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Mechanism of Asp24 Upregulation in *Brucella abortus* Rough Mutant with a Disrupted O-Antigen Export System and Effect of Asp24 in Bacterial Intracellular Survival

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We previously showed that *Brucella abortus* rough mutant strain 2308 Δ*ATP* (called the Δ*rfbE* mutant in this study) exhibits reduced intracellular survival in RAW264.7 cells and attenuated persistence in BALB/c mice. In this study, we performed microarray analysis to detect genes with differential expression between the Δ*rfbE* mutant and wild-type strain S2308. Interestingly, acid shock protein 24 gene (*asp24*) expression was significantly upregulated in the Δ*rfbE* mutant compared to S2308, as confirmed by quantitative reverse transcription-PCR (qRT-PCR) and Western blotting. Further studies using additional strains indicated that the upregulation of *asp24* occurred only in rough mutants with disrupted O-antigen export system components, including the ATP-binding protein gene *rfbE* (*bab1_0542*) and the permease gene *rfbD* (*bab1_0543*), while the Δ*wboA* rough mutant (which lacks an O-antigen synthesis-related glycosyltransferase) and the RB51 strain (a vaccine strain with the rough phenotype) showed no significant changes in *asp24* expression compared to S2308. In addition, abolishing the intracellular O-antigen synthesis of the Δ*rfbE* mutant by deleting the *wboA* gene (thereby creating the Δ*rfbE* Δ*wboA* double-knockout strain) recovered *asp24* expression. These results indicated that *asp24* upregulation is associated with intracellular O-antigen synthesis and accumulation but not with the bacterial rough phenotype. Further studies indicated that *asp24* upregulation in the Δ*rfbE* mutant was associated neither with bacterial adherence and invasion nor with cellular necrosis on RAW264.7 macrophages. However, proper expression of the *asp24* gene favors intracellular survival of *Brucella* in RAW264.7 cells and HeLa cells during an infection. This study reveals a novel mechanism for *asp24* upregulation in *B. abortus* mutants.

Brucella spp. are facultative intracellular bacteria that infect both animals and humans (1–3). Brucellosis is one of the most widespread zoonotic diseases in the world, especially in developing countries (2, 4). The *Brucella* genus is currently divided into 10 species according to preference for specific animal hosts, including the six classical species (*Brucella abortus*, *B. suis*, *B. melitensis*, *B. neotomae*, *B. canis*, and *B. ovis*) and newly recognized species (*B. ceti*, *B. microti*, *B. pinnipedialis*, and *B. inopinata*) (5, 6). *Brucella* has no classical virulence factors, such as exotoxins, cytolysins, capsules, fimbriae, plasmids, lysogenic phages, drug-resistant forms, antigenic variations, or endotoxic lipopolysaccharide (LPS) molecules (7); its virulence relies on the ability to invade and multiply intracellularly in both phagocytic cells and nonphagocytic cells (8).

Brucella LPS is recognized as a main virulence factor for resisting phagocytosis and enhancing survival in macrophages (9–11). The LPS consists of three key components, namely, lipid A, core sugar, and O-antigen (12), among which the O-antigen is critical for the virulence of classical *Brucella* species (*B. melitensis*, *B. abortus*, and *B. suis*) (13, 14). *B. abortus* lipid A possesses a diaminoglucose backbone, and the acyl groups are longer (C_{18}-C_{19} and C_{28}) and are linked to the core only by amide bonds (12). The O-antigen of *Brucella* is a homopolymer of 4,6-dideoxy-4-formamido-α-D-mannopyranosyl residues joined by an α-1,2 linkage in A-epitope-dominant strains, but every fifth residue is joined by an α-1,3 linkage in M-epitope-dominant strains (15, 16). The *Brucella wboA* gene is capable of encoding a glycosyltransferase that has been demonstrated to be essential for the biosynthesis of the *Brucella* O-antigen (17). Disruption of the *wboA* gene in *B. abortus* 2308, *B. melitensis* 16M, and *B. suis* resulted in rough mutants that were unable to synthesize the O-antigen (18). The main genes involved in LPS biosynthesis in *Brucella* spp. include those for GDP-mannose dehydratase (Gmd), perosamine synthetase (Per), phosphoglucomutase (Pgm), phosphomannomutase (Pmm), mannose isomerase (ManB), mannose guanylyltransferase (ManC), O-antigen export permease (Wzm), ATP-binding protein (Wzt), WbkB, methionyl tRNA formyltransferase (WbkC), and *N*-formylperosaminyltransferase (WbkA). Among these genes, *wzm* and *wzt* (called *rfbD* and *rfbE* in this study) encode putative integral membrane components of ATP-binding cassette (ABC) transporters (12, 19) that flip the O-antigen from the cytoplasmic face to the periplasmic face of the inner membrane (20).

Our previous study showed that *B. abortus* rough mutant strain 2308 Δ*ATP* (called the Δ*rfbE* mutant in this study) exhibits reduced intracellular survival in RAW264.7 cells and attenuated persistence in BALB/c mice. In this study, we performed microarray analysis to detect genes with differential expression between the Δ*rfbE* mutant and wild-type (WT) strain S2308. Interestingly, acid shock protein 24 gene (*asp24*) expression was significantly upregulated in the Δ*rfbE* mutant compared to S2308, as con-

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Characteristics	Source or reference
Strains		
B. abortus strains		
S2308	WT; parental strain; smooth phenotype	ATCC
RB51	Vaccine strain; rough phenotype	Q. Wu
Δ*rfbE*	Deletion strain for *rfbE* gene; rough phenotype	23
Δ*rfbE*(pBBR-*rfbE*)	Cm^r; Δ*rfbE* strain carrying the complementary plasmid pBBR-*rfbE*; smooth phenotype	This study
Δ*rfbD*	Deletion strain for *rfbD* gene; rough phenotype	This study
Δ*wboA*	Deletion strain for *wboA* gene; rough phenotype	This study
Δ*rfbE* Δ*wboA*	Deletion strain for *rfbE* and *wboA* genes; rough phenotype	This study
Δ*rfbE* Δ*asp24*	Deletion strain for *rfbE* and *asp24* genes; rough phenotype	This study
Δ*rfbE* Δ*asp24*(pBBR-*asp24*)	Cm^r; Δ*rfbE* Δ*asp24* strain carrying the complementary plasmid pBBR-*asp24*; rough phenotype	This study
S2308(pBBR-*asp24*)	Cm^r; WT carrying the plasmid pBBR-*asp24*; smooth phenotype	This study
Δ*rfbE*(pBBR-*asp24*)	Cm^r; Δ*rfbE* strain carrying the plasmid pBBR-*asp24*; rough phenotype	This study
E. coli strains		
DH5α	F^- ϕ80*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_K^- m_K^+) *phoA supE44 thi-1 gyrA96 relA1* λ^-	Invitrogen
BL21(DE3)	F^- *ompT hsdSB*(r_B^- m_B^-) *gal dcm*(DE3)	Invitrogen
Plasmids		
pBBR1MCS1	Cm^r; Broad-host-range cloning vector	25
pET28a(+)	Kan^r, prokaryotic expression vector	Novagen
pSC	Amp^r; pUC19 plasmid containing *sacB* gene	23
pSCΔ*wboA*	Amp^r; pSC plasmid containing the Δ*wboA* fragment; used to construct deletion strain	This study
pSCΔ*asp24*	Amp^r; pSC plasmid containing the Δ*asp24* fragment; used to construct deletion strain	This study
pBBR-*rfbE*	Cm^r; pBBR1MCS1 containing the *rfbE* gene flanked by its upstream and downstream regions	This study
pBBR-*asp24*	Cm^r; pBBR1MCS1 containing the *asp24* gene flanked by its upstream and downstream regions	This study

firmed by quantitative reverse transcription-PCR (qRT-PCR) and Western blotting. Acid shock protein 24 (Asp24) is a protein previously reported to be induced in acidic environments; in *Brucella*, Asp24 expression is optimal at pH values below 4.0 and within the first 3 h of acid exposure (21). The exact role of Asp24 in pathogenesis remains unknown. In this study, we demonstrated upregulated *asp24*/Asp24 expression in *B. abortus* O-antigen transporter mutants. Further study revealed that the upregulation is associated with intracellular O-antigen synthesis and accumulation. Therefore, we discovered a novel mechanism for *asp24* upregulation and showed that its altered expression affects intracellular survival of *Brucella* in host cells.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions. All strains and plasmids used in the study are listed in Table 1. *B. abortus* S2308 and its derivatives were cultured on tryptic soy agar (TSA) (Difco) or in tryptic soy broth (TSB) at 37°C with 5% CO_2. *Escherichia coli* strains were cultured at 37°C in Luria Broth (LB). When appropriate, 100 μg/ml ampicillin or 20 μg/ml chloramphenicol (Sigma) was added.

DNA microarray analysis. Total RNAs were extracted from S2308 and the Δ*rfbE* mutant by use of TRIzol RNA isolation reagents (Invitrogen). Genomic DNA contamination was removed through treatment with a Turbo DNA-free kit (Ambion). The *B. abortus* bv. 1 strain 9-941 gene expression array was manufactured by Agilent (Agilent Technologies) and consisted of 3,334 genes. Sample preparation and microarray hybridization were performed based on the manufacturer's standard protocols. Briefly, 1 μg of total RNA from each sample was amplified and transcribed into fluorescent cRNA according to the Agilent Quick Amp labeling protocol (version 5.7; Agilent Technologies). Labeled cRNAs were hybridized onto a whole-genome oligonucleotide array (8 × 15K; Agilent Technologies). After slide washes, the arrays were scanned using an Agilent G2505B scanner. Agilent Feature Extraction software (version 10.7.3.1) was used to analyze acquired array images. Quantile normalization and subsequent data processing were performed using the GeneSpring GX v11.5.1 31 software package (Agilent Technologies). Differentially expressed genes were identified through volcano plot filtering.

Real-time PCR assay. Validation of the microarray data was performed using qRT-PCR. Genes which showed expression differences of >5-fold in the microarray assay were selected for validation. Primers used are listed in Table 2. Total RNA was extracted as described above. RNA (1 μg) was reverse transcribed into cDNA by using a PrimeScript RT-PCR kit (TaKaRa) according to the manufacturer's instructions. One microliter of cDNA was used in a 20-μl RT-PCR mixture containing 10 μl 2× GoTaq qPCR master mix (Promega), 0.5 μl (each) forward and reverse primers (10 μM [each]), and 8 μl double-distilled water (ddH_2O). The mixture was incubated at 95°C for 2 min, and then 40 cycles of 95°C for 15 s and 60°C for 1 min were carried out with a Mastercycler ep Realplex system (Eppendorf). For each gene, PCRs were performed in triplicate, and relative transcription levels were determined by the $2^{-\Delta\Delta CT}$ method, using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control for data normalization.

Plasmid construction. All primers used in the study are listed in Table 2. Suicide plasmids were constructed using an overlap PCR assay as previously reported (22). Briefly, the upstream and downstream fragments of the target gene were amplified by independent PCRs and purified by gel extraction before being used as templates for a second round of PCR. The resultant product, containing joined flanking sequences, was gel purified, digested with appropriate enzymes, and cloned into the pSC plasmid (pUC19-sacB plasmid) (22, 23). The recombinant suicide plasmids were transformed into DH5α cells (Invitrogen) for propagation and then extracted to construct the mutants.

The complementation plasmids pBBR-*rfbE* and pBBR-*asp24* were constructed using conventional methods (23, 24). The *rfbE* and *asp24* fragments, containing respective promoter and terminator regions, were amplified by PCR. The joined flanking sequences for *rfbE* and *asp24* were digested by use of BamHI/KpnI and BamHI/SalI, respectively, and then

TABLE 2 Primers used in this study

Primer	Oligonucleotide sequence (5′ to 3′)[a]	Target gene[b]	Product size (bp)
C*rfbE*-F	CGCGTCGACCACAGGATCTTACCCTGTTG	*rfbE* containing the promoter region	1,712
C*rfbE*-R	CGCGGATCCTCATGCTATAGCTCCCATTC	*rfbE* containing the promoter region	1,712
rfbD-UF	TGCACTGCAGTGCACGAAATAGGTCCAGGTCTCG	Upstream fragment of *rfbD*	1,250
rfbD-UR	TACGCCACTTCGGGATCCCGTGAGCCTCCTCCTAACCTG	Upstream fragment of *rfbD*	1,250
rfbD-DF	AGGAGGCTCACGGGATCCCGAAGTGGCGTACCTTCCAG	Downstream fragment of *rfbD*	1,140
rfbD-DR	TCCCCCGGGGGAGTGAACGAATGCCTTGATAC	Downstream fragment of *rfbD*	1,140
wboA-UF	GCTCTAGAGCAATTCCGGTGGGTTGATT	Upstream fragment of *wboA*	1,013
wboA-UR	AACCTACATCTATTGGCGTCTTCCGCCTCGGTACTTAA	Upstream fragment of *wboA*	1,013
wboA-DF	TTAAGTACCGAGGCGGAAGACGCCAATAGATGTAGGTT	Downstream fragment of *whoA*	1,091
wboA-DR	GCTCTAGAGCTTGATAAAGGCATGTAATGTG	Downstream fragment of *whoA*	1,091
asp24-UF	GCTCTAGAGCAGCCACCAGCCAAGAAGATAGG	Upstream fragment of *asp24*	1,153
asp24-UR	CGGGATCCCGTACTCTTTTACAGGTGCGCCGC	Upstream fragment of *asp24*	1,153
asp24-DF	CGGGATCCCGCCCAGAAGCCGCAGGAATAAG	Downstream fragment of *asp24*	1,089
asp24-DR	GCTCTAGAGCGGGTGCGATTGTGAATGTCTCCT	Downstream fragment of *asp24*	1,089
Asp24-F	CGCGGATCCAATACGGATAAACCAGCAGC	Coding region of *asp24*	474
Asp24-R	CCGCTCGAGTTATTCCTGCGGCTTCTGG	Coding region of *asp24*	474
C*asp24*-F	GGGGTACCCCAAGACGAGCGGAAACGAC	*asp24* containing the promoter region	1,467
C*asp24*-R	GCTCTAGAGCCATTGGACGACTTTGTTGAC	*asp24* containing the promoter region	1,467
RT-0004F	CGTCCTATCGCGATCTTTTC	BruAb1_0004	189
RT-0004R	GGACATTCAGCGCATTGTAG	BruAb1_0004	189
RT-1070F	CCTCGCAGTTACAGGCTTTC	BruAb2_1070	216
RT-1070R	TGCCGGTAATCATCAACTCA	BruAb2_1070	216
RT-1334F	GCGGTCCTATCGATCTTGAG	BruAb1_1334	178
RT-1334R	GTTACCTTGCCGTCACCATT	BruAb1_1334	178
RT-1735F	ATGCCTGTTTCAATCCGAAC	BruAb1_1735	156
RT-1735R	ATGCCTGTTTCAATCCGAAC	BruAb1_1735	156
RT-0035F	AGCTTCGGCTGATGAATGAC	BruAb1_0035	115
RT-0035R	TCGAAAGTCAGCATGGTCTG	BruAb1_0035	115
RT-1556F	AAGCTTTGGCATGATTACCG	BruAb1_1556	87
RT-1556R	TCCAGAACGCAAACATCAAC	BruAb1_1556	87
RT-1726F	CACGCTCGAAAAGAAGCAC	BruAb1_1726	96
RT-1726R	CGCTTGAGAGATGCGATATG	BruAb1_1726	96
RT-1735F	ATGCCTGTTTCAATCCGAAC	BruAb1_1735	156
RT-1735R	AATTCACTTGTCCGCAAACC	BruAb1_1735	156
RT-0400F	AGAGCAAGCAGCTCTCGAAC	BruAb2_0400	185
RT-0400R	CGGGGATTCTTAAACCCAAT	BruAb2_0400	185
RT-0811F	GTTCGATTGCGAGGTGTCTT	BruAb2_0811	156
RT-0811R	TTCGGAAAACAGGAACATGA	BruAb2_0811	156
RT-1055F	ATCGCAAAATCCTCAAATCG	BruAb2_1055	189
RT-1055R	CGGGATTATAGAAGCCGTCA	BruAb2_1055	189
RT-1241F	CGTATATCCAGCTCGGTGGT	BruAb1_1241	231
RT-1241R	CGATGTGGTAGTTGGTCGTG	BruAb1_1241	231
RT-0657F	CGTATATCCAGCTCGGTGGT	BruAb1_0657	231
RT-0657R	CGATGTGGTAGTTGGTCGTG	BruAb1_0657	231
RT-0822F	ATACGCCGATGGTCAAGAAG	BruAb1_0822	169
RT-0822R	TCAACAGCACGCTTGTTTTC	BruAb1_0822	169
RT-1878F	GGCAAGAACAAGGACCACAT	BruAb1_1878	184
RT-1878R	CCCAATAGTTGGTCGGAATG	BruAb1_1878	184
RT-1239F	CGTTTGATCATCGGATCCTT	BruAb1_1239	192
RT-1239R	TATCGAGAAGACGCTTGTGC	BruAb1_1239	192
RT-1255F	ACGACGAAGACCACGGTTAC	BruAb1_1255	228
RT-1255R	GTTGGTGAAGAACGGCGTAT	BruAb1_1255	228
RT-1238F	GTCATAACTTCGGCGGTCAT	BruAb1_1238	106
RT-1238R	TTGCCCTTGAACACCTTACC	BruAb1_1238	106
RT-1242F	AGACGCCATAAGGCAGAGAA	BruAb1_1242	99
RT-1242R	CTTGCCATGAAGCATGACTG	BruAb1_1242	99
RT-1243F	TGCCTACCGTAAACCAGCTT	BruAb1_1243	168
RT-1243R	GACGAACCTTGGCAACCTTA	BruAb1_1243	168
RT-0505F	ACCTCGTTTTCGTCGAATTG	BruAb2_0505	140
RT-0505R	GACAGCATCGTTGACTTCCA	BruAb2_0505	140
RT-GAPDH-F	GACATTCAGGTCGTCGCCATCA	GAPDH	188
RT-GAPDH-R	TCTTCCTTCCACGGCAGTTCGG	GAPDH	188

[a] Underlining indicates restriction endonuclease recognition sequences.
[b] *B. abortus* locus tags listed are for genes in *B. abortus* strain 9-941.

inserted into the same digested sites of the pBBR1MCS1 plasmid (25). The pBBR-*rfbE* or pBBR-*asp24* plasmid was propagated in *E. coli* DH5α cells.

Mutants and complemented strain construction. The *B. abortus* mutants were constructed by allelic replacement, using a two-step strategy as previously reported (26). Briefly, *B. abortus* strain 2308 competent cells were prepared through two washes with ice-cold sterile water. A suicide plasmid (0.5 to 1.0 μg) was transformed into competent cells by electroporation. The singly exchanged recombinants were selected by plating on

TSA containing ampicillin, and then colonies were inoculated into TSB without antibiotics. The second exchanged recombinants were selected by plating on TSA containing 5% sucrose. All colonies were selected and verified by PCR or Western blotting.

The complemented strains were also constructed by electroporation. The recombinants were then selected by plating on TSA containing chloramphenicol. The colonies were verified through PCR or Western blotting.

Asp24 protein expression and rabbit antiserum preparation. The coding region of the *asp24* gene was amplified from the *B. abortus* S2308 genome by PCR using the primers Asp24-F and Asp24-R (Table 2) and then cloned into pET28a(+) (Novagen). The plasmid was introduced into *E. coli* BL21(DE3) for IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible expression of recombinant proteins and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue staining. The recombinant proteins were purified using HisTrap chelating high-performance columns (Amersham) and were assayed quantitatively using a bicinchoninic acid (BCA) protein assay kit (Beyotime). The rabbit antiserum was prepared as reported previously (27). Samples with enzyme-linked immunosorbent assay (ELISA) titers over 1:10,000 qualified for Western blotting.

Extraction and Western blotting of *Brucella* total protein and LPS. *B. abortus* S2308 and its derivatives were cultured in TSB medium, and the bacterial whole cells were obtained by centrifugation. Bacterial pellets were then diluted to an optical density at 600 nm (OD_{600}) of 10 and inactivated at 80°C for 1 h. Total bacterial proteins were extracted by boiling with SDS sample loading buffer (Beyotime). LPS was isolated by SDS-proteinase K extraction as described previously (28). Samples were loaded onto a 12% polyacrylamide gel for SDS-PAGE analysis. The gels were then transferred onto a nitrocellulose membrane (Whatman) for Western blotting.

The membranes were blocked for 1 h in phosphate-buffered saline (PBS) with 5% skimmed milk at room temperature. After being washed four times in PBS-0.1% Tween 20 (PBST) for 5 min, the membranes were incubated overnight with primary antibody (*Brucella* O-antigen monoclonal antibody A76 12G12 F12, rabbit anti-Asp24 polyclonal antibody, or mouse anti-D15 monoclonal antibody) diluted in PBST, washed four times in PBST for 5 min, incubated for 1 h with secondary antibody (IRDye 800CW-conjugated donkey anti-rabbit polyclonal antibody or IRDye 680RD-conjugated donkey anti-mouse polyclonal antibody) (Li-Cor) in PBST-0.02% SDS, and finally washed four times in PBST for 5 min. The blots were visualized with an Odyssey two-color infrared imaging system (Li-Cor). Quantitative analysis of the Asp24 protein was performed by gray scanning, using the *Brucella* surface antigen D15 as the internal control.

Bacterial adherence, invasion, and intracellular survival assays. RAW264.7 cells were used to determine the effects of the *Brucella asp24* gene on the bacterial adherence, invasion, and intracellular survival capacities. The cells were seeded at about 2×10^5 cells per well in 24-well plates and grown in Dulbecco modified Eagle medium (DMEM) (Biowest) with 10% fetal bovine serum (FBS) (Biowest) at 37°C with 5% CO_2 for 24 h. For the adherence assay, the cell monolayer was washed twice with DMEM and infected with *B. abortus* S2308 or its derivatives at a multiplicity of infection (MOI) of 200, as previously described (29). Bacteria were centrifuged onto the cells at $400 \times g$ for 5 min, and cells were then incubated at 37°C for 1 h. Nonadherent bacteria were removed by rinsing the wells twice with PBS (HyClone). The cells were released from the plate by adding 100 μl of 0.2% Triton X-100 in sterile water, and subsequently 900 μl of DMEM was added. This cell suspension was 10-fold serially diluted with PBS and spread onto TSA plates to determine the number of viable bacteria.

For the invasion assay, cell culture, bacterial infection, and bacterial counting were performed as described above for the bacterial adherence assay, except that the extracellular bacteria were killed by incubation of the monolayers with DMEM containing gentamicin (100 μg/ml) for 1 h after incubation with bacteria and three washes with PBS.

For the bacterial intracellular survival assay, cell culture and bacterial counting were performed as described above for the bacterial adherence assay. RAW264.7 cells were infected with *B. abortus* S2308 or its derivatives at an MOI of 100 and incubated in DMEM with 3% FBS and 50 μg/ml gentamicin. The cells were then washed and lysed at 1, 12, 24, 36, 48, and 60 h postinfection (hpi) to determine the amount of bacterial recovery. HeLa cells were also used to investigate the effect of *asp24* on bacterial intracellular survival. The cells were infected with S2308, the Δ*rfbE* mutant, or S2308(pBBR-*asp24*) at a high infection dose (MOI of 500) to increase the number of invasive bacteria, with the bacterial CFU determined at 2, 10, 24, and 48 hpi, or infected with the Δ*rfbE*, Δ*rfbE* Δ*asp24*, or Δ*rfbE*(pBBR-*asp24*) strain, with the bacterial CFU determined at 1, 12, 24, and 48 hpi.

All assays were performed in triplicate wells, and the results are averages for at least three independent experiments.

Immunofluorescence assay. RAW264.7 cells cultured on glass coverslips with a diameter of 15 mm (Thermo Fisher Scientific) were infected with *B. abortus* S2308 or the Δ*rfbE* mutant at a low infection dose (MOI of 30) to conveniently observe intracellular survival of individual bacteria. The infected cells were fixed with 3.7% (wt/vol) paraformaldehyde at 0, 6, 12, 24, 36, 48, 60, and 72 hpi. Indirect immunofluorescence assay was performed as previously reported (29), using rabbit anti-*B. abortus* serum (diluted 1:500) as the primary antibody and Alexa Fluor 488-conjugated goat anti-rabbit IgG (diluted 1:1,000) (Invitrogen) as the secondary antibody.

Determination of *Brucella*-induced macrophage death. RAW264.7 cells in 24-well plates were infected with *B. abortus* S2308 or derivatives at an MOI of 200. Culture supernatants were collected at 8 and 24 hpi, and levels of lactate dehydrogenase (LDH) were determined by CytoTox 96 nonradioactive cytotoxicity assay (Promega) according to the manufacturer's instructions. *Brucella*-induced macrophage death was further analyzed using a propidium iodide (PI)-Hoechst 33342 staining kit (Beyotime) and a fluorescein isothiocyanate (FITC)-annexin-PI staining kit (Vazyme) at 4, 8, or 15 hpi.

Acid induction and tolerance assay. *B. abortus* S2308 or its derivatives were cultured to mid-logarithmic phase ($OD_{600} = 1.0$), and then a bacterial suspension (10^7 CFU/ml) was prepared in TSB with a pH of 7.3, 5.5, 4.5, or 3.8. After 2 h of incubation at 37°C, cells were serially diluted and plated on TSA to determine the bacterial CFU. The bacterial survival percentages were calculated with respect to numbers of CFU obtained from bacteria incubated in TSB at pH 7.3 (100% survival).

Statistical analysis. Statistical analysis was performed using GraphPad Prism software. All results are presented as means ± standard deviations (SD) for at least three independent experiments. Statistical significance was determined by either an unpaired, two-tailed Student *t* test or, in the case of groups, one-way analysis of variance (ANOVA) followed by Tukey's test.

RESULTS

***asp24* expression is significantly upregulated in the Δ*rfbE* mutant.** Genes with differential expression between *B. abortus* S2308 and the Δ*rfbE* mutant were identified using a *B. abortus* gene expression array containing 3,334 genes. A total of 283 genes that exhibited altered expression (>2-fold difference) were identified, among which 114 genes (114/3,334 genes [3.42%]) were upregulated and 169 genes (169/3,334 genes [5.07%]) were downregulated in the Δ*rfbE* mutant relative to *B. abortus* S2308 (see Table S1 in the supplemental material). Among these, 21 genes with at least 5-fold increases in expression in the microarray analysis were further validated using the qRT-PCR assay. The results revealed that only *asp24* gene expression was significantly changed in the Δ*rfbE* mutant. Compared to the case in *B. abortus* S2308, the *asp24* gene showed 24-fold more expression in the Δ*rfbE* mutant. Other se-

TABLE 3 Transcriptional levels of *Brucella* genes examined using qRT-PCR

B. abortus 9-941 ORF	Predicted protein function	$2^{-\Delta\Delta CT}$ value[a]
BruAb1_0004	Molybdopterin biosynthesis protein MoeB	1.08
BruAb2_1070	Flagellar biosynthesis protein FliQ	1.17
BruAb1_1334	Acid shock protein	24.41
BruAb1_1735	Hypothetical protein	1.46
BruAb1_0035	Hypothetical protein	1.02
BruAb1_1556	Hypothetical protein	0.82
BruAb1_1726	Hypothetical protein	1.63
BruAb1_1735	Hypothetical protein	1.49
BruAb2_0400	Hypothetical protein	1.63
BruAb2_0811	Hypothetical protein	0.34
BruAb2_1055	IS*3* family transposase OrfB	2.14
BruAb1_1241	Elongation factor G	0.93
BruAb1_0657	Omp2b, porin	1.26
BruAb1_0822	NADH dehydrogenase subunit G	1.10
BruAb1_1878	Succinate dehydrogenase flavoprotein subunit	1.21
BruAb1_1239	30S ribosomal protein S10	1.25
BruAb1_1255	Elongation factor Tu	1.00
BruAb1_1238	50S ribosomal protein L3	1.28
BruAb1_1242	30S ribosomal protein S7	2.04
BruAb1_1243	30S ribosomal protein S12	0.90
BruAb2_0505	Glycine cleavage system protein H	1.44

[a] A value of 1 indicates that the gene is similarly expressed under both conditions (Δ*rfbE* mutant versus S2308), a value of >1 indicates that the gene is overexpressed in the Δ*rfbE* mutant, and a value of <1 indicates that the gene is expressed less in the mutant.

lected genes showed changes of <3-fold by qRT-PCR analysis (Table 3).

The expression of *asp24* in the Δ*rfbE* mutant was then investigated at 6, 12, 24, 36, and 48 h of bacterial growth in TSB to determine whether the increase in *asp24* expression depended on different bacterial growth phases. qRT-PCR showed that *asp24* expression was upregulated in the Δ*rfbE* mutant throughout its entire growth cycle. At 6, 12, 24, 36, and 48 h of growth, 8.7-, 14.3-, 49.6-, 447.8-, and 582.1-fold increases, respectively, were observed (Fig. 1A). Furthermore, enhanced Asp24 protein levels in the Δ*rfbE* mutant were also determined by Western blotting, using the surface antigen D15 as a reference protein (Fig. 1B). *asp24* expression in the Δ*rfbE*(pBBR-*rfbE*) complemented strain exhibited levels similar to those in *B. abortus* S2308 for the entire growth cycle (Fig. 1A and B). Overall, the results indicated that disruption of the O-antigen export system *rfbE* gene in *B. abortus* progressively upregulated bacterial *asp24* gene expression and its protein level throughout the bacterial growth cycle.

***asp24*-upregulated expression in the Δ*rfbE* mutant is associated with intracellular O-antigen synthesis and accumulation.** To determine whether *asp24* upregulation depends on the *Brucella* rough phenotype, the level of *asp24* expression was determined by qRT-PCR analysis of different rough phenotype strains, including the Δ*rfbE*, Δ*rfbD*, Δ*wboA*, and RB51 strains. Interestingly, *asp24* was upregulated only in the Δ*rfbE* and Δ*rfbD* mutants, the O-antigen export system-disrupted mutants, with 29-fold and 49-fold increases, respectively (Fig. 2A). *asp24* expression was unchanged in the Δ*wboA* and RB51 strains (Fig. 2A). The results suggested that *asp24* upregulation is not associated with the bacterial rough phenotype.

The *Brucella* O-antigen export system is an ATP-binding cassette (ABC) transporter system, encoded by the *rfbE* and *rfbD* genes, which flips the O-antigen from the cytoplasmic face to the periplasmic face of the inner membrane; disruption of this system affects the flipping of O-antigen, resulting in an accumulation of intracellular O-antigen (20). Western blotting was further used to detect intracellular O-antigen synthesis in the rough phenotype strains and showed that O-antigen was detected in the Δ*rfbE* and Δ*rfbD* mutants but absent in the Δ*wboA* and RB51 strains (Fig. 3A), suggesting that disruption of the O-antigen export system does not abolish intracellular O-antigen synthesis.

To determine whether *asp24*-upregulated expression is associated with the accumulation of intracellular O-antigen, a glycosyltransferase gene involved in O-antigen synthesis, *wboA* (20, 24), was knocked out of the Δ*rfbE* mutant, thereby generating a double-knockout strain (Δ*rfbE* Δ*wboA*). Western blotting showed that no O-antigen was detected in the Δ*rfbE* Δ*wboA* strain, suggesting that O-antigen synthesis was abolished due to *wboA* deletion (Fig. 3B). Furthermore, qRT-PCR and Western blotting results showed that *asp24*/Asp24 expression in the Δ*rfbE* Δ*wboA* strain was restored to levels comparable to those in *B. abortus* S2308 (Fig. 2B and C). This indicates that *asp24* upregulation in the Δ*rfbE* mutant is associated with the intracellular accumulation of O-antigen.

***asp24* upregulation is not the cause of Δ*rfbE* mutant-induced oncotic and necrotic macrophage death.** The number of CFU recovered from infected RAW264.7 cells was dramatically reduced for the Δ*rfbE* mutant compared to WT strain S2308, as previously reported (23); however, the Δ*rfbE* mutant did multiply in macrophages, as suggested by enhanced bacterial staining postinfection by fluorescence microscopy (Fig. 4A). This result is

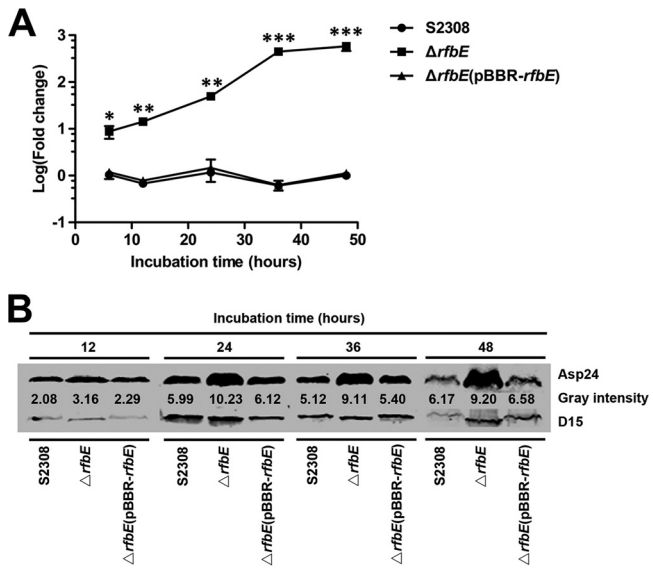

FIG 1 *asp24* expression is significantly upregulated in the Δ*rfbE* mutant. (A) *asp24* expression in the Δ*rfbE* mutant was significantly upregulated at the indicated incubation times in TSB compared to that in *B. abortus* S2308, as measured using qRT-PCR. The Δ*rfbE*(pBBR-*rfbE*) complemented strain had recovered *asp24* expression. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (B) Western blotting revealed an enhanced Asp24 protein level in the Δ*rfbE* mutant compared to that in *B. abortus* S2308. The Δ*rfbE*(pBBR-*rfbE*) complemented strain had a recovered Asp24 protein level. Protein levels are indicated by gray-scanning intensity values.

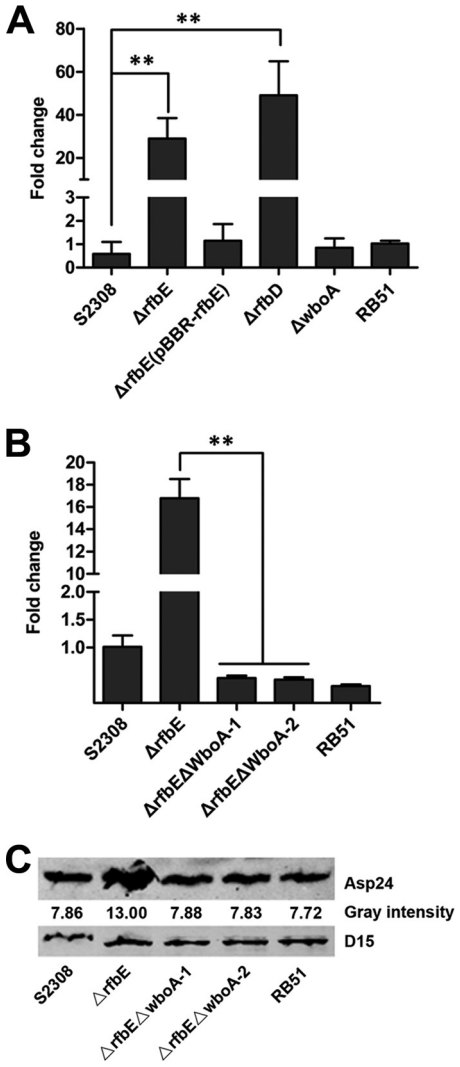

FIG 2 Intracellular O-antigen synthesis and accumulation are responsible for *asp24* upregulation in the Δ*rfbE* mutant. (A) qRT-PCR showed enhanced *asp24* expression in the Δ*rfbE* and Δ*rfbD* mutants, but not in the Δ*wboA* and RB51 strains, compared to WT strain S2308. The Δ*rfbE*(pBBR-*rfbE*) complemented strain had recovered *asp24* expression. **, $P < 0.01$. (B) Deletion of the *wboA* gene from the Δ*rfbE* mutant resulted in recovered *asp24* expression as determined using qRT-PCR. **, $P < 0.01$. (C) Western blot showing the recovered Asp24 protein level in the *wboA* gene-deleted Δ*rfbE* mutant (lanes Δ*rfbE*Δ*wboA*-1 and Δ*rfbE*Δ*wboA*-2). Protein levels are indicated by gray-scanning intensity values.

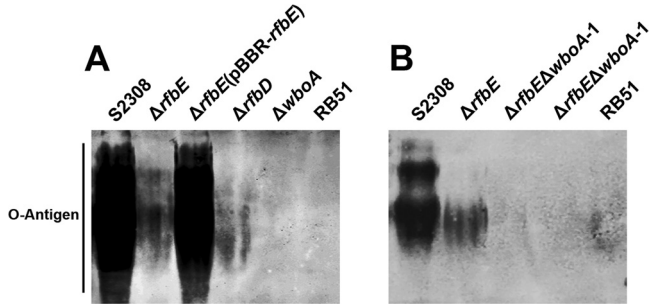

FIG 3 Western blotting identifies O-antigen synthesis in bacteria. (A) O-antigen was synthesized in the Δ*rfbE* and Δ*rfbD* mutants but not the Δ*wboA* and RB51 strains. (B) Deletion of the *wboA* gene from the Δ*rfbE* mutant abolished O-antigen synthesis; no O-antigen was detected in lanes Δ*rfbE*Δ*wboA*-1 and Δ*rfbE*Δ*wboA*-2.

consistent with that for the rough mutant CA180 infecting RAW264.7 cells (29). Hoechst 33342 (blue) and PI (red) staining showed that more than 60% and 80% of the cells infected by the Δ*rfbE* mutant were dead at 8 and 15 hpi, respectively (Fig. 4B). A marked cytopathic effect was observed in the infected cells, as evaluated by phase-contrast microscopy (Fig. 4C).

Brucella strains with the rough phenotype can induce macrophage oncosis and necrosis but not apoptosis (29). Apoptosis is a process of programmed cell death which leads to cell death with karyorrhexis and cell shrinkage; necrosis is a form of traumatic cell death that results from acute cellular injury; and oncosis precisely means cell death by swelling and leads to necrosis with karyolysis, standing in contrast to apoptosis. To determine whether *asp24* upregulation affects Δ*rfbE* mutant-induced macrophage death, the *asp24* gene was knocked out of the Δ*rfbE* mutant to produce the Δ*rfbE* Δ*asp24* strain, and a complemented strain [Δ*rfbE* Δ*asp24*(pBBR-*asp24*)] was constructed using the pBBR-*asp24* plasmid. RAW264.7 cells were infected with the S2308, Δ*rfbE*, Δ*rfbE* Δ*asp24*, and Δ*rfbE* Δ*asp24*(pBBR-*asp24*) strains, and cell death was analyzed by annexin V (green) and PI (red) staining. Annexin V staining was used to detect translocation of phosphatidylserine from the inner cell membrane to the outer cell membrane of cells during the early stage of apoptosis. PI stains the DNA of necrotic cells and/or cells at the late stage of apoptosis. The results showed that infections with the Δ*rfbE*, Δ*rfbE* Δ*asp24*, and Δ*rfbE* Δ*asp24*(pBBR-*asp24*) strains exhibited similar characteristics of necrosis, indicating that these mutants induced macrophage oncosis and necrosis but not apoptosis (Fig. 4C). In addition, levels of LDH released from RAW264.7 cells infected with the Δ*rfbE*, Δ*rfbE* Δ*asp24*, and Δ*rfbE* Δ*asp24*(pBBR-*asp24*) mutants showed no significant differences (Fig. 4D). These results indicated that *asp24* upregulation is not the cause of Δ*rfbE* mutant-induced oncotic and necrotic macrophage death.

***asp24* expression is induced under acidic conditions but not associated with bacterial acid resistance.** To determine *asp24* expression under acidic conditions, S2308 and the Δ*rfbE* mutant were grown to log phase and exposed to TSB at pH 7.3, 5.5, 4.5, and 3.8 for 2 h. qRT-PCR and Western blotting were performed to determine *asp24* expression, revealing that *asp24* upregulation was induced in both strains at pH 4.5 and 3.8 (Fig. 5A and B).

To determine whether *asp24*-upregulated expression in the Δ*rfbE* mutant enhances bacterial acid resistance, the S2308, Δ*rfbE*, Δ*rfbE*(pBBR-*rfbE*), and Δ*rfbE* Δ*asp24* strains were recovered after exposure to TSB at pH 7.3, 5.5, 4.5, and 3.8 for 2 h. The survival ratio of the Δ*rfbE* mutant was decreased significantly at pH 5.5 and 4.5 ($P < 0.05$) compared to that of WT strain S2308, but it recovered when the strain was complemented with the plasmid pBBR-*rfbE* (Fig. 5C). However, *asp24* deletion in the Δ*rfbE* mutant (Δ*rfbE* Δ*asp24*) did not weaken its acid resistance (Fig. 5C). This implies that the lack of O-antigen in *B. abortus* significantly decreases bacterial acid resistance in an *asp24*-independent manner.

Altered *asp24* expression in *B. abortus* strains reduces intracellular survival within RAW264.7 and HeLa cells. RAW264.7 cells were infected with the Δ*rfbE* or Δ*rfbE* Δ*asp24* mutant to determine the functions of *asp24* in bacterial adherence and invasion capacities. The results showed that no significant difference be-

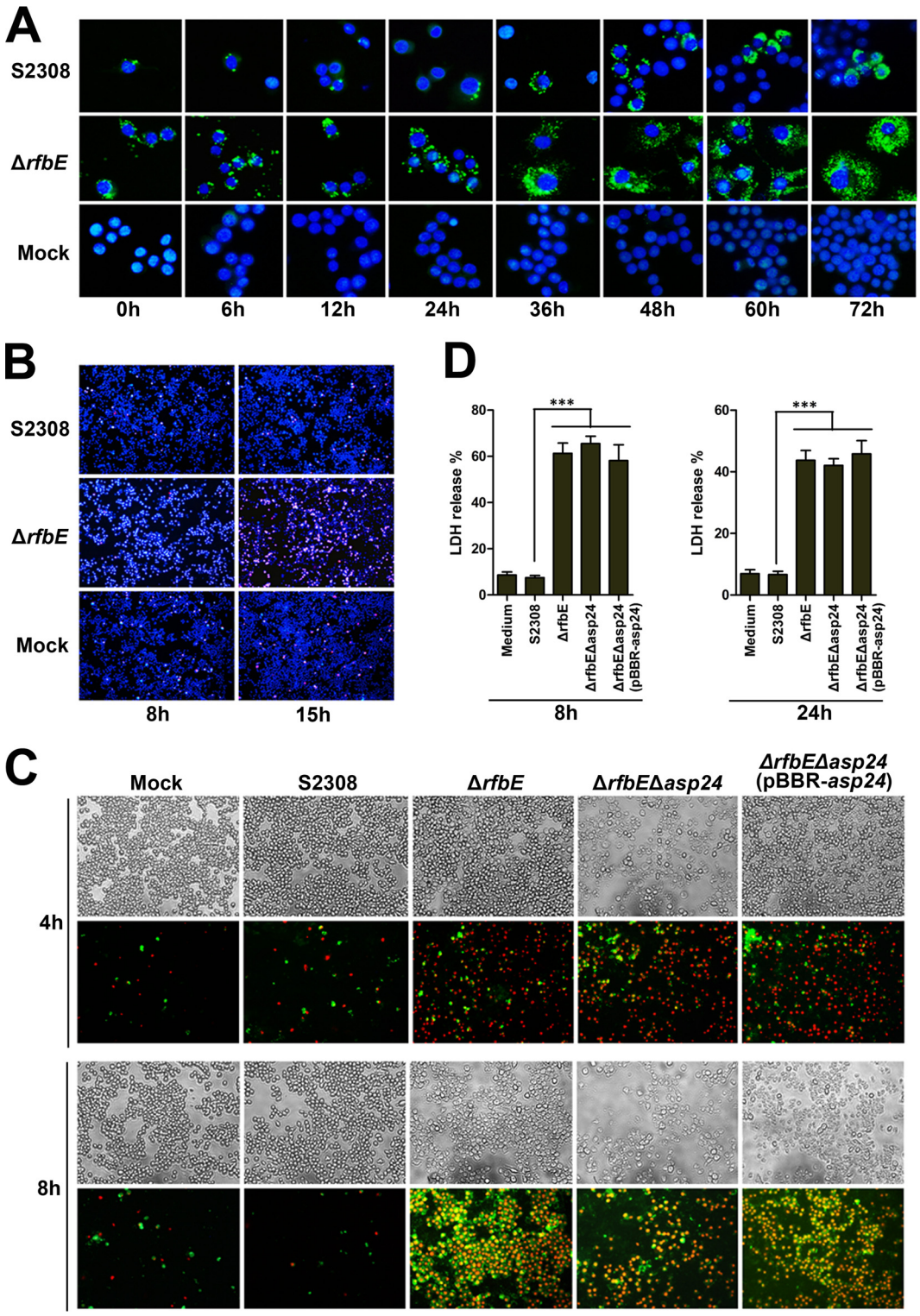

FIG 4 *Brucella* rough mutants induce necrosis in RAW264.7 cells. (A) RAW264.7 cells were infected with S2308 or the Δ*rfbE* mutant at an MOI of 30. Intracellular bacteria were stained at different time points with primary antibody (rabbit anti-*Brucella* polyclonal antibody) and secondary antibody (Alexa Fluor 488-conjugated goat anti-rabbit IgG) and then observed under a fluorescence microscope (magnification, ×10,000). Uninfected RAW264.7 cells were used as negative controls (Mock). (B) RAW264.7 cells cultured in a 24-well plate were infected with S2308 or the Δ*rfbE* mutant at an MOI of 200. The cells were stained at 8 and 15 hpi with Hoechst 33342 (blue) and PI (red) and then observed using fluorescence microscopy (magnification, ×100). Uninfected RAW264.7 cells were used as negative controls (Mock). (C) RAW264.7 cells cultured in a 24-well plate were infected with S2308, the Δ*rfbE* mutant, the double-knockout Δ*rfbE* Δ*asp24* strain, or the Δ*rfbE* Δ*asp24*(pBBR-*asp24*) complemented strain at an MOI of 200. The cells were stained at 4 and 8 hpi with FITC-annexin (green) and PI (red) and observed by phase-contrast (upper panels) or fluorescence (lower panels) microscopy at a magnification of ×200. Uninfected RAW264.7 cells were used as negative controls (Mock). (D) RAW264.7 cells were infected with S2308 or the Δ*rfbE*, Δ*rfbE* Δ*asp24*, or Δ*rfbE* Δ*asp24*(pBBR-*asp24*) mutant at an MOI of 200. The supernatants were collected at 8 and 24 hpi, and LDH release was detected using the CytoTox 96 nonradioactive cytotoxicity assay. The supernatants of uninfected RAW264.7 cells were used as negative controls (medium). ***, $P < 0.001$.

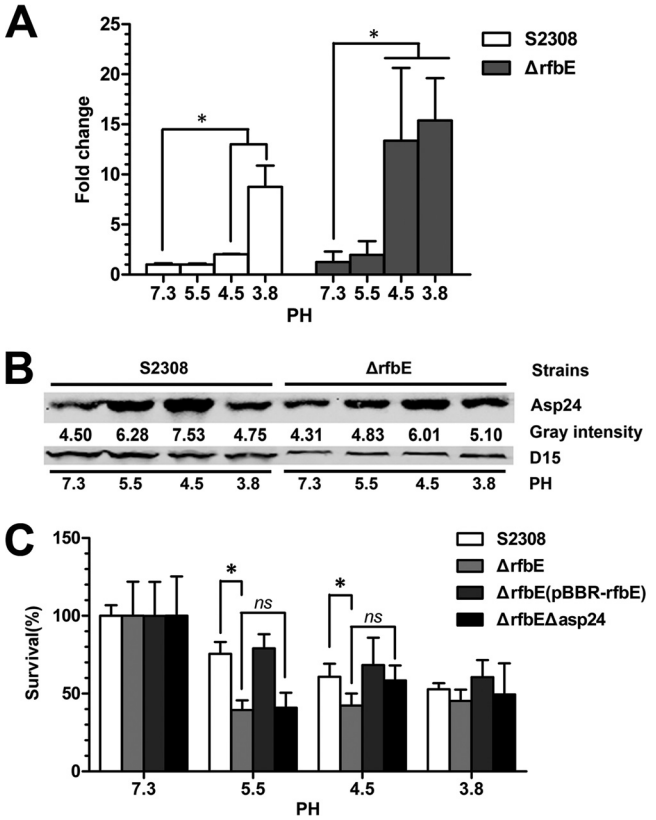

FIG 5 *asp24*/Asp24 expression is induced under acidic conditions but not associated with acid resistance. (A) *asp24* expression in the S2308 and Δ*rfbE* strains was upregulated at pH 4.5 and 3.8 compared to that at pH 7.3, as determined by qRT-PCR. *, $P < 0.05$. (B) Western blot showing enhanced Asp24 protein levels in S2308 at pH 5.5 and 4.5 and in the Δ*rfbE* mutant at pH 5.5, 4.5, and 3.8. Protein levels are indicated by gray-scanning intensity values. (C) The survival percentages for the Δ*rfbE* Δ*asp24* mutant showed no significant changes for TSB at pH 7.3, 5.5, 4.5, and 3.8 compared to those for the Δ*rfbE* mutant. *ns*, no significant difference. The survival percentages for S2308 were significantly higher at pH 5.5 and 4.5 than those for the Δ*rfbE* mutant. *, $P < 0.05$.

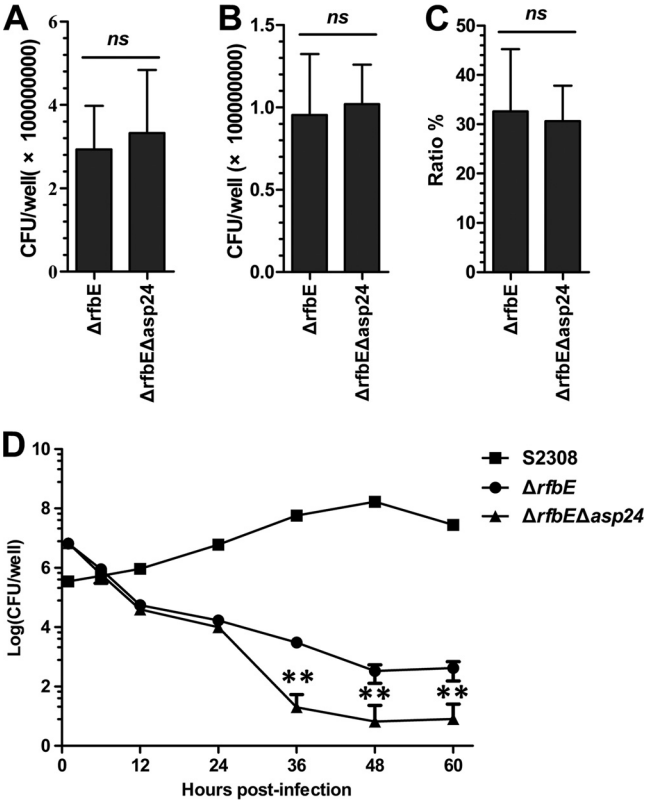

FIG 6 *asp24* deletion from the Δ*rfbE* mutant affects intracellular survival, but not adherence and invasion capacities, in RAW264.7 cells. (A to C) Adherence (A), invasion (B), and invasion ratios (C) for the Δ*rfbE* and Δ*rfbE* Δ*asp24* mutants in RAW264.7 cells, with no significant changes seen for infections at an MOI of 200. The invasion ratio was evaluated as the number of internalized bacteria/number of adherent bacteria. *ns*, no significant difference. (D) Bacterial intracellular survival of the Δ*rfbE* Δ*asp24* mutant was significantly decreased compared to that of the Δ*rfbE* mutant at 36, 48, and 60 hpi, for infections at an MOI of 50. **, $P < 0.01$.

tween the two mutants was found for bacterial adherence (Fig. 6A), invasion (Fig. 6B), or invasion ratio (number of invasive bacteria/number of adherent bacteria) (Fig. 6C). Thus, *asp24* does not appear to have a role in *B. abortus* adherence to and invasion into macrophages. An intracellular survival assay demonstrated reduced survival of both the Δ*rfbE* and Δ*rfbE* Δ*asp24* mutants in RAW264.7 cells compared to that of S2308. Intracellular survival of the Δ*rfbE* Δ*asp24* mutant was significantly worse than that of the Δ*rfbE* mutant after 36 hpi (Fig. 6D), indicating a role of *asp24* in intracellular survival within macrophages.

HeLa cells were also used to evaluate mutant intracellular survival, in addition to using macrophages. The S2308 and Δ*rfbE* strains were made to overexpress *asp24* through electroporation of the pBBR-*asp24* plasmid, generating strains S2308(pBBR-*asp24*) and Δ*rfbE*(pBBR-*asp24*), respectively (Fig. 7A). Both *asp24*-overexpressing strains showed attenuated survival in HeLa cells compared with that of the respective S2308 (24 and 48 hpi) and Δ*rfbE* (12, 24, and 48 hpi) strains (Fig. 7B and C). The results implied that appropriate expression of *asp24* plays a pivotal role in maximizing virulence for *B. abortus*. Deletion or overexpression of *asp24* in the S2308 and Δ*rfbE* strains affects their intracellular survival within host cells.

DISCUSSION

Brucella LPS is an important virulence determinant with important roles in host immune system evasion and bacterial survival (30–32). LPS with a complete O-antigen is crucial for *Brucella* virulence in humans (33). Disruption of the *rfbE* or *rfbD* gene induces the transformation of smooth-type *Brucella* strains into rough-type strains due to the creation of an incomplete LPS lacking O-antigen (19). The Δ*rfbE* mutant shows a rough phenotype, which enhances internalization into the host cell and induces macrophage oncosis and necrosis, like the case with other *Brucella* rough mutants (29, 34, 35). The mutant also has reduced survival in the host cell and fails to establish chronic infection in a mouse model (23). However, the mechanisms behind attenuated infection by the rough mutant have not been deciphered completely. In a previous study, artificial *Brucella* rough mutants were found to be cytopathic for macrophages and dependent on the type 4 secretion system (T4SS) (36, 37), but the effectors delivered by the T4SS are still unknown.

In this study, *asp24*-upregulated expression was found in rough mutants with a disrupted O-antigen export system ABC

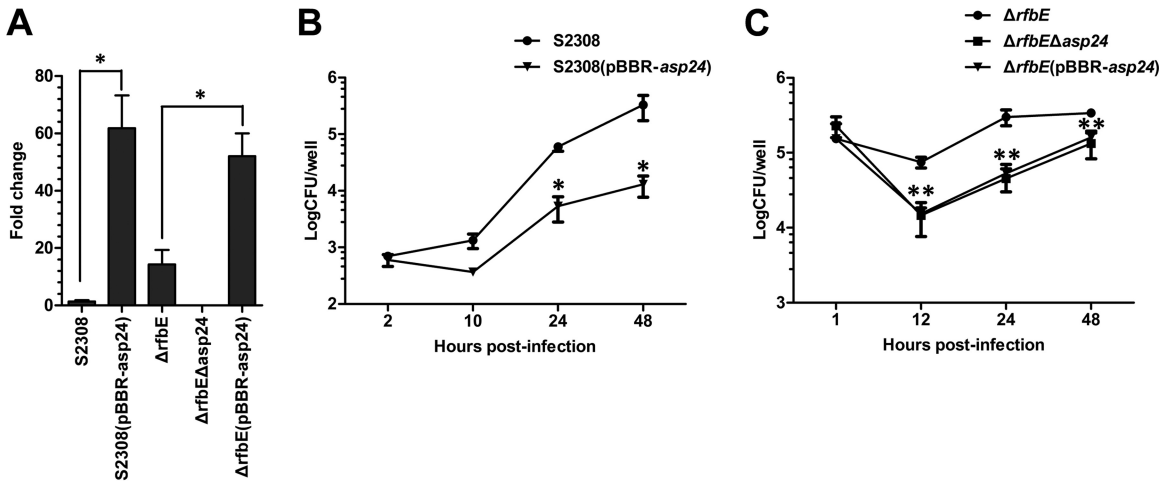

FIG 7 *asp24* deletion or overexpression in the S2308 and Δ*rfbE* strains results in reduced intracellular survival. (A) *asp24* expression in the S2308(pBBR-*asp24*) and Δ*rfbE*(pBBR-*asp24*) strains was upregulated compared to that in the respective S2308 and Δ*rfbE* strains, as determined using qRT-PCR. ∗, $P < 0.05$. (B) The intracellular survival of S2308(pBBR-*asp24*) (*asp24*-overexpressing S2308 strain) was significantly decreased at 24 and 48 hpi compared to that of S2308 in HeLa cells infected at an MOI of 500. ∗, $P < 0.05$. (C) The intracellular survival of the Δ*rfbE* Δ*asp24* (*asp24* deletion Δ*rfbE* strain) and Δ*rfbE*(pBBR-*asp24*) (*asp24*-overexpressing Δ*rfbE* strain) strains was significantly decreased at 12, 24, and 48 hpi compared to that of the Δ*rfbE* strain in HeLa cells infected at an MOI of 500. ∗∗, $P < 0.01$.

transporter, and further study confirmed that intracellular O-antigen synthesis and accumulation induced *asp24* upregulation in the mutant. In *Brucella*, O-antigen is synthesized via a Wzy-independent mechanism, and after its assembly onto bactoprenol phosphate at the cytoplasmic face of the inner membrane, the complete lipid-linked O-antigen is transported across the inner membrane by an ABC transporter system. *rfbD* and *rfbE* were identified and predicted to form the transmembrane and ATPase domains, respectively, of an ABC transporter required for the translocation of the full-length homopolymeric O-antigen from the cytoplasmic to the periplasmic face of the inner membrane (38). In the study, the deletion of *rfbD*/*rfbE* resulted in the accumulation of intracellular synthesized O-antigen, which may induce *asp24* upregulation by increasing intracellular pressure. The Asp24 protein was initially identified from its enhanced expression in macrophages and under low-pH conditions *in vitro* (21); its exact role in pathogenesis is unknown. In *B. melitensis* or *B. abortus*, *asp24* deletion mutants showed attenuated persistence in mice compared to the WT strains, and the *B. melitensis* Δ*asp24* strain failed to cause abortion or to colonize fetal tissues in pregnant goats (26, 39). This suggests that *asp24* plays an important role in ensuring *Brucella*'s full virulence in infecting mice and goats.

asp24 expression is stimulated under acidic conditions. Asp24 contains an EF-hand motif found in a large family of calcium-binding proteins (40, 41). This motif appears to have a specific role in acid shock adaptation; however, the function of Asp24 has not been defined completely. In a previous report, rough mutants were cytopathic toward macrophages; the Δ*rfbE* mutant was also found to have this characteristic. In this study, we report that *asp24* expression is upregulated in rough mutants with a compromised O-antigen export system, but this upregulation is not the cause of macrophage cytotoxicity. In addition, the acid resistance assay revealed that *asp24* deletion did not affect Δ*rfbE* mutant survival at low pH, even though survival of both the Δ*rfbE* and Δ*rfbE* Δ*asp24* mutants was significantly lower than that of WT strain S2308 under acidic conditions.

asp24 upregulation is induced not only under acidic conditions but also inside macrophages (21). In this study, *asp24* upregulation was found in the Δ*rfbE* and Δ*rfbD* mutants. To explore the function of *asp24* in *B. abortus* intracellular survival, mutants with altered *asp24* expression were constructed, and intracellular survival was assessed using a gentamicin protection assay. In RAW264.7 cells, *asp24* deletion from the Δ*rfbE* mutant reduced its intracellular survival, but both the Δ*rfbE* and Δ*rfbE* Δ*asp24* mutants were cytopathic to the macrophages. To circumvent this cytopathic effect, the experiments were repeated in HeLa cells, because neither strain was cytopathic to HeLa cells. Interestingly, both abolished and overexpressed *asp24* reduced Δ*rfbE* mutant intracellular survival in HeLa cells. Similar results were obtained with HeLa cells infected with the WT strain S2308 overexpressing *asp24*.

Nonopsonized *Brucella* entry into macrophages is mediated through lipid rafts (29, 42–44). Once inside cells, *Brucella* organisms reside in the *Brucella*-containing vacuole (BCV), which interacts with components of endocytic pathways to ensure bacterial survival (45–48). Inhibition of BCV acidification at the early stages of infection completely abolishes *Brucella* intracellular survival (49). We hypothesized that Asp24 plays a role in BCV acidification, but the role may not be indispensable at this stage, because *asp24* deletion in the Δ*rfbE* mutant did not completely abolish intracellular survival. Intriguingly, overexpressing *asp24* in the Δ*rfbE* and S2308 strains also reduced intracellular survival, indicating that proper and moderate *asp24* expression is necessary to ensure intracellular survival. How the *asp24* gene is precisely regulated and expressed during infection inside host cells is still unknown.

In conclusion, *asp24* expression is induced in rough mutants with a disrupted O-antigen export system ABC transporter. Its upregulation is not the cause of rough mutant-induced oncosis and necrosis in macrophages, but irregular *asp24* expression in WT or mutant strains affects *B. abortus* intracellular survival within host cells. In the present study, we (i) illustrated that *Brucella asp24* upregulation in specific mutants is associated with in-

tracellular O-antigen synthesis and accumulation, (ii) discovered a novel *asp24* upregulation mechanism, and (iii) indicated the role of proper Asp24 expression in bacterial intracellular survival. Further studies will investigate the possible mechanism relating Asp24 expression to O-antigen secretion during the normal course of infection.

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