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*Brucella abortus*ΔcydCΔcydD and ΔcydCΔpurD double-mutants are highly attenuated and confer long-term protective immunity against virulent *Brucella abortus*

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ABSTRACT

We constructed double deletion (Δ*cydC*Δ*cydD* and Δ*cydC*Δ*purD*) mutants from virulent *Brucella abortus* biovar 1 field isolate (BA15) by deleting the genes encoding an ATP-binding cassette-type transporter (*cydC* and *cydD* genes) and a phosphoribosylamine–glycine ligase (*purD*). Both BA15Δ*cydC*Δ*cydD* and BA15Δ*cydC*Δ*purD* double-mutants exhibited significant attenuation of virulence when assayed in murine macrophages or in BALB/c mice. Both double-mutants were readily cleared from spleens by 4 weeks post-inoculation even when inoculated at the dose of 10^8 CFU per mouse. Moreover, the inoculated mice showed no splenomegaly, which indicates that the mutants are highly attenuated. Importantly, the attenuation of *in vitro* and *in vivo* growth did not impair the ability of these mutants to confer long-term protective immunity in mice against challenge with *B. abortus* strain 2308. Vaccination of mice with either mutant induced humoral and cell-mediated immune responses, and provided significantly better protection than commercial *B. abortus* strain RB51 vaccine. These results suggest that highly attenuated BA15Δ*cydC*Δ*cydD* and BA15Δ*cydC*Δ*purD* mutants can be used effectively as potential live vaccine candidates against bovine brucellosis.

1. Introduction

Brucella abortus is a Gram-negative and facultative intracellular bacterium that causes bovine brucellosis, a zoonotic disease of worldwide importance. Infection with *B. abortus* leads to abortions and reduced fertility in cattle and severe illness in humans [1,2]. Due to this serious economic loss and public health risk, extensive eradication programs for brucellosis have been conducted to prevent, control and eliminate the disease in animals [3]. Among these, vaccination has been shown to be a critical factor in control and preventative programs for bovine brucellosis [3]. Vaccination with live attenuated *B. abortus* strains such as smooth strain 19 and rough strain RB51 have been widely used for several decades, and have proven effective against bovine brucellosis in the field. However, these vaccines have the limitations of antibiotic resistance, diagnosis interference, and residual virulence in the host, and are pathogenic for humans [4,5].

To overcome these drawbacks, extensive efforts have been made to identify additional vaccine targets for the development of potential live attenuated vaccines [6–8]. Among these, a systematic search for virulence factors in *Brucella* has identified the genes encoding the purine biosynthetic enzyme (*purE*) and cytochrome *bd* terminal oxidase (*cydB*, *cydX*). These genes are preferentially used inside macrophages and contributed to stress adaptation, intracellular survival, and replication [9–11]. These genes also appear to be crucial for the virulence of *Brucella* in mouse models, and have been used for construction of vaccine candidates [12–14]. However, these attenuated mutants are considered either unsafe or insufficiently studied for use as possible vaccines in animals.

Previously, we have identified *B. abortus cydC* (ATP-binding/permease protein, a component of the *cydDC* operon encoding an ATP-binding cassette (ABC)-type transporter) [15] and *purD* (phosphoribosylamine–glycine ligase) [16] using mini-Tn5Km2 transposon mutagenesis. Similar to *Brucella cydB* and *purE* [9,10], interruption of *cydC*::Tn5 and *purD*::Tn5 genes in *B. abortus* was found to significantly attenuate virulence in a mouse model of infection. Immunization of mice with *cydC*::Tn5 and *purD*::Tn5 mutants conferred protection against *B. abortus* 544 challenge strain. These data implied that the *cydDC* operon and *purD* gene may be the ideal targets for development of new

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Table 1
Bacterial strains, plasmids and primers used in this study.

Strains/plasmids /Primers	Relevant characteristics	Source /reference
Bacterial strains		
E. coli DH5α	F-φ80 *lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *recA1 endA1 hsdR17*(r_k-, m_k +) *gal*$^-$ *phoA supE44* λ$^-$ *thi*$^-$1 *gyrA96 relA1*	Invitrogen
BA15	The virulent *B. abortus* biovar 1 field strain isolated from aborted bovine fetus in South Korea (IVKB90015)	This study
RB51	Rough *B. abortus* strain RB51 vaccine	This study
S2308	Smooth, virulent *B. abortus* strain 2308	[38]
BA15Δ*cydD*	Unmarked deletion of the *cydD* gene in wild-type BA15	This study
BA15Δ*cydC*	Unmarked deletion of the *cydC* gene in wild-type BA15	This study
BA15Δ*cydC*Δ*cydD*	Unmarked deletion of the *cydD* gene in BA15Δ*cydC* mutant	This study
BA15Δ*cydC*Δ*purD*	Unmarked deletion of the *purD* gene in BA15Δ*cydC* mutant	This study
Plasmids		
pEX18Ap	*sacB* bla, Amp^R	[18]
pEX18Δ*cydD*	D1F-D2R/D3F-D4R cloned into pEX18Ap for *cydD* deletion	This study
pEX18Δ*cydC*	C1F-C2R/C3F-C4R cloned into pEX18Ap for *cydC* deletion	This study
pEX18Δ*purD*	PD1F-PD2R/PD3F-PD4R cloned into pEX18Ap for *purD* deletion	This study
Primers[a]		
C1F	GG**AATTCC**CTTCAATCGCAAGCGGTGC (***EcoR*I**)	Upstream of *cydC*
C2R	GCATTCACG**GGATCC**CGCATGAGGTGAGTCATGCAGG (***BamH*I**)	
C3F	CCTCATGCG**GGATCC**CGTGAATGCAAACCCTTCATTCC (***BamH*I**)	Downstream of *cydC*
C4R	GC**TCTAGA**GCTGTTGCTCTTCGCAAGAGA (***Xba*I**)	
C5F	AACGGTTTCCATGATGGCCA	Detecting *cydC*
C6R	CGACGCATGATGAAACGCTG	
D1F	GG**AATTCC**AATGCAAACCCTTCATTCCG (***EcoR*I**)	Upstream of *cydD*
D2R	TGTTTCACG**GGATCC**CGTGTTGCTCTTCGCAAGAGA (***BamH*I**)	
D3F	TTGGATGCG**GGATCC**CGTGAAACATTGCCCCCCGTTT (***BamH*I**)	Downstream of *cydD*
D4R	GC**TCTAGA**GCACGATCCAACGTTTCCATG (***Xba*I**)	
D5F	CTGCCATCAGCAGTTCGGT	Detecting *cydD*
D6R	CGTACGGTGGGGCAGATTTA	
PD1F	GG**AATTCC**TTTACAATCGCCGTGGCTT (***EcoR*I**)	Upstream of *purD*
PD2R	AAAACTACG**GGATCC**CGCATCGACTGGCCGGAAGGTT (***BamH*I**)	
PD3F	GTCGATGCG**GGATCC**CGTAGTTTTTCAAGCAGGGGC (***BamH*I**)	Downstream of *purD*
PD4R	GC**TCTAGA**GCTCATTATAGGTGCAGCCTGC (***Xba*I**)	
PD5F	CGCACCATGTTGCGCATATT	Detecting *purD*
PD6R	CAGTCATAGCCGAATAGCCT	

[a] Bold letters indicate the sequence of restriction sites inserted.

safe and efficacious live vaccine strains. In the present study, we describe the construction of double-gene deletion (Δ*cydC*Δ*cydD* and Δ*cydC*Δ*purD*) mutants from *B. abortus* strain, and evaluate their intracellular survival in murine macrophages and their virulence and immunogenicity in mice, together with their ability to induce long-term protection against challenge with virulent *B. abortus* strain 2308 (S2308).

2. Materials and methods

2.1. Bacterial strains and media

B. abortus IVKB90015 strain (BA15) was isolated in our laboratory from an aborted bovine fetus from South Korea. This strain was identified and is denoted as the smooth virulent *B. abortus* biovar 1. All *Brucella* strains and mutants (Table 1) were routinely grown on tryptic soy agar (TSA) or broth (TSB) (Difco, MI, USA) at 37 °C in 5% CO_2. *Escherichia coli* DH5α was cultivated in Luria Bertani (LB) broth or agar (Difco). Electro-competent cells of BA15 and mutants were prepared as previously described by McQuiston [17].

2.2. Construction of the BA15 single- and double-mutants

To construct the plasmids for deleting the *cydD*, *cydC*, and *purD* genes, we designed two sets of primers to amplify DNA fragments in the upstream and downstream regions of each gene of interest from BA15. The sequences of each primer pair are listed in Table 1. The construction of an unmarked gene deletion mutants was performed according to previously published methods [18,19]. Briefly, the respective plasmids pEX18Δ*cydD*, pEX18Δ*cydC* and pEX18Δ*purD* were created by two-round PCR amplification, restricted digestion and ligation, and then introduced into BA15 and BA15Δ*cydC* via electroporation. Transformants were selected in the presence of 100 μg/ml carbenicillin for the first screening and 5% sucrose for the second screening. The deletions of *cydD* (D5F/D6R), *cydC* (C5F/C6R) and *purD* (PD5F/PD6R) genes in BA15 or BA15Δ*cydC* were then verified by PCR amplification and DNA sequencing analysis, and the mutants are referred to as BA15Δ*cydD*, BA15Δ*cydC*, BA15Δ*cydC*Δ*cydD*, and BA15Δ*cydC*Δ*purD*. A crystal violet method [20] was also used to confirm that the BA15 mutants maintained a smooth phenotype.

2.3. Intracellular growth of BA15 mutants in murine macrophages

Preparation and the intracellular bacterial growth assays within RAW 264.7 cells (RAW cells) and bone marrow-derived macrophages (BMDM) was performed as described previously [21,22]. Briefly, the RAW cells and BMDM were seeded into tissue culture plates at 2×10^5 cells/well in complete media [CM; RPMI 1640 (GenDepot, USA), 10 mM HEPES and 10% FBS] and infected with BA15, or its isogenic mutants at a multiplicity of infection (MOI) of 50. The plates were centrifuged at 250 × *g* for 5 min, and incubated at 37 °C for 30 min. After washing twice with phosphate-buffered saline (PBS), the infected cells were incubated for 1 h in the presence of 50 μg/ml of gentamicin (Sigma, USA) to kill extracellular bacteria. The media in the wells were then replaced by CM containing 10 μg/ml gentamicin, and incubated at 37 °C for 0, 6, 24

and 48 h. At different times post-infection (p.i.), the infected cells were washed three times with PBS and lysed with 0.1% Triton X-100 in sterile double distilled water. Ten-fold serial dilutions of the lysates were plated on TSA plates to enumerate the colony forming units (CFU).

2.4. Evaluation of virulence of BA15 mutants in BALB/c mice

Seven-week-old female BALB/c mice were purchased from Orients (Daejeon, Korea), and housed in individually ventilated cage rack systems in the BL2+ facilities. All animal handling and protocols were reviewed and approved by Kangwon University Institutional Animal Care and Use Committee (permit no. KW-150527-1). Groups of mice ($n = 30$ per group) were inoculated intraperitoneally (i.p.) with 2.4–3.1 $\times 10^8$ CFU of BA15 mutants and *B. abortus* strain RB51 vaccine (RB51), or 2×10^5 CFU of wild-type BA15 in 0.2 ml of PBS. At 1, 2, 3, 4, 6, and 8 weeks post-inoculation (WPI), five mice from each group were sacrificed by the time point, and spleens were aseptically removed, weighed, and homogenized in PBS. Ten-fold serial dilutions of spleen homogenates were plated on TSA plates and incubated for 3–5 days at 37 °C. The CFU were enumerated to evaluate colonization efficiency of each strain in mice. The results are presented as the mean ± standard deviation (SD) of the $\log_{10}$ (log) CFU/spleen for each group.

2.5. Immunization of mice

Seven-week-old female BALB/c mice were randomly devided into five treatment groups ($n = 22$ per group) and vaccinated i.p. with a single dose of 2.4–3.1 $\times 10^8$ CFU of BA15Δ*cydD*, BA15Δ*cydC*, BA15Δ*cydC*Δ*cydD*, and BA15Δ*cydC*Δ*purD* mutants and RB51, respectively. An equal number of mice were given only PBS and were used as unvaccinated controls in all the experiments performed.

2.6. Protective efficacy incuded by BA15 mutants in BALB/c mice

At 6 and 20 weeks post-vaccination (WPV), all groups of mice ($n = 6$ per group) were challenged i.p. with 1.5×10^5 CFU of virulent S2308 per mouse. One week after the virulent challenge, the mice were sacrificed, and the spleens were collected, weighed, and homogenized in PBS. The numbers of CFU recovered from spleens were determined as described above. The levels of infection were expressed as the mean ± SD of log CFU/spleen of S2308 for each mouse group obtained after challenge. Mean log CFU reductions or log units of protection were obtained by subtracting the mean log CFU for the vaccinated group from the mean log CFU for the unvaccinated control group.

2.7. Evaluation of serum antibody response

Mice ($n = 5$ per group) were bled from the orbital sinus during the immunization schedule and sera were stored at −70 °C until tested. The IgG and IgG subclass antibodies against heat-killed and sonicated *Brucella* (HKSB) were measured by ELISA [23]. Briefly, ELISA plates were coated with 25 μg/well HKSB in carbonate buffer at 4 °C overnight. The plates were then washed with PBS containing 0.05% Tween 20 (PBS-T) and blocked with blocking buffer (1% bovine serum albumin in PBS). Subsequently, triplicate samples of serum diluted 1:200 in blocking buffer were added and incubated at 37 °C for 1 h. After three washes with PBS-T, the goat anti-mouse IgG-, IgG1-, and IgG2a horseradish peroxidase conjugate (Fitzgerald, MA, USA) at a dilution of 1:2000 was added and incubated at 37 °C for 1 h. After extensive washing, *O*-phenylenediamine dihydrochloride peroxidase substrate (Sigma) was added, and incubated for 20 min and then the absorbance at 450 nm was measured using an ELISA reader (Model 680, Bio-Rad, USA).

2.8. Cytokine detection in spleen cell culture supernatants

Splenocytes from PBS- and immunized-mice ($n = 5$ per group) were prepared as described previously [19]. At 6 and 20 WPV, the splenocytes were isolated, counted and plated at 2×10^6 cells/well in CM, and then stimulated with 1×10^8 CFU heat-killed BA15 strain, 2 μg/ml concanavalin A (ConA, Sigma), or media alone. After 72 h incubation at 37 °C in 5% CO_2, the culture supernatants were collected and the concentrations of IFN-γ and IL-10 were determined using mouse ELISA MAX kits according to the manufacturer's instructions (Bio-Legend, CA, USA).

2.9. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad software, CA, USA). The data from assays for bacterial survival in murine macrophages and BALB/c mice at different time points and from efficacy studies were expressed as the mean log CFU ± SD for each group and analyzed by Student's *t* test. The data for antibody responses and cytokine production were expressed as mean OD_{450} or mean cytokine concentration ± SD for each group. The significance of differences between the experimental groups was analyzed by analysis of variance (ANOVA) followed by Turkey's post-test. For all statistical analyses, *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. Mutants are incapable of intracellular survival and replication within macrophages

The intracellular growth of BA15 and its isogenic BA15Δ*cydD*, BA15Δ*cydC*, BA15Δ*cydC*Δ*cydD*, and BA15Δ*cydC*Δ*purD* mutants within RAW264.7 cells and BMDM were evaluated at different time points. As shown in Fig. 1, the number of intracellular bacteria recovered at 0 h p.i. did not differ significantly between BA15 and mutants. This indicates that *cydD*, *cydC*, and *purD* genes play no role in the internalization of *B. abortus* by either RAW264.7 cells or BMDM. However, a decrease in the intracellular numbers of mutants compared with that of BA15 was observed in RAW264.7 cells and BMDM at 6 h after infection ($P < 0.05$). At later time points p.i. (24 h and 48 h), BA15 was able to survive and replicate inside RAW264.7 cells and BMDM, while the intracellular numbers of mutants had dramatically decreased in both cell types, with approximately 3.5- and 5-log reduction relative to BA15 ($P < 0.001$). These results indicate that both single- (BA15Δ*cydD* and BA15Δ*cydC*) and double-mutants (BA15Δ*cydC*Δ*cydD* and BA15Δ*cydC*Δ*purD*) are impaired in their ability to survive and replicate within murine macrophages.

3.2. Mutants are highly attenuated in mice

To determine the rate of clearance of BA15Δ*cydD*, BA15Δ*cydC*, BA15Δ*cydC*Δ*cydD*, and BA15Δ*cydC*Δ*purD* mutants *in vivo*, BALB/c mice were inoculated i.p. with 10^5 CFU of wild-type BA15 and 10^8 CFU of mutants and RB51. The persistence of all strains was monitored by the enumeration of CFU in the spleens at different time points post-inoculation. As shown in Fig. 2A, mice given BA15 showed significant differences in CFU compared with mice inoculated with the mutants ($P < 0.001$, Fig. 2A). By 4 WPI, no CFU of BA15Δ*cydC*, BA15Δ*cydC*Δ*cydD*, or BA15Δ*cydC*Δ*purD* mutants were detected in the spleens, in contrast to mice infected with the BA15, which remained infected ($P < 0.001$, Fig. 2A). BA15Δ*cydD*

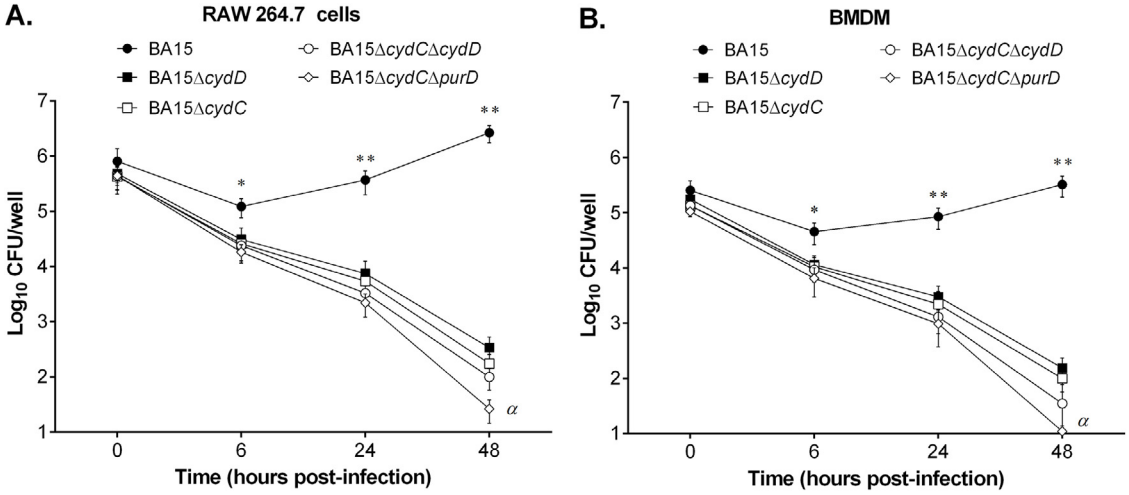

Fig. 1. The intracellular growth of wild-type BA15 and its isogenic BA15Δ*cydC*, BA15Δ*cydD*, BA15Δ*cydC*Δ*cydD* and BA15Δ*cydC*Δ*purD* mutants in RAW 264.7 cells (A) and BMDM (B). Data points and error bars represent the mean and SD of triplicate determinations of CFU. The asterisks denote a significant difference compared with the values for the parental BA15 ($^{*}P < 0.05$; $^{**}P < 0.001$) or for the BA15Δ*cydD* mutant ($^{\alpha}P < 0.05$).

mutant and RB51 were cleared at 6 and 8 WPI, respectively. Analysis of spleen weights showed that mice inoculated with the mutants exhibited significantly decreased spleen weights compared with mice inoculated with either BA15 or RB51 (Fig. 2B). These results indicate that the mutants, particularly the double-mutants, are more attenuated than RB51.

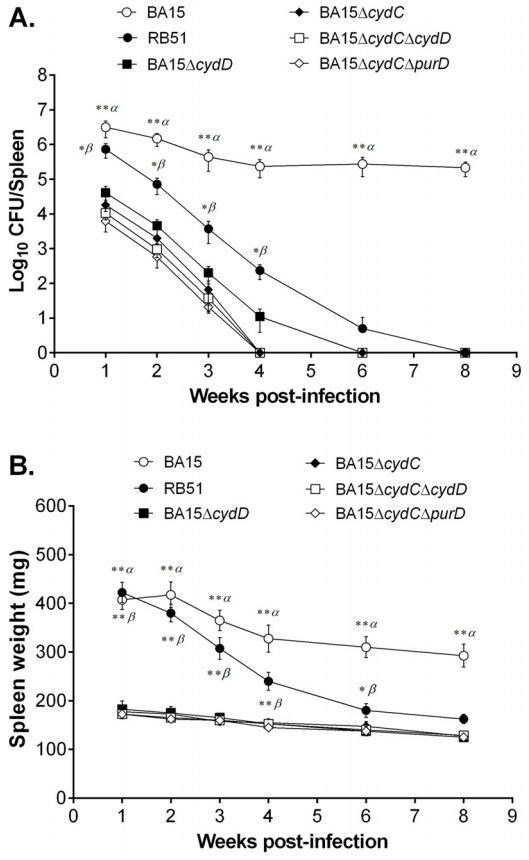

Fig. 2. Persistence of BA15Δ*cydC*, BA15Δ*cydD*, BA15Δ*cydC*Δ*cydD* and BA15Δ*cydC*Δ*purD* mutants and parental BA15 in BALB/c mice. The mouse groups ($n = 30$ per group) were inoculated i.p. with 10^5 CFU of wild-type BA15, and 10^8 CFU of the mutants and the RB51. At 1, 2, 3, 4, 6 and 8 WPI, five mice from each group were euthanized, and individual spleens were removed and assessed for colonization levels (A) and weights (B). Error bars represent the SD and the asterisks denote values that differ significantly between BA15 ($^{**\alpha}P < 0.001$) or RB51 ($^{*\beta}P < 0.01$; $^{**\beta}P < 0.001$) and BA15 mutants at each time point.

3.3. Vaccination with mutants confers long-term protection in mice

Having demonstrated above that the BA15 mutants are highly attenuated *in vitro* and *in vivo*, we next evaluated the efficacy of these mutants as vaccine strains. The mutants were used at a high dose (10^8 CFU/dose) for i.p. immunization to reflect that used in livestock. At 6 and 20 WPV, the mice were challenged i.p. with 1.5×10^5 CFU of S2308. The level of protection against S2308 provided by vaccination with BA15 mutants and RB51 was evaluated 1 WPC. As shown in Fig. 3A, at 7 WPV (1 WPC), groups of vaccinated mice demonstrated a significant decrease in bacterial load in the spleen relative to that in PBS-dosed mice, with a 2.61-, 2.50-, 2.29-, 2.37-, and 1.48-log reduction for BA15Δ*cydD*, BA15Δ*cydC*, BA15Δ*cydC*Δ*cydD*, and BA15Δ*cydC*Δ*purD* mutants and RB51, respectively, compared with PBS-dosed mice ($P < 0.001$). At 21 WPV (1 WPC), mice vaccinated with the BA15Δ*cydD* mutant exhibited the highest level of protection against S2308 infection, with a 2.92-log reduction relative to PBS-dosed mice ($P < 0.001$), followed by BA15Δ*cydC* with a 2.81-log reduction, BA15Δ*cydC*Δ*purD* with a 2.72-log reduction, BA15Δ*cydC*Δ*cydD* with a 2.64-log reduction, and RB51 with a 1.09-log reduction ($P < 0.001$). Remarkably, vaccination with BA15 mutants induced protection superior to that generated by RB51 following challenge at either 6 WPV or 20 WPV ($P < 0.01$). In addition, the presence of fewer CFU following challenge in mice vaccinated with BA15 mutants was also correlated with decreased spleen weights (Fig. 3B).

3.4. Humoral and cell-mediated immune responses induced by mutants

Sera were collected from immunized mice at 0, 2, 4, 6, 8, 12, 16 and 20 WPV, and assayed for the presence of *Brucella*-specific IgG, IgG2a and IgG1 antibodies by ELISA. As shown in Fig. 4, significant levels of IgG antibodies were detected in sera of mice vaccinated with the mutants or RB51, but not in those inoculated with PBS, at 2 WPV, indicating that the mutants could induce *Brucella*-specific antibodies. The IgG levels peaked at 4 and 6 WPV, and remained consistent in subsequent weeks. There was no significant difference in IgG levels between the vaccinated groups (Fig. 4A). Similar

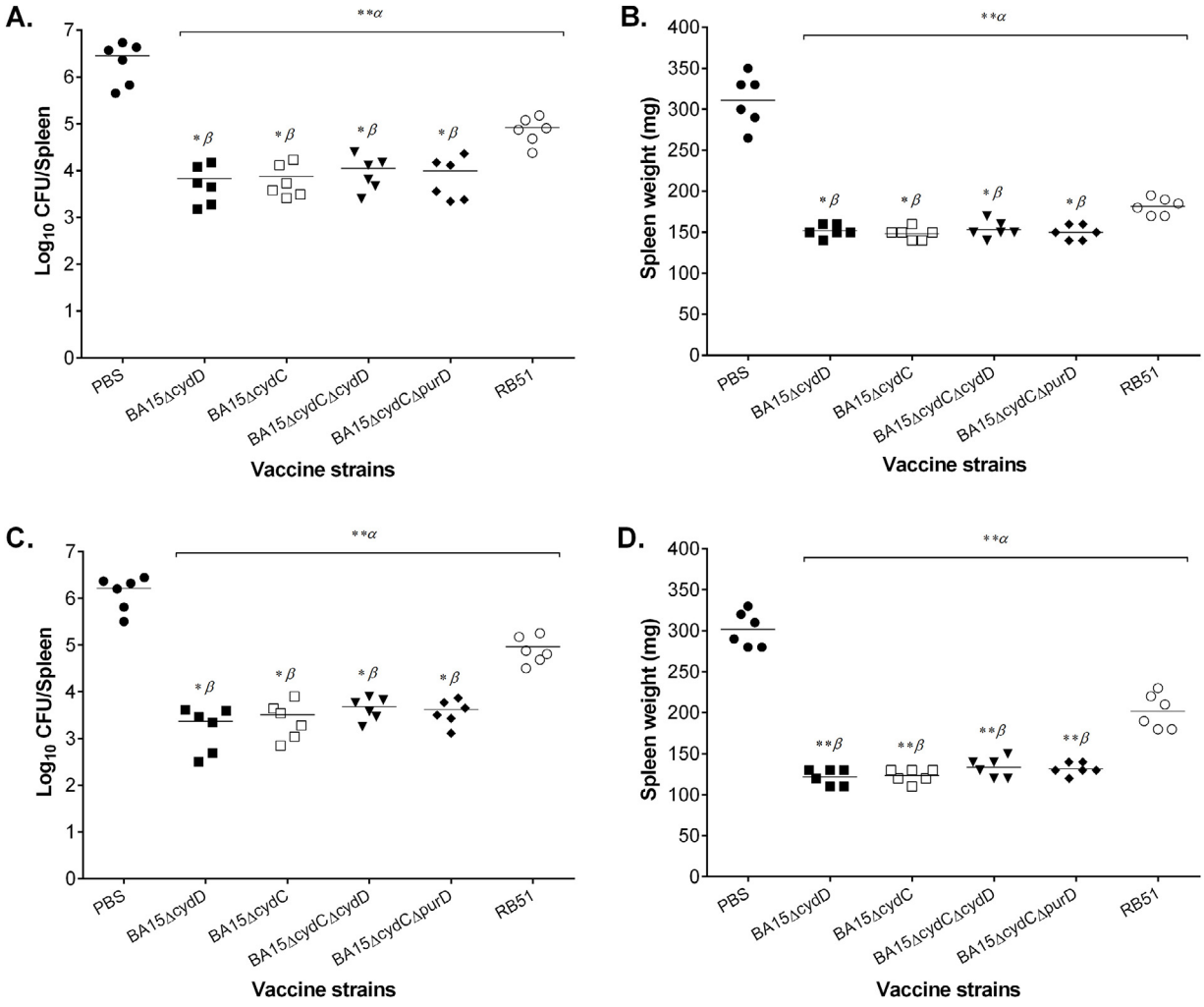

Fig. 3. Protection induced by immunization with BA15Δ*cydC*, BA15Δ*cydD*, BA15Δ*cydC*Δ*cydD* and BA15Δ*cydC*Δ*purD* mutants and the strain RB51 vaccine in BALB/c mice. Mouse groups ($n = 12$ per group) were immunized i.p. with 10^8 CFU of the BA15 mutants and the RB51. The control group received 200 μl of PBS. At 6 (A and B) and 20 (C and D) weeks post-vaccination, mice ($n = 6$ per group) were challenged with 10^5 CFU of virulent *B. abortus* strain 2308, respectively. At 1 week post-challenge, the spleens from each mouse group were assessed for changes in CFU levels (A and C) and weights (B and D). Data points and error bars represent the mean and SD of splenic CFU and spleen weights from mice at the indicated times. The asterisks indicate a significant difference in mean values between PBS-immunized and mutant- and RB51-immunized mice ($^{**\alpha}P < 0.001$), and between RB51-immunized mice and mutant-immunized mice ($^{*\beta}P < 0.01$; $^{**\beta}P < 0.001$).

to the trends observed for the IgG response, mice immunized with either single- or double-mutants or RB51 produced significant levels of IgG1 and IgG2a antibodies (Fig. 4B and 4 C). Moreover, these responses were marked by a predominance of IgG2a, suggesting a bias towards a Th1-type response.

To characterize the cellular immune response, culture supernatants from stimulated splenocytes collected from immunized mice at 6 and 20 WPV were assessed for the Th1-type cytokine IFN-γ and the Th2-type cytokine IL-10 (Fig. 5). As expected, splenocytes from immunized mice that were stimulated with ConA produced significant amounts of IFN-γ and IL-10, whereas no cytokine production was induced by culture medium. When stimulated with heat-killed BA15, splenocytes from BA15Δ*cydD*-, BA15Δ*cydC*-, and BA15Δ*cydC*Δ*cydD*-, BA15Δ*cydC*Δ*purD*-immunized mice at 6 and 20 WPV showed significantly enhanced productions of IFN-γ and IL-10 relative to that of PBS-dosed mice ($P < 0.001$). Production of these cytokines was also higher than in RB51-immunized mice (Fig. 5). Altogether, these results indicated that immunization with BA15 mutants could induce robust humoral and cellular immune responses with a predominantly Th1-type response.

4. Discussion

In previous studies, we identified the *cyd*C and *purD* genes that are required for survival and virulence of *B. abortus* [15,16]. Characterization and complementation of *cydC*::Tn5 and *purD*::Tn5 mutants demonstrated that the *cydC* gene is essential for functional cytochrome terminal oxidases [24–27] which are important for protecting *B. abortus* against metal toxicity, oxidative stress, and acidity in the harsh environmental conditions encountered within host macrophages [15]. The *purD* gene is involved in the response to oxidative stress and in *de novo* purine nucleotide biosynthesis by *Brucella* [16]. These mutants were found to be significantly attenuated *in vitro* and *in vivo*, and could induce protection in mice. These data indicated that the *cydDC* operon and *purD* are probably ideal candidate genes for construction of new attenuated vaccine strains. Based on this, we hypothesized that single or combined deletions of *cydC*, *cydD* and *purD* genes in *B. abortus* would result in severe virulence attenuation while possibly maintaining the desired efficacy as an attenuated vaccine.

With the aim of generating an effective and safe vaccine, in the present study, we constructed smooth, unmarked double deletion

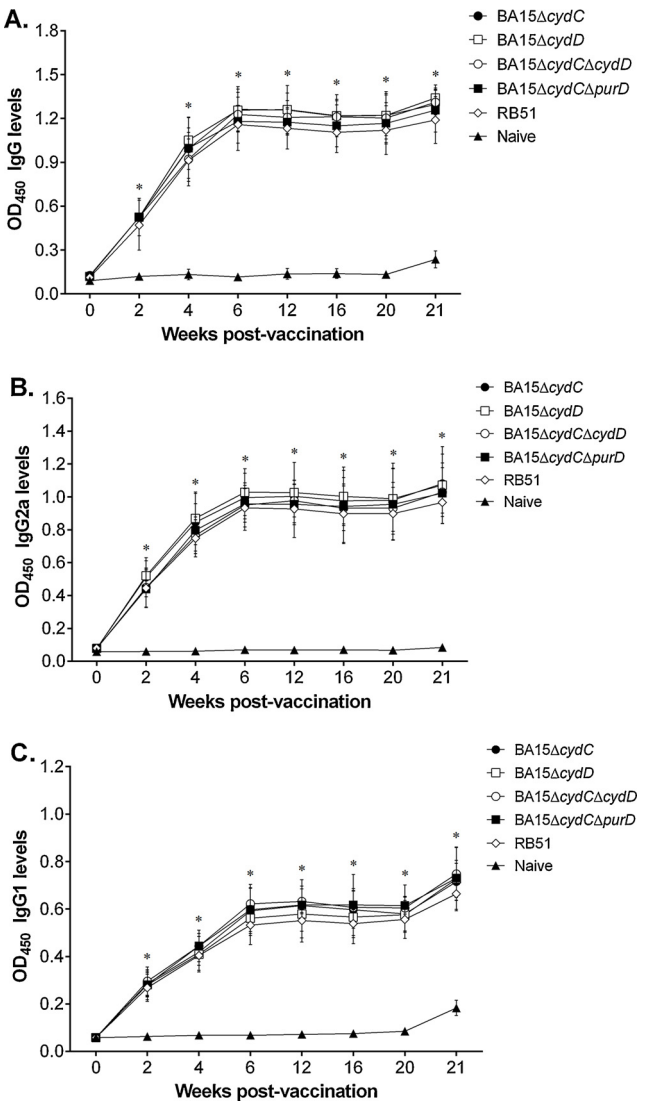

Fig. 4. The IgG, IgG2a and IgG1 anti-*Brucella* antibodies in sera from mice immunized with BA15Δ*cydC*, BA15Δ*cydD*, BA15Δ*cydC*Δ*cydD*, BA15Δ*cydC*Δ*purD* mutants, RB51 and PBS alone. At 0, 2, 4, 6, 12, 16, and 20 weeks post-vaccination and 1 week post-challenge, serum samples ($n = 5$ per group) were collected and analyzed for IgG- (A), IgG2a- (B) and IgG1-specific (C) antibody responses using indirect ELISA. Results are shown as the means ± SD of the absorbance at 450 nm, and asterisks symbolize the significant differences between the mice immunized with mutants or RB51 and those immunized with PBS (*$P < 0.001$).

mutants (BA15Δ*cydC*Δ*cydD* and BA15Δ*cydC*Δ*purD*), and evaluated their survival and virulence in macrophages and BALB/c mice as well as their ability to induce long-term protective immunity. The utility of these mutants arises from the fact that they have a limited ability to undergo replication in the harsh or purine-limiting environments of the host cells but maintain their ability to stimulate the immune system. In fact, targeted deletions of virulence-associated genes including purine biosynthetic genes have long been used for generating live attenuated strains of intracellular pathogens [12,14,28,29]. Moreover, incorporating additional genetically defined mutations into attenuated *Brucella* strains may be an ideal way to improve their safety and maintain sufficient efficacy [14,18].

As shown in the present study, deletion of either *cydD* or *cydC* in BA15 or the additional deletion of *cydD* and *purD* genes in the BA15Δ*cydC* mutant conferred attenuated phenotypes in cultured macrophages and BALB/c mice. In particular, the BA15 mutants were capable of entering RAW cells and BMDM to the same extent as wild-type BA15, but these mutants were severely defective in intracellular survival and replication within these cell types. These defects were more apparent in the BA15Δ*cydC*Δ*purD* mutant than in the other mutants. As described in our previous study [15], mutation of the *cydC* gene severely impairs the ability of *B. abortus* to survive inside macrophages by decreasing its resistance to the hostile intracellular environment of macrophages. Therefore, it is not surprising that deletion in BA15 of either *cydD* gene (ATP-binding transporter protein) alone or both *cydC* and *cydD* genes had the same attenuated phenotype *in vitro* as did the *cydC* mutant, because both genes encode an ABC-type transporter that is required for the activity of cytochrome *bd* and *c* oxidases, similar to that seen in *E. coli* [24,26,27]. Meanwhile, the effect of *purD* deletion in BA15Δ*cydC* mutant may account for the BA15Δ*cydC*Δ*purD* phenotype, because *B. abortus* with mutated *purD* is more sensitive to oxidative stress and requires purine bases for growth [16]. Consistent with the phenotypes observed for mutants in murine macrophages, our results showed that even after inoculation of BALB/c mice with a high dose (10^8 CFU), BA15 mutants were also highly attenuated and were cleared faster than RB51. Remarkably, BA15Δ*cydC*, BA15Δ*cydC*Δ*cydD* and BA15Δ*cydC*Δ*purD* mutants were completely eliminated from mouse spleens by 4 WPI. Moreover, the markedly reduced splenomegaly associated with vaccination in mice suggests that BA15 mutants are more attenuated for virulence than RB51, which induces splenomegaly in mice. These results collectively indicated that the attenuated virulence and limited persistence in the host of BA15 mutants, particularly BA15Δ*cydC*, BA15Δ*cydC*Δ*cydD* and BA15Δ*cydC*Δ*purD* mutants, make them an attractive live vaccine candidates from a safety perspective.

A variety of strategies have been described for developing attenuated *Brucella* mutants that are able to persist for extended period in host tissues following immunization, and a correlation in terms of protection was found between enhanced clearance and persistence [6,19]. However, although mutants that can persist for an extended period of time may provide better protection against a challenge strain, a potential drawback of using these vaccines in animals or humans is the possibility of incomplete clearance of the vaccine strains [30]. Our findings were particularly encouraging in that both single- and double-mutants were effectively cleared from the spleens of mice within 4–6 weeks and yet, as evident from the challenge studies at 6 and 20 WPV, were still able to sustain a long-term protective immunity. We observed no significant differences between the mutants tested in the present study in their ability to engender protective immunity. In fact, vaccination with single-mutants (BA15Δ*cydC* and BA15Δ*cydD*) offered a significantly higher protective efficacy against *B. abortus* challenge than RB51, but it was not significantly different to that induced by the double-mutants (BA15Δ*cydC*Δ*cydD* and BA15Δ*cydC*Δ*purD*). Our results suggest that both highly attenuated BA15 single- and double-mutants could be used as ideal attenuated vaccine strains against bovine brucellosis, because they are less virulent and provide significantly better protection than the current strain RB51 vaccine.

Protection by *Brucella* live vaccines has been shown to be mediated by the induction of humoral and cell-mediated immune responses. In particular, a Th1-biased immune response is known to play a major role in the establishment of a protective response against *Brucella* [31,32]. To identify any correlates of protective immunity that could be used to predict the vaccine efficacy of BA15 mutants, the humoral and cellular immune responses induced by the mutants were characterized by analyzing the antibody response and the cytokines produced after immunization, respectively. Mice immunized with BA15 mutants are capable of inducing consistently higher levels of *Brucella*-specific IgG antibodies than unvaccinated mice. The presence of specific IgG1 and IgG2a antibodies

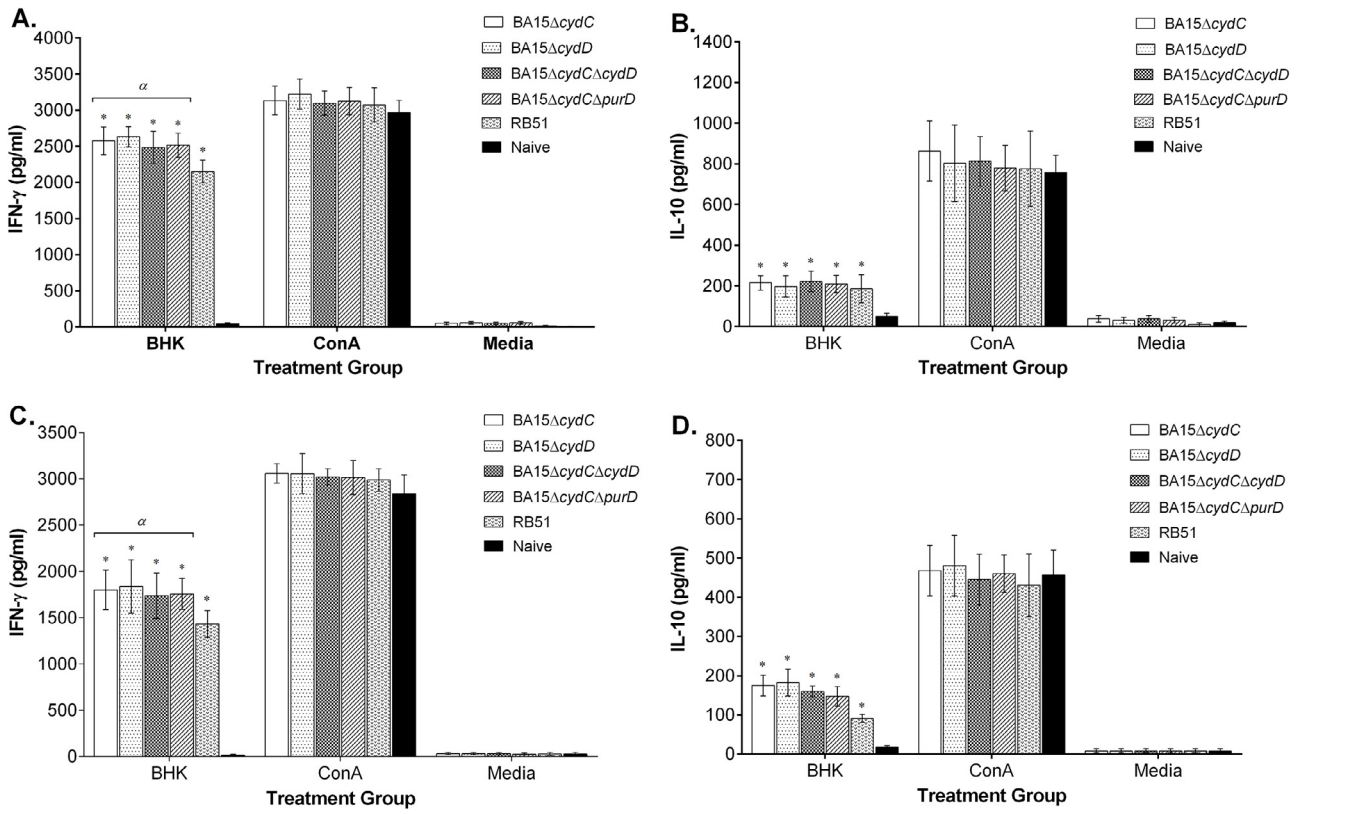

Fig. 5. Production of cytokines in stimulated splenocytes from mice immunized with BA15Δ*cydC*, BA15Δ*cydD*, BA15Δ*cydC*Δ*cydD*, and BA15Δ*cydC*Δ*purD* mutants, RB51 strain and PBS alone. At 6 (A and B) and 20 (C and D) weeks post-vaccination, mice ($n = 5$ per group) were euthanized and splenocytes were isolated and stimulated with heat-killed *B. abortus* (BHK) and ConA or medium alone as control. IFN-γ (A and C) and IL-10 (B and D) production was measured after 72 h stimulation using capture ELISA. Cytokine production was expressed as the mean cytokine concentration ± SD for each mouse group. Asterisks symbolize a significant difference in cytokine concentration of splenocytes from immunized mice with BA15 mutants and RB51 compared with PBS group ($^*P < 0.001$), and from mice immunized with BA15 mutants compared with those immunized with RB51 ($^\alpha P < 0.05$).

in the serum of the vaccinated mice suggests that vaccination with BA15 mutants induced a mix of Th1- and Th2-type immune responses. Moreover, we found that IgG2a predominated in all of the immunized groups, suggesting that the response was biased towards a Th1-type response. The analysis of cytokine secretion by splenocytes suggested that in immunized mice the Th1-type cytokine IFN-γ was more prominently expressed than the Th2-type cytokine IL-10. Therefore, these data indicate that vaccination with BA15 mutants favors a Th1-biased cellular and humoral immune responses.

As shown in this study, vaccination with RB51 was also able to induce significant levels of IgG antibodies and IFN-γ production. However, previous studies have demonstrated that protection induced by vaccination with RB51 against challenge with smooth S2308 is based upon cell-mediated immunity, and that antibodies play a minor or no role in protection [33–36]. The lower IFN-γ level and the lack of protective immunity to the O side chain of the LPS in mice immunized with RB51 may explain in part why this rough vaccine strain induces lower protection against S2308 infection than do the smooth BA15 mutants [34,37]. Taken together, the observed protection and protective immunity induced by BA15 mutants, especially BA15Δ*cydC*, BA15Δ*cydC*Δ*cydD*, and BA15Δ*cydC*Δ*purD* mutants, demonstrate that these mutants persisted in mice for a sufficient time to permit induction of effective protective immunity against virulent *B. abortus* challenge.

In conclusion, the results reported here demonstrate that immunization with the BA15 mutants induces strong humoral and cellular immunity, and provides long-term protection in mice against S2308 challenge. Furthermore, the major advantage of BA15 mutants, especially BA15Δ*cydC*, BA15*cydC*Δ*cydD*, and BA15Δ*cydC*Δ*purD* mutants is that they only persist for a short period of time, thus reducing the chance of residual virulence. Therefore, the BA15 mutants should be considered as potential vaccine candidates against bovine brucellosis that deserve further investigation for the suitability, efficacy and safety in cattle.

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References

[1] Ficht TA. Intracellular survival of Brucella: defining the link with persistence. Vet Microbiol 2003;92(April (3)):213–23.
[2] Corbel MJ. Brucellosis: an overview. Emerg Infect Dis 1997;3(April–June (2)):213–21.
[3] Olsen SC, Stoffregen WS. Essential role of vaccines in brucellosis control and eradication programs for livestock. Expert Rev Vacc 2005;4(December (6)):915–28.
[4] Ashford DA, di Pietra J, Lingappa J, Woods C, Noll H, Neville B, et al. Adverse events in humans associated with accidental exposure to the livestock brucellosis vaccine RB51. Vaccine 2004;22(September (25–26)):3435–9.
[5] Schurig GG, Sriranganathan N, Corbel MJ. Brucellosis vaccines: past, present and future. Vet Microbiol 2002;90(December (1–4)):479–96.
[6] Oliveira SC, Giambartolomei GH, Cassataro J. Confronting the barriers to develop novel vaccines against brucellosis. Expert Rev Vacc 2011;10(September (9)):1291–305.

[7] Ko J, Splitter GA. Molecular host-pathogen interaction in brucellosis: current understanding and future approaches to vaccine development for mice and humans. Clin Microbiol Rev 2003;16(January (1)):65–78.
[8] Wang Z, Wu Q. Research progress in live attenuated Brucella vaccine development. Curr Pharm Biotechnol 2013;14(10):887–96.
[9] Crawford RM, Van De Verg L, Yuan L, Hadfield TL, Warren RL, Drazek ES, et al. Deletion of purE attenuates Brucella melitensis infection in mice. Infect Immun 1996;64(June (6)):2188–92.
[10] Endley S, McMurray D, Ficht TA. Interruption of the cydB locus in *Brucella abortus* attenuates intracellular survival and virulence in the mouse model of infection. J Bacteriol 2001;183(April (8)):2454–62.
[11] Sun YH, de Jong MF, den Hartigh AB, Roux CM, Rolan HG, Tsolis RM. The small protein CydX is required for function of cytochrome bd oxidase in *Brucella abortus*. Front Cell Infect Microbiol 2012;2:47.
[12] Hoover DL, Crawford RM, Van De Verg LL, Izadjoo MJ, Bhattacharjee AK, Paranavitana CM, et al. Protection of mice against brucellosis by vaccination with Brucella melitensis WR201(16MDeltapurEK). Infect Immun 1999;67(November (11)):5877–84.
[13] Ko J, Gendron-Fitzpatrick A, Ficht TA, Splitter GA. Virulence criteria for *Brucella abortus* strains as determined by interferon regulatory factor 1-deficient mice. Infect Immun 2002;70(December (12)):7004–12.
[14] Yang XH, Thornburg T, Walters N, Pascual DW. Delta znuA Delta purE *Brucella abortus* 2308 mutant as a live vaccine candidate. Vaccine 2010;28(January (4)):1069–74.
[15] Truong QL, Cho Y, Barate AK, Kim S, Hahn TW. Characterization and protective property of *Brucella abortus* cydC and looP mutants. Clin Vacc Immunol 2014;21(November (11)):1573–80.
[16] Truong QL, Cho Y, Barate AK, Kim S, Watarai M, Hahn TW. Mutation of purD and purF genes further attenuates *Brucella abortus* strain RB51. Microb Pathog 2015;February (79):1–7.
[17] McQuiston JR, Schurig GG, Sriranganathan N, Boyle SM. Transformation of *Brucella* species with suicide and broad host-range plasmids. Methods Mol Biol 1995;47:143–8.
[18] Wang Z, Niu J, Wang S, Lv Y, Wu Q. In vivo differences in the virulence, pathogenicity, and induced protective immunity of wboA mutants from genetically different parent *Brucella* spp. Clin Vacc Immunol 2013;20(February (2)):174–80.
[19] Kahl-McDonagh MM, Ficht TA. Evaluation of protection afforded by *Brucella abortus* and *Brucella melitensis* unmarked deletion mutants exhibiting different rates of clearance in BALB/c mice. Infect Immun 2006;74(July (7)):4048–57.
[20] White PG, Wilson JB. Differentiation of smooth and nonsmooth colonies of *Brucellae*. J Bacteriol 1951;61(February (2)):239–40.
[21] Kim S, Kurokawa D, Watanabe K, Makino S, Shirahata T, Watarai M. *Brucella abortus* nicotinamidase (PncA) contributes to its intracellular replication and infectivity in mice. FEMS Microbiol Lett 2004;234(May (2)):289–95.
[22] Trant CG, Lacerda TL, Carvalho NB, Azevedo V, Rosinha GM, Salcedo SP, et al. The *Brucella abortus* phosphoglycerate kinase mutant is highly attenuated and induces protection superior to that of vaccine strain 19 in immunocompromised and immunocompetent mice. Infect Immun 2010;78(May (5)):2283–91.
[23] Arenas-Gamboa AM, Ficht TA, Kahl-McDonagh MM, Rice-Ficht AC. Immunization with a single dose of a microencapsulated *Brucella melitensis* mutant enhances protection against wild-type challenge. Infect Immun 2008;76(June (6)):2448–55.
[24] Goldman BS, Gabbert KK, Kranz RG. The temperature-sensitive growth and survival phenotypes of *Escherichia coli* cydDC and cydAB strains are due to deficiencies in cytochrome bd and are corrected by exogenous catalase and reducing agents. J Bacteriol 1996;178(November (21)):6348–51.
[25] Edwards SE, Loder CS, Wu G, Corker H, Bainbridge BW, Hill S, et al. Mutation of cytochrome bd quinol oxidase results in reduced stationary phase survival, iron deprivation, metal toxicity and oxidative stress in *Azotobacter vinelandii*. FEMS Microbiol Lett 2000;185(April (1)):71–7.
[26] Poole RK, Hatch L, Cleeter MW, Gibson F, Cox GB, Wu G. Cytochrome bd biosynthesis in *Escherichia coli*: the sequences of the cydC and cydD genes suggest that they encode the components of an ABC membrane transporter. Mol Microbiol 1993;10(October (2)):421–30.
[27] Poole RK, Gibson F, Wu G. The cydD gene product, component of a heterodimeric ABC transporter, is required for assembly of periplasmic cytochrome c and of cytochrome bd in *Escherichia coli*. FEMS Microbiol Lett 1994;117(April (2)):217–23.
[28] Jackson M, Phalen SW, Lagranderie M, Ensergueix D, Chavarot P, Marchal G, et al. Persistence and protective efficacy of a *Mycobacterium tuberculosis* auxotroph vaccine. Infect Immun 1999;67(June (6)):2867–73.
[29] O'Callaghan D, Maskell D, Liew FY, Easmon CS, Dougan G. Characterization of aromatic- and purine-dependent *Salmonella typhimurium*: attention, persistence, and ability to induce protective immunity in BALB/c mice. Infect Immun 1988;56(February (2)):419–23.
[30] Perkins SD, Smither SJ, Atkins HS. Towards a *Brucella* vaccine for humans. FEMS Microbiol Rev 2010;34(May (3)):379–94.
[31] Stevens MG, Pugh Jr GW, Tabatabai LB. Effects of gamma interferon and indomethacin in preventing *Brucella abortus* infections in mice. Infect Immun 1992;60(October (10)):4407–9.
[32] Jiang X, Baldwin CL. Effects of cytokines on intracellular growth of *Brucella abortus*. Infect Immun 1993;61(January (1)):124–34.
[33] Jimenez de Bagues MP, Elzer PH, Jones SM, Blasco JM, Enright FM, Schurig GG, et al. Vaccination with *Brucella abortus* rough mutant RB51 protects BALB/c mice against virulent strains of *Brucella abortus*, *Brucella melitensis*, and *Brucella ovis*. Infect Immun 1994;62(November (11)):4990–6.
[34] Stevens MG, Olsen SC, Pugh Jr GW, Brees D. Comparison of immune responses and resistance to brucellosis in mice vaccinated with *Brucella abortus* 19 or RB51. Infect Immun 1995;63(January (1)):264–70.
[35] Montaraz JA, Winter AJ, Hunter DM, Sowa BA, Wu AM, Adams LG. Protection against *Brucella abortus* in mice with O-polysaccharide-specific monoclonal antibodies. Infect Immun 1986;51(March (3)):961–3.
[36] Surendran N, Hiltbold EM, Heid B, Sriranganathan N, Boyle SM, Zimmerman KL, et al. Heat-killed and gamma-irradiated Brucella strain RB51 stimulates enhanced dendritic cell activation, but not function compared with the virulent smooth strain 2308. FEMS Immunol Med Microbiol 2010;60(November (2)):147–55.
[37] Campos MA, Rosinha GM, Almeida IC, Salgueiro XS, Jarvis BW, Splitter GA, et al. Role of Toll-like receptor 4 in induction of cell-mediated immunity and resistance to *Brucella abortus* infection in mice. Infect Immun 2004;72(January (1)):176–86.
[38] Lee JJ, Simborio HL, Reyes AWB, Kim DG, Hop HT, Min W, et al. Immunoproteomic identification of immunodominant antigens independent of the time of infection in *Brucella abortus* 2308-challenged cattle. Vet Res 2015;46:1–13.