FULL PAPER *Bacteriology*

Zinc Uptake System (*znuA* Locus) of *Brucella abortus* is Essential for Intracellular Survival and Virulence in Mice

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(Received 20 February 2004/Accepted 15 April 2004)

ABSTRACT. *Brucella* spp. are facultative intracellular pathogens that have the ability to survive and multiply in professional and nonprofessional phagocytes, and cause abortion in domestic animals and undulant fever in humans. The mechanism and factors of virulence are not fully understood. High-affinity zinc uptake system protein mutant (*znuA* mutant) showed reduced growth in zinc chelated medium, and failed to replicate in HeLa cells and mouse bone marrow-derived macrophages. Transformation of *znuA* mutant with a shuttle vector pBBR1MCS-4 containing *znuA* gene restored the growth in zinc chelated medium and intracellular replication in HeLa cells and macrophages to a level comparable to that of wild-type strain. Bacterial internalization into HeLa cells and macrophages and co-localization with either late endosomes or lysosomes of *znuA* mutant were not different from those of wild-type strain. These results suggest that *znuA* does not contribute to intracellular trafficking of *B. abortus*, but contributes to utilization of zinc required for intracellular growth.

KEY WORDS: *Brucella abortus*, intracellular growth, zinc, *znuA*.

J. Vet. Med. Sci. 66(9): 1059–1063, 2004

Brucella abortus, a facultative, gram-negative, intracellular pathogen, is the etiologic agent of brucellosis, a widely distributed zoonosis [22]. The establishment of chronic infection depends on the ability of brucellae to survive within phagocytes [9]. In contrast to other intracellular pathogens, *Brucella* species do not produce exotoxins, antiphagocytic capsules or thick cell walls, resistance forms or fimbriae and do not show antigenic variation [7]. Intracellular survival and replication are key virulence features of *Brucella* because mutants defective for these attributes are also avirulent [10, 14]. A key aspect of the virulence of *Brucella* successfully bypasses the bactericidal effects of phagocytes, and their virulence and chronic infections are thought to be due to their ability to avoid the killing mechanisms within host cells [5, 20].

The genetic basis of *Brucella* virulence is still poorly understood. The VirB type IV secretion system of *Brucella* has been identified recently [16]. This operon is composed of 13 open reading frames (ORFs) that share homology with other bacterial type IV secretion systems in the intracellular trafficking of pathogens. Deletion or polar and non-polar mutations of these ORFs were not able to replicate and survive within phagocytes [19, 21], and internalization and uptake pathway in phagosome trafficking between wild-type and *virB* mutant showed different patterns [11]. Thus, the VirB proteins of *B. abortus* are thought to be constituents of the secretion apparatus. The genus *Brucella* does not contain plasmids naturally, and it is therefore possible that the proteins encoded by *virB* genes are involved in protein secretion rather than conjugation. A possible role in virulence is to inject effector molecules, which help in the establishment of replication niche into the host cell.

Zinc plays an important role in living organisms for the functioning of many enzymes and structural proteins. In *Eschrerichia coli*, two systems, a high-affinity system and a low-affinity system, have been described for zinc uptake [2, 17], and the high-affinity zinc uptake system of *Salmonella enterica* serovar Typhimurium is important for virulence in mouse model [3]. However, *B. abortus* does not contain low-affinity zinc uptake system [6] and high-affinity zinc uptake system is still not understood.

A preliminary characterization of *B. abortus znuA* (high-affinity zinc uptake system protein) mutant showed that it had reduced intracellular growth within HeLa cells [10], thus suggesting that this zinc uptake system protein might be a virulence-associated factor. In this study, we examined the growth of *znuA* mutant in zinc chelated medium and their intracellular replication in HeLa cells and macrophages. The intracellular trafficking and virulence of *znuA* mutant were also evaluated in mice.

METERIALS AND METHODS

Bacterial strains and media: All *B. abortus* derivatives were from 544 (ATCC23448), smooth virulent *B. abortus* biovar 1 strain. Isogenic mutants of *B. abortus* 544 *znuA*::Tn5Km2 and *ΔvirB4* have been described previously [10, 20]. *B. abortus* strains were maintained as frozen glycerol stocks and were cultured in *Brucella* broth (Becton Dickinson, Sparks, MD) or *Brucella* broth containing 1.5% agar. Kanamycin (30 μg/ml) and ampicillin (100 μg/ml) were used, when necessary.

pSK*znuA* (*znuA*$^+$) was constructed by cloning a PCR fragment into *Kpn*I/*Sac*I-cleaved pBBR1MCS-4 [12]. The

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1496 bp *Kpn*I-*Sac*I PCR fragment spanned a site located 25 bp upstream of the 5' end of *znuA* to a position 450 bp downstream from the 3' end [6] and amplified using primers 5'-GGTACCCCGGCTTGAGAATATGCAGCG-3' (*Kpn*I site underlined) and 5'-GAGCTCCGTTGCCTGCCACGGAAAGGG-3' (*Sac*I site underlined).

Cell culture: HeLa cells were grown at 37°C in a 5% CO_2 atmosphere in Eagle's minimum essential medium (MEM) (Sigma, St. Louis, MO) containing 10% fetal bovine serum (FBS). Bone marrow-derived macrophages from female BALB/c mice were prepared by the method described previously [10]. After culturing in L-cell conditioned medium, the macrophages were replated for use by lifting cells in phosphate-buffered saline (PBS) on ice for 5 to 10 min, harvesting by centrifugation and resuspending in RPMI 1640 (Sigma) containing 10% FBS. The HeLa cells or macrophages were seeded ($2–3 \times 10^5$ per well) in 24-well tissue culture plates one day before infection for all assays.

Comparative growth of the mutants in minimal medium: *In vitro* growth of the *znuA* mutant in Luria-Bertani (LB) broth was performed as the modified method described previously [3]. Briefly, $2–3 \times 10^{10}$ colony forming units (CFU) of wild-type *B. abortus*, *znuA* mutant and complemented strain were pelleted at 8,000 rpm for 15 min, washed 3 times with PBS and resuspended to $2–3 \times 10^7$ CFU/m*l* in LB broth alone, LB broth with zinc-chelating agent (1.5 mM EDTA) or LB broth with 1.5 mM EDTA and 1 mM $ZnCl_2$, and then were incubated at 37°C for 0, 2, 6 , 24 and 48 hr with shaking. To measure the CFU of brucellae, equal portions were withdrawn from each culture, serially diluted into PBS, and spread on the surface of *Brucella* plates to determine the number of viable cells at intervals up to 48 hr.

Determination of efficiency of intracellular growth by cultured HeLa cells and macrophages: Bacterial infection and intracellular survival assay were done by using the modified method described previously [10]. Briefly, *B. abortus* strains were deposited onto HeLa cells or mouse bone marrow-derived macrophages grown on 24-well microtiter plates filled with MEM or RPMI 1640 with 10% FBS at a multiplicity of infection (MOI) of 20 by centrifugation at $150 \times$ g for 10 min at room temperature, were then incubated at 37°C in a 5% CO_2 for 30 min. Both types of cells were washed twice with 0.5 m*l* of sterile PBS and incubated with MEM or RPMI 1640 with gentamicin (30 μg/m*l*). At different time points, cells were washed three times with PBS and were lysed with distilled water. CFU were measured by serial dilutions on *Brucella* plates.

LAMP-1 staining: LAMP-1 staining was performed by using the method described previously [15]. Briefly, infected macrophages were fixed in 4% periodate-lysine-paraformaldehyde (PLP)-sucrose for 1 hr at 37°C. All antibody-probing steps were for 1 h at 37°C. Samples were washed three times in PBS for 5 min and then were permeabilized at –20°C in methanol for 10 s. After incubating three times for 5 min with a blocking buffer (2% goat serum in PBS), the samples were stained with anti-LAMP-1 rat monoclonal antibody 1D4B diluted 1:100 in blocking buffer. After washing three times for 5 min in blocking buffer, the samples were stained with Texas red-goat anti-rat IgG. Then the samples were stained with anti-*B. abortus* polyclonal rabbit serum and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG in blocking buffer to identify the bacteria, and then they were placed in mounting medium and were visualized by fluorescence microscopy. One hundred bacteria within macrophages were selected randomly and the LAMP-1 acquisition of bacteria was counted.

Virulence in mice: The virulence was determined by quantifying survival of the strains in spleen after ten days. Six-week-old female BALB/c mice were infected intraperitoneally with approximately 10^4 CFU of brucellae in 0.1 m*l* saline. Groups of five mice were infected with each strain. At 10 days post-infection, the mice were sacrificed by decapitation and their spleens were removed, weighed and homogenized in saline. Tissue homogenates were serially diluted with PBS and were plated on *Brucella* agar to count the number of CFU in each spleen.

RESULTS

Effect of disruption of the high-affinity zinc uptake system in vitro and in vivo: As *B. abortus* cannot grow in poor medium such as M9 minimal medium, bacterial growth was analyzed by LB broth in the presence or absence of EDTA. Wild-type *Brucella*, *znuA* mutant and complemented strain showed almost the same growth yield in LB broth, LB broth in 1.5 mM EDTA and 1.0 mM $ZnCl_2$ (Fig. 1A and C). In LB broth with 1.5 mM EDTA, *znuA* mutant grew until 24 hr but the bacterial numbers decreased after that as compared to that of wild-type strain (Fig. 1B). This was probably due to the fact that *znuA* mutant enables to achieve small amount of zinc in zinc-chelated LB broth for bacterial survival [3], but after using these elements completely, may show decreasing growth pattern (Fig. 1B).

As survival and multiplication in professional and non-professional phagocytic host cells is an important virulence of *Brucella*, we examined the intracellular replication of *Brucella* strains in HeLa cells and mouse bone marrow-derived macrophages. The results showed that *znuA* mutant failed to replicate in HeLa cells and macrophages (Fig. 2A and B). It is likely that different amounts of zinc uptake from media of macrophages and HeLa cells might be different which affect intracellular growth of *znuA* mutant in both cells.

Phagosomes containing virulent *B. abortus* are reluctant to fuse with lysosomes, whereas dead *B. abortus* phagosomes co-localize with endocytic compartments in the early stage of infection in macrophages [1]. To test the ability of *B. abortus* to target properly within macrophages early in infection, interaction of the mutants with the endocytic pathway was quantified by immunofluorescence localization of LAMP-1, a membrane protein of late endosomes and lysosomes [4]. Most phagosomes containing wild-type strain did not co-localize with the LAMP-1 (18.6 ± 4.2% positive). In

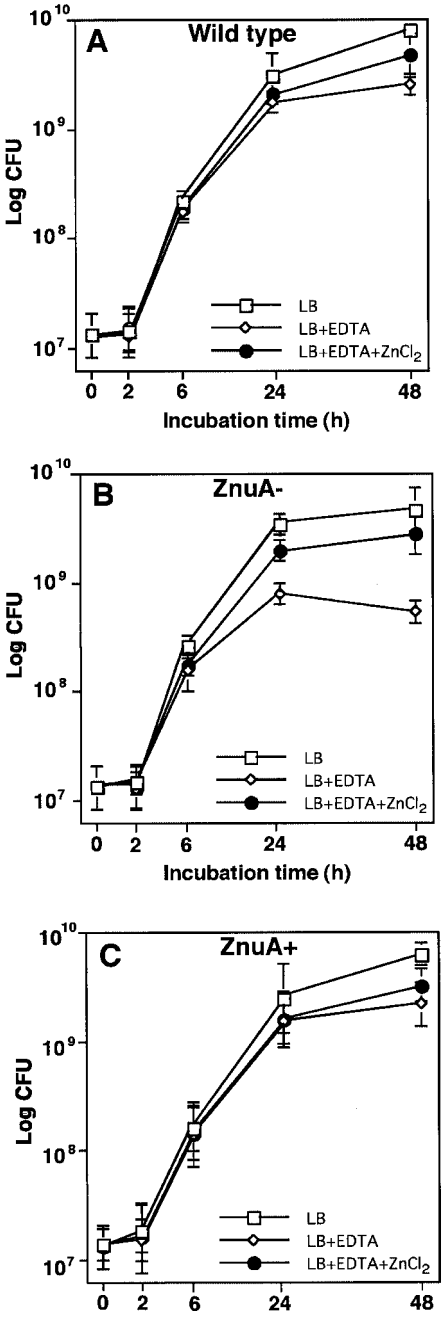

Fig. 1. Comparative growth of *Brucella* wild-type and *znuA* mutants in the presence and absence of the chelating agent EDTA. Wild-type (A), *znuA* mutant (*znuA*-) (B), and complemented strain (*znuA*+) (C) were grown on LB broth, LB broth in the presence of EDTA with or without $ZnCl_2$ as described in Materials and Methods. Concentrations of EDTA and $ZnCl_2$ used were 1.5 and 1.0 mM, respectively. The numbers of viable bacteria at each time point were determined by making serial dilutions in PBS and plating on *Brucella* plates. Data points and error bars represent the mean CFU of triplicate samples from a typical experiment (performed at least four times) and their standard deviation.

contrast, phagosomes containing *virB4* mutant with severe intracellular growth defects were frequently stained brightly by an antibody specific for LAMP-1 (78.6 ± 5.3% positive) [20]. Most phagosomes containing *znuA* mutant or complemented strain resided in a LAMP-1-positive compartment (27.3 ± 4.8% or 31.2 ± 5.9% positive, respectively). It suggests that *znuA* does not contribute to intracellular trafficking of *B. abortus*, rather contributes to utilization of nutrients required for intracellular growth.

Virulence in mice: To assess whether the *znuA* mutant exhibits impaired internalization and intracellular replication within HeLa cells and macrophages and whether or not it correlates with an inability to establish infection in the host, we experimentally infected mice with wild-type *B. abortus* and *znuA* mutant. Many bacteria were recovered from the spleen of mice infected with the wild-type strain at ten days after infection (6.5×10^5 CFU/spleen), but fewer bacteria were recovered from mice infected with *znuA* mutant (6.6×10^2 CFU/spleen). Wild-type strain induced splenomegaly (430 ± 41 mg) as a consequence of inflammatory response, but *znuA* mutant induced a reduced response (130 ± 20 mg) whereas showed same induction in uninfected control (140 ± 35 mg).

DISCUSSION

Zinc is an essential trace element, probably for all organisms. A large number of enzymes and proteins with very diverse functions contain zinc as a structural or catalytic cofactor [16, 17]. Some zinc transport systems have been studied in prokaryotes such as *Enterobacteriaceae, Pseudomonadaceae, Neisseriaceae* and *Pasteurellaceae*, among others [3, 8, 16, 17]. *Escherichia coli* cells present two independent systems to zinc uptake encoding *znuABC* and *pitA* genes, respectively [2, 17]. *E. coli znuA* and *znuCB* operons (encoding a periplasmic-binding protein, an ATPase and an integral membrane protein, respectively) are divergently oriented, and their transcriptional starting points are separated by 24 bp [17]. The genomes of *B. melitensis* and *B. suis* contain similar *znuACB* systems of *E. coli* [6] and *B. abortus* also contain the *znuACB* system [10].

Few data exist about the importance of the zinc-uptake system in bacterial virulence. In fact, it has only been reported that *H. ducreyi znuA*, *P. multocida* and *S. typhimurium znuC* mutants are less virulent than their wild-type strains [3, 8, 14]. A preliminary characterization of *B. abortus znuA* mutant showed that it had reduced intracellular growth within HeLa cells [10], therefore, we constructed *znuA* complemented strain and analyzed their characterizations. No relevant differences in bacterial growth *in vitro* and intracellular replication rates *in vivo* were detected for the wild-type and complemented strain, whereas *znuA* mutant showed a different characterization. *Brucella* strains cultured in LB broth containing about 10 μM zinc [3], showed the same growth yield. Presence of the chelating agent EDTA dramatically decreased the growth of the *znuA* mutant, whereas wild-type and complemented strains were

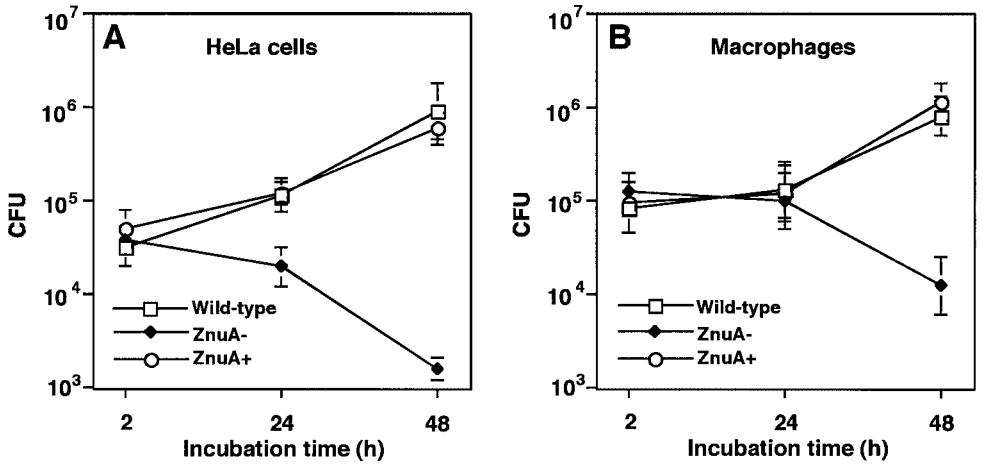

Fig. 2. Intracellular replication of wild-type and *znuA* mutants within HeLa cells and mouse bone marrow-derived macrophages. HeLa cells (A), and macrophages (B) were infected with wild-type *B. abortus*, *znuA* mutant (*znuA*-) and complemented strain (*znuA*+) as described in Materials and Methods. At different times of incubation, the cells were lysed, and the numbers of viable intracellular bacteria were determined. Data points and error bars represent the mean CFU of triplicate samples from a typical experiment (performed at least four times) and their standard deviation.

practically not affected. Addition of $ZnCl_2$ (1.0 mM) restored the wild-type growth yield of *znuA* mutant in the presence of EDTA, indicating that this growth defect was zinc specific. Furthermore, wild-type and complemented strains showed the same intracellular replication, whereas *znuA* mutant failed to replicate in HeLa cells and macrophages.

No relevant differences, however, in bacterial internalization and phagosome-lysosome fusion after uptake *in vivo* were detected for all strains, indicating that zinc uptake systems may have important roles for virulence, contribute to utilization of nutrients required for intracellular growth but may not affect bacterial internalization or intracellular trafficking of *B. abortus*.

In conclusion, data reported in this work concerning virulence of the *Brucella abortus znuA* mutant indicate that the zinc uptake *znuACB* system may have important roles by zinc uptake during host infection and give support to the protective role that zinc-chelating systems of mammalian organisms may play against bacterial infection [17].

ACKNOWLEDGEMENTS. We wish to thank Drs. A. R. Ghosh and Neeraj Rana for critical reading of the manuscript. This study was supported, in part, by a grant from The 21st Century COE Program (A-1), Ministry of Education, Culture, Sports, Science, and grant from Inamori Foundation.

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