INFECTION AND IMMUNITY, July 2006, p. 3874–3879 Vol. 74, No. 7
0019-9567/06/$08.00+0 doi:10.1128/IAI.01957-05

Deletion of *znuA* Virulence Factor Attenuates *Brucella abortus* and Confers Protection against Wild-Type Challenge

Xinghong Yang, Todd Becker, Nancy Walters, and David W. Pascual*

Veterinary Molecular Biology, Montana State University, Bozeman, Montana 59717-3610

Received 1 December 2005/Returned for modification 26 January 2006/Accepted 18 April 2006

***znuA* is known to be an important factor for survival and normal growth under low Zn^{2+} concentrations for *Escherichia coli*, *Haemophilus* spp., *Neisseria gonorrhoeae*, and *Pasteurella multocida*. We hypothesized that the *znuA* gene present in *Brucella melitensis* 16 M would be similar to *znuA* in *B. abortus* and questioned whether it may also be an important factor for growth and virulence of *Brucella abortus*. Using the *B. melitensis* 16 M genome sequence, primers were designed to construct a *B. abortus* deletion mutant. A *znuA* knockout mutation in *B. abortus* 2308 (Δ*znuA*) was constructed and found to be lethal in low-Zn^{2+} medium. When used to infect macrophages, Δ*znuA B. abortus* showed minimal growth. Further study with Δ*znuA B. abortus* showed that its virulence in BALB/c mice was attenuated, and most of the bacteria were cleared from the spleen within 8 weeks. Protection studies confirmed the Δ*znuA* mutant as a potential live vaccine, since protection against wild-type *B. abortus* 2308 challenge was as effective as that obtained with the RB51 or S19 vaccine strain.**

Zn^{2+} is an essential mineral required by bacteria as either a structural or catalytic cofactor (32). Bacterial survival and proliferation in the environment and within animal hosts are critically dependent on the uptake and sequestration of transition metals, such as Zn^{2+} (4). This is problematic, because free Zn^{2+} concentrations in mammalian hosts are very low, so as to prevent bacterial colonization. To acquire the necessary Zn^{2+} for its metabolism, bacteria have evolved several types of proteins that are involved in binding and transporting zinc (9).

The translation products of the *znuABC* operon found in *Escherichia coli* (5, 31), *Haemophilus* spp. (27), *Neisseria gonorrhoeae* (8), *Pasteurella multocida* (15), and *Synechocystis* sp. strain 6803 (4) constitute a high-affinity periplasmic binding protein-dependent and ATP-binding cassette (ABC) transport system for Zn^{2+}. In gram-negative bacteria, ABC transporters are involved in the active transport of molecules from periplasm to the cytosol (19). In addition, *znuA* mutants in *H. ducreyi* (27) and *P. multocida* (15) were found to be significantly less virulent than wild-type strains when tested in animal models.

B. abortus is a gram-negative facultative, intracellular pathogen capable of infecting both wildlife and livestock (7), and it is able to cause severe zoonotic infection in humans (3, 34). Currently, there are no human *Brucella* vaccines, and current livestock vaccines such as S19 and RB51 are virulent in humans. Attempts to develop live brucellae vaccines have met with varied success. For instance, inactivation of the amino acid biosynthesis pathway genes *pheA*, *trpB*, and *dagA* displayed little or no attenuation in cultured murine macrophages or in mice (1). The mutants of purine biosynthesis pathway genes *purL*, *purD*, and *purE* (1) displayed significant attenuation in BALB/c mice, but live brucellae remained viable after 12 weeks, suggesting that their virulence was not sufficiently attenuated for adoption as a livestock vaccine. The aromatic amino acid pathway *aroC* mutant of *B. suis* (14), the *B. abortus* lipid A fatty acid-transporting gene *bacA* mutant (12, 26), the type IV secretion *virB* mutant (11), and the ferrochelatase *hemH* mutant (2) showed highly attenuated virulence, but their protective efficacy has yet to be reported. *B. abortus* nicotinamidase *pncA* (22) and exopolysaccharide transporter *exsA* (33) mutants showed substantial protection, but these vaccine strains still colonized the host even after 6 weeks. One promising live brucellae vaccine candidate was shown with the cyclic β-1,2-glucan *B. abortus cgs* mutant of S19, which effectively cleared within 4 weeks and conferred complete protection against low-dose, not high-dose, challenge with wild-type *B. abortus* 2308 (6). Collectively, these studies show that defined *Brucella* mutants can be effective, but their potential efficacy remains difficult to predict.

MATERIALS AND METHODS

Genetic methods. Isolation of plasmid and bacterial chromosomal DNA, restriction enzyme digests, and agarose gel electrophoresis were performed according to standard molecular biological techniques. Plasmids were transformed into *E. coli* strains by standard techniques. Restriction endonucleases, T4 DNA ligase, calf intestinal alkaline phosphatase, the plasmid Miniprep kit, and the DNA fragment gel extraction kit were purchased from New England Biolabs and used according to the manufacturer's specifications.

Bacterial strains. *B. abortus* strain 2308 and the vaccine strain RB51 were obtained from the National Veterinary Services Laboratory, USDA (Ames, IA). *B. abortus* vaccine strain S19 was obtained from Colorado Serum Co. (Denver).

Suicide plasmid and *B. abortus* mutant construction. A pair of primers was designed for *znuA* DNA fragment amplification. The primers' designations were based on *B. melitensis* 16 M, whereas the template DNA used for PCR was from *B. abortus* 2308. Primer sequences were the following: Z-F, TTTAAGCTTCG GTTGCCCGGATGCTG; and Z-R, GCGTCTAGATTGAAGGCAAGTGTC GTG. Restriction enzyme sites of HindIII and XbaI (underlined nucleotides in the above sequences) were integrated to the 5′ end of primers Z-F and Z-R, respectively. This pair of primers was designed for amplifying *znuA* and its upstream (164 bp) and downstream (126 bp) DNA sequences. A total of 1,277 bp was expected to be amplified. The 1,277-bp PCR product was cloned into pUC18 for sequencing, and differences with *B. melitensis* and *B. suis* were annotated. Two amino acid differences were determined (GenBank accession no. AY941821). This pair of primers was also used to verify the deleted sequence in the knockout mutant.

For the mutant construction, two pairs of primers were designed for the

* Corresponding author. Mailing address: Veterinary Molecular Biology, Montana State University, Bozeman, MT 59717-3610. Phone: (406) 994-6244. Fax: (406) 994-4303. E-mail: dpascual@montana.edu.

upstream (1,818 bp) and downstream (1,538 bp) regions of the *B. abortus* 2308 *znuA* DNA fragment in which SacI, XbaI, XbaI, and SalI (underlined) sites were integrated into both of the PCR fragment ends. Primer sequences were the following: up-F, TTAGAGCTCACCGTTGCTGCGAGGAG; up-R, GTATCTAGAAGCCAGAAAGGCAGAAGCAAG; and dn-F, CTCTCTAGATCGCTGAAAGACTGCCTG; dn-R, TTTGTCGACTTGGATGTGCGGGAAGCC.

Upstream and downstream *znuA* DNA fragments from *B. abortus* 2308 were then cloned into pUC18 and subcloned into the suicide vector pCVD442 (Amp^r) between SacI and SalI to yield suicide plasmid pCznuA, which is capable of propagating in *E. coli* S17-1 λ*pir*$^+$. pCznuA from donor *E. coli* S17-1 λ*pir*$^+$ was transferred to *B. abortus* 2308 by conjugation. Cells were spread onto Bacto Potato Infusion Agar (PIA) (DIFCO, Sparks, MD) plates, which contained 100 μg/ml ampicillin plus 7 μg/ml nalidixic acid (*E. coli* is sensitive to nalidixic acid but *B. abortus* is not), and incubated at 37°C in 5% CO_2. After 5 days, one colony from the selection plate was chosen and inoculated into *Brucella* Broth (BB) (DIFCO) medium and shaken at 160 rpm, 37°C overnight. Cells were harvested and spread onto PIA plates containing 6% sucrose and incubated at 37°C in 5% CO_2. After 4 days, six individual colonies were selected and screened by PCR analysis using primers Z-F and Z-R. A crystal violet test, as described by White and Wilson (38), was performed to confirm that the Δ*znuA* mutant maintained its smooth phenotype.

Complementation in *trans* of the Δ*znuA* mutation. To restore *znuA* activity, PCR amplification fragments with primers Z-F and Z-R and vector pBBR1MCS2 (Kan^r) (25) were double digested with restriction enzymes HindIII and XbaI and ligated to yield pBznuA, which was electroporated into *E. coli* S17-1. Transformants were recovered on Luria-Bertani agar containing 10 μg/ml kanamycin. Plasmids from these transformants were isolated (QIAprep Miniprep; QIAGEN Inc., Valencia, CA) and analyzed by agarose gel electrophoresis after restriction digestion. *B. abortus* Δ*znuA* was electroporated with pBznuA. Transformants were selected on PIA containing 10 μg/ml kanamycin.

Evaluation of *B. abortus* Δ*znuA* attenuation in RAW 264.7 macrophages. RAW 264.7 cells (American Type Culture Collection, Manassas, VA) were used to assess Δ*znuA* mutant survival in macrophages compared to that of the *B. abortus* wild-type 2308 strain, S19, and the complemented mutant, Δ*znuA* (pBznuA). Infections were conducted in a fashion similar to that previously described (30). Briefly, 1.25×10^6 cells/well in complete medium (CM; RPMI 1640, 10% fetal bovine serum [Atlanta Biologicals, GA], 10 mM HEPES buffer, 10 mM nonessential amino acids, 10 mM sodium pyruvate) without antibiotics were allowed to adhere to plastic in 24-well microtiter dishes (B-D Labware, Franklin, NJ) for 3 h at 37°C. Wells were washed, and the nonadherent cells were collected and counted to determine cell numbers that remained plastic adherent. After overnight culture, cells were infected with varying bacteria-to-macrophage ratios (5:1, 10:1, and 20:1) for 1 h at 37°C. Wells were washed twice with CM without antibiotics and then incubated with 50 μg/ml of gentamicin (Life Technologies) for 30 min at 37°C. After washing twice, as described above, fresh CM without antibiotics (1.0 ml/well) was added, and cells were incubated for an additional 4, 24, or 48 h.

Immunization and challenge of mice. All experiments with live brucellae were performed in biosafety level 3 facilities. Female BALB/c mice (Frederick Cancer Research Facility, National Cancer Institute, MD) were maintained at the Montana State University Animal Resource Center. All animals were maintained in individually ventilated cages under HEPA-filtered barrier conditions of 12 h of light and 12 h of darkness in the animal biosafety level 3 facility, and they were provided with food and water ad libitum. Experiments were conducted with 7- to 9-week-old age-matched mice. All animal care and procedures were in accordance with institutional policies for animal health and well-being.

Wild-type 2308, Δ*znuA* mutant, RB51, and S19 *Brucella* strains were grown overnight in BB at 37°C. Cells were pelleted, washed twice in sterile phosphate-buffered saline (PBS), and diluted to 1×10^8 cells/200 μl in sterile PBS. The actual viable inoculum CFU was confirmed by serial dilution tests on PIA, and 0.2 ml of this suspension was administered to mice via intraperitoneal (i.p.) injection. For challenge study, *B. abortus* 2308 was diluted in sterile PBS, in which 100 μl of bacterial suspension contained 5×10^4 CFU bacteria, and immunized and naive mice were subsequently i.p. challenged. The challenge dose was confirmed by plating *B. abortus* on PIA.

Enumeration of brucellae in spleens. For the in vivo Δ*znuA* mutant colonization studies, splenic CFU were assessed at 1, 4, and 8 weeks and compared to those from in vivo colonization by RB51, S19, and 2308. Individual spleens were removed and mechanically homogenized in 1 ml of sterile Milli-Q water. After incubation for 3 to 5 days at 37°C in 5% CO_2 on PIA, *Brucella* colonies were enumerated and CFU per spleen were calculated.

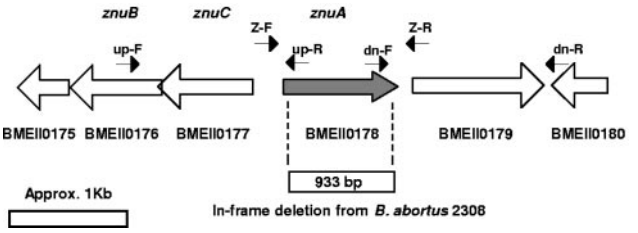

FIG. 1. Schematic representation of the *B. melitensis* 16 M chromosome II region around *znuA* (10) used for *B. abortus* 2308 mutant construction. The orientation of the different genes is indicated by large arrows. The orientations of the different primers used in this study are indicated by small arrows. The position of in-frame deletion of a 933-bp *znuA* inner DNA fragment from *B. abortus* 2308 during the construction of the *znuA* mutant by allelic replacement is indicated.

Statistical analysis. The Student *t* test was used to evaluate differences between variations of in vitro and in vivo colonization by Δ*znuA B. abortus*, Δ*znuA*-(pBzunA) *B. abortus*, strain 2303, RB51, or S19 at the 95% confidence interval.

RESULTS

Isolation of *B. abortus* 2308 *znuA*. Zn^{2+} is an essential element for some bacteria (32), and we hypothesized that a defective Zn^{2+} transport system may attenuate *B. abortus*. At the time of these studies, the *B. abortus* 2308 *znuA* DNA sequence had not been released, but the *B. melitensis* 16 M genome sequence was published in 2002 (10). The 16 M genome sequence was referred to for designing primers, utilizing *B. abortus* strain 2308 as a template for PCR amplification with Z-F and Z-R primers (Fig. 1). The PCR fragment was cloned into pUC18, and three positive clones were chosen for sequencing analysis. DNA sequencing results showed that all three clones were identical and that there were only two 1-bp differences from *B. melitensis* 16 M and *B. suis* 1330 *znuA*. These two 1-bp differences represented two amino acid changes: I265 in *B. melitensis* and *B. suis* became T265 in *B. abortus*, and S333 became P333 in *B. abortus*. These results confirmed our hypothesis that it would be possible to clone *znuA* from strain 2308 by using the *B. melitensis* 16 M genome sequence and allowing for the development of Δ*znuA* in a *B. abortus* mutant (Fig. 1).

***B. abortus znuA* mutant and its complementation in *trans*.** Out of six clones randomly selected from PIA supplemented with 6% sucrose, one clone was confirmed as a positive in-frame knockout mutant, as selected by PCR with primers Z-F and Z-R, and subsequently was confirmed by sequencing. A 933-bp inner DNA fragment of *znuA* was deleted from *B. abortus* 2308 genome chromosome II, and only 78 bp of this gene sequence remained.

To verify that Δ*znuA* attenuation was due to gene replacement and not to a secondary mutation, we complemented the *B. abortus* Δ*znuA* mutant with pBznuA plasmid. pBznuA was constructed by PCR amplification of the wild-type *B. abortus* *znuA* gene with primers Z-F and Z-R, including 164 bp upstream and 126 bp downstream of the coding sequence. The 1,277-bp amplification product was cloned into vector pBBR1MCS2 (25) and transferred into *B. abortus* Δ*znuA* mutant for complementation analysis.

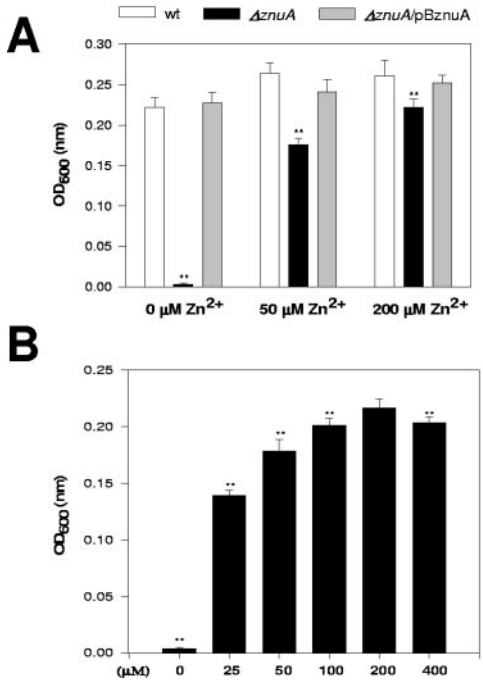

FIG. 2. *B. abortus* 2308 Δ*znuA* is unable to grow in low-Zn^{2+} liquid GMM. (A) Optical densities at 600 nm (OD_{600}) attained by wild-type *B. abortus* 2308, the *B. abortus* Δ*znuA* mutant, and recombinant strain *B. abortus* Δ*znuA* with *znuA* restored in *trans*, Δ*znuA*(pBznuA), at 120 h postinoculation of GMM containing 0, 50, or 200 μM Zn^{2+}. Values are the means of three independent experiments ± standard errors of the means; significant differences are compared to optical densities obtained with wild-type *B. abortus*. **, $P = 0.01$. (B) Growth (OD_{600} at 120 h postinoculation) of *B. abortus* Δ*znuA* mutant in liquid GMM supplemented with increasing Zn^{2+} concentrations. Values are the means of three independent experiments ± standard errors of the means, and significant differences are compared to the results for the 200 μM dose of Zn^{2+}. **, $P = 0.01$. wt, wild type.

***znuA* is required for *B. abortus* growth in low-Zn^{2+} medium.** Δ*znuA* mutations in *E. coli* (31), *H. ducreyi* (27), *N. gonorrhoeae* (8), *P. multocida* (15), and *Synechocystis* sp. strain 6803 (4) have been shown to be defective in growth medium lacking Zn^{2+}. For comparison, we investigated the growth of *B. abortus* Δ*znuA* in different concentrations of Zn^{2+} in Gerhardt's minimal medium (GMM). Without Zn^{2+}, *B. abortus* Δ*znuA* showed no growth, while wild-type 2308 and Δ*znuA*(pBznuA) grew to similar concentrations. With increasing Zn^{2+} concentrations, the *B. abortus* Δ*znuA* mutant recovered growth that approached that of wild-type strain 2308 and recombinant strain *B. abortus* Δ*znuA*(pBzunA) (Fig. 2A). Further testing showed that 200 μM Zn^{2+} is required for optimal growth of *B. abortus* Δ*znuA* (Fig. 2B). These data suggest that *znuA* is required for normal growth in low-Zn^{2+} medium.

Δ*znuA* mutant's growth is arrested in RAW 264.7 macrophages. To further assess the Δ*znuA* mutant's attenuation, RAW 264.7 macrophages were infected with Δ*znuA* mutant and compared to other *B. abortus* strains to determine their ability to replicate in macrophages. Three ratios of bacteria to macrophage were tested at three time points, 4, 24, and 48 h (Fig. 3). At 0 h, there were no differences in the amount of bacteria that infected the RAW 264.7 cells (Fig. 3). By 4 h postinfection, the macrophages contained equivalent bacterial loads, but by 24 h the *znuA* mutant's growth was significantly arrested compared to that of macrophages infected with wild-type 2308, the Δ*znuA*(pBznuA) strain ($P \leq 0.001$), or the S19 strain ($P \leq 0.005$), and results were similar at 48 h. These results show that the Δ*znuA* mutant has a limited capability of replicating in RAW 264.7 macrophages compared to that of virulent *B. abortus* or the vaccine S19 strain. In addition, these results show enhanced attenuation in macrophages, as opposed to that reported using a transposon-induced mutant of the *znuA* gene of *B. abortus* using bone marrow-derived macrophages (22).

Δ*znuA* mutant is attenuated in BALB/c mice. To evaluate Δ*znuA* mutant virulence in vivo, BALB/c mice were i.p. in-

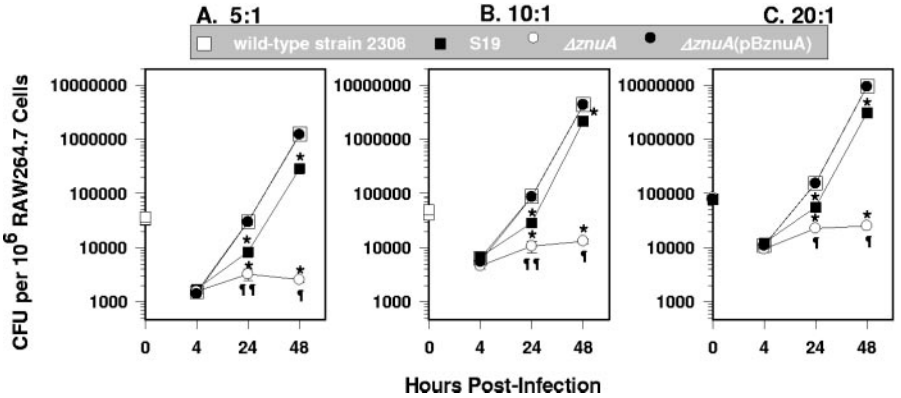

FIG. 3. Growth of the *B. abortus* Δ*znuA* mutant is attenuated in RAW 264.7 macrophages. Wild-type strain 2308, live S19 vaccine, *B. abortus* Δ*znuA* mutant, and the *B. abortus* recombinant strain Δ*znuA*(pBznuA) were used to infect RAW 264.7 macrophages at a bacteria-to-macrophage ratio of (A) 5:1, (B) 10:1, or (C) 20:1. After 1 h of incubation followed by 30 min of treatment with gentamicin, infected RAW 264.7 cells were incubated in fresh medium for 0, 4, 24, or 48 h. Infected macrophages were water lysed, and supernatants were diluted for CFU enumeration. The level of initial infection was the same for all *B. abortus* strains (time = 0 h). The results show that the Δ*znuA* mutant was unable to achieve the level of colonization reached by the wild type, Δ*znuA*(pBznuA), or S19. Values are the means of two independent experiments ± standard errors of the means. Differences in macrophage colonization by strain 2308 or Δ*znuA*(pBznuA) versus Δ*znuA* mutant or S19 are indicated (*, $P < 0.001$); differences in macrophage colonization by S19 versus the Δ*znuA* mutant are also indicated (¶, $P < 0.001$; ¶¶, $P \leq 0.005$).

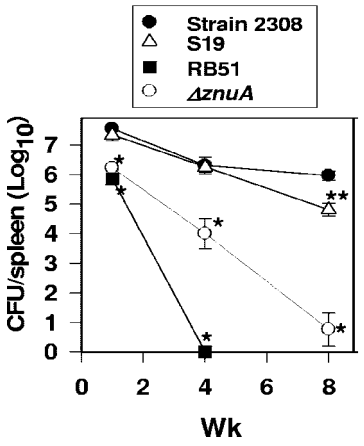

FIG. 4. *B. abortus* Δ*znuA* is effectively cleared by 8 weeks after infection. BALB/c mice (5 mice/group/time point) were i.p. dosed with 1×10^8 CFU of wild-type *B. abortus* 2308, *B. abortus* Δ*znuA* mutant, and RB51 or S19 vaccine. At weeks 1, 4, and 8, individual spleens were assessed for colonization. Values are the means of individual mice ± standard errors of the means, and differences in colonization were determined in comparison to results for the wild-type 2308 strain. *, $P < 0.001$; **, $P = 0.003$. Wk, week.

fected with 1×10^8 CFU of *B. abortus* Δ*znuA*, wild-type 2308, the smooth vaccine strain S19, or the rough vaccine strain RB51. Compared to wild-type strain 2308, splenic CFU in *B. abortus* Δ*znuA*-dosed mice were significantly decreased ($P < 0.001$) at weeks 1, 4, and 8. Importantly, by week 8, in three of the five *B. abortus* Δ*znuA*-dosed mice, splenic CFU could not be detected (Fig. 4). These results show that the Δ*znuA* strain is attenuated. In addition, in this study RB51 was cleared by week 4, which is consistent with what has been previously shown (21, 35). While an in vivo splenic colonization study has been previously reported (22), no kinetic study was described using the transposon-induced *znuA* mutant.

***B. abortus* Δ*znuA* confers protection to BALB/c mice.** Due to decreased virulence, the Δ*znuA* mutant strain is a potential live vaccine candidate, and past works have not tested its potential efficacy (22). To assess its protective efficacy, four groups of BALB/c mice were immunized i.p. with 1×10^8 CFU of *B. abortus* Δ*znuA*, RB51, or S19 or with sterile PBS. This immunization dose was similar to what others have used to determine protective efficacy (21, 29, 37) for rough *Brucella* mutants, due to their rapid clearance in animal hosts. Since our Δ*znuA* mutant is also highly attenuated in mice (Fig. 4), although bearing a smooth phenotype, we elected to use a high dose for immunization to reflect what may be used in livestock. Eight weeks postimmunization, mice were i.p. challenged with wild-type strain 2308. Four weeks postchallenge, mice vaccinated with *B. abortus* Δ*znuA* showed significantly fewer splenic CFU than mice given PBS. *B. abortus znuA* was as protective as RB51 and S19 vaccines (Fig. 5A).

We showed that the Δ*znuA* mutant was mostly cleared by 8 weeks postinfection. To verify if any Δ*znuA* mutant remained after the strain 2308 challenge that may confound the splenic CFU determinations, Δ*znuA* mutant-immunized BALB/c mice were evaluated by PCR 4 weeks after wild-type 2308 challenge (12 weeks after immunization). Spleens were removed, and the homogenized tissues were spread onto PIA plates for selecting individual bacterial colonies. Fifty colonies were randomly chosen from PIA plates and mixed together to extract genomic DNA, which was used as a template for PCR amplification. The primers used were Z-F and Z-R. A 1,277-bp DNA fragment was expected to be amplified for the wild-type strain, and 344 bp (1,277 bp minus 933 bp) was expected to be amplified for the Δ*znuA* mutant strain. Wild-type 2308 genomic DNA was used as positive control I, and 2308 genomic DNA from 49 mixed colonies plus 1 colony from Δ*znuA* mutant genomic DNA was used as positive control II. None of the isolates from Δ*znuA* mutant-immunized and challenged mice showed a detectable 344-bp fragment following PCR, despite the experiments being repeated five times per mouse (Fig. 5B). These results confirmed that the splenic CFU detected 4 weeks postchallenge were all wild type, and none were from the Δ*znuA* mutant.

DISCUSSION

As with *E. coli*, *Haemophilus* spp., *N. gonorrhoeae*, *P. multocida*, and *Synechocystis* sp. strain 6803, *znuA* is also required by *B. abortus* for growth in low-Zn^{2+} medium. These observations suggest that *znuA* proteins from these bacteria share a similar function, i.e., providing the capacity to grow in a low-Zn^{2+} environment. The earliest report on *znuA* virulence was regarding *H. ducreyi* (27). Subsequently, it was demonstrated that *znuA* and *znuBC* transcriptional units in *P. multocida* are required for virulence in the mouse model (15). As shown in this present study, our *znuA* mutant's growth depends on environmental Zn^{2+} concentration, and its growth is compromised when it is cultured under no or little Zn^{2+}. Similar results were obtained when Zn^{2+} was chelated from the growth medium for the transposon-induced mutant (22). In addition, we assessed the potential of using the Δ*znuA* mutant as a live vaccine. Since site-directed mutagenesis was done, no antibiotic selection was used to develop this vaccine strain, and our mutation was limited solely to the *znuA* gene. Thus, the strain described in our study is different from the previously described *znuA* mutant (22), in which a polar mutation was produced by the insertion of a kanamycin resistance gene into the *znuA* gene. Because of this approach, downstream gene expression was affected, unlike the results of our approach, in which an in-frame disruption was used to limit the effects to a single gene, *znuA*.

A successful infection involves *Brucella* survival in the phagosomes of macrophages (18, 28). However, in phagosomes the low pH, limited nutrition, and O_2 tension adds more pressure for bacterial survival (13, 17, 20, 23, 24). Thus, to counteract this inhospitable environment, bacteria develop a number of survival mechanisms. One strategy involves the *znuABC* operon for high-affinity binding of Zn^{2+} to allow survival where Zn^{2+} levels are limiting. This requirement for zinc is evident by serving as a cofactor for the Cu,Zn superoxide dismutase (*sodC* gene) and enabling *Brucella* to resist oxidation by the host phagosome (16). In addition, Zn^{2+} is an essential structural or catalytic cofactor for many enzymes, and losing its ability to acquire the limited Zn^{2+} levels from phagosomes would inhibit the activity of enzymes such as alkaline phosphatase, RNA polymerase, aspartate transcar-

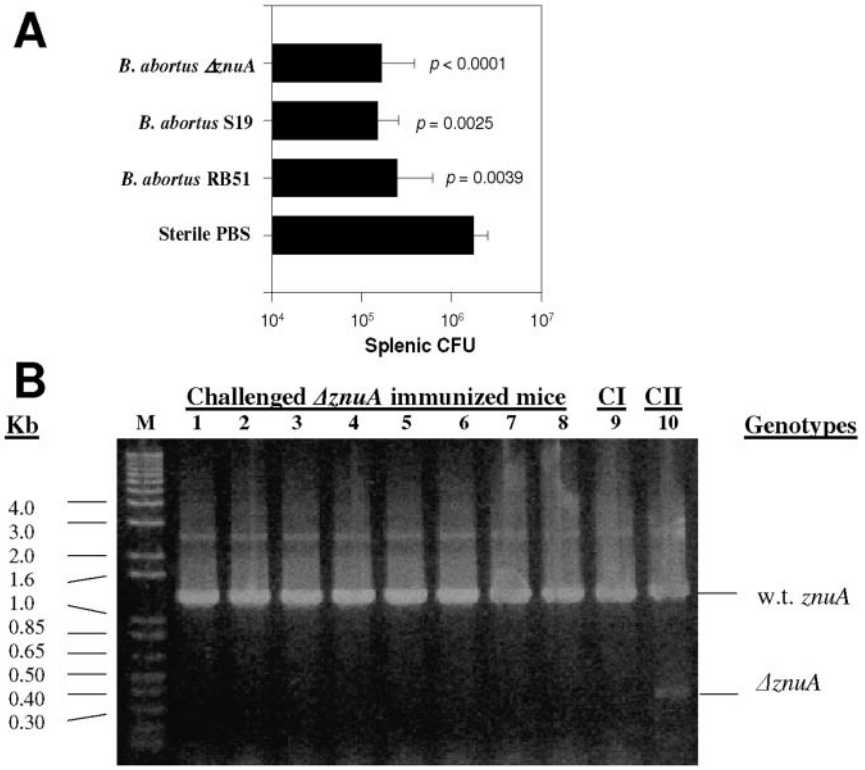

FIG. 5. *B. abortus* Δ*znuA* is protective against wild-type *B. abortus* challenge. (A) BALB/c mice immunized with *B. abortus* Δ*znuA* (15 mice/group), RB51 (5 mice/group), S19 (5 mice/group), or sterile PBS (11 mice/group). After 8 weeks, mice were challenged with 5×10^4 CFU wild-type *B. abortus* 2308. Four weeks postchallenge, their spleens were assessed for CFU levels. Values are the means of individual mice ± standard errors of the means from two experiments. (B) Remaining CFU in challenged mice are the wild-type strain 2308. Lanes 1 to 8 depict the results for 50 colonies' DNA obtained from the spleens of 8 individual BALB/c mouse spleens used as a template for PCR amplification. Lane 9 is positive control I (CI) for wild-type 2308 genomic DNA template amplified as a 1,277-bp DNA fragment. Lane 10 is positive control II (CII), representing 49 colonies of wild-type 2308 and 1 colony of mutant Δ*znuA* genomic DNA used as a template for amplifying both 1,277-bp and 344-bp DNA fragments. These results show that no Δ*znuA* genomic DNA can be amplified from any of the splenic *Brucella* colonies taken from the Δ*znuA*-vaccinated mice. Thus, the Δ*znuA* mutant is cleared from the spleen before the 4-week-postchallenge CFU enumeration was conducted. Each mouse was evaluated by this method five times. w.t., wild type.

bamylase, FtsH (Zn^{2+}-dependent protease), and zinc finger proteins (31).

Because of this importance of Zn^{2+}, we sought the development of the Δ*znuA* mutant for *B. abortus*. As a result, we observed diminished splenic CFU displayed by the *B. abortus* Δ*znuA* mutant in BALB/c mice and in RAW 264.7 macrophages. Although dosages of the mutant were different from those in a previous study (22), it was apparent that our mutant was eventually cleared within 8 weeks. Moreover, the clearance of our Δ*znuA* mutant from RAW 264.7 macrophages was an order of magnitude greater than that observed with the polar Δ*znuA* mutant (22). Such development of a vaccine exhibiting less virulence but higher protective efficacy is desired. As such, the *B. abortus pgm* mutant also showed low virulence, but its protective efficacy was only as good as that of S19 (36). The advantage of this mutant, like RB51, is its lack of the lipopolysaccharide O side chain, which enables us to distinguish wild-type *Brucella*-infected animals from those that have been vaccinated.

Our findings show that the *znuA* mutant may be another suitable live vaccine candidate for *B. abortus*, because of its low virulence in BALB/c mice while maintaining a protective efficacy similar to that of RB51 and S19 vaccine strains. Further testing in livestock will determine whether the *B. abortus* Δ*znuA* mutant will be a promising live vaccine candidate, since it is proposed that some immunity to lipopolysaccharide is desirable for stimulating optimal immunity in livestock. Current studies are evaluating whether additional mutations will enhance the Δ*znuA* mutant's efficacy and determining how to distinguish vaccinated from naturally infected animals.

ACKNOWLEDGMENTS

We thank Michael Donnenberg and Xiaolin Wang from the University of Maryland, School of Medicine, Division of Infectious Diseases, for kindly providing us with the suicide plasmid pCVD442; Gao Weimin from the Oak Ridge National Laboratory for kindly providing us with the *E. coli* S17-1 λpir strain; and Nancy Kommers for her assistance in preparing the manuscript.

This work was supported by a grant from the USDA (2005-06034). It was also supported in part by the Montana Agricultural Station and USDA Formula Funds as well as NIH/NCRR COBRE-supported (P20 RR-020185) biosafety level 3 facilities.

REFERENCES

1. **Alcantara, R. B., R. D. Read, M. W. Valderas, T. D. Brown, and R. M. Roop, Jr.** 2004. Intact purine biosynthesis pathways are required for wild-type

virulence of *Brucella abortus* 2308 in the BALB/c mouse model. Infect. Immun. **72:**4911–4917.
2. **Almirón, M., M. Martínez, N. Sanjuan, and R. A. Ugalde.** 2001. Ferrochelatase is present in *Brucella abortus* and is critical for its intracellular survival and virulence. Infect. Immun. **69:**6225–6230.
3. **Ashford, D. A., J. di Pietra, J. Lingappa, C. Woods, H. Noll, B. Neville, R. Weyant, S. L. Bragg, R. A. Spiegel, J. Tappero, and B. B. Perkins.** 2004. Adverse events in humans associated with accidental exposure to the livestock brucellosis vaccine RB51. Vaccine **22:**3435–3439.
4. **Banerjee, S., B. Wei, M. Bhattacharyya-Pakrasi, H. B. Pakrasi, and T. J. Smith.** 2003. Structural determinants of metal specificity in the zinc transport protein *ZnuA* from Synechocystis 6803. J. Mol. Biol. **333:**1061–1069.
5. **Beard, S. J., R. Hashim, G. Wu, M. R. Binet, M. N. Hughes, and R. K. Poole.** 2000. Evidence for the transport of zinc(II) ions via the pit inorganic phosphate transport system in *Escherichia coli*. FEMS Microbiol. Lett. **184:**231–235.
6. **Briones, G., N. Iñón de Iannino, M. Roset, A. Vigliocco, P. S. Paulo, and R. A. Ugalde.** 2001. *Brucella abortus* cyclic β-1,2-glucan mutants have reduced virulence in mice and are defective in intracellular replication in HeLa cells. Infect. Immun. **69:**4528–4535.
7. **Cao, T. B., and M. H. Saier, Jr.** 2001. Conjugal type IV macromolecular transfer systems of gram-negative bacteria: organismal distribution, structural constraints and evolutionary conclusions. Microbiology **147:**3201–3214.
8. **Chen, C. Y., and S. A. Morse.** 2001. Identification and characterization of a high-affinity zinc uptake system in *Neisseria gonorrhoeae*. FEMS Microbiol. Lett. **202:**67–71.
9. **Claverys, J. P.** 2001. A new family of high-affinity ABC manganese and zinc permeases. Res. Microbiol. **152:**231–243.
10. **Del Vecchio, V. G., V. Kapatral, R. J. Redkar, G. Patra, C. Mujer, T. Los, N. Ivanova, I. Anderson, A. Bhattacharyya, A. Lykidis, G. Reznik, L. Jablonski, N. Larsen, M. D'Souza, A. Bernal, M. Mazur, E. Goltsman, E. Selkov, P. H. Elzer, S. Hagius, D. O'Callaghan, J. J. Letesson, R. Haselkorn, N. Kyrpides, and R. Overbeek.** 2002. The genome sequence of the facultative intracellular pathogen *Brucella melitensis*. Proc. Natl. Acad. Sci. USA **99:**443–448.
11. **den Hartigh, A. B., Y. H. Sun, D. Sondervan, N. Heuvelmans, M. O. Reinders, T. A. Ficht, and R. M. Tsolis.** 2004. Differential requirements for VirB1 and VirB2 during *Brucella abortus* infection. Infect. Immun. **72:**5143–5149.
12. **Ferguson, G. P., A. Datta, J. Baumgartner, R. M. Roop, Jr., R. W. Carlson, and G. C. Walker.** 2004. Similarity to peroxisomal-membrane protein family reveals that *Sinorhizobium* and *Brucella* BacA affect lipid-A fatty acids. Proc. Natl. Acad. Sci. USA **101:**5012–5017.
13. **Finlay, B. B., and S. Falkow.** 1997. Common themes in microbial pathogenicity revisited. Microbiol. Mol. Biol. Rev. **61:**136–169.
14. **Foulongne, V., K. Walravens, G. Bourg, M. L. Boschiroli, J. Godfroid, M. Ramuz, and D. O'Callaghan.** 2001. Aromatic compound-dependent *Brucella suis* is attenuated in both cultured cells and mouse models. Infect. Immun. **69:**547–550.
15. **Garrido, M. E., M. Bosch, R. Medina, M. Llagostera, A. M. Pérez de Rozas, I. Badiola, and J. Barbé.** 2003. The high-affinity zinc-uptake system *znuACB* is under control of the iron-uptake regulator (fur) gene in the animal pathogen *Pasteurella multocida*. FEMS Microbiol. Lett. **221:**31–37.
16. **Gee, J. M., M. W. Valderas, M. E. Kovach, V. K. Grippe, G. T. Robertson, W. L. Ng, J. M. Richardson, M. E. Winkler, and R. M. Roop, Jr.** 2005. The *Brucella abortus* Cu,Zn superoxide dismutase is required for optimal resistance to oxidative killing by murine macrophages and wild-type virulence in experimentally infected mice. Infect. Immun. **73:**2873–2880.
17. **Grinberg, O. Y., P. E. James, and H. M. Swatz.** 1998. Are there significant gradients of pO_2 in cells? Adv. Exp. Med. Biol. **454:**415–423.
18. **Harmon, B. G., L. G. Adams, and M. Frey.** 1988. Survival of rough and smooth strains of *Brucella abortus* in bovine mammary gland macrophages. Am. J. Vet. Res. **49:**1092–1097.
19. **Higgins, C. F.** 1992. ABC transporters: from microorganisms to man. Annu. Rev. Cell Biol. **8:**67–113.
20. **James, P. E., O. Y. Grinberg, and H. M. Swartz.** 1988. Superoxide production by phagocytosing macrophages in relation to the intracellular distribution of oxygen. J. Leukoc. Biol. **64:**78–84.
21. **Jiménez de Bagues, M. P., P. H. Elzer, S. M. Jones, J. M. Blasco, F. M. Enright, G. G. Schurig, and A. J. Winter.** 1994. Vaccination with *Brucella abortus* rough mutant RB51 protects BALB/c mice against virulent strains of *Brucella abortus*, *Brucella melitensis*, and *Brucella ovis*. Infect. Immun. **62:**4990–4996.
22. **Kim, S., K. Watanabe, T. Shirahata, and M. Watarai.** 2004. Zinc uptake system (*znuA* locus) of *Brucella abortus* is essential for intracellular survival and virulence in mice. J. Vet. Med. Sci. **66:**1059–1063.
23. **Ko, J., and G. A. Splitter.** 2000. Residual virulence of *Brucella abortus* in the absence of the cytochrome bc(1) complex in a murine model in vitro and in vivo. Microb. Pathog. **29:**191–200.
24. **Kohler, S., V. Foulongne, S. Ouahrani-Bettache, J. Bourg, G., Teyssier, M. Ramuz, and J. P. Liautard.** 2002. The analysis of the intramacrophagic virulome of *Brucella suis* deciphers the environment encountered by the pathogen inside the macrophage host cell. Proc. Natl. Acad. Sci. USA **99:**15711–15716.
25. **Kovach, M. E., P. H. Elzer, D. S. Hill, G. T. Robertson, M. A. Farris, R. M. Roop, Jr., and K. M. Peterson.** 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene **166:**175–176.
26. **LeVier, K., R. W. Phillips, V. K. Grippe, R. M. Roop, Jr., and G. C. Walker.** 2000. Similar requirements of a plant symbiont and a mammalian pathogen for prolonged intracellular survival. Science **287:**2492–2493.
27. **Lewis, D. A., J. Klesney-Tait, S. R. Lumbley, C. K. Ward, J. L. Latimer, C. A. Ison, and E. J. Hansen.** 1999. Identification of the *znuA*-encoded periplasmic zinc transport protein of *Haemophilus ducreyi*. Infect. Immun. **67:**5060–5068.
28. **Liautard, J. P., A. Gross, J. Dornald, and S. Kohler.** 1996. Interactions between professional phagocytes and *Brucella* spp. Microbiologia **12:**197–206.
29. **Oñate, A., S. Céspedes, A. Cabrera, R. Rivers, A. González, C. Muñoz, H. Folch, and E. Andrews.** 2003. A DNA encoding Cu,Zn superoxide dismutase of *Brucella abortus* induces protective immunity in BALB/c mice. Infect. Immun. **71:**4857–4861.
30. **Pascual, D. W., T. Trunkle, and J. Sura.** 2002. Fimbriated *Salmonella enterica* serovar Typhimurium abates the initial inflammatory responses by macrophages. Infect. Immun. **70:**4273–4281.
31. **Patzer, S. I., and K. Hantke.** 1998. The *znuABC* high-affinity zinc uptake system and its regulator Zur in *Escherichia coli*. Mol. Microbiol. **28:**1199–1210.
32. **Ratledge, C., and L. G. Dover.** 2000. Iron metabolism in pathogenic bacteria. Annu. Rev. Microbiol. **54:**881–941.
33. **Rosinha, G. M., D. A. Freitas, A. Miyoshi, V. Azevedo, E. Campos, S. L. Cravero, O. Rossetti, G. Splitter, and S. C. Oliveira.** 2002. Identification and characterization of a *Brucella abortus* ATP-binding cassette transporter homolog to *Rhizobium meliloti* ExsA and its role in virulence and protection in mice. Infect. Immun. **70:**5036–5044.
34. **Smith, L. D., and T. A. Ficht.** 1990. Pathogenesis of *Brucella*. Crit. Rev. Microbiol. **17:**209–230.
35. **Stevens, M. G., S. C. Olsen, G. W. Pugh, Jr., and M. V. Palmer.** 1994. Immune and pathologic responses in mice infected with *Brucella abortus* 19, RB51, or 2308. Infect. Immun. **62:**3206–3212.
36. **Ugalde, J. E., D. J. Comerci, M. S. Leguizamon, and R. A. Ugalde.** 2003. Evaluation of *Brucella abortus* phosphoglucomutase (pgm) mutant as a new live rough-phenotype vaccine. Infect. Immun. **71:**6264–6269.
37. **Vemulapalli, R., Y. He, L. S. Buccolo, S. M. Boyle, N. Sriranganathan, and G. G. Schurig.** 2000. Complementation of *Brucella abortus* RB51 with a functional *wboA* gene results in O-antigen syntheses and enhanced vaccine efficacy but no change in rough phenotype and attenuation. Infect. Immun. **68:**3927–3932.
38. **White, P. G., and J. B. Wilson.** 1951. Differentiation of smooth from nonsmooth colonies of brucellae. J. Bacteriol. **61:**239–240.

Editor: V. J. DiRita