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A Potent *Brucella abortus* 2308 Δ*ery* Live Vaccine Allows for the Differentiation between Natural and Vaccinated Infection

Junbo Zhang[1†], Shuanghong Yin[2†], Fei Guo[2], Ren Meng[1], Chuangfu Chen[1*], Hui Zhang[1], Zhiqiang Li[1], Qiang Fu[1], Huijun Shi[1], Shengwei Hu[1], Wei Ni[3], Tiansen Li[1], and Ke Zhang[1]

[1]College of Animal Science and Technology, [2]College of Medicine, Shihezi University, Shihezi 832003, P. R. China
[3]College of Life Sciences, Shihezi University, Xinjiang 832000, P. R. China

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***Brucellosis* is a globally distributed zoonotic disease that causes animal and human diseases. However, the current *Brucella abortus* vaccines (S19 and RB51) are deficient; they can cause abortion in pregnant animals. Moreover, when the vaccine S19 is used, tests cannot differentiate natural from vaccinated infection. Therefore, a safer and more potent vaccine is needed. A *Brucella abortus* 2308 *ery* promoter mutant (Δ*ery*) was constructed to overcome these drawbacks. The growth of the Δ*ery* mutant was significantly attenuated in macrophages and mice and induced high protective immunity in mice. Moreover, Δ*ery* induced an anti-*Brucella*-specific IgG (immunoglobulin G) response and stimulated the expression of interferon-gamma (INF-γ) and interleukin-4 (IL-4). Furthermore, the expression of EryA antigen allowed for the serological differentiation between natural and vaccinated infection in mice. These results indicate that the Δ*ery* mutant is a potential attenuated live vaccine candidate against virulent *Brucella abortus* 2308 (S2308) infection.**

***Keywords*:** *Brucella abortus* 2308, *ery* promoter mutant, live attenuated vaccine, EryA

Introduction

Brucella spp. are Gram-negative and facultative intracellular pathogens that can multiply within professional and non-professional phagocytes of both humans and animals, resulting in heavy economic losses and human suffering (Lacerda *et al*., 2010). *Brucella* spp. cause acute transmissible infections in animal reservoirs and abortion in pregnant animals (Ficht, 2003). *Brucella abortus*, *Brucella melitensis*, and *Brucella suis* can also cause human diseases. Infection in humans can cause fever, arthritis, spondylitis, dementia, and meningitis, and endocarditis (Elzer *et al*., 2002; Hamdy *et al*., 2002; Godfroid *et al*., 2005), and the symptoms of acute brucellosis can endure over one year and eventually result in chronic persistence (Castaño and Solera, 2009). Currently, there is no effective or safe *Brucella* vaccine for humans and animals. Therefore, new vaccines with high protective efficiency and low virulence are needed to overcome these drawbacks.

The current two live vaccines available for the control of brucellosis in cattle are *B. abortus* S19 and RB51. S19 is a spontaneously attenuated strain discovered by Dr. John Buck in 1923 (Nicoletti, 1990; Crasta *et al*., 2008). RB51 is a laboratory-derived mutant of the virulent strain of *B. abortus* 2308 (Schurig *et al*., 1991). RB51 is similar to S19 and S2308, which contain the same outer membrane proteins (Schurig *et al*., 1991; Elzer *et al*., 1998), but unlike S19 and S2308, RB51 lacks virtually all of the LPS (lipopolysaccharide) O side chains (Schurig *et al*., 1991). The two vaccines are effective in conferring protection against virulent strains of S2308. However, S19 may cause abortion in pregnant cattle and induce LPS antibodies that interfere with the serological testing. RB51 has the limitation of rifampin resistance (Elzer *et al*., 1998; Poester *et al*., 2006), and it may also cause abortion in pregnant animals, depending on the dosage and route of delivery (Cheville *et al*., 1996; Olsen *et al*., 1999). Therefore, one potential approach to these problems is to develop a marker vaccine by deleting the virulence from parental vaccine strains with good immunogenicity and vaccine efficacy. In-depth research on the deletion of virulence genes is required for the generation of new live vaccines against S2308 infection that are superior to S19 or RB51.

Erythritol is utilized by *Brucella* in preference to glucose (Anderson and Smith, 1965), and erythritol promotes the growth of some *Brucella* strains (Meyer, 1967). Erythritol utilization is a characteristic of pathogenic *B. abortus* strains. The degradation pathways of erythritol, maltose, ribose, arabinose, galactose, glucose, glycerol and rhizopine appear to be needed for the intracellular survival of *Brucella* (Delrue *et al*., 2004). The *ery* operon is formed by four genes: *eryA*, *eryB*, *eryC*, and *eryD*. The *eryA* gene encodes a putative erythritol kinase. The *eryB* gene encodes an erythritol phosphate dehydrogenase. The *eryC* gene encodes a D-erythrulose 1-phosphate dehydrogenase, and the *eryD* gene encodes a regulator of the *ery* operon (Sangari *et al*., 2000; Eoh *et al*., 2010). The enzyme D-erythrulose-1-phosphate dehydrogenase plays a crucial role in erythritol metabolism and is absent in the S19 vaccine strain (Sperry and Robertson, 1975; Burkhardt *et al*., 2005). The goal of our study was to investigate the role of the *ery* promoter in the virulence of S2308 in macrophages and mice. Our results indicate that the *ery*

[†]These authors contributed equally to the work.
*For correspondence. E-mail: chuangfu_chen@163.com; Tel.: +86-0993-2058002; Fax: +86-0993-2058612

promoter has an important function in S2308 virulence. When the *ery* promoter gene was disrupted, Δ*ery* exhibited a major decrease in virulence. These results indicate, for the first time, that Δ*ery* may be useful as an attenuated live S2308 vaccine candidate.

Materials and Methods

Mice

Eight-week-old female BALB/c mice were obtained from the Experimental Animal Center of Academy of Military Medical Science (China). All experimental procedures and animal care were performed in compliance with institutional animal care regulations.

Bacterial strains, plasmids, and growth conditions

Brucella abortus 2308 and S19 were obtained from the Center of Chinese Disease Prevention and Control (China). *Brucella* was cultured in TSB (tryptic soy broth) or TSA (tryptic soy agar) (Sigma, USA). *E. coli* strain DH5a was grown on an LB (Luria-Bertani) medium. Plasmid pGEM-7Zf$^+$ was purchased from Promega (USA). The culture media were supplemented with appropriated antibiotics (100 μg/ml ampicillin for S2308 or 50 μg/ml kanamycin for *E. coli*).

Construction of Δ*ery*

The *ery* promoter region was predicted using Neural Network Promoter Prediction software (http://www.fruitfly.org/seq_tools/promoter.html). A pair of primers was designed for the amplification of part of the *ery* promoter DNA fragment (1,500 bp). The primer sequences were as follows:

E-F, GGATCCTCAGCCATGCGTGAAAAAGG
(containing a *Xho*I site);
E-R, CTCGAGGGAAGCGAGTTTGTCCCAGA
(containing a *Bam*HI site).

Two pairs of primers with restriction sites at the 5′ ends were designed for the amplification of the upstream (1,227 bp) and downstream (1,037 bp) arms of the S2308 *ery* promoter, in which the *Sph*I, *Xho*I, and *Bam*HI (underlined) sites were integrated into both PCR fragment ends. The primer sequences were as follows:

up-F, GCATGCGCGAGGCTTAATTCCTGATGG;
up-R, CTCGAGCACGCCGCTATCATAAGTTTCT;
dn-F, CTCGAGGCGTGAAAAAGGTGACATC;
dn-R, GGATCCCGTTGAACCAGTCGTCAAG;

The two upstream (1,227 bp) and downstream (1,037 bp) arms of the S2308 *ery* promoter were cloned into pMD18-T Simple Vector for sequencing and then subcloned into pGEM-7Zf$^+$ to generate the suicide plasmid pGEM-7Zf$^+$-*ery*. A pair of primers was designed for *sacB* DNA fragment amplification:

S-F, GGATCCGGGCTGGAAGAAGCAGACCGCTA
(containing a *Bam*HI site)
S-R, GGATCCTTATTTGTTAACTGTTAATTGTCC
(containing a *Bam*HI site)

A 1,475-bp fragment was amplified by PCR from *Bacillus subtilis*. The PCR-amplified DNA fragment was subcloned into the plasmid pGEM-7Zf$^+$-*ery* to generate the plasmid pGEM-7Zf$^+$-*ery*-SacB. Competent strain S2308 was electroporated with pGEM-7Zf$^+$-*ery*-SacB, and transformants of *Brucella* containing a single-crossover plasmid insertion were isolated on plates containing 100 μg/ml ampicillin for the first screening and then selected using the SacB-based sucrose sensitivity counter-selection system for the second screening. The deletion mutant was further confirmed by PCR amplification and DNA sequencing. Then, a pair of primers was designed for the amplification of part of the *ery* promoter DNA fragment (257 bp). The primer sequences were as follows:

P-F, CGAAATCCACCGCTAAAT;
P-R, CATAACGCTGGCGAACTG.

Growth curve and erythritol sensitivity of Δ*ery*

The bacterial growth was measured at an optical density at 600 nm (OD_{600}). For growth curve analysis, *B. abortus* S2308 and Δ*ery* were cultured in TSB for 48 h and then diluted with TSB to an OD600 of 0.05 and cultured in a rotary shaker (250 rpm) at 37°C. Aliquots of the cultures were taken at an interval of 2 h, and OD_{600} was recorded.

For erythritol sensitivity analyses, stationary-phase pre-cultures of *B. abortus* S2308 and Δ*ery* were diluted in TSB containing erythritol (80 mM) for 48 h.

Evaluation of Δ*ery* survival and growth in macrophages

RAW 264.7 macrophages were used to assess Δ*ery* survival by comparing its survival with the survival of S2308 and S19. Briefly, 5×10^5 cells/well were cultured in a 24-well plate for 24 h at 37°C and infected with *Brucella* at 50 MOI. Culture plates were centrifuged for 5 min at 1,000 rpm in a Jouan centrifuge at room temperature and then placed in a 5% CO_2 atmosphere at 37°C. At 1 h post-infection, the macrophages were washed twice with medium without antibiotics and incubated with 50 μg/ml gentamicin (Invitrogen, USA) for 30 min to kill any extracellular bacteria. Subsequently, the culture was replaced with DMEM containing 25 μg/ml gentamicin. At 4, 12, 24, and 48 hr post-infection, the macrophages were lysed, and the live bacteria were enumerated by plating on TSA plates. All assays were performed in triplicate and repeated at least three times.

Evaluation of Δ*ery* survival and growth in mice

Female BALB/c mice were used to evaluate the survival of Δ*ery*. Briefly, 8-week-old mice (n=10 per group) were intraperitoneally (i.p.) inoculated with a total of 1×10^6 CFU (colony-forming-units) of Δ*ery*, S2308, S19 or PBS. The survival of the bacteria in mice was evaluated by bacterial enumeration in the spleens at different time points post inoculation. At 1, 2, 4, 6, 8, and 10 weeks after the inoculation, the mice were euthanized, and their spleens were collected aseptically for quantitation of bacterial burden. The speen weight was measured. Splenocytes were lysed, and the suspensions were diluted in sterile saline and plated on TSA. The plates were incubated at 37°C, and the number of bacterial CFU was counted after three days. The experiments were repeated twice.

Immuno-protection induced by Δ*ery* in BALB/c mice

Protective activity was evaluated by comparing the abilities of mice receiving Δ*ery* (experimental group), S19 (vaccine control group), or PBS (unvaccinated control group) to prevent detectible spleen infection after virulent challenge with S2308 (a standardized virulent, strain). Eight-week-old-female BALB/c mice (n=10 per group) were vaccinated i.p. with 1×10^6 CFU of Δ*ery* or S19. Ten unvaccinated mice were injected i.p. with 200 µl of PBS as controls. The mice were challenged i.p. with 1×10^6 CFU (200 µl) per mouse of virulent strain S2308 at 11 weeks after vaccination. All mice were euthanized by cervical dislocation 2 and 4 weeks post-challenge, and the bacterial CFUs in the spleen were determined. A mean value for each spleen count was obtained after logarithmic conversion. Log_{10} units of protection were obtained by subtracting the mean log_{10} CFU of the experimental group from the mean log_{10} CFU of the control group, as described previously (Adone *et al*., 2005). The experiment was repeated twice.

Evaluation of antibody production

Serum samples were obtained from immunized mice 2, 4, 6, and 10 weeks after the immunization, and IgG levels were determined by ELISA as described previously (Goel and Bhatnagar, 2012). Serum samples were diluted with PBS (1:100), and then, serial two-fold dilutions were prepared. The levels of IgG in the supernatants were measured using an ELISA Quantikine Mouse kit (R&D Systems, USA).

Cytokine measurement

Ten weeks after the immunization, BALB/c mice (n=10 per group) were sacrificed, and their spleens were removed under aseptic conditions. Single-cell suspensions from the spleens were obtained by homogenization. The cells were suspended in RPMI 1640 medium supplemented with 2 mM L-glutamine and 10% (v/v) FBS. Erythrocytes were eliminated with an ACK lysis solution (150 mM NH_4Cl, 1 mM Na_2-EDTA, pH 7.3). Splenocytes (4×10^5 cells/well) were cultured in

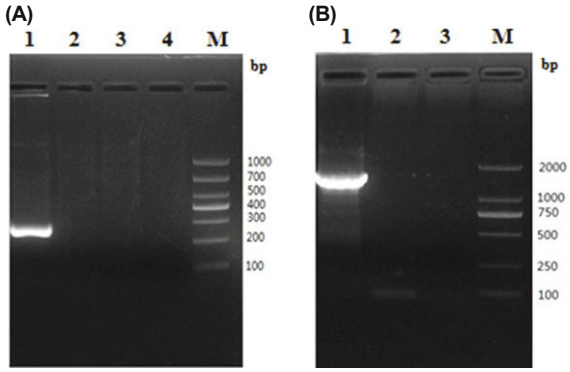

Fig. 1. Identification of construction of the Δ*ery*. (A) PCR identification of Δ*ery*. Lanes: 1, the strain S2308; 2 and 3, Δ*ery* mutant strain; 4, negative control; M, DNA marker. (B) RT-PCR identification of Δery. Lanes: 1, the strain S2308; 2, Δ*ery* mutant strain; 3, negative control; M, DNA marker.

96-well plates; the culture was stimulated by adding 25 µg of S19 or heat-killed S2308 lysate/well, 0.5 µg of ConA (concanavalinA) (as a positive control), or medium alone (as a negative control). The cells were then incubated with 5% CO_2 for 72 h at 37°C. The interferon-gamma (IFN-γ) and interleukin-4 (IL-4) levels in the supernatants were measured using an ELISA Quantikine Mouse kit (R&D Systems).

Expression and purification of EryA

EryA open reading frames were amplified by PCR from the S2308 genome. The amplified DNA fragments were then subcloned into the pET28a vector (Novagen, USA) and expressed in *Escherichia coli* BL21 (DE3 {*Escherichia coli* BL21 (DE3)}) as N-terminally His-tagged fusion proteins. The expression of the recombinant EryA protein was confirmed by SDS-PAGE. The EryA protein was purified by affinity chromatography with Ni^{2+}-conjugated Sepharose. The purified recombinant EryA protein was separated by SDS-PAGE using 12% gels and electrotransferred to a nitrocellulose membrane with a Mini Trans-Blot Cell (Bio-Rad). Unbound sites on the membrane were blocked overnight with 10% dry

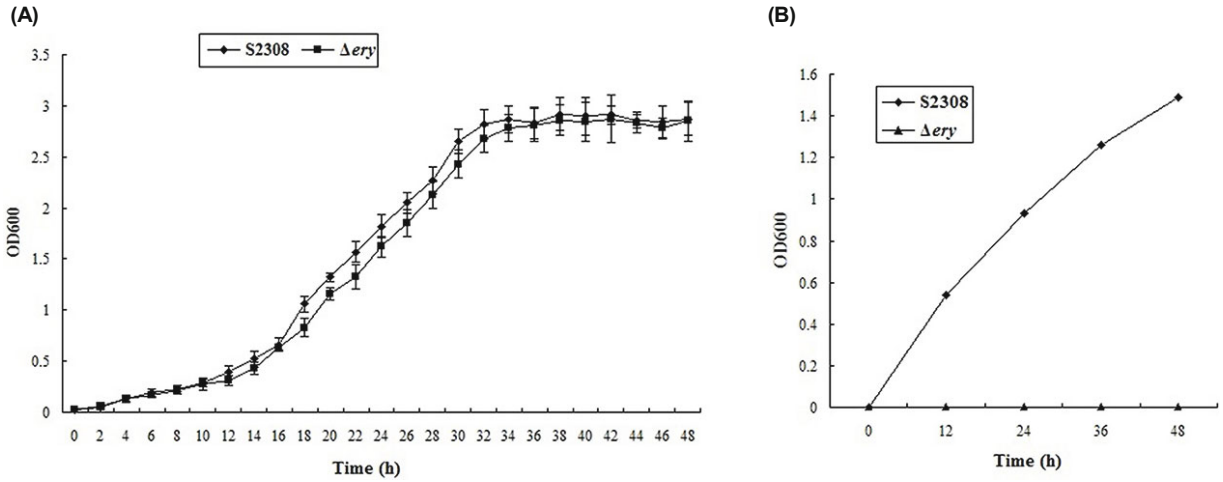

Fig. 2. Growth curves of the Δ*ery* in nutrient-replete (TSB 7.0) media (A) or broth containing erythritol (80 mM) (B). The OD_{600} value was measured in the experiment.

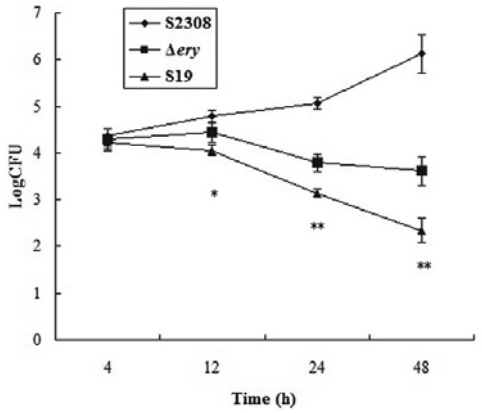

Fig. 3. Intracellular replication of Δ*ery* within RAW264.7 macrophages. The level of initial infection was the same for Δ*ery*, S19, and S2308. The results showed that the Δ*ery* failed to achieve the level of colonization reached by the wild type. At 4, 12, 24, and 48 h post-infection, infected cells were lysed, and supernatants were diluted for CFU enumeration. Significant differences between the mutant and S2308 are indicated as follows: * $P < 0.05$, ** $P < 0.001$.

milk in TBST buffer (100 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.2). The membrane was washed three times with TBST buffer and incubated with S2308-infected serum (diluted 1:300). The bands were incubated with rabbit anti-mouse IgG (peroxidase conjugated) for 1 h at room temperature. Bound conjugate was visualized with a DAB substrate kit (Beyotime) after further washing.

EryA indirect ELISA

Serum samples were obtained from *Brucella*-infected mice. Antibody responses to the purified recombinant EryA protein were evaluated via EryA-based indirect ELISA, as described previously (Liu *et al.*, 2012).

Statistical analysis

The data were analysed using Student's *t*-test and expressed as the mean value±standard error of the mean (S.E.M.). *P* values of less then 0.05 were considered statistically significant.

Results

Construction of Δ*ery*

The *ery* gene deletion mutants of the 2308 strain were successfully obtained. The *ery* promoter was correctly knocked out and subsequently confirmed by PCR and RT-PCR. The PCR and RT-PCR results indicated that a 257-bp or 1,500-bp product were amplified from S2308 using the primers P-F and P-R and E-F and E-R, respectively, but nothing was amplified from the mutant strain Δ*ery* (Figs. 1A and 1B). These data indicate that Δ*ery* was constructed correctly.

Growth curve and erythritol sensitivity of Δ*ery*

The effects of the *ery* promoter on S2308 growth in nutrient-replete (TSB 7.0) media were then examined. When cultured in rich TSB media, Δ*ery* displayed a similar lag phase and reached the stationary phase at a similar optical density compared with the wild-type strain (Fig. 2A). We next tested whether the Δ*ery* mutant was sensitive to erythritol when growing the strain in broth containing erythritol (80 mM). The wild-type strain S2308 grew well. In contrast, no growth of the Δ*ery* mutant was detected in broth containing erythritol (Fig. 2B). These results indicate that the *ery* promoter does not affect the growth of S2308 in TSB 7.0 media, that Δ*ery* was sensitive to erythritol, and that the wild-type strain S2308 was tolerant to erythritol.

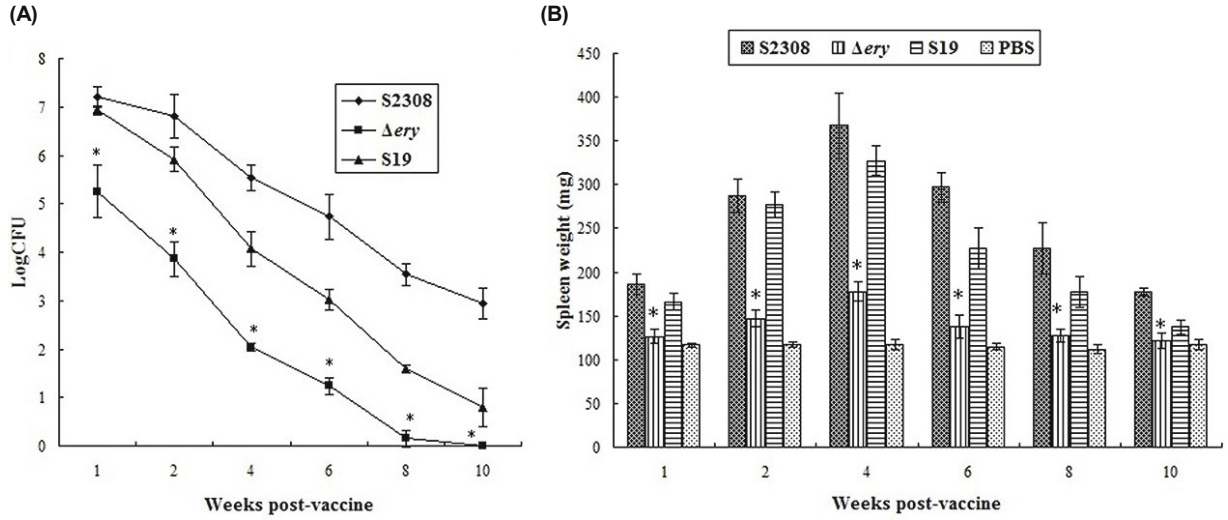

Fig. 4. Clearance of Δ*ery* after infection. BALB/c mice were infected with 1×10^6 CFU/mouse of Δ*ery*, S19, S2308, and S2308-*ery*. At 1, 2, 4, 6, 8, and 10 weeks post-infection, the spleens were harvested at different time points and individual spleens were assessed for colonization (A) and spleen weight (B). Results are the means ± SD, and Significant differences between Δ*ery* and S2308 are indicated as follows: * $P < 0.001$.

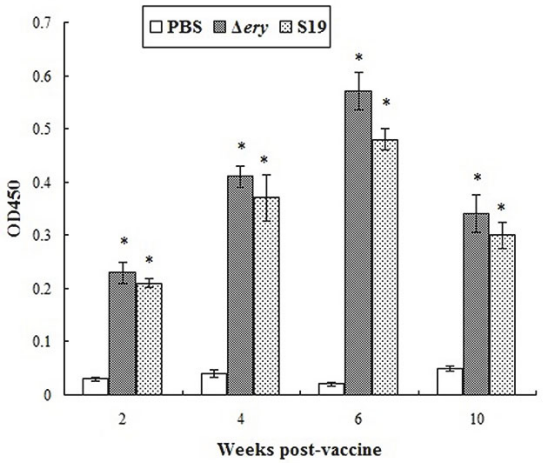

Fig. 5. Immunization with Δ*ery* induces antibody responses. BALB/c mice were immunized with 1×10^6 CFU of either Δ*ery* or S19. The control groups received PBS. At 2, 4, 6, and 10 weeks post-immunization, serum samples were collected and analyzed for IgG levels by ELISA. Results are means ± SD (n=10) of the absorbance at 450 nm (OD450). Significant differences between Δ*ery* and PBS are indicated as follows: * $P < 0.001$.

Intracellular growth of Δ*ery* was attenuated in macrophages

Intracellular survival and replication within macrophages is an indispensable characteristic of *Brucella* pathogenesis. RAW 264.7 macrophages were infected with Δ*ery,* S19 and S2308 to compare their intracellular growth capability. Four hours post-infection, no significant difference was observed among the bacterial growth in the macrophages, indicating the similar ability of the three strains to invade macrophages. Furthermore, the *ery* promoter did not affect the entry of *Brucella* into the macrophages (Fig. 3). At 12 h post-infection, a 0.73-log decrease ($P<0.05$) was observed in the number of Δ*ery* inside of the macrophages compared with that of S2308. At 48 h post-infection, the decrease in the number of Δ*ery* was more significant (3.78-log fold; $P<0.001$) compared with S19 and S2308. These results indicate that the *ery* promoter was involved in *Brucella* chronic infection and that the growth of Δ*ery* was significantly attenuated in RAW 264.7 macrophages.

Table 1. Protection conferred by Δ*ery* against S2308

Vaccine	Log_{10}CFU Brucella per spleen (mean±SD) at wk postchallenge		Log_{10} units of potection[a,b] at wk	
	2	4	2	4
PBS	7.36±0.67	6.53±0.33		
Δ*ery*	5.08±0.48	4.81±0.25	2.28*	1.72*
S19	5.49±0.88	5.42±0.74	1.87*	1.11*

[a] Significance is indicated as follows: *$P \le 0.05$ compared with the PBS control group.
[b] Log_{10} units of protection = Average of the log_{10} CFU in spleens of control unvaccinated mice minus the average of log_{10} CFU in spleens of vaccinated mice.

Δ*ery* is attenuated in BALB/c mice

BALB/c mice were vaccinated i.p. with 1×10^6 CFU of Δ*ery*, S2308, and S19. The number of *Brucella* in the spleen was evaluated 1, 2, 4, 6, 8, and 10 weeks post-infection. From weeks 1 to 10, the bacterial loads in the Δ*ery*-vaccinated mice displayed a significant reduction compared with that of the S2308-vaccinated mice ($P<0.001$; Figs. 4A and 4B), which was lower than that of the S19-vaccinated mice, and the Δ*ery* mutant was cleared by week 10. In addition, as demonstrated by the comparative splenic weights, at most time points, there was less splenic inflammation in the animals that had received the Δ*ery* mutant than in those that had received S2308 and S19.

Δ*ery* immunization induced humoral immune and cytokine responses

Serum from mice inoculated i.p. with 1×10^6 CFU of Δ*ery*, S19 or PBS were collected to monitor total IgG levels by ELISA. The Δ*ery*-vaccinated mice expressed significantly higher IgG levels than that in the PBS-infected mice and slightly higher IgG levels than that in the S19-infected mice (Fig. 5).

To examine the cytokine responses to Δ*ery*, the levels of IFN-γ and IL-4 in the splenocytes of the Δ*ery*- and S19-vaccinated mice were assessed at 10-weeks post-immunization. Mice inoculated with homologous immunogen ConA and heat-inactivated S2308 were used as controls. Splenocytes of the Δ*ery*- and S19-vaccinated mice expressed significantly higher levels of IFN-γ and IL-4 than those of the

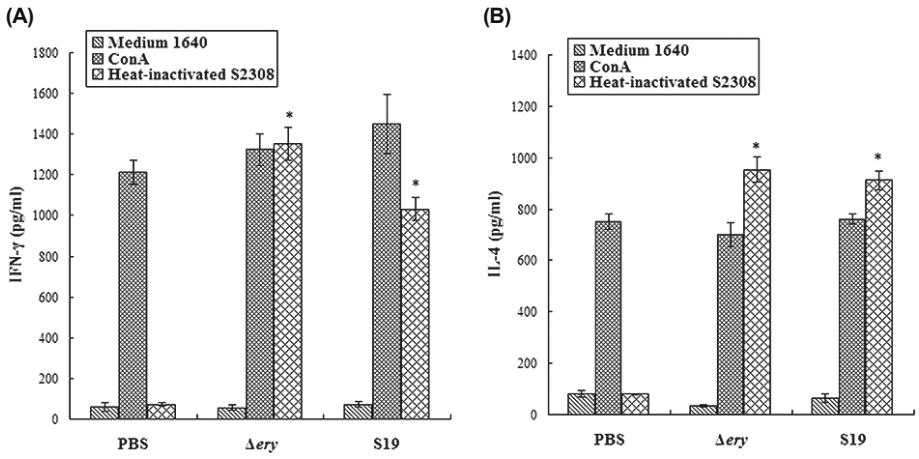

Fig. 6. Splenocyte production of IFN-γ and IL-4 induced by Δ*ery*. Spleens from BALB/c mice were inoculated intraperitoneally with 1×10^6 CFU of either Δ*ery*, S19, or PBS. Splenocyte culture supernatants were harvested after 72 h of culture. The analysis of IFN-γ (A) and IL-4 (B) secretion was measured by ELISA from the supernatant. Significantly different from the same stimulus in PBS-immunized mice are indicated as follows: * $P < 0.001$.

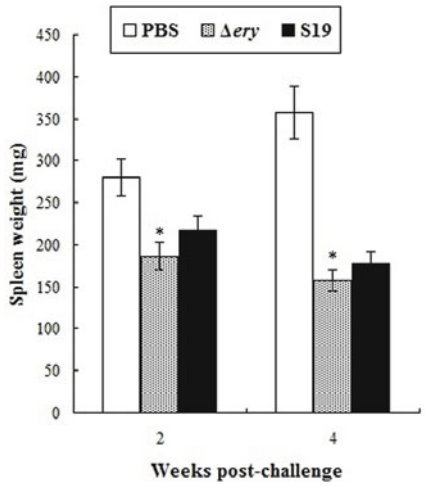

Fig. 7. The result of spleen weight in the mice inoculated with Δ*ery* mutant, S19 and PBS after 2 and 4 weeks post-challenge. Significant differences between Δ*ery* and PBS are indicated as follows: * $P < 0.001$.

PBS-injected mice ($P < 0.001$; Figs. 6A and 6B), and the splenocytes of the Δ*ery*-vaccinated mice expressed higher levels of IFN-γ and IL-4 than that of the S19-vaccinated mice.

Δ*ery*-vaccination provided better immune protection against S2308 infection

Mice were vaccinated i.p. with 1×10^6 CFU of Δ*ery*, S19 or PBS to determine whether Δ*ery* vaccination can provide protection against wild-type S2308 infection. Eleven weeks after vaccination, mice were challenged with 1×10^6 CFU of S2308. Mice immunized with the Δ*ery* mutants exhibited significantly fewer splenic *Brucella* than the non-immunized mice at 2 (2.28 log unit) and 4 (1.72 log unit) weeks after challenge ($P<0.001$; Table 1). As expected, S19 also induced significant protection at 2 (1.87 log unit) and 4 (1.11 log unit) weeks after challenge, and the protective efficacy of S19 was lower than that in the Δ*ery*-vaccinated mice. Moreover, inflammation in the spleen was diminished in the animals that received Δ*ery* compared with that in the PBS-infected animals, as was evident by the lack of splenomegaly (Fig. 7). The results indicate that the Δ*ery* vaccination can provide stronger protection than the S19 vaccination against the virulent S2308.

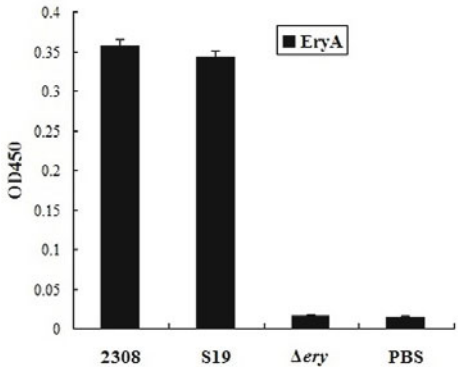

Fig. 8. Reaction of EryA to Δ*ery* immunization sera. Antibodies to EryA protein were not detected in the serum from Δ*ery* immunized mice by EryA-iELISA, but they were detected in the serum from S2308 and S19 immunized mice.

Expression and purification of EryA protein

To express the *eryA* gene, the constructed pET28a-*EryA* was transformed into the *E. coli* BL21(DE3) expression host. A high level of 76-kDa recombinant protein was obtained after induction with 1 mM IPTG for 4 and 6 h. The EryA proteins were then purified by SDS-PAGE (data not shown). The purified protein could react with mouse anti-S2308 immune serum by Western blot (data not shown). As expected, the control (non-immune) serum exhibited no reactivity with the recombinant EryA protein (data not shown).

Differentiation of *Δery*

Serum from mice vaccinated with Δ*ery*, S2308, S19 or PBS were collected to determine whether the EryA protein can be used as a diagnostic antigen. Antibodies against the EryA protein could be detected in the serum of the S2308- or S19- vaccinated mice and were not detected in the serum of the Δ*ery*- or PBS-vaccinated mice, indicating that the Δ*ery* vaccination did not induce antibodies against EryA (Fig. 8). Furthermore, serum from the S2308-vaccinated mice produced a positive reaction, and serum from the Δ*ery*-vaccinated mice displayed a negative reaction according to an EryA-iELISA using His-EryA as a solid-phase antigen. These results indicate that the EryA protein can be used to differentiate between natural and vaccinated infections by EryA-iELISA after the confirmation of *Brucellosis* infection using LPS-based serological tests.

Discussion

The generation of an efficacious vaccine for *Brucellosis* has been a challenge for scientists for several years. Most of the present licensed vaccines have several limitations, such as residual virulence, splenomegaly, and interference of serodiagnosis (Schurig *et al.*, 2002; Berkelman, 2003; Ashford *et al.*, 2004). One of the limitations in the development of new *Brucella* vaccines is the limited knowledge of the virulence factors. Serological interference of classical vaccines is also a significant problem. Therefore, the ideal vaccine must be protective and should carry a genetic marker without antibiotic resistance and be non-virulent for the host and the gene being defined (Moriyón *et al.*, 2004). Δ*ery* was constructed, and its virulence and protective efficacy were assessed in macrophages and mice to investigate the ability of Δ*ery* to maintain protective efficacy.

The Δ*ery* mutant was constructed to confirm that the reduced survival capability of the mutant was directly related to the deleted gene *eryA*. The Δ*ery* mutants were confirmed by PCR and transcription analyses (data not shown). After the deletion of the *ery* promoter, the mRNAs of *eryABC* were unexpressed according to the RT-PCR analysis (data not shown), indicating that the *eryABC* operon was inactive.

The two genes *eryB* and *eryC* are part of the same metabolic pathway and are virulence factors for *Brucella*, depending on large-scale screens (Delrue *et al*., 2004). The *eryB* and *eryC* mutants of *Brucella suis* and *Brucella melitensis* are attenuated in macrophage models (Delrue *et al*., 2004). The vaccine strain *Brucella abortus* B19 is erythritol sensitive, which is defective in *eryB* and is attenuated and induces only few or no abortions (Burkhardt *et al*., 2005), but complementation experiments with a murine model demonstrated that the defect in erythritol metabolism of *Brucella abortus* B19 is not associated with its attenuated virulence in mice (Sangari *et al*., 1998). Our results revealed that Δ*ery* was sensitive to erythritol and defective for survival in macrophages and BALB/c mice. The lack of splenomegaly in inoculated mice indicates the increased safety of Δ*ery*.

Vaccinations with Δ*ery* offered significant protection against challenge inoculation based on the observation of the number of bacteria in the spleen. An ideal live vaccine strain combines efficient immunogenicity with minimal reactogenicity. Therefore, we performed the protection experiment in BALB/c mice and found that Δ*ery* can elicit good protective efficacy against a subsequent challenge with the virulent strain S2308.

The cell-mediated and humoral responses were evaluated to evaluate the protection conferred by Δ*ery*. Cytokine-induced IFN-γ secretion can locate to the infection site and enhance bacterial killing. We detected antigen-specific production of IFN-γ (Th1 cytokine) and IL-4 (Th2 cytokine) in the splenocytes from *Δery*-immunized mice. The Th1 immune responses characterized by IFN-γ production are associated with protective immunity to *Brucella* (Golding *et al*., 2001). Previous studies revealed that IFN-γ is a critical cytokine required for macrophage bactericidal activity (Sathiyaseelan *et al*., 2006). IL-4 is a Th2 cytokine that is considered an important microbial molecule. IL-4 is a mediator of CD4/CD8 cross-talk, leading to the development of immunity against an infectious pathogen (Carvalho *et al*., 2002). Thus, the mixed Th1/Th2 responses can be demonstrated through the detection of IFN-γ and IL-4. Our results indicate that Δ*ery* immunization induced higher levels of IFN-γ and IL-4 than those observed in S19. The vaccination strategy should lead to mixed Th1/Th2 responses. Δ*ery*-vaccinated mice produced IgG antibodies to the LPS O side chain, which was conducive to serological testing.

Current serological diagnostic tests include the RBPT (Rose Bengal Plate Test), SAT (standard tube agglutination test), CFT (complement fixation test), iELISA or cELISA, using hot saline extract and smooth LPS antigens. The LPS of smooth *Brucella* species is by far the strongest antigen compared with other antigenic molecules (Wang *et al*., 2011). However, LPS-based serological tests have difficulty differentiating between the serum of vaccinated animals and those infected naturally. Thus, we evaluated the possibility of using EryA protein as a diagnostic antigen. The results indicated that the humoral immune response to EryA can be detected in the infected serum but not in Δ*ery*-immunized serum, indicating that immunization serum can be differentiated from vaccination serum using EryA as a diagnostic antigen. Therefore, *Brucellosis* was first confirmed by LPS-based serological tests, and then, Δ*ery* vaccination was detected by EryA-ELISA. The results revealed that mice infected with S2308 were positive in the EryA-iELISA based on a recombinant His-fusion protein His-EryA as the solid-phase antigen, whereas the animals vaccinated with the Δ*ery* were negative. Therefore, Δ*ery* provides a choice for differentiation between infected and vaccinated animals. The proteins EryB and EryC may allow for a reliable distinction between infected and vaccinated animals, but this requires further investigation.

In conclusion, our results indicate that Δ*ery* is another suitable live vaccine candidate for *B. abortus* because of its low virulence in RAW 264.7 macrophages and BALB/c mice while maintaining higher protective efficacy than that of the S19 vaccine strain at 4 weeks post-challenge with S2308 infection. Post-vaccination humoral responses indicated that the vaccine candidate could elicit an anti-*Brucella*-specific IgG response, providing an ideal diagnostic EryA antigen for the differentiation of immunization from infection using an EryA-iELISA. The overall results indicated that Δ*ery* offered good protection against virulent S2308 in this study. This finding should be considered as a new potential vaccine candidate against *Brucellosis*. In future studies, comprehensive protection experiments must be conducted to determine whether the measurable immune responses in systemic compartments via different routes confer detectable protection against *Brucella* infections. In addition, further insight into the mechanisms that contribute to the humoral immune response (IgG1 and IgG2a) in mice will be evaluated in this study, and further testing in livestock will determine whether Δ*ery* is a promising live vaccine candidate.

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