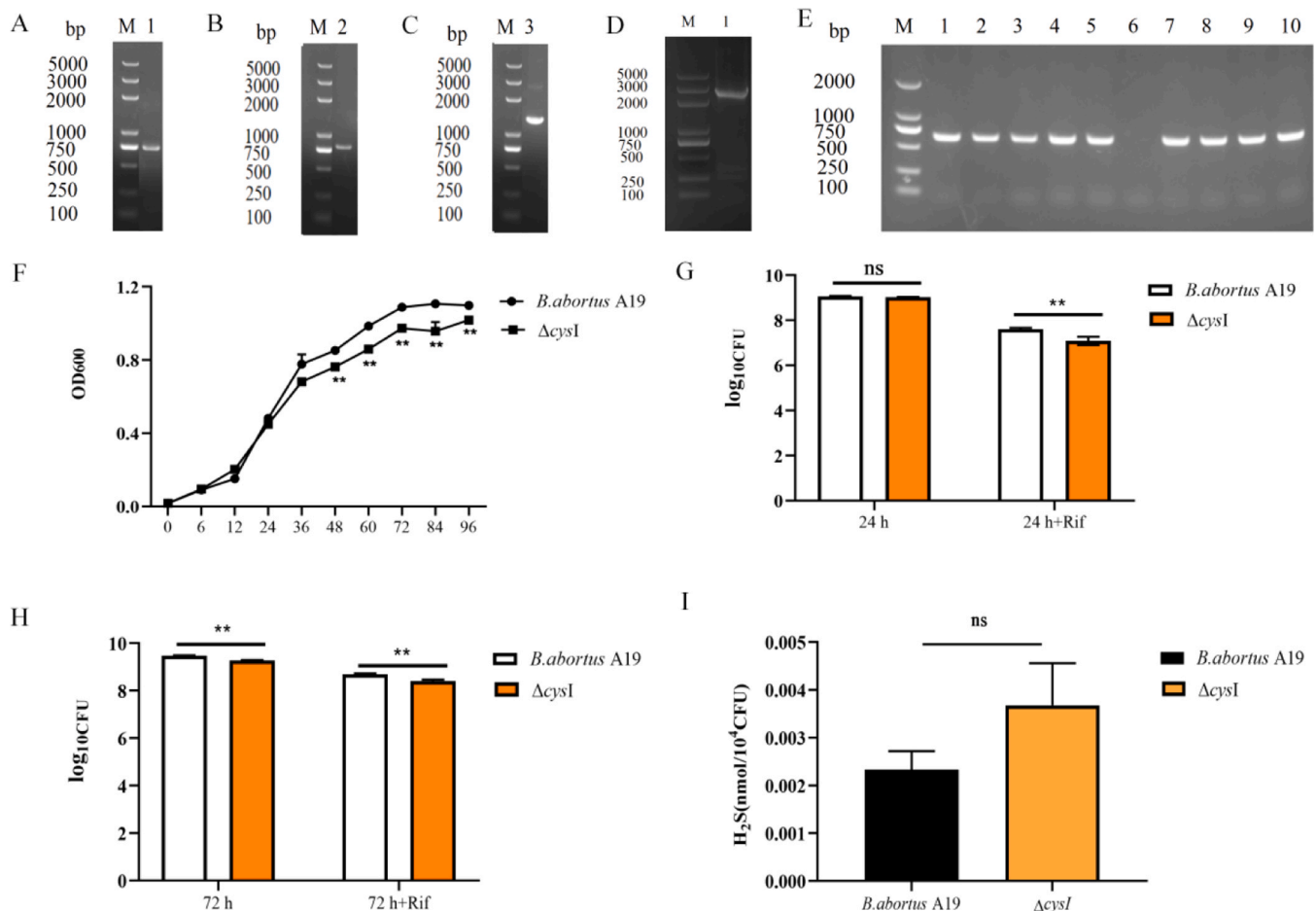


Fig. 9. The *CysI* promotes persister cells formation and sulfur metabolism in *B. abortus*. (A) Upstream fragment of *cysI*. M: DNA marker, 1: upstream; (B) Downstream fragment of *cysI*. M: DNA marker, 2: Downstream; (C) Kanamycin resistance gene. M: DNA marker, 3: Kanamycin resistance gene; (D) PCR identification of 19T-*cysI*. M: DNA marker, 1: construction of *cysI* deletion plasmids; (E) PCR identification of the *Rsh* deletion strain; M: DNA marker 2000, 1–5: positive, 6: candidate strains of the *Rsh* deletion strain, 7–10: positive; (F) Growth curve of *cysI* in *Brucella* (OD₆₀₀); (G) Persister cells formation of *cysI* deletion strain at exponential phase; (H) Persister cells formation of *cysI* deletion strain at stationary phase; (I) Consumption of hydrogen sulfide *cysI* promoting sulfur metabolism in *Brucella*. Data are shown as mean $\pm$ SEM. “ns”, $P > 0.05$; “*”, $P < 0.05$; “***”, $P < 0.01$.

5. Discussion

Most bacterial pathogens are microorganisms capable of colonize permanently their host and establish chronic or recurrent infections in which immune system and antimicrobial therapies struggle to eliminate them. The persister cells are believed to be regarded as a major cause of treatment failure of persistent infections and main culprit behind relapse that make these infections challenging (Niu et al., 2024). Persister cells refer to a subpopulation of bacterial cells and possess the non-heritable character of surviving prolonged that are detected by bacterial cultures exposure to a wide variety of antibiotics (Urbaniec et al., 2022). The persister cells has been experimentally identified for major bacterial pathogens including *Staphylococcus aureus* (Conlon et al., 2016), *Salmonella enterica* (Hill et al., 2021). Our previous studies have demonstrated that *Brucella* can form persister cells that is consistent with Mode's study (Mode et al., 2022). The mechanism of persister cells with focuses on the toxin-antitoxin modules, stringent response, bacterial communication, drug efflux and others. And we have demonstrated that (p)ppGpp synthase Rsh promotes persister cells by regulating toxin-antitoxin system *mbcTA* in *B. abortus* A19 and ATP levels of Δ *rsh* strain increased significantly compared with WT in stationary phase (Liu et al., 2024).

The RNA-sequencing show that persister cells maintains metabolic activity by reprogramming their central carbon metabolic pathways during prolonged intracellular residence within host cells in *S. aureus* (Peyrusson et al., 2020). Beyond carbon metabolism, amino acid metabolism and energy metabolism are also vital for persister cells in a bacterial population (Amato et al., 2014). In this study, we aim to validate whether Rsh promotes persister cells formation by influencing metabolic pathway in *B. abortus* during the rifampicin exposure. Proteomics revealed that Rsh was linked to multiple metabolic pathways during persister cells. These DEPs were enriched in secondary bile acid biosynthesis, thiamine metabolism, vitamin B6 metabolism, D-Glutamine and D-glutamate metabolism, drug metabolism - other enzymes, phosphotransferase system (PTS), purine metabolism, fructose and mannose metabolism, sulfur metabolism and D-Alanine metabolism. Metabolomics revealed that Rsh was linked to multiple metabolic pathways during persister cells. These differential metabolites were enriched in inositol phosphate metabolism, vitamin B6 metabolism, purine metabolism, pyrimidine metabolism, carbon metabolism, phosphotransferase system (PTS), cysteine and methionine metabolism. We integrated analyzed the proteome and metabolome of persister cells during rifampicin exposure to examine icinal metabolites and their regulatory mechanism between WT and Δ *rsh*. We found that 7- α -hydroxysteroid dehydrogenase (WP_002964715.1), IolB protein (WP_002965925.1), inositol 2-dehydrogenase (WP_004682008.1), sulfite reductase (WP_006097741.1) and lithocholic acid, phytic acid, L-cysteine, 4-pyridox acid are key proteins and metabolites in KEGG enrichment that they might play a key regulatory role in persister cells formation of Rsh involved in *B. abortus*. And we confirmed that the L-cysteine and sulfite reductase CysI exhibit a positive correlation in sulfur metabolism in this study. In agreement with our result, the knockout mutants of the sulfite reductase genes (Δ *cysI* and Δ *cysJ*) and the L-cysteine synthase gene (Δ *cysK*) decrease the L-cysteine production in each compared with wild type strain (Nakatani et al., 2012).

Although the (p)ppGpp synthetase Rsh is involved in multiple metabolic pathways during persister cell formation across various bacteria (Haurlyiuk et al., 2015), whether Rsh promotes *Brucella* persister formation through modulating sulfite reductase CysI remains unknown. The sulfite reductase CysI participates in sulfur compound metabolic process which can catalyse the six-electron reduction reactions of sulphite to sulphide and involve in the nonmetallic element sulfur or compounds that contain sulfur, such as the amino acids methionine and cysteine or the tripeptide glutathione (Ostrowski et al., 1989). Here, we firstly demonstrated that Rsh, as a bifunctional (p)ppGpp synthetase, either influenced sulfur metabolism or upregulated expression the CysI

during persister cells formation in *B. abortus*. Notably, we further confirmed the Rsh positively regulated the promoter of *cysI* gene in *B. abortus*. and the promoter region was identified in this study. Our result further shows that the (p)ppGpp synthetase Rsh did influence the sulfite reductase CysI and sulfur metabolism in persister cells formation of *B. abortus*.

Next, we further constructed the deletion strain of *cysI* and found that Δ *cysI* can also significantly reduced persister cells either in exponential phase or stationary phase in *B. abortus*. In addition, the CysI was also involved in the consumption of hydrogen sulfide the sulfur metabolic pathway in *B. abortus*. The production of hydrogen sulfide may help bacteria enter a low-energy metabolic state, thereby tolerating the bactericidal effect of antibiotics in persister cells formation (Shatalin et al., 2011); Furthermore, hydrogen sulfide activates the SOS response which can promote persister cells formation, enabling bacteria to survive under antibiotic stress (Pu et al., 2016); Some metabolites in the sulfur metabolic pathway can also enhance the antibiotic excretion capacity of bacteria. For example, hydrogen sulfide can activate the excretion pump of bacteria and reduce the concentration of antibiotics in bacteria and thereby enhance bacterial tolerance (Pu et al., 2016). The metabolites in the sulfur metabolic pathway could participate in the metabolic reprogramming of bacteria, enabling them to enter a low-metabolic state. For instance, the production of hydrogen sulfide can inhibit the respiratory chain of bacteria and reduce the generation of ATP, thereby putting the bacteria into a low-energy metabolic state and enabling them to tolerate the bactericidal effect of antibiotics. In conclusion, we firstly confirmed that (p)ppGpp synthetase Rsh promotes persister cells formation by regulating sulfur metabolism mediated by sulfate reductase CysI in *B. abortus*. These finding provide additional knowledge on the manufacture of therapeutic metabolites and the eradication of *Brucella* persister cells. Meanwhile, these findings could also provide a theoretical reference for the treatment and eradication of other bacterial persister cells.

6. Conclusion

This study demonstrates that the (p)ppGpp synthetase Rsh promotes persister cell formation in *B. abortus* by regulating sulfur metabolism. Integrated omics analyses reveal that Rsh up-regulates the sulfate reductase CysI and enhances hydrogen sulfide (H₂S) production. And Rsh positively regulates the *cysI* promoter whose core region lies within 122 bp upstream of the coding sequence. In addition, Deletion of *cysI* significantly reduces both persister formation and H₂S levels. We confirm that Rsh drives persister cells formation via *cysI* - mediated sulfur metabolism in *Brucella*. While the primary findings rely on *in vitro* models which may not fully replicate the complex host environment during infection. Future work should validate this regulatory mechanism in relevant animal models. Additionally, the precise signaling cascade and potential interaction partners through which Rsh regulates the *cysI* promoter remain to be elucidated.

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CRedit authorship contribution statement

Yuanhao Yang: Methodology, Investigation, Formal analysis. **Yunyi Zhai:** Software, Methodology, Investigation. **Wei Liu:** Formal analysis, Data curation. **Jialu Chen:** Methodology, Investigation. **Chenyang Cui:** Supervision, Software, Resources, Methodology, Investigation, Formal analysis, Data curation. **Aihua Wang:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Xiaofang Liu:** Writing – review & editing, Writing – original draft, Supervision, Software, Methodology, Investigation, Formal analysis, Data

curation, Conceptualization. **Hui Wang:** Methodology, Investigation. **Pingping Wang:** Validation, Supervision, Software, Resources, Methodology, Investigation, Formal analysis, Data curation. **Zheng Dong:** Methodology, Investigation, Formal analysis. **Yaping Jin:** Writing – review & editing, Writing – original draft, Validation, Resources, Methodology, Investigation. **Dong Zhou:** Supervision, Software, Resources.

Declaration of Competing Interest

The authors declare that there is no known competing interests or personal relationships that could have appeared to influence the work reported in this paper.

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