Comparative proteomic and genomic analyses of *Brucella abortus* biofilm and planktonic cells

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Abstract. The present study aimed to explore the differences in protein and gene expression of *Brucella abortus* cultured under biofilm and planktonic conditions. The proteins unique to biofilms and planktonic *B. abortus* were separated by two-dimensional (2-D) electrophoresis and then identified by matrix-assisted laser desorption/ionization-tandem time of flight-mass spectrometry (MALDI-TOF/TOF-MS). High-throughput sequencing and bioinformatic analyses were performed to identify differentially expressed genes between *B. abortus* cultured under biofilm and planktonic conditions. The proteins and genes identified by proteomic and genomic analyses were further evaluated via western blot and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analyses. 2-D electrophoresis identified 20 differentially expressed protein spots between biofilms and planktonic cells, which corresponded to 18 individual proteins (12 downregulated and 6 upregulated) after MALDI-TOF/TOF-MS analysis, including elongation factor Tu and enolase. RT-qPCR analysis revealed that all of the 18 genes were downregulated in biofilms compared with planktonic cells. Western blot analysis identified 9 downregulated and 3 upregulated proteins. High-throughput sequencing and bioinformatic analyses identified 14 function and pathway-associated genes (e.g., BAbS19_I14970). RT-qPCR analysis of the 14 genes showed that they were upregulated in biofilm compared with in planktonic state. In conclusion, these differentially expressed genes may play important roles in bacterial defense, colonization, invasion, and virulence.

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Introduction

Brucella is a group of α-2 *Proteobacteria* that has a great impact on animal and human health worldwide (1). Infection with *Brucella* results in brucellosis, one of the most common bacterial zoonotic diseases in humans and cattle globally (2). An estimated 500,000 cases of brucellosis occur each year globally (3). Brucellosis can not only lead to the reproductive failure of livestock but also decrease human productivity. As a result, *Brucella* species have been regarded as potential agricultural, animal husbandry, civilian and even bioterrorism agents (4,5).

During chronic infection, bacteria can organize themselves into matrix-enclosed microcolonies or aggregates, termed biofilms (6,7). Biofilm formation is a critical survival mechanism for bacteria in the environment (8). Altered gene and protein expression in biofilms is responsible for cell virulence, adherence and drug resistance (9,10). Additionally, biofilm-grown microorganisms have an inherent lack of susceptibility to antibiotics (11-13). *Brucella melitensis* (*B. melitensis*) has been suggested to form biofilms during its life cycle (14). Wild-type *B. abortus* can also develop biofilms under nutritionally deficient, microaerobic conditions (15). Previous studies have investigated several virulence and drug resistance-associated proteins from planktonic *Brucella*, such as lipopolysaccharide (16), B lymphocyte mitogen (17) and outer membrane proteins (18). However, numerous different types of bacterial infections are presumed to be due to bacteria growing in a biofilm state including cystic fibrosis-related lung infections, biomaterial-related infections, chronic wounds,

and endocarditis. The USA National Institutes of Health has estimated that 80% of all infections are biofilm-related (19). However, little is known concerning the proteins associated with biofilm-mediated infections.

The present study investigated differences in protein and gene expression of *B. abortus* cultured under biofilm compared with planktonic conditions. The differential proteins unique to biofilms and planktonic *B. abortus* were identified by employing two-dimensional (2-D) electrophoresis and matrix-assisted laser desorption/ionization-tandem time of flight-mass spectrometry (MALDI-TOF/TOF-MS) analyses. The differential genes were identified by high-throughput sequencing and bioinformatic analysis. Findings of the current study may help to understand the underlying molecular mechanisms that control biofilm formation in *B. abortus*.

Materials and methods

Bacterial strains and culture conditions. B. abortus strain isolate A3313 was used in this study, which was isolated from the abortus of dairy cows in Hohhot District, Inner Mongolia, China. The A3313 strain was grown in *Brucella* broth medium (BD Biosciences) at 37˚C with 5% CO_2.

All the experiments related to the cultivation of *Brucella* and its biofilms, as well as the operation of viable bacteria were conducted in a Biosafety Level 3 Laboratory in the College of Veterinary Medicine, Huazhong Agricultural University (Wuhan, China). For the experiments of electron microscope observation, 2-D electrophoresis, high-throughput sequencing and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis, the *Brucella* and its biofilm were effectively inactivated with glutaraldehyde or bacterial lysate before being removed from the Biosafety Level 3 Laboratory.

Biofilm culture and microscopic observation. Brucella broth was added to 6-well cell culture plates. A clean coverslip sterilized by autoclaving (121˚C, 20 min) was then put in each well, and the A3313 bacterial suspension was inoculated on the coverslip at 2 ml/well. The culture plate was placed at 37˚C with 5% CO_2, and the culture medium was changed every 48 h until a complete biofilm was formed. The coverslips were taken out, gently washed three times with phosphate-buffered saline (PBS; 30 mM, pH 7.4), and then fixed immediately with 2.5% glutaraldehyde for 6-8 h at 4˚C. After being washed with PBS, biofilms were stained with 200 μl 1% crystal violet (Ding Bei Biological Technology Co., Ltd.) for 20 min at room temperature. These procedures were conducted to protect biofilms from falling off from the abiotic surfaces. The biofilms were observed under a phase-contrast light microscope (magnification, x20) (Axiovert 135; Zeiss AG).

For scanning electron microscope observation, biofilms were fixed with 2% osmic acid at room temperature until black. After washing with 0.1 M PBS for three times, the samples underwent sequential dehydration with gradient ethanol solutions (30, 50, 70 and 90%) for 15 min each. Then, samples were dehydrated with 100% ethanol twice (15 min each), and dried with a critical point dryer. The dry samples were fixed on the sample stage with conducting resin, and sprayed gold with ion sputtering equipment (15 mA) for 2 min. The biofilms were observed under a scanning electron microscope.

2-D electrophoresis. Biofilms and planktonic bacteria were used for 2-D electrophoresis. For biofilm culture, the A3313 strain was grown in *Brucella* broth medium in Petri dishes at 37˚C and 5% CO_2. The culture medium was changed every 48 h for 8 days. After removing the supernatant, the plates were rinsed twice with PBS. Biofilms were detached by scraping. Planktonic *B. abortus* was cultured in the same condition. The culture medium was collected and centrifugally washed twice with PBS. The planktonic *B. abortus* was resuspended with PBS.

Protein was precipitated as previously described (10,20). The biofilm and planktonic bacteria were harvested by centrifugation (6,000 x g) at 4˚C for 10 min. The bacteria were washed four times with a solution containing 3 mM KCl, 68 mM NaCl, 9 mM NaH_2PO_4 and 115 mM KH_2PO_4, and then resuspended in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 10 mM DTT and 2% Pharmalyte pH 3-10) with protease inhibitor. After ultrasonic decomposition on ice at 40% maximum power for 90 cycles (5 sec on, 10 sec off; the ultrasound conditions were the same for biofilm disruption and planktonic cells), unbroken cells and the cell debris were incubated at 25˚C for 30 min, and removed by centrifugation (10,000 x g) for 30 min at 25˚C. Proteins in the supernatant were precipitated in 10% trichloroacetic acid (TCA) on ice for 30 min. Precipitated proteins were washed with chilled acetone and then centrifuged at 10,000 x g for 10 min at 4˚C. The air-dried proteins were dissolved in 400 μl sample preparation solution [2-D lysate: 9.5 M urea, 4% CHAPS, 2% (v/v) ampholytes, and 60 mM DTT; 10 μl 50 XProtein Inhibitor Cocktail Set I (Merck KGaA) was added into 500 μl 2-D lysate] at 25˚C for 30 min and centrifuged at 10,000 x g for 20 min at 25˚C. The protein concentration was determined via a Bradford protein assay.

Proteins (200 μg) were separated by 2-D electrophoresis. Isoelectric focusing for the first dimension was performed in precast Immobiline DryStrips (GE Healthcare Life Sciences) with a nonlinear gradient of pH 3 to 10 in an Ethan IPGphor Isoelectric Focusing System (GE Healthcare Life Sciences) according to the manufacturer's instructions. The electrophoresis conditions were 30 V for 12 h, 500 V for 1 h, 1,000 V for 1 h, 8,000 V for 8 h and 500 V for 4 h. The second dimension (SDS-PAGE) was conducted vertically in a Hofer SE 600 (GE Healthcare Life Sciences) using 12.5% polyacrylamide gels. The resolved proteins were then stained with silver for 30 min at room temperature and scanned with UMax Powerlook 2110Xl (GE Healthcare Life Sciences). All experiments were performed in triplicate. The gels were analyzed with the Image Master Platinum version 5.0 software (GE Healthcare Life Sciences). The normalized protein amount for each protein spot was calculated as the ratio of that spot volume to the total spot volume on the gel. Significant differences between two groups were determined using the Student's t-test, and a fold change ≥1.5 was considered the threshold value.

MS analysis. The differentially expressed protein spots were excised from the 2-D gels and then subjected to MALDI-TOF/TOF-MS analysis (Shanghai Applied Protein Technology Co. Ltd.). Before MS analysis, the protein spots were digested in-gel by 0.1 mg/ml trypsin for 2 h at 37˚C

and desalinated by Ziptip (EMD Millipore). The digested samples were then freeze-dried. After being re-dissolved, 1 μl samples were spotted on the target plate (Immobiline DryStrips; 13 cm, pH 3-10 NL) with air drying, and then 0.5 μl supersaturated α-cyano-4-hydroxycinnamic acid matrixes (Sigma-Aldrich; Merck KGaA) prepared in 50% acetonitrile with 0.1% trifluoroacetic acid were spotted and naturally dried. All MALDI-MS and MS/MS data were acquired in the positive reflectron ion mode on a 4800 Plus MALDI TOF/TOF Analyzer (AB Sciex LLC). Samples were irradiated by a 355-nm Nd:YAG laser (355 nm), and the acceleration voltage was 2 kV. The scanned area for MS was 800-4,000 Da, and the parent ion with signal:noise ratio >50 was selected for MS/MS analysis. Data from MALDI-MS and MS/MS were subjected to a Database Search from NCBI or Uniprot (NCBI preferentially) using Mascot version 2.2 software (Matrix Science), and a Mascot score was calculated. The MS/MS spectra were subjected to similarity searches using the BLASTX algorithm. The parameter settings were trypsin digestion, fixed modification of carbamidomethyl, dynamical modification of oxidation (M), unrestricted protein mass, peptide mass tolerance for monoisotopic data of ±100 ppm, fragment mass tolerance of ±0.4 Da, peptide charge state of 1+, and one maximum missed cleavage.

High-throughput sequencing. RNA sequencing of the biofilms and planktonic bacteria were performed at Genergy Biotechnology Co., Ltd. Total RNA was isolated and examined by NanoDrop spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.) and 1% agarose gel electrophoresis (Tanon Science and Technology Co., Ltd.). An RNA library was then constructed and sequenced on the Illumina HiSeq 2500 platform (Illumina, Inc.). The data are available at National Center for Biotechnology Information under the accession numbers SRX1604658 (biofilm conditions; http://www.ncbi.nlm.nih.gov/sra/?term=SRX1604658) and SRX1604659 (planktonic conditions; http://www.ncbi.nlm.nih.gov/sra/SRX1604659/).

Bioinformatics analyses. The raw reads were evaluated using RSeQC 2.3.2 (http://rseqc.sourceforge.net/) (21), and sequence alignment was conducted with TopHat 2.0.10 (http://ccb.jhu.edu/software/tophat/index.shtml) (22). The remaining reads were used for the following analyses.

The mRNA expression levels were detected based on Cufflinks 2.2.1 (http://cole-trapnell-lab.github.io/cufflinks/) software (23). Differentially expressed mRNAs were defined based on strict criteria [q value ≤0.05, and log2(fold change) ≥1] using the Cuffdiff program (23). Functional annotation of differentially expressed genes was carried out using various bioinformatics procedures, including Gene Ontology (GO) (24) and Kyoto Encyclopedia of Genes and Genomes (KEGG, Kolmogorov-Smirnov value <0.05) (25).

RT-qPCR. The mRNA levels of differential proteins identified through 2-D electrophoresis and function-associated genes identified by high-throughput sequencing and bioinformatics analyses were detected via RT-qPCR according a previously described method (26). In brief, total RNA was isolated from biofilms and planktonic cells ($1x10^6$ cells) of the A3313 strain using a Takara MiniBEST Universal RNA Extraction kit (Takara Biotechnology Co., Ltd.). The RNA quality and concentration were determined by a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Inc.). The RNA was reverse-transcribed into cDNA using a PrimeScript RT reagent kit (Takara Biotechnology Co., Ltd.; 42˚C for 15 min). The 16S rRNA housekeeping gene was amplified as the internal control. The specific primers are listed in Table SI. The SYBR Green PCR method was performed using an SYBR Premix Ex Taq kit (Takara Biotechnology Co., Ltd.). The qPCR reaction was carried out under the following conditions: 95˚C for 30 sec, 40 cycles of 95˚C for 3 sec and 60˚C for 30 sec. Relative mRNA expression ratios of selected genes were calculated with the $2^{-\Delta\Delta Cq}$ method (27). The experiment was performed with three replications.

Western blot analysis. The levels of differentially expressed proteins identified through 2-D electrophoresis, described above, were measured by western blotting. Briefly, cells were lysed in RIPA buffer (Sigma-Aldrich; brand of Merck KGaA), and the protein concentration was measured using BCA Protein Assay Kit (Thermo Fisher Scientific, Inc.). Protein samples (20 μg) were separated using 12% SDS-PAGE and transferred onto a polyvinylidene fluoride membrane (GE Healthcare), and the membrane was blocked with 100 mM Tris, 150 mM NaCl, 0.05% Tween-20 (TBST), containing 5% dry milk powder for 2 h at room temperature. Then, the blocked membrane was incubated with sera from primary antibodies [hypothetical protein BAbS19_I16470 (1:1,000; cat. no. orb309412; Biorbyt Ltd.); chaperone protein DnaJ (1:1,000; cat. no. PA3-018; Invitrogen; Thermo Fisher Scientific, Inc.); elongation factor Tu (1:1,000; cat. no. ab210089; Abcam); Chaperonin Cpn60/TCP-1 (1:1,000; cat. no. 3094R-100; BioVision, Inc.); polyprenyl synthetase (1:1,000; cat. no. ab80647; Abcam); periplasmic binding protein (1:1,000; cat. no. M30934-1; Wuhan Boster Biological Technology, Ltd.); enolase (1:1,000; cat. no. sc-271384; Santa Cruz Biotechnology, Inc.); acetyl-CoA carboxylase, a subunit (1:1,000; cat. no. MAB6898; R&D Systems, Inc.); tryptophanyl-tRNA synthetase (1:1,000; cat. no. ab31536; Abcam); aspartate-semialdehyde dehydrogenase (1:1,000; cat. no. EM1708-10a; Jingke Huaxue; exosporium protein B (1:1,000; cat. no. ab92932; Abcam); enoyl-(acyl carrier protein) reductase (1:1,000; cat. no. abx109426; Abbexa Ltd.); Omp16 (1:1,000; cat. no. ab93127; Abcam)] for 2 h at room temperature and then incubated with horseradish peroxidase-labeled secondary antibodies (1:5,000; cat. no. 29139; Invitrogen; Thermo Fisher Scientific, Inc.) in blocking buffer for 1 h at room temperature. After washing with 0.05% Tween-20 (TBST), the membranes were incubated with DAB substrate (Tiangen Biotech Co., Ltd.) for 10 min at room temperature. Outer membrane protein 16 was used as a loading control. The western blot bands were visualized using the Millipore ECL Western Blotting Detection System (EMD Millipore).

Statistical analysis. All experiments were repeated three times except for high-throughput sequencing, and the results were presented as the mean ± standard deviation. Statistical analyses were performed using GraphPad 6.0 (GraphPad

Software, Inc.). P-values were calculated using Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Biofilm observation. Biofilm formation was observed under a phase-contrast light microscope. A large number of bacteria adhered to the coverslips and formed a community (Fig. 1). The structure of biofilms on coverslips was observed under a scanning electron microscope. The biofilms formed flake or microcolony clusters on the coverslips. Colonies were wrapped together by the mucus they secreted, forming an uneven, dense membrane structure (Fig. 2).

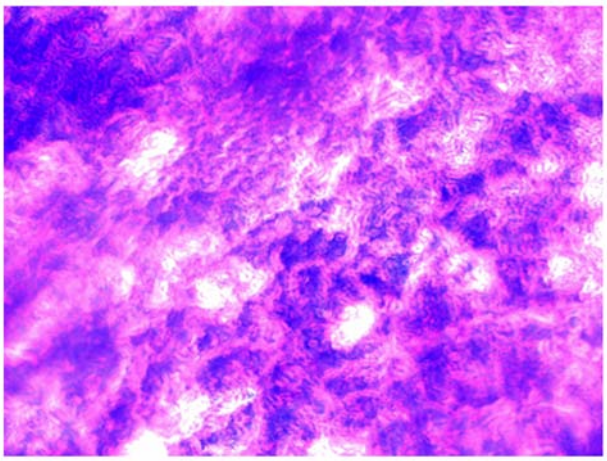

Figure 1. Observation of *Brucella abortus* strain isolate A3313 biofilm by crystal violet staining under a phase-contrast light microscopy. Magnification, x200.

Comparative proteomics. A total of 1,930 protein spots were detected in all gels. The representative 2-D electrophoresis images of 20 differentially expressed protein spots (7 upregulated and 13 downregulated protein spots) between biofilms and planktonic cells are presented in Fig. 3. Most proteins were distributed in the range of isoelectric point 4-7. MALDI MS and MS/MS analysis identified 20 protein spots corresponding to 18 individual proteins, including 6 upregulated (including catalase, extracellular solute-binding protein and ubiquinol-cytochrome C reductase iron-sulfur subunit) and 12 downregulated ones (including elongation factor Tu, enolase, isocitrate dehydrogenase and Chaperonin Cpn60/TCP-1; Table I).

RT-qPCR and western blot analysis. The mRNA and protein expression levels of the 18 identified proteins in 2-D electrophoresis were detected by RT-qPCR and western blot analyses, respectively. RT-qPCR analysis revealed that all of the 18 genes were downregulated in biofilms compared with planktonic cells, including elongation factor Tu (fold change = -100.0), enolase (fold change = -8.6), isocitrate dehydrogenase (fold change = -9.9), and Chaperonin Cpn60/TCP-1 (fold change = -12.5; Fig. 4A). The consistency rate with the 2-D electrophoresis results at the transcriptional level was 66.67% (12/18). Thus, the proteins of the 12 genes that had consistent expression trends in 2-D electrophoresis and RT-qPCR were further detected by western blot analysis. The results showed that 9 proteins were significantly downregulated and 3 were upregulated in biofilms (Fig. 5). Therefore, the 9 downregulated proteins were considered differentially expressed proteins between biofilms and planktonic cells of the A3313 strain. The fold changes of the 18 mRNAs are shown in Table II.

High-throughput sequencing and bioinformatics analyses. A total of 22,039,653 and 37,506,048 reads were generated from biofilms and planktonic cells, respectively. After pre-processing, 16,624,091 and 34,222,222 aligned reads were obtained from biofilms and planktonic cells respectively.

Based on the thresholds of q value ≤0.05 and log2(fold change) ≥1, 157 differentially expressed mRNAs were identified. These mRNA species were grouped in three categories defined by GO, including biological processes (including protein glycosylation, nitrogen compound metabolic process, and cell wall organization), cellular compartment (such as integral component of membrane, plasma membrane, and cytoplasm), and molecular function (including DNA binding, ATP binding, and oxidoreductase activity; Fig. 6A). In addition, these differentially expressed mRNAs were significantly involved in six pathways (annotated by KEGG), including RNA degradation, sulfur metabolism, butanoate metabolism, aminoacyl-tRNA biosynthesis, aminobenzoate degradation, and selenocompound metabolism (Fig. 6B).

Confirmation of function and pathway-associated genes. RT-qPCR was performed to confirm 14 function and pathway-associated genes identified by bioinformatics analyses, including BAbS19_I10210 [log2(fold change) = 2.67], BAbS19_I13070 [log2(fold change) = 2.51], BAbS19_I02060 [log2(fold change) = 1.92], BAbS19_I03220 [log2(fold change) = 2.79], and the results demonstrated that all of the 14 genes were upregulated in biofilms, in accordance with the sequencing results (Fig. 4B). For instance, the fold changes for the genes above (BAbS19_I10210, BAbS19_I13070, BAbS19_I02060, and BAbS19_I03220) were 13.9, 1.4, 2.3 and 9.9, respectively (Table III). These genes were considered differentially expressed genes between biofilms and planktonic cells of A3313 strain.

Discussion

Bacteria can produce an extracellular matrix that helps them adhere to inert or biological surfaces. Bacteria that colonize different surfaces and invade susceptible hosts to cause infections predominantly grow in biofilms (28). Biofilm formation is a developmental process characterized by altered expression of structural and regulatory genes (28). Most bacteria grow in biofilms, and only a small portion grow in planktonic mode (29). There have been previous studies regarding the differences between biofilms and planktonic cells for bacteria, including *Lactobacillus plantarum* (30), swine *Brodetella bronchiseptica* (31), *Porphyromonas gingivalis* (32) and *Clostridium perfringens* (33). Nevertheless, the majority of the studies focus only one aspect, either proteomic analysis or transcriptomic analysis. In the present study, the differences in both protein and gene expression levels in *B. abortus* cultured

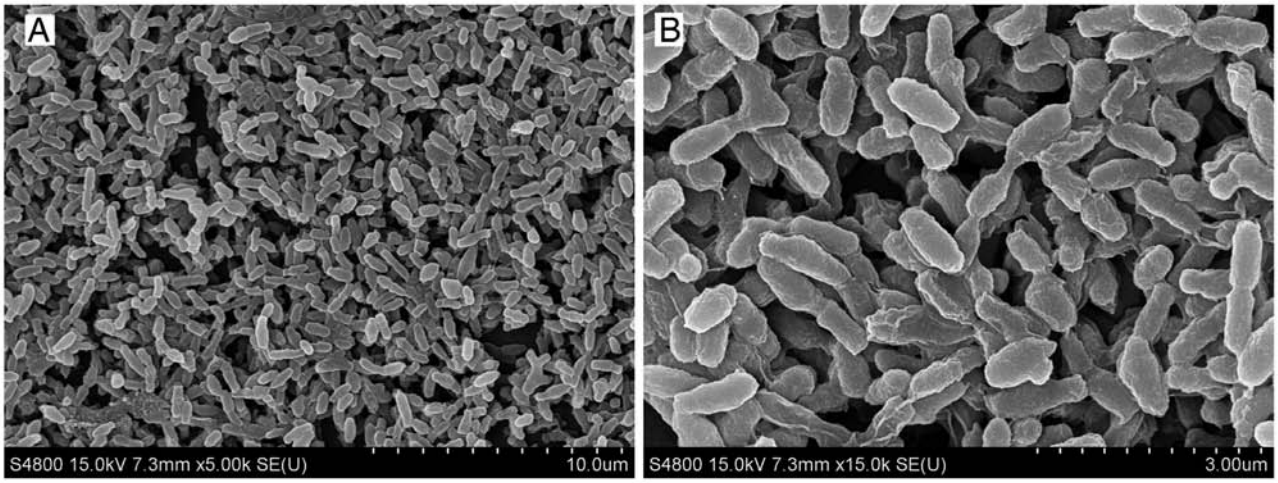

Figure 2. Scanning electron microscope images of *Brucella abortus* strain isolate A3313 biofilm. Magnifications, (A) x1,500 and (B) x4,000.

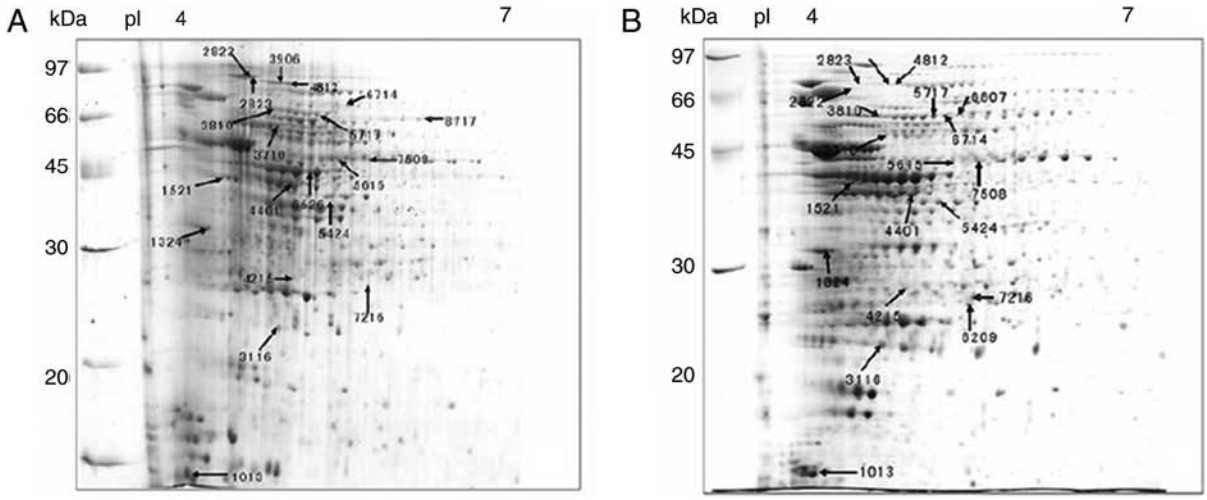

Figure 3. 2-D electrophoresis patterns of *Brucella abortus* A3313 from whole cell lysate proteins. *B. abortus* was cultured in biofilm or planktonic conditions, and the proteins were separated via 2-D electrophoresis. The proteins were separated in the first dimension by IEF and in the second dimension by SDS-PAGE. Molecular weight markers are shown in the left lane (kDa). (A) Protein pattern in the biofilm culture. (B) Protein pattern in the planktonic culture. 2-D, two-dimensional; IEF, isoelectric focusing; pI, isoelectric point.

under either biofilm or planktonic conditions were analyzed. A total of 9 downregulated proteins under conditions of biofilm growth were identified by proteomics, and 14 upregulated genes were identified in biofilm via high-throughput sequencing.

Bacteria in biofilms exhibit persistence in spite of sustained host defense (8); however, little is known regarding the host immune response to biofilm infections. The protein expression in biofilms grown *in vivo* is difficult to study due to the difficulty of extracting bacterial proteins from *in vivo* biofilms (10). The present study separated proteins via 2-D electrophoresis and analyzed with MALDI-TOF/TOF-MS to identify the differentially expressed protein spots, which included elongation factor Tu, enolase, chaperone protein DnaJ and periplasmic binding protein.

Among the differentially expressed protein spots, elongation factor Tu had a higher fold change. It was also found to have the greatest fold change in mRNA expression, which suggested the potential role of elongation factor Tu in *B. abortus*. Elongation factor Tu is one of the most abundant proteins in bacterial cells, involved in critical steps in protein biosynthesis and forming structural filaments *in vitro* (34). It has been observed on the surface of several pathogenic bacteria, including *Burkholderia pseudomallei* and the closely-related *Pseudomonas aeruginosa* (35,36). It has also been demonstrated that elongation factor Tu may play a role as a bacterial virulence factor. Barel *et al* (37) reported that elongation factor Tu can facilitate invasion of host cells by *Francisella tularensis* via interaction with nucleolin. Notably, it also acts as a biofilm component in *Serratia aureus* (38). Thus, it was hypothesized that elongation factor Tu may be associated with virulence in *B. abortus* biofilm.

Enolase is an enzyme involved in the glycolytic pathway, catalyzing the reversible conversion of 2-phosphoglycerate to phosphoenolpyruvate (39). Enolase was previously regarded as a soluble glycolytic enzyme, present in cytosol exclusively (39). Enolase has been found to be a multifaceted protein with sub-cellular localizations and diverse biological functions (40,41). It acts as a plasminogen receptor on the cell

Table I. Differentially expressed proteins in the *Brucella abortus* A3313 biofilm, identified by MALDI-TOF/TOF-MS analysis.

Target no.	Spot no[a]	Protein identified	BLASTX similarity matched protein/species/identify score	Theoretical MW/pI[b]	Experimental MW/pI	Mascot score[c]	No. of peptides matched[d]	Coverage (%)[e]	Fold change[f]		
									Mean	Standard deviation	P-value
E11	1916	gil189024835	hypothetical protein BAbS19_I16470	17153.2/4.96	18000/5.20	102	5	60	-6.84	0.06	0.005
E12	873	gil189020752	DnaJ, chaperone protein DnaJ	41595.3/6.51	43000/7.10	336	13	8	-5.55	0.14	0.003
E13	1275	gil189019956	elongation factor Tu	42749/5.29	35000/5.10	132	4	37	-4.32	0.15	0.005
J12	534	gil189020977	Chaperonin Cpn60/TCP-1	57479.4/5.08	60000/5.20	133	14	25	-3.16	0.17	0.008
E15	998	gil189023639	polyprenyl synthetase	36201.5/5.04	42000/5.70	309	13	38	-2.39	0.12	0.015
E16	1054	gil189022660	Periplasmic binding protein	37595.3/5.85	41000/6.00	105	12	52	-2.19	0.17	0.006
E17	701	gil189019858	Enolase	45404.2/5.03	49000/5.20	430	15	57	-1.95	0.05	0.030
E18	1220	gil189020664	Acetyl-CoA carboxylase, a subunit	35075.2/6.14	35000/7.10	659	20	39	-1.95	0.09	0.014
E19	1007	gil189023395	tryptophanyl-tRNA synthetase	39230.9/5.49	42000/6.20	109	12	43	-1.90	0.09	0.007
E20	875	gil189022544	aspartate-semialdehyde dehydrogenase	37701.4/5.37	43000/6.10	131	9	38	-1.88	0.06	0.002
E21	1481	gil189020611	ExsB protein	25638.7/6.1	30000/7.30	178	11	40	-1.82	0.10	0.014
C24	1378	gil189023658	enoyl-(acyl carrier protein) reductase	29205/5.61	33000/7.00	191	7	23	-1.76	0.04	0.024
E4	467	gil189022983	catalase	56525.1/6.52	56000/8.00	423	18	35	2.41	0.26	0.011
E6	984	gil189022336	extracellular solute-binding protein	39181.9/4.88	42000/5.00	316	10	31	1.78	0.01	0.002
E7	1666	gil189024657	Ubiquinol-cytochrome C reductase iron-sulfur subunit	20053.9/5.35	25000/5.60	162	6	36	1.70	0.04	0.032
E8	2126	gil189020978	Chaperonin Cpn10	10386.6/5.41	12000/5.80	98	8	69	1.68	0.05	0.004
E9	1308	gil189024343	Tetracycline resistance protein TetB	34252.1/6.92	32000/5.80	390	10	28	1.67	0.03	0.020
E10	1775	gil189019259	Ribosomal protein L9	20957.7/4.86	22000/5.70	353	14	57	1.51	0.09	0.032

[a]GenInfo Identifier number in National Center for Biotechnology Information. [b]Theoretical isoelectric point was calculated using AnTheProt, an integrated protein sequence analysis software (http://anthe-prot-pbil.ibcp.fr/). [c]Mascot score obtained for the peptide mass fingerprint. The significance threshold was 70. [d]Number of peptides that match the predicted protein sequence. [e]Percentage of predicted protein sequence covered by matched peptides. [f]Differential protein expression (fold change) of corresponding protein between *Brucella abortus* planktonic and biofilm proteome. MALDI-TOF/TOF-MS, matrix-assisted laser desorption/ionization-tandem time of flight-mass spectrometry; MW, molecular weight; pI, isoelectric point.

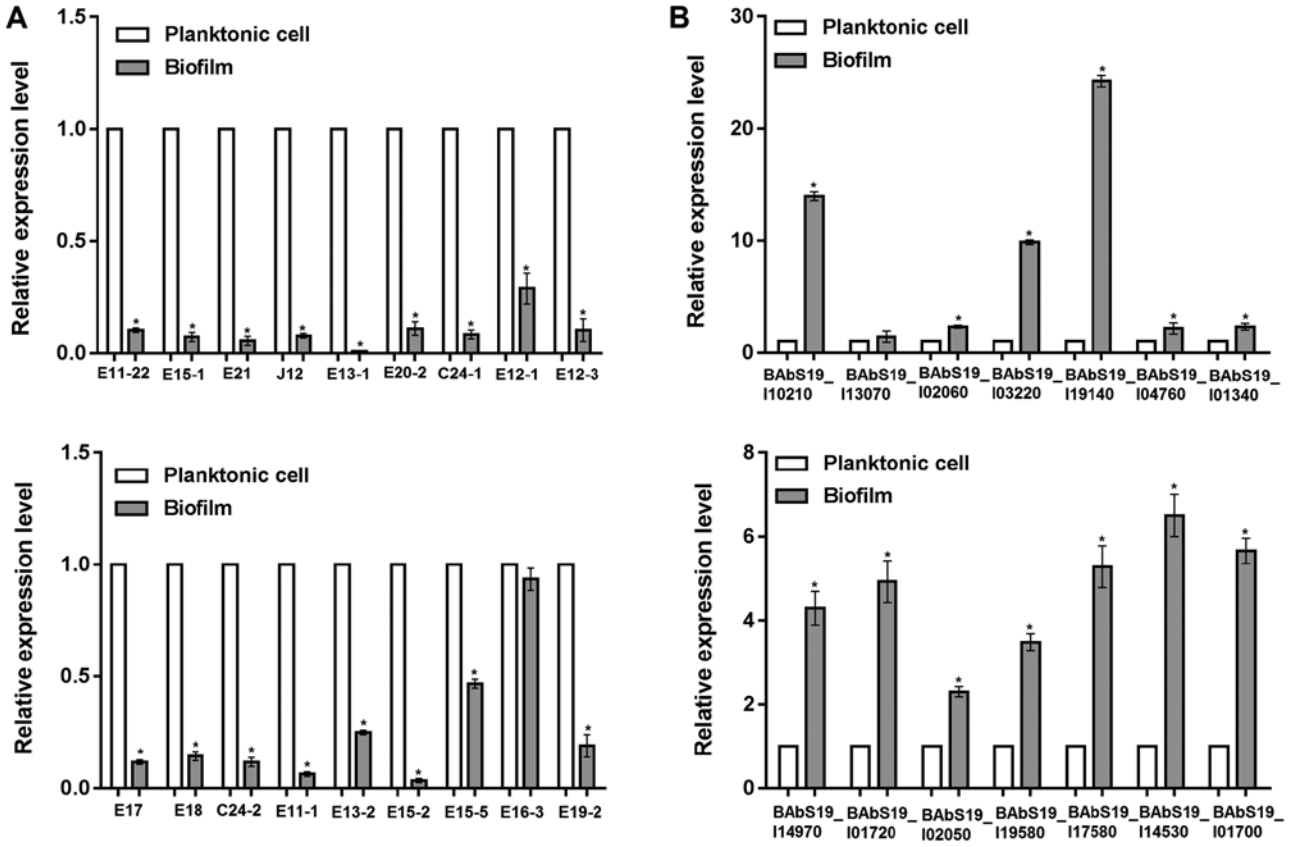

Figure 4. Differential expression of mRNAs between *Brucella abortus* cultured under planktonic or biofilm conditions. The relative expression levels of (A) 18 genes encoding proteins identified by 2-D electrophoresis and (B) 14 genes identified via high-throughput sequencing. 2-D, two-dimensional. *P<0.05 vs. control (planktonic cells). E11-22, hypothetical protein BAbS19_I16470; E15-1, polyprenyl synthetase; E21, ExsB protein; J12, Chaperonin Cpn60TCP-1; E13-1, elongation factor Tu; E20-2, aspartate-semialdehyde dehydrogenase; C24-1, enoyl-(acyl carrier protein) reductase; E12-1, DnaJ, chaperone protein DnaJ; E12-3, isocitrate dehydrogenase; E17, Enolase; E18, Acetyl-CoA carboxylase, alpha subunit; C24-2, putative sulfite oxidase subunit YedY; E11-1, Bacterial protein export chaperone SecB; E13-2, Antifreeze protein, type I; E15-2, Lactatemalate dehydrogenase; E15-5, Phosphoribosylformylglycinamidinecyclo-ligase; E16-3, Periplasmic binding protein; E19-2, tryptophanyl-tRNAsynthetase.

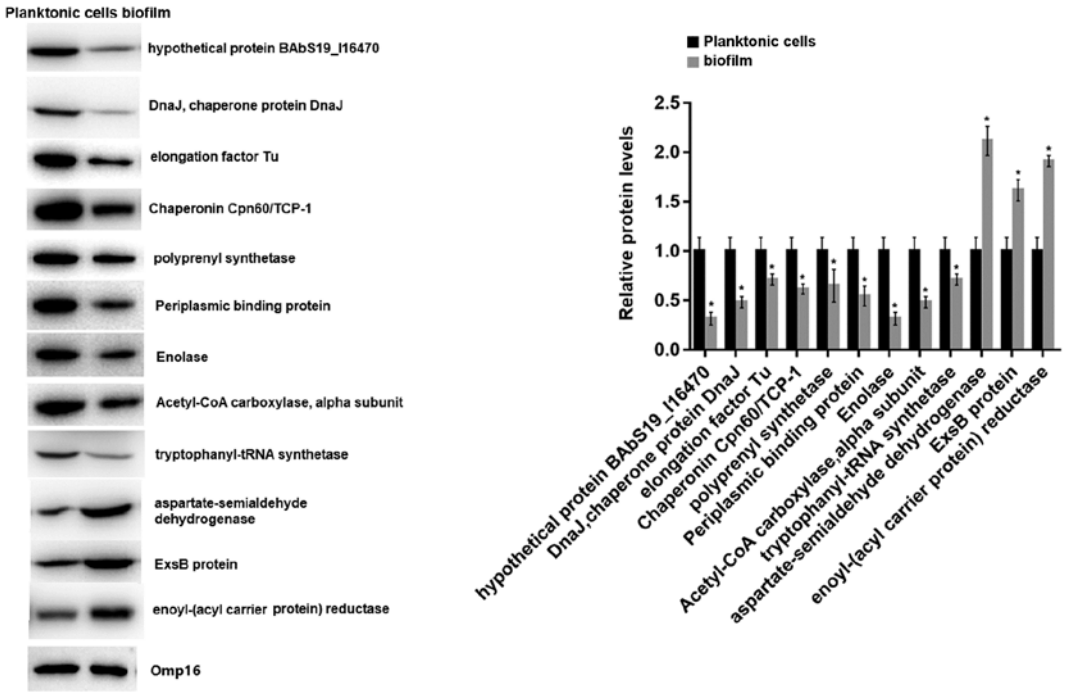

Figure 5. Analysis of differential protein expression in *Brucella abortus* cultured under planktonic or biofilm conditions. Western blot analysis of 12 proteins showed that 9 proteins were downregulated and 3 were upregulated between biofilms and planktonic cells. Omp16, outer membrane protein 16. *P<0.05 vs. control (planktonic cells).

Table II. RT-qPCR identification of the mRNA expression levels of 18 proteins identified in 2-D electrophoresis.

Protein spot	Protein	FC[a]	FC[b]
E11	hypothetical protein BAbS19_I16470	-10.0	-6.84
E21	ExsB protein	-20.0	-1.82
C24	enoyl-(acyl carrier protein) reductase	-12.5	-1.76
J12	Chaperonin Cpn60TCP-1	-12.5	-3.16
E20	aspartate-semialdehyde dehydrogenase	-9.1	-1.88
E13	elongation factor Tu	-100	-4.32
E15	polyprenyl synthetase	-14.3	-2.39
E12	DnaJ, chaperone protein DnaJ	-3.5	-5.55
E17	Enolase	-8.6	-1.95
E18	Acetyl-CoA carboxylase, a subunit	-7.0	-1.95
E16	Periplasmic binding protein	-1.1	-2.19
E19	tryptophanyl-tRNAsynthetase	-5.3	-1.90
E4	catalase	-33.3	2.41
E6	extracellular solute-binding protein	-22.6	1.78
E7	Ubiquinol-cytochrome C reductase iron-sulfur subunit	-25.0	1.70
E8	Chaperonin Cpn10	-14.3	1.68
E9	Tetracycline resistance protein TetB	-12.5	1.67
E10	Ribosomal protein L9	-33.3	1.51

[a]Expression level was determined by RT-qPCR; [b]expression level was determined by 2-D electrophoresis. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; 2-D, two-dimensional; FC, fold change.

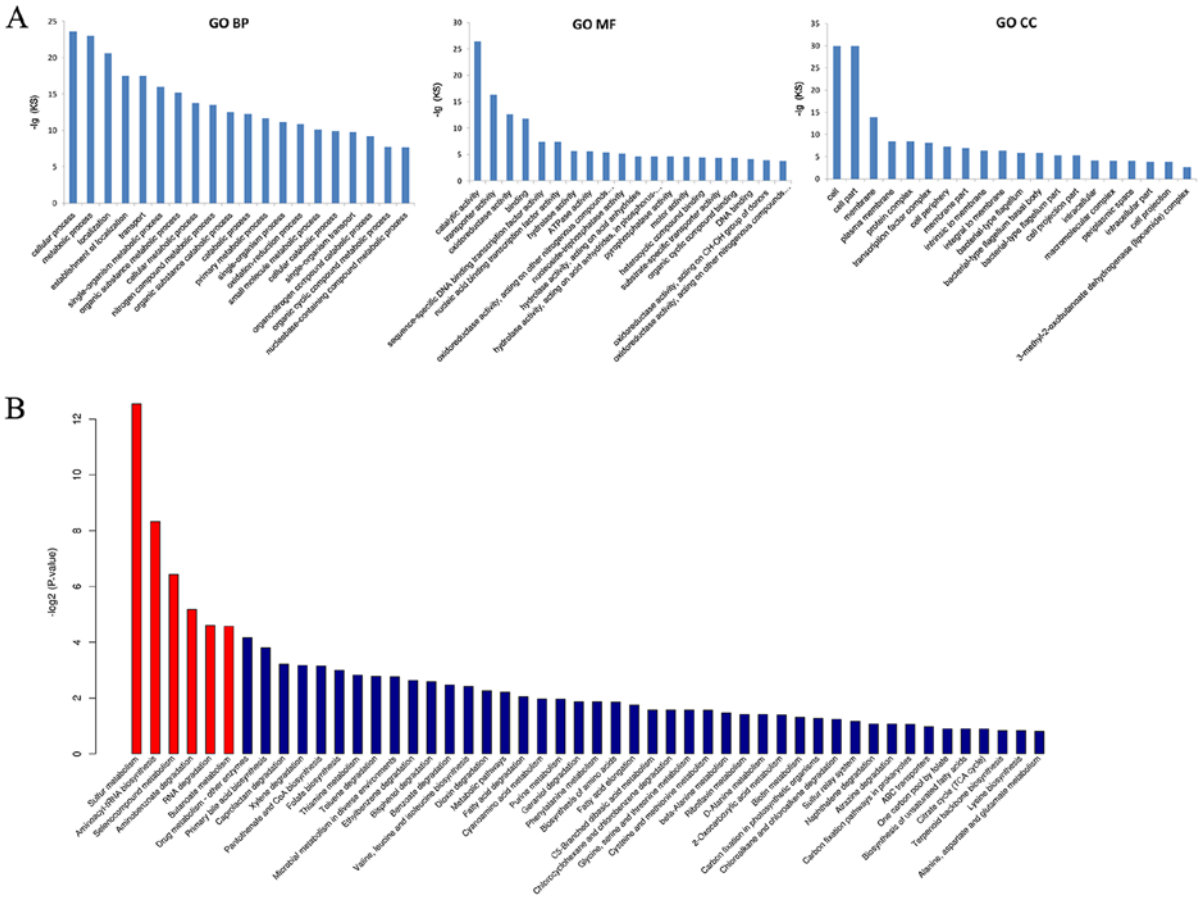

Figure 6. Functional and pathway analysis of differentially expressed genes in *Brucella abortus* biofilm. The significant (A) GO terms (BP, CC and MF) and (B) pathways [significant (red); not significant (blue)] enriched by differentially expressed genes. The horizontal axis represents significant GO terms and pathways, and the vertical axis represents the percentage of genes. GO, Gene Ontology; BP, biological process; MF, molecular function; CC, cellular compartment; KS, Kolmogorov-Smirnov.

Table III. Confirmation of the expression levels of function-associated genes identified by high-throughput sequencing.

Target gene	Gene name	Enriched function (cellular component)	Enriched function (molecular function)	Enriched function (biological process)	Pathway	FC[a]	log2 (FC)[b]
16S rRNA							
BAbS19_I10210	Phage integrase		DNA binding [GO:0003677]	DNA integration [GO:0015074]; DNA recombination [GO:0006310]		13.9	2.67
BAbS19_I13070	Ketol-acid reductoisomerase (EC 1.1.1.86; acetohydroxy-acid isomeroreductase; α-keto-β-hydroxylacyl reductoisomerase)		ketol-acid reductoisomerase activity [GO:0004455]	isoleucine biosynthetic process [GO:0009097]; valine biosynthetic process [GO:0009099]		1.4	2.51
BAbS19_I02060	Phosphomethylpyrimidine kinase		ATP binding [GO:0005524]; phosphomethylpyrimidine kinase activity [GO:0008972]	thiamine biosynthetic process [GO:0009228]		2.3	1.92
BAbS19_I03220	Uncharacterized protein		sialyltransferase activity [GO:0008373]	protein glycosylation [GO:0006486]		9.9	2.79
BAbS19_I19140	Short-chain dehydrogenase/ reductase SDR		oxidoreductase activity [GO:0016491]			24.3	3.71
BAbS19_I04760	EAL domain protein				Aminobenzoate degradation [path:ko00627]	2.1	2.38
BAbS19_I01340	Putative lipid II flippase MurJ	integral component of membrane [GO:0016021]; plasma membrane [GO:0005886]		cell wall organization [GO:0071555]; peptido glycan biosynthetic process [GO:0009252]; regulation of cell shape [GO:0008360]; transport [GO:0006810]		2.3	2.87
BAbS19_I14970	Binding-protein-dependent transport systems inner membrane component	integral component of membrane [GO:0016021]; plasma membrane [GO:0005886]		transport [GO:0006810]		4.3	3.12

Table III. Continued.

Target gene	Gene name	Enriched function (cellular component)	Enriched function (molecular function)	Enriched function (biological process)	Pathway	FC[a]	log2 (FC)[b]
BAbS19_I01720	Sulfite reductase (NADPH) hemoprotein β-component		4 iron, 4 sulfur cluster binding [GO:0051539]; heme binding [GO:0020037]; metal ion binding [GO:0046872]; oxidoreductase activity [GO:0016491]			4.9	2.64
BAbS19_I02050	D-amino acid oxidase family protein		D-amino-acid oxidase activity [GO:0003884]; FAD binding [GO:0071949]	D-amino acid metabolic process [GO:0046416]		2.3	3.37
BAbS19_I19580	Phosphoenolpyruvate carboxykinase [ATP]	cytoplasm [GO:0005737]	ATP binding [GO:0005524]; kinase activity [GO:0016301]; metal ion binding [GO:0046872]; phosphoenolpyruvate carboxykinase (ATP) activity [GO:0004612]	gluconeogenesis [GO:0006094]		3.5	4.59
BAbS19_I17580	Nitrilase/cyanide hydratase/ apolipoprotein N-acyltransferase		hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds [GO:0016810]; transferase activity, transferring acyl groups [GO:0016746]	nitrogen compound metabolic process [GO:0006807]		5.3	2.02
BAbS19_I14530	MaoC-like dehydratase				Butanoate metabolism [path:ko00650]	6.5	1.58
BAbS19_I01700	CysG, siroheme synthase		NAD binding [GO:0051287]; precorrin-2 dehydrogenase activity [GO:0043115]; sirohydrochlorin ferrochelatase activity [GO:0051266]; uroporphyrin-III C-methyltransferase activity [GO:0004851]	cobalamin biosynthetic process [GO:0009236]; siroheme biosynthetic process [GO:0019354]		5.7	2.07

[a]Expression level was determined by reverse transcription-quantitative polymerase chain reaction; [b]expression level was determined by high-throughput sequencing. FC, fold change.

surface of various microorganisms and pathogens, and serves an important role in bacterial colonization, persistence and host tissue invasion (42,43). It has been reported that in *Candida albicans*, enolase expresses on the surface of biofilm-forming cells and contributes to the adhesion of *Candida albicans* to different substrates with potential implications for biofilm adhesion and formation (44). Importantly, enolase has been cloned in *B. abortus* A19 and exhibits critical roles in the colonization and invasion of this pathogen (45). Specially, Han *et al* (45) also find that enolase protein can bind to *B. abortus*-positive sera, indicating that enolase may serve as a useful diagnostic marker for brucellosis.

DnaJ chaperone is a prototypical member of the Hsp40 family, which is important for numerous cellular functions, such as membrane lipid composition and cell division (46,47). Grudniak *et al* (48) demonstrated that this chaperone was involved in the biofilm formation of *Escherichia coli*. Periplasmic binding proteins have been introduced into bacteria to function in synthetic signal transduction pathways that respond to extracellular ligands and act as biologically active enzymes (49). As for the other differentially expressed proteins, their roles in biofilm formation have not been reported to the best of the authors' knowledge. Given their differential expression between biofilm and planktonic cells, it can be hypothesized that they may be associated with the biofilm characteristics of *B. abortus*.

From high-throughput sequencing, 14 function- and pathway-associated genes were identified. For instance, BAbS19_I19580 was enriched in functions related to phosphoenolpyruvate carboxykinase activity [GO:0004612] and gluconeogenesis [GO:0006094]. In most organisms, phosphoenolpyruvate carboxykinase can catalyze the formation of phosphoenolpyruvate via the phosphorylation and decarboxylation of oxaloacetate, which is the first step in the gluconeogenic pathway (50). A previous study by Li *et al* (51) suggested that gluconeogenesis serves a key role in the development of *Saccharomyces cerevisiae* biofilms. It was found that during the attachment period of biofilms, the expression of gluconeogenesis pathway-associated genes was upregulated, which was consistent with the findings of the present study. Viadas *et al* (52) found that the expression level of phosphoenolpyruvate carboxykinase gene was increased in *B. abortus* with bvrR mutant compared with wild type cells. Notably, phosphoenolpyruvate carboxykinase has been reported to be an acid-induced virulence factor in *Agrobacterium tumefaciens* (53). Therefore, it was proposed that phosphoenolpyruvate carboxykinase serves an important role in the virulence of *B. abortus* through the functions described above.

Binding protein-dependent transport systems have been found to be closely associated with structure, organization, mechanism and evolutionary origin (54). Numerous binding protein-dependent transport systems have been identified in Gram-negative bacteria (54). The major role of the binding protein systems is to recapture substrates that leak from the cell and retain them near the cell (55). In the present study, BAbS19_I14970 (binding-protein-dependent transport systems inner membrane component) was differentially expressed between biofilm and planktonic bacteria and enriched in an integral component of membrane (GO:0016021), indicating an important role in biofilm function.

Despite the identification of differentially expressed proteins and genes between biofilm and planktonic cells, no common protein or gene was identified, which may be attributed to several reasons. First, the detectability and abundance of proteomic and genomic analyses were different. Second, only 9 differentially expressed proteins were validated in proteomics, which may affect the consistency between proteomic and genomic analyses. Third, the thresholds used in proteomic and genomic analyses were different. Of note, there were certain common genes between proteomic and genomic analyses when significant differences were disregarded. Finally, there were differences in expression between transcriptional and protein levels due to post-transcriptional and posttranslational modifications. Furthermore, there is a limitation in the present study in that only one isolate was used for the analysis. Further isolates of *B. abortus* will be applied in future studies to further investigate the differentially expressed proteins and genes between the two culture conditions.

In conclusion, differential expression analysis at the protein and genomic levels suggested that the proteins and genes differentially expressed in *B. abortus* biofilms may serve important roles in bacterial defense, colonization, invasion, and virulence.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TT, CL and YJ participated in the design of this study. TT, MW, LZ an AG performed the experiments. TT, GC, YX and CZ analyzed the data. CL, LZ and AG obtained funding. TT and YX drafted the manuscript. GC and CZ participated in revision of manuscript for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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