Veterinary Microbiology 230 (2019) 130–137

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Identification of novel genes essential for *Brucella abortus* to establish infection by signature-tagged mutagenesis

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ARTICLE INFO

Keywords:
Brucella abortus
Virulence-related gene
Signature-tagged mutagenesis
Pyruvate carboxylase
Pathogenesis

ABSTRACT

Brucella is a facultative intracellular bacterium, causing brucellosis, an important zoonosis worldwide. *Brucella* has no classic virulence factors, thus virulence is dependent on invasion of host cells and subsequent intracellular replication. Identification of key genes involved in *Brucella* virulence is important to further elucidate its pathogenesis. In this study, signature-tagged mutagenesis was used to identify novel genes involved in *B. abortus* infection in a mouse model. In total 3600 mutants were obtained, of which 56 were identified as attenuated mutants. Furthermore, 53 genes were identified to be inactivated by transposon insertion, including 19 genes previously reported to be essential for *Brucella* virulence and 34 others that were newly identified in this study. These genes were catalogued into 16 functional classifications, except for three that were not cited in the Clusters of Orthologous Groups database. Bioinformatics analysis revealed that energy production and conversion, amino acid transport and metabolism, as well as inorganic ion transport and metabolism were predominant functional classifications, suggesting that genes involved in these functions were crucial for *Brucella* virulence. In addition, the function of the identified pyruvate carboxylase (*pyc*) gene in bacterial virulence was confirmed using an allelic replacement *pyc* mutant and a mouse model. These findings provide novel genetic information associated with *Brucella* infection.

1. Introduction

Brucella is a genus of Gram-negative facultative intracellular pathogens, causing brucellosis, an important bacterial zoonosis worldwide (Boschiroli et al., 2001; von Bargen et al., 2012). In contrast to other pathogenic bacteria, *Brucella* has no classic virulence factors, such as exotoxins, cytolysins, capsules, fimbria or endotoxic lipopolysaccharide (LPS) (Seleem et al., 2008). A key aspect of *Brucella* virulence is the ability to replicate within host cells. Upon invasion of the host cell, *Brucella* uses several sophisticated strategies for intracellular survival, including resistance to the bactericidal effects of phagocytes, inhibition of host cell apoptosis, adaption to acidified membrane-bound vesicles and prevention of phagosome-lysosome fusion (Martirosyan et al., 2011). Together, these properties endow *Brucella* with the ability to establish infection in preferred hosts. Hence, it is very important to identify key genes necessary for *Brucella* infection and to further elucidate the mechanisms of *Brucella* pathogenesis.

To date, several key factors of *Brucella* virulence have been identified, which include LPS (Lapaque et al., 2005), the type IV secretion system (T4SS) (Celli et al., 2003; O'Callaghan et al., 1999) and the two-component regulatory system BvrS/BvrR (Sola-Landa et al., 1998). Besides, many other components are reportedly involved in *Brucella* virulence, including cyclic β-1,2-glucans, superoxide dismutase, catalase, urease and cytochrome oxidase (Seleem et al., 2008). So far, based on a platform of the *Brucella* Bioinformatics Portal, 245 genes involved in *Brucella* virulence have been collected in a database (Xiang et al., 2006). However, with the development of molecular genetic techniques, more and more genes associated with *Brucella* virulence continue to be discovered, thereby offering further insight into *Brucella* pathogenesis.

In this study, a PCR-based signature-tagged mutagenesis technique was used to identify attenuated mutants in a mouse model of *Brucella* infection. Finally, 53 genes were identified as necessary for *Brucella* infection, including 34 that are newly reported in this study, thereby providing further insight into *Brucella* pathogenesis.

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https://doi.org/10.1016/j.vetmic.2019.02.005
Received 24 November 2018; Received in revised form 15 January 2019; Accepted 3 February 2019

Table 1
Strains, plasmids and primers used in this study.

Strains, plasmids and primers	Description or sequences (5'–3')	Sources or characteristics
B.abortus		
S2308	Wild-type *Brucella abortus* strain; Smooth phenotype	Chinese Veterinary Culture Collection Center
S2308 Nal^R	Nalidixic acid resistance WT strain; Smooth phenotype	This study
E. coli		
S17-1 λ*pir*	Tp^RSm^R*recAthi pro hsdR*$^-$M$^+$ RP4::2-Tc::Mu-*km*::Tn7 λ*pir*	Biomedal Life Science
Plasmids		
pUTmini-Tn5 Cm	Cm^R; Containing mini-Tn5 mobile elements able to transpose to bacterial chromosome.	Biomedal Life Science
pUTmini-Tn5-tag1-tag8	Cm^R; pUTmini-Tn5 Cm containing tag1-tag8, respectively	This study
Primers		
Tag1	GGCCGCCTGAACGTTTAAGCGCGGTACGC	Construction of tag1 transposon plasmid
Tag1-com	GGCCGCGTACCGCGCTTAAACGTTCAGGC	
Tag2	GGCCGCCAGGCTATTTAAGCGCGGTACGC	Construction of tag2 transposon plasmid
Tag2-com	GGCCGCGTACCGCGCTTAAATAGCCTGGC	
Tag3	GGCCGCCGAGACTTTTAAGCGCGGTACGC	Construction of tag3 transposon plasmid
Tag3-com	GGCCGCGTACCGCGCTTAAAAGTCTCGGC	
Tag4	GGCCGCCCACGTTATTAAGCGCGGTACGC	Construction of tag4 transposon plasmid
Tag4-com	GGCCGCGTACCGCGCTTAATAACGTGGGC	
Tag5	GGCCGCCTACCAGTTTAAGCGCGGTACGC	Construction of tag5 transposon plasmid
Tag5-com	GGCCGCGTACCGCGCTTAAACTGGTAGGC	
Tag6	GGCCGCCACGATTGTTAAGCGCGGTACGC	Construction of tag6 transposon plasmid
Tag6-com	GGCCGCGTACCGCGCTTAACAATCGTGGC	
Tag7	GGCCGCCGTCTTGATTAAGCGCGGTACGC	Construction of tag7 transposon plasmid
Tag7-com	GGCCGCGTACCGCGCTTAATCAAGACGGC	
Tag8	GGCCGCCCTACTAGTTAAGCGCGGTACGC	Construction of tag8 transposon plasmid
Tag8-com	GGCCGCGTACCGCGCTTAACTAGTAGGGC	
Tag1-F	GTACCGCGCTTAAACGTTCAG	Forward primer for PCR identification of Tag 1
Tag2-F	GTACCGCGCTTAAATAGCCTG	Forward primer for PCR identification of Tag 2
Tag3-F	GTACCGCGCTTAAAAGTCTCG	Forward primer for PCR identification of Tag 3
Tag4-F	GTACCGCGCTTAATAACGTGG	Forward primer for PCR identification of Tag 4
Tag5-F	GTACCGCGCTTAAACTGGTAG	Forward primer for PCR identification of Tag 5
Tag6-F	GTACCGCGCTTAACAATCGTG	Forward primer for PCR identification of Tag 6
Tag7-F	GTACCGCGCTTAATCAAGACG	Forward primer for PCR identification of Tag 7
Tag8-F	GTACCGCGCTTAACTAGTAGG	Forward primer for PCR identification of Tag 8
Tag-R	GTCCCCCGCAGTTGGTAATA	Universal reverse primer for PCR identification of eight tags
SP1	ATGCTGGCAATCACTTTGACGTG	Genome walking PCR primer
SP2	CTTGAGGCGTTGCATCCAGGTC	Genome walking PCR primer
SP3	CACCGAACAAGTGATCCCGATGT	Genome walking PCR primer
Tn5-F	TTTGACGGATTTAACCTG	Preparation of DNA probe
Tn5-R	TATACTGAACAGAAAAGCATG	
pyc-UF	GCTCTAGAGCGACCAACCCGACCTTTACC	PCR amplification of the upstream fragment of *pyc* gene
pyc-UR	GTTTGGCACCTTGATGCGCTTGTCTTCCTCAGCCCATA	
pyc-DF	TATGGGCTGAGGAAGACAAGCGCATCAAGGTGCCAAAC	PCR amplification of the downstream fragment of *pyc* gene
pyc-DR	GCTCTAGAGCTGCCAGCCATCGAGAAAG	

2. Materials and methods

2.1. Ethics statement

This study was performed in strict accordance with the guidelines of the Care and Use of Laboratory Animals of the Institutional Animal Care and Use Committee of Shanghai Veterinary Research Institute, the Chinese Academy of Agricultural Sciences (CAAS). Mice (SLAC Experimental Animal Inc., Shanghai, China) were housed in cages with *ad libitum* access to food and water under biosafety conditions. All animal handling procedures were approved by the Committee on the Ethics of Animal Experiments of Shanghai Veterinary Research Institute, CAAS (permit no. SHVRI-MO-0126).

2.2. Bacterial strains and cultural conditions

All strains used in this study are listed in Table 1. The wild-type (WT) *B. abortus* strain S2308 or its derivatives were cultured on tryptic soy broth (TSB) (Difco Laboratories, Franklin Lakes, NJ, USA) or tryptic soy agar (TSA) (Difco Laboratories) at 37 °C under an atmosphere of 5% CO_2. Manipulation of *B. abortus* was performed in a biosafety level 3 laboratory facility at the CAAS. *Escherichia coli* strain S17-1 λ*pir* was cultured on Luria-Bertani medium at 37 °C. When appropriate, 100 μg/mL of ampicillin (Amp), 20 μg/mL of chloramphenicol (Cm) or 50 μg/mL of nalidixic acid (NA) (Sigma-Aldrich Corporation, St. Louis, MO, USA) were added to the medium.

2.3. Development of the NA-resistant B. abortus strain S2308

The WT strain S2308 was cultured on TSB supplemented with 5% heat-killed foetal bovine serum (FBS) (Hyclone™, GE Healthcare Life Sciences, Logan, UT, USA). FBS was add to TSB to provide more abundant nutrition for *Brucella* growth. NA was gradually added to the medium to a final concentration of 1, 2, 4, 6, 8, 10, 15, 20, 30, 40 or 50 μg/mL. The resistant strain was successively cultured for 10 generations in TSB supplemented with 5% FBS and 50 μg/mL of NA. Finally, the FBS was removed from TSB, which still contained 50 μg/mL of NA, and the resistant strain was cultured for 20 generations. The achieved resistant strain was designated as S2308 Nal^R.

2.4. Determination of bacterial virulence

Bacterial virulence was determined using 6–8-week-old BALB/c female mice as described elsewhere (Gao et al., 2016). Briefly, each mouse was intraperitoneally inoculated with the WT strain, the S2308 Nal^R strain or the *pyc* mutant in 0.1 mL of bacterial suspension

containing 1×10^6 colony-forming units (CFU). Afterward, six mice from each group were sacrificed by cervical dislocation at 1, 3 or 5 weeks post-infection (p.i.). Bacteria were recovered from the spleens of the infected mice. The bacterial load was determined by serial dilutions of spleen homogenates on TSA plates, which were incubated for 72 h at 37 °C under an atmosphere of 5% CO_2. The spleens were weighed to evaluate the degree of splenomegaly.

2.5. Construction of tagged pUTmini-Tn5 plasmids

pUTmini-Tn5 plasmids (Biomedal Life Science, Seville, Spain) with each of the eight tags were constructed for signature-tagged mutagenesis. The tags were designed according to previously reported sequences (Lehoux and Levesque, 2002). Eight pairs of 21-mer oligonucleotides were designed (Table 1) to synthesize the complementary DNA strands, which were cloned into the pUTmini-Tn5 plasmid. Pairs of complementary oligonucleotides were designed with the same sticky ends digested by the *Not*I enzyme. Annealing reactions contained 10 μM concentrations of both complementary oligonucleotides in 100 μL of 1× annealing buffer for DNA oligos (Beyotime Institute of Biotechnology, Haimen, China). The oligonucleotide mixture was heated for 5 min at 95 °C and slowly cooled to room temperature in a block heater and then kept on ice.

The pUTmini-Tn5 Cm plasmid was digested with the restriction enzyme *Not*I (Takara Bio, Inc., Shiga, Japan) and dephosphorylated with shrimp alkaline phosphatase (Takara Bio, Inc.). The annealed oligonucleotide mixture was ligated with linearized pUTmini-Tn5 Cm using a DNA ligation kit (Takara Bio, Inc.). The constructed plasmids with eight different tags were designated as pUTmini-Tn5-tag1–8, respectively. The pUTmini-Tn5 plasmids with eight tags were transformed into *E. coli* S17-1 *λpir* cells as donor strains.

2.6. Verification of the specificity of eight tags by polymerase chain reaction (PCR)

Tag design and PCR amplification were performed as described previously (Lehoux and Levesque, 2002). The cross-reactions among the eight tags were detected by PCR for verification of the specificity of eight tags as follows: pUTmini-Tn5-tag1–8 plasmids were extracted as templates, respectively. The tag was identified by PCR using the counterpart primers, such as with tag1 detection using the specific forward primer Tag1-F and the universal reverse primer Tag-R. PCR specificity was verified using a mixture of templates. For example, the PCR specificity of tag1 was confirmed using the other seven plasmid tags in a mixture of templates, while PCR was performed using the primer pair Tag1-F and Tag-R. A specific fragment was not amplified by PCR from the other seven tag templates, thereby qualifying the specificity of the tag.

2.7. Mutant library construction

To construct the mutant library, a bacterial conjugation assay was used to transform the S2308 Nal^R strain with the eight specifically tagged pUTmini-Tn5 Cms. Briefly, *E. coli* S17-1 cells harbouring the target plasmids as the donor strain and the 2308 Nal^R derivative as the receptor strain were cultured to the early log phase (optical density at 600 nm = 0.6–0.8), the bacteria were washed twice with 10 mM $MgSO_4$ buffer and adjusted to a concentration of 1×10^9 CFU/mL of *E. coli* and 1×10^{10} CFU/mL of *B. abortus*. Mating was performed by mixing equal volumes (1 mL) of suspensions of S17-1 donor cells and the S2308 Nal^R recipient strain on a 0.22-μM filter, which was placed on a TSA plate without antibiotics at 28 °C for 8 h. Then, the mixed cells on the filter were washed down with 5 mL of 10 mM $MgSO_4$ buffer and 100 μL of the washed cells were plated on a TSA plate containing 20 μg/mL of NA and 20 μg/mL of Cm. After incubation for 3–5 days at 37 °C, the exconjugants were replicated on TSA plates containing NA and Cm or containing NA and Amp. The Amp-resistant clones were discarded, while the Cm-resistant and Amp-sensitive clones were transferred to 2-mL microtubes for proliferation to construct the mutant library.

2.8. Screening of attenuated B. abortus mutant strains

A mouse infection assay was used to screen the attenuated mutants. Briefly, the mutants were grown at 37 °C in 400 μL of TSB in 2-mL microtubes for 36–48 h. The bacteria were then pooled with eight mutants harbouring the different tags and the mixed bacterial suspension was diluted to a final concentration of approximately 10^7 CFU (about 10^6 CFU of each tag mutant) in 200 μL of phosphate-buffered saline (PBS). A 200-μL aliquot of the suspension was injected intraperitoneally into 6–8-week-old female BALB/c mice, in total, 450 mice were used for infection. The remaining bacterial suspension was plated on TSA. After culturing for 3–5 days, the bacteria were washed down from the TSA plate with 5 mL of PBS for DNA extraction using the TIANamp bacteria DNA kit (Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer's instructions. At 5 weeks p.i., the mice were sacrificed and the spleens were removed aseptically. To recover the bacteria, the spleens were homogenized in 5 mL of PBS-0.2% Triton X-100 (Sigma-Aldrich Corporation). Then, a 100-μL aliquot of the suspension was plated on TSA with appropriate antibiotics for 3–5 days. The bacterial clones were washed down from TSA and used for DNA extraction. The mutants that were positive in culture and negative in spleen recovery by the PCR assay were designated as attenuated strains. PCR reactions were performed in 20-μL reaction volumes containing 100 ng of bacterial genomic DNA.

After the first round of screening, a second round of screening was performed to exclude the false positives. Finally, 468 mutants that were not isolated from the infected spleens were identified as attenuated strains. For further confirmation, a second round of screening was performed as described above. Briefly, the mutants that were not recovered from the first round of screening were pooled again and screened with the mouse infection assay as with the first round of screening. In total, about 60 mice were used for infection.

After the second round of screening, 115 mutants that were not isolated from the infected spleens were identified as attenuated strains. Of these, 29 mutants were not further assayed because of obvious impaired growth in TSB, while the remaining 89 mutants showed similar growth capabilities as the S2308 strain. To determine the virulence of the mutants, the 89 attenuated mutants from two rounds of screening were injected intraperitoneally (about 10^6 CFU) into 6–8-week-old female BALB/c mice (n = 2), in total, 180 mice were used for infection. At 5 weeks p.i., the spleens of the infected mice were collected and homogenized in 5 mL of PBS-0.2% Triton X-100. Then, serial dilutions of the homogenates were plated on TSA plates to determine the bacterial loads. The 'virulence ratio' was used to evaluate the attenuation rate of the mutant's virulence and calculated as: $\log_{10}$ CFU (recovered from the mutant-infected spleens)/$\log_{10}$ CFU (recovered from the WT strain S2308-infected spleens). A virulence ratio < 0.9 [($\log_{10}$ CFU recovered from the non-attenuated mutant-infected spleens/$\log_{10}$ CFU recovered from the WT strain S2308-infected spleens) – 3× standard deviation] indicates that the virulence of the mutant was attenuated.

2.9. Southern blot analysis

Southern blot analysis was performed to confirm the copy number of the miniTn5 transposons inserted into the genomes of the mutants. A DNA probe was prepared by PCR amplification of a 630-bp fragment from the pUTmini-Tn5 Cm plasmid using the primers Tn5-F and Tn5-R (Table 1). The probe was labelled with digoxigenin (DIG) using the DIG-High Prime DNA Labeling and Detection Starter Kit I (Roche Diagnostics GmbH, Penzberg, Upper Bavaria, Germany). Genomic DNA was extracted from the attenuated mutants using the TIANamp Bacteria DNA Kit (Tiangen Biotech Co., Ltd.), digested with the *Eco*RI restriction

endonuclease, separated by gel electrophoresis and transferred to a nylon membrane. Hybridization and immunological detection were conducted using a standard assay according to manufacturer's instructions.

2.10. Transposon insertion sites and bioinformatic analyses

The site of transposon insertion in the attenuated mutant was determined by genomic walking PCR, which was performed using a genome walking kit (Takara Bio, Inc.) according to the manufacturer's instructions. Arbitrary primers (AP1, AP2, AP3 and AP4) provided with the kit, as well as the specific primers SP1, SP2 and SP3 (Table 1), were used to amplify DNA adjacent to the insertion site. After purifying the PCR products from agarose gels with the GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Waltham, MA, USA), the PCR products were sequenced (Shanghai Invitrogen Biotechnology Co., Ltd., Shanghai, China). The obtained sequences were compared to that of *B. abortus* strain S2308 (accession numbers: NC_007618 and NC_007624) in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) using the nucleotide BLAST algorithm (https://blast.ncbi.nlm.nih.gov). The site of transposon insertion was confirmed in the *Brucella abortus* genome and then the inactivated genes were identified in each mutant. Afterward, the encoding protein sequences were downloaded from the GenBank database and their functional annotation and classification were searched against the eggNOG (version 4.5) database (http://eggnog.embl.de/version_3.0/).

2.11. Construction of the pyc deletion mutant

The *Brucella* deletion mutant was constructed as described previously (Gao et al., 2016). Briefly, based on the S2308 genome template, the upstream and downstream fragments of the *pyc* gene were amplified by PCR using the primers pyc-UF/UR and pyc-DF/DR, respectively, and then fused by overlap-PCR using the primers pyc-UF and pyc-DR. The fused DNA fragments were recovered from an agarose gel, digested with the *Xba*I restriction enzyme and ligated into the *Xba*I-digested pSC plasmid (Bao et al., 2017; Gao et al., 2016). The suicide plasmid pSC-pyc was constructed successfully and proliferated in *E. coli* DH5α cells.

The *pyc* mutant was constructed based on the SacB-assisted allelic replacement suicide plasmid by electroporation as reported previously (Tian et al., 2018, 2019). The electro-cells of the WT strain S2308 were prepared by washing twice with pre-chilled sterile deionized water and then suspended in a 10% (v/v) solution of sterile glycerol and water. Next, 5 μg of the pSC-pyc was added to the suspension of electro-cells and electroporation was conducted at 2.4 kV and 400 Ω. Positive clones were selected on TSA plates containing Amp. Afterward, the *pyc* mutant strains were further screened on TSA plates containing 5% sucrose based on the *sacB* gene in the pSC plasmid. The *pyc* mutant was verified by PCR.

2.12. Cell infection assay

RAW 264.7 macrophages were used to assess the ability of the *pyc* mutant to survive intracellularly. A gentamicin protection assay was performed as previously reported (Gao et al., 2016). A cell monolayer was prepared and infected with the WT strain or the *pyc* mutant at a multiplicity of infection of 100. At 4, 24 and 48 h p.i., the macrophages were lysed with 0.2% Triton X-100 in sterile water and the live bacteria were enumerated on TSA plates. All assays were performed in triplicate and repeated at least three times. The results are presented as the averages of triplicate infection samples ± standard deviation of one independent experiment.

2.13. Statistical analysis

Statistical analysis was performed using GraphPad Prism software 6.0 (GraphPad Software Inc., La Jolla, CA, USA). Statistical significance was determined using the two-tailed Student's *t*-test. A probability (p) value of ≤ 0.05 was considered statistically significant.

3. Results

3.1. Virulence was similar between the NA-resistant and WT B. abortus strains

To construct a mutagenesis library of *B. abortus*, the bacterial strain requires an antibiotic resistant marker to screen for mutants. According to a previous report by Kim et al. (2003), NA resistance is easily induced in *Brucella*. A NA-resistant strain of *B. abortus* S2308 was successfully constructed in this study. The WT *B. abortus* strain S2308 was inoculated into TSB with 5% FBS and NA, and a gradually increasing concentration of antibiotics. *Brucella* was grown in TSB containing 50 μg/mL of NA. Then, FBS was removed from the medium and the *Brucella* strain was cultured for an additional 20 generations. Finally, a NA-resistant strain was acquired, which was designated as strain S2308 Nal^R.

To confirm the virulence of strain S2308 Nal^R, BALB/c mice were infected with either the WT S2308 or S2308 Nal^R strain and the bacterial load in the mouse spleen was calculated at the indicated time. As shown in Fig. 1, no significant difference on bacterial load was observed between the S2308 Nal^R and the WT strains at 1, 3 and 5 weeks p.i., suggesting that the virulence of the S2308 Nal^R strain was similar to that of the WT strain.

3.2. Eight different tags of the mini-Tn5 transposons showed no cross-reaction in a PCR assay

Eight pairs of complementary oligonucleotides were designed (Table 1) and ligated with pUTmini-Tn5 Cm plasmids in accordance with the manufacturer's instructions. Then, the eight tagged transposon plasmids (pUTmini-Tn5-tag1-8) were constructed as described in the Materials and Methods section. The specificity of the eight tags was verified by PCR using the primer pair of one tag to detect the other seven tags. The tag transposon was identified by PCR using relative tag primer pairs (Table 1) and 1% agarose electrophoresis clearly revealed an objective band of about 1200 bp (Fig. 2A). However, PCR with selected tag primer pairs failed to amplify the other tags in the mixed template (Fig. 2B). These results indicated that the eight tag primer

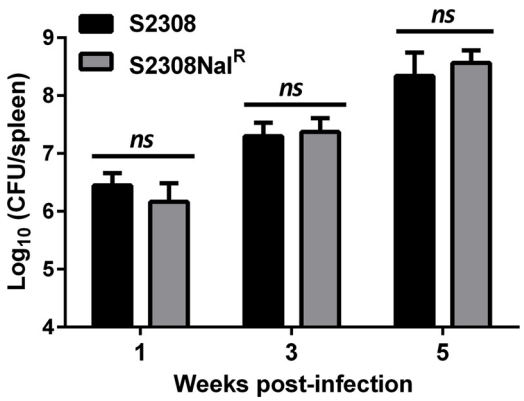

Fig. 1. Virulence determination of the WT S2308 and the NA-resistant S2308 Nal^R strains. Thirty-six 6–8-week-old BALB/c mice were intraperitoneally injected with S2308 or S2308 Nal^R at a dose of 10^6 CFU/mouse. At 1, 3 and 5 weeks p.i., six mice in each group were sacrificed and the bacterial load in the spleen was quantified and analysed. *ns*, not significant.

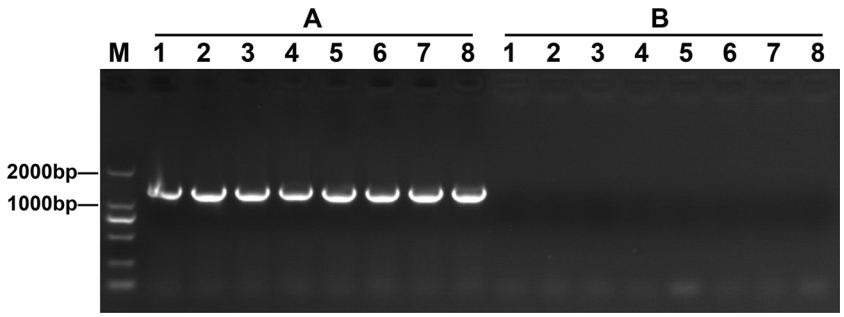

Fig. 2. Determination of the specificity of eight tags using PCR analysis. (A) PCR products of eight tags (about 1200 bp) were amplified using each specific tag forward primer and the universal reverse primer Tag-R. Lanes 1–8 showed the products of tags 1–8, respectively, amplified with respective forward primers and the universal reverse primer Tag-R. (B) PCR specificity was verified using specific forward primers to amplify a mixture of seven non-matched tags. For example, no product was obtained using the Tag1-F and Tag-R primers to amplify the template of a mixture of tags 2–8 (Lane 1). Lanes 1–8 showed the specificity for tags 1–8, respectively. Lane M, DNA marker DL2000 (Takara Bio, Inc.).

pairs specifically amplified only the respective tag in the transposons. The PCR results showed no cross-reaction among the eight tags.

3.3. Construction of the random mutant library

Escherichia coli S17-1 λ*pir* cells harbouring the pUTmini-Tn5 plasmid with eight different tags were conjugated with the S2308 Nal^R strain as described in the Materials and Methods section. The transformants were isolated by plating on TSA supplemented with Cm and NA. The Amp-resistant mutants were discarded. Finally, a total of 3600 mutants were produced by transposon mutagenesis, consisting of 450 mutants for each tag of the mutant library.

3.4. The attenuated mutants were identified from the mutant library

Each pool of mutants with eight tags were cultured in TSB *in vitro* and used for infection of mice. The mutants cultured *in vitro* and recovered from the spleens of infected mice were collected for extraction of genomic DNA, which was amplified by PCR. The mutant was designated as the attenuated strain when a positive reaction in culture and negative reaction in recovery were shown by PCR. As shown in Fig. 3, three pools of mutants were identified by PCR using eight different primer pairs. Then, eight attenuated mutants were screened by PCR analysis. After two rounds of screening, 89 attenuated strains were identified and further investigated to confirm their roles in *Brucella* virulence. The 'virulence ratio' was used to assess the virulence of the mutants, as described in the Materials and Methods section. Among these, 77 mutants were identified as attenuated strains (virulence ratio < 0.9), while virulence was not significantly reduced in 12 mutants with a virulence ratio ≥ 0.9. Strain S2308 (virulence ratio = 1) was used as a positive control. In total, 77 mutants were attenuated in the library of 3600 mutants, accounting for 2.1% of the total number. Of these, 68 were verified by Southern blot analysis to confirm that only one copy of the mini-Tn5 transposon was inserted into the genome. More than one copy of the transposon was inserted into the genomes of nine mutants, which were not used for the further analysis. As shown by the representative results of the Southern blot analysis in Fig. 4, most mutant genomes contained one copy of the transposon insertion, while the E10 and H10 mutants contained two. The S2308 Nal^R strain, which contained no transposon insertion, was used as a negative control. Furthermore, genome walking PCR was used to identify the disrupted genes of the attenuated mutants. The results showed that a transposon was inserted in the gene open reading frame of 56 attenuated strains and into the spacer region of two genes in 10 attenuated strains. The transposon insertion site could not be identified in two mutants. In all, 56 attenuated strains containing one transposon within the functional open reading frame were obtained, in which 53 genes were inserted and identified to be associated with *B. abortus* virulence (Table 2). Among these genes, 34 were newly identified to be important for *Brucella* virulence, while the other 19 were previously reported as virulence-related genes.

3.5. Bioinformatics analysis revealed important functional classifications of genes involved in Brucella virulence

Of the 3600 mutants in the mutant library, 56 were identified as attenuated strains, which included three with the same inactivated gene. So, a total of 53 genes were confirmed to be associated with *Brucella* virulence (Table 2). By functional classification, these 53 genes were classified into 16 groups based on the Clusters of Orthologous Groups database (Table 2). Three groups were predominant by functional classification, being involved in energy production and conversion, amino acid transport, metabolism, and organic ion transport and catabolism.

3.6. Brucella pyruvate carboxylase was verified as a virulence-associated gene

To further confirm the role of the identified genes in *Brucella* virulence, a pyruvate carboxylase (*pyc*) deletion mutant (*pyc* mutant) was

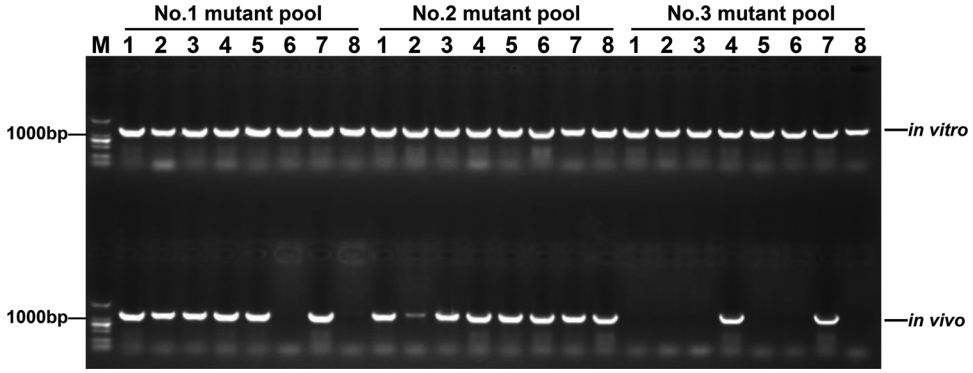

Fig. 3. Representative image of PCR screening of the attenuated strains. Data from three mutant pools are shown. Each pool contained eight mutants with different tags (1–8). The mutants in each pool cultured in TSA (*in vitro*) and recovered from infected mice spleens (*in vivo*) were subjected to genomic DNA extraction and PCR amplification. The mutant was designated as the attenuated strain when a positive reaction from *in vitro* cultured bacteria and negative reaction from *in vivo* recovered bacteria by PCR amplification. The mutants 6 and 8 in mutant pool 1 and mutants 1, 2, 3, 5, 6 and 8 in mutant pool 3 were designated as the attenuated strains. Lane M, DNA marker DL2000 (Takara Bio, Inc.). Lanes 1–8, PCR was performed using respective eight primer pairs for eight different tags.

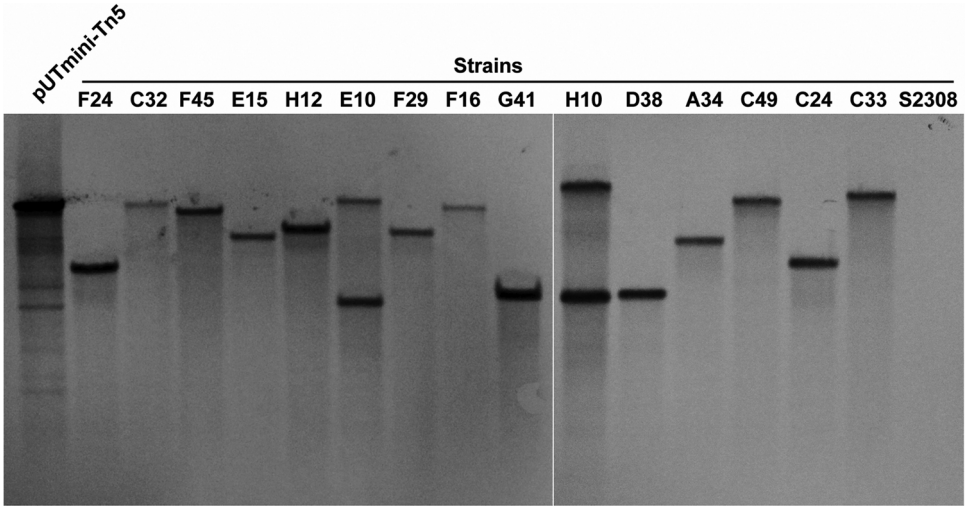

Fig. 4. Southern blot analysis. Most representative mutants had one transposon insertion site. However, the E10 and H10 mutants had two transposon insertion sites. pUTmini-Tn5 was used as a positive control and the S2308 Nal^R strain as a negative control.

constructed using the SacB-assisted allelic replacement suicide vector pSC as described previously (Gao et al., 2016). A cell infection experiment showed that the ability of the *pyc* mutant to survive within RAW264.7 cells was significantly reduced at both 24 and 48 h p.i. (Fig. 5A), as compared with the WT strain. However, at 4 h p.i., there was no significant difference in the bacterial load of the *pyc* mutant and the WT strain in the infected cells (Fig. 5A), suggesting that Pyc is necessary for intracellular survival of *Brucella*, but not invasion into host cells. The results of the animal infection experiments showed no significant difference in the CFU number between the *pyc* mutant and WT strain recovered from the spleens of infected mice at 1 week p.i., although spleen colonization levels were significantly reduced at 5 weeks p.i. (Fig. 5B). Furthermore, the mice infected with the *pyc* mutant had slight splenomegaly at 5 weeks p.i., while those infected with the WT strain exhibited severe splenomegaly (Fig. 5C). Together, these results suggest that Pyc plays an important role in chronic *B. abortus* infection. These data are in accordance with the results of mutant library screening, as strains A37 and D38 were both attenuated in the mouse model, in which the *pyc* gene was disrupted by the mini-Tn5 insertion.

4. Discussion

Brucella virulence is associated with genes involved in energy production and metabolism. In this study, the *pyc* (BAB1_1791), *pyk* (BAB1_1761) and *ppdk* (BAB1_0525) genes associated with pyruvate metabolism were identified, suggesting that the node of pyruvate catabolism may play an important role in *Brucella* virulence. Pyc is a ligase that irreversibly catalyses carboxylation of pyruvate to form oxaloacetate, which is important to carbon metabolism of the cyclic pyruvate-tricarboxylic acid pathway. In *Listeria monocytogenes*, Pyc plays a crucial role in carbon metabolism required for extra- and intracellular replication (Schar et al., 2010). The present results confirmed that Pyc plays an important role in the intracellular survival of *Brucella* and the establishment of infection in a mouse model. Ppdk belongs to a family of transferases that catalyse pyruvate to phosphoenolpyruvate. A *ppdk* deletion mutant reduces *Brucella* multiplication within host cells and had reduced virulence in a mouse model (Zuniga-Ripa et al., 2014). Pyk is an enzyme that catalyses the final step of glycolysis, where a phosphate group is transferred from phosphoenolpyruvate to adenosine diphosphate, yielding one molecule of pyruvate and one molecule of adenosine triphosphate. Pyk is necessary for pyruvate synthesis under nutrition deprivation conditions in *B. abortus*, while a deletion to the coding region of Pyk affected *Brucella* colonization in a mouse model (Gao et al., 2016). Together, these data revealed that pyruvate metabolism plays a significant role in *Brucella* virulence.

Brucella virulence is also associated with genes involved in amino acid transport. Amino acid metabolism is widely believed to be the predominant source of carbon and nitrogen of *Brucella* in the intracellular environment. In this study, disruption of glycine dehydrogenase (GcvP; BAB2_0515) significantly reduced *Brucella* virulence in mice, consistent with the findings of a previous report (Hong et al., 2000). GcvP along with an aminomethyltransferase (GcvT, BAB2_0513), acyltransferase (GcvH, BAB2_0514) and dihydrolipoyl dehydrogenase (GcvL, not found in *Brucella*) form a complex of the glycine cleavage system (GCS), which facilitates the degradation of glycine to acquire 5, 10-methylenetetrahydrofolate, a one-carbon donor utilized in the production of serine, thymidine and purines (Kikuchi, 1973). Although the contributions of the GCS to *Brucella* virulence have not been well investigated, it has been confirmed that the GCS contributes to the intracellular replication of virulent *Francisella tularensis* in serine-limited environments, is involved in the serine biosynthetic pathway and plays a significant role in pathogenesis *in vivo* (Brown et al., 2014). Besides, *hisC* and *hisD* encode a histidinolphosphate transaminase and histidinol dehydrogenase, respectively, which are involved in histidine biosynthesis. A study of miniTn5 mutants of *B. suis* that constitutively express *gfp* indicated that *hisD* and *hisF* (BAB1_2086) are required for intracellular multiplication in human macrophage THP-1 cells (Kohler et al., 2002). In this study, the *hisC* disrupted by miniTn5, significantly reduced bacterial colonization in the spleens of *B. abortus*-infected mice, suggesting that histidine biosynthesis is necessary for intracellular survival of *Brucella* and to establish infection in mice.

Brucella virulence is associated with genes involved in transcription, translation and post-translational modification. BAB2_0143, BAB1_1894 and BAB1_1605 encode for DeoR family, GntR family and ArsR family transcriptional regulators, respectively. In a previous report, Haine et al. identified a series of transcriptional regulators involved in *Brucella* virulence by systematic targeted mutagenesis (Haine et al., 2005). Of these, BAB1_1894 and BAB1_1605, named as BMEI0169 and BMEI0430 in *B. melitensis*, respectively, were identified as important regulators of *Brucella* virulence, in accordance with the results of the present study (Haine et al., 2005). However, BAB2_0143 is a homologue of BMEII0430, which was reported to be not involved in *B. melitensis* virulence (Haine et al., 2005). Analysis of conserved protein domains indicated that the C-terminal of the BAB2_0143 protein is a sensor domain of diverse sugar derivatives, such as deoxyribose

Table 2
B. abortus necessary genes for virulence in a mouse model at 5 weeks post-infection.

Strain	Gene locus	Gene	Description	Virulence ratio[a]	Reference
Energy production and conversion					
A29	BAB1_0977	*fumB*	Fumarate hydratase	0.43	This study
A37, D38	BAB1_1791	*pyc*	Pyruvate carboxylase	0.68	(Kohler et al., 2002)
B6	BAB2_0728	*cydA*	Cytochrome bd ubiquinol oxidase, subunit I	No test	(Endley et al., 2001)
B19	BAB1_1761	*pyK*	Pyruvate kinase	0.71	(Gao et al., 2016)
F6	BAB1_0525	*ppdK*	Pyruvate phosphate dikinase	No test	(Zuniga-Ripa et al., 2014)
Carbohydrate transport and metabolism					
A40	BAB2_0551	*ugpC*	Sugar ABC transporter ATP-binding protein	0.40	This study
A34	BAB1_1123	*phnN*	Ribose 1,5-bisphosphate phosphokinase	0.49	This study
F29	BAB1_1692		Major facilitator superfamily/transporter	0.60	This study
Amino acid transport and metabolism					
A4	BAB2_0515	*gcvP*	Glycine dehydrogenase	0.72	(Hong et al., 2000)
A6	BAB1_0657	*hisC*	Histidinol-phosphate aminotransferase	0.36	This study
B14	BAB2_0286	*aapJ2*	Amino acid ABC transporter substrate-binding protein	0.40	(Tian et al., 2018a, 2018b)
B21	BAB1_0285	*hisD*	Histidinol dehydrogenase	No test	(Kohler et al., 2002)
C4	BAB1_1798	*livM*	Branched-chain amino acid ABC transporter permease protein	0.24	This study
C23	BAB1_0009		ABC transporter substrate-binding protein	0.48	This study
C6	BAB1_1624	*potC*	Putrescine/spermidine ABC transporter permease	0.38	This study
C24	BAB1_1792		Branched chain amino acid ABC transporter substrate-binding protein	0.56	This study
Inorganic ion transport and metabolism					
B45	BAB1_1481	*hcaE*	(2Fe-2S) binding protein	0.39	This study
C33	BAB1_1483		PhnA protein	0.45	This study
E19	BAB1_1500	*nptA*	Na/Pi-cotransporter protein	0.44	This study
H12	BAB2_0226		Hypothetical protein	0.60	This study
E30	BAB2_1079	*znuA*	Zinc uptake system protein	0.00	(Kim et al., 2013)
Transcription					
A52	BAB2_0143		DeoR family transcriptional regulator	0.38	This study
C34	BAB1_1894	*gntR4*	GntR family transcriptional regulator	0.38	(Haine et al., 2005)
F24	BAB1_1605	*arsR6*	ArsR family transcriptional regulator	0.45	(Haine et al., 2005)
C9	BAB1_1512		Cold-shock DNA-binding domain	0.36	This study
Translation, ribosomal structure and biogenesis					
C28	BAB1_2171		N-6 adenine-specific DNA methylase	0.39	This study
G51	BAB1_0159	*raiA*	Hypothetical protein	0.00	This study
Post-translational modification, protein turnover, and chaperones					
A20	BAB1_0474	*radA*	DNA repair protein RadA	0.37	(Roux et al., 2006)
C27	BAB1_1382	*ureD*	Urease accessory protein	0.46	(Sangari et al., 2007)
C49	BAB1_0837	*RNaseJ*	mRNA degradation ribonuclease	0.53	This study
Cell wall/membrane/envelope biogenesis					
E34, E39	BAB1_1466		Lytic transglycosylase	0.28	This study
E43	BAB1_0561	*manC*	Mannose-6-phosphate isomerase	No test	(Cardoso et al., 2006)
F5	BAB1_0553	*wbkA*	Mannosyltransferase	No test	(Cardoso et al., 2006)
H6	BAB1_0545	*gmd*	GDP-mannose 4,6-dehydratase	No test	(Cardoso et al., 2006)
Intracellular trafficking, secretion, and vesicular transport					
E15, F45	BAB2_0065	*virB4*	Type IV secretion system protein VirB4	No test	(Kohler et al., 2002)
F16	BAB2_0067	*virB2*	Type IV secretion system protein VirB2	No test	(Kohler et al., 2002)
Replication, recombination and repair					
C45	BAB2_1075		Transposase	0.48	This study
Cell cycle control, cell division, chromosome partitioning					
E27	BAB1_1996		Cell division protein FtsX	0.53	This study
Nucleotide transport and metabolism					
F1	BAB1_2156	*ybeY*	16S rRNA maturation RNase YbeY	0.41	(Budnick et al., 2018)
Lipid transport and metabolism					
A33	BAB1_0440	*caiA*	Acyl-CoA dehydrogenase	0.34	This study
Secondary metabolites biosynthesis, transport, and catabolism					
B27	BAB1_0111		SAM-dependent methyltransferase	0.36	This study
Defense mechanisms					
C48	BAB1_0340		XRE family transcriptional regulator	0.41	This study
C21	BAB1_1112		Acriflavin resistance protein	0.41	This study
Function unknown					
A42	BAB1_1725		MotA/TolQ/ExbB proton channel family protein	0.40	This study
B20	BAB2_0130		Hypothetical protein	0.60	This study
C20	BAB2_0479		Fusaric acid resistance protein family	0.28	This study
C47	BAB1_1774	*yiiM*	Molybdenum cofactor sulfurase	0.42	This study
E12	BAB1_2077	*mutS*	DNA mismatch repair protein	0.00	This study
E16	BAB1_1280	*vhpA/romA*	Hypothetical protein	0.00	(Tian et al., 2018, 2019; Valguarnera et al., 2018)
H8	BAB2_1078		Hypothetical protein	0.28	This study
Not in the Clusters of Orthologous Groups database					
B52	BAB1_1207		Hypothetical protein	0.38	This study
E37	BAB1_1108		Pseudo	0.24	This study
G41	BAB2_0332		Pseudo	0.39	This study

[a] Virulence ratio = $\log_{10}$ CFU (recovered from the mutant-infected spleens)/$\log_{10}$ CFU (recovered from the WT S2308-infected spleens).

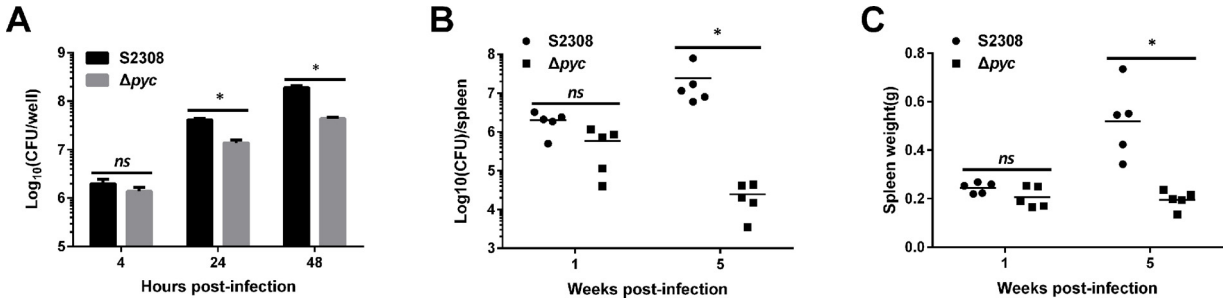

Fig. 5. Pyc is crucial for the *Brucella* survival in the RAW 264.7 cells and mouse infection models. (A) Intracellular survival within RAW 264.7 cells of the WT strain and the *pyc* mutant. (B) Bacterial loads of the WT strain and the *pyc* mutant in the spleens of BALB/c mouse at weeks 1 and 5 p.i. (C) Spleen weights of mice infected with the WT strain and *pyk* mutant at weeks 1 and 5 p.i. *ns*, not significant; *, $p < 0.05$; **, $p < 0.01$; **, $p < 0.001$.

nucleoside and tagatose phosphate, suggesting that BAB2_0143 may regulate the expression of various genes by sensing changes in carbon metabolism.

In all, 56 attenuated strains were screened, which identified 53 genes associated with *Brucella* virulence. Of these, 19 have previously been reported to be associated with virulence. Importantly, 34 genes were identified as novel genes related to *Brucella* infection in a mouse model. Further investigations are needed to elucidate the function of these novel genes in *Brucella* virulence and to gain additional insight into *Brucella* pathogenesis.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This work was supported by funds from the National Natural Science Foundation of China (31602070), the Shanghai Sailing Program (16YF1414600), the Scientific and Technical Innovation Project of the Chinese Academy of Agricultural Sciences (SHVRI-ASTIP-2014-8) and the National Key Research and Development Program of China (2018YFD0500500).

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