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Brucella abortus deficient in copper/zinc superoxide dismutase is virulent in BALB/c mice

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The gene encoding the Cu/Zn superoxide dismutase (SOD) of *Brucella abortus* strain 2308 was identified in a *Brucella* genomic library utilizing a combination of Western blotting and native gel electrophoresis. The Cu/Zn SOD gene was inactivated *in vitro* by ligation of a kanamycin resistance gene into the open reading frame encoding SOD. The plasmid born construct was introduced back into *B. abortus* by electroporation. Replacement of the wild-type Cu/Zn SOD by recombination was demonstrated by showing that both the KnR gene and the Cu/Zn SOD gene hybridized to the same band in a Southern analysis of genomic DNA. In addition, KnR strains were deficient in Cu/Zn SOD activity as assessed by lack of Cu/Zn SOD activity on a native gel and by lack of reactivity with specific serum in a Western analysis. Either strain 2308 or the Cu/Zn SOD deficient mutant injected intraperitoneally into BALB/c mice, exhibited no differences in their ability to colonize the spleen at 7 and 28 days post-inoculation. Thus, the inability to produce Cu/Zn SOD by *B. abortus* does not significantly impair its virulence in mice.

Key words: *Brucella abortus*; copper/zinc superoxide dismutase mutant.

Introduction

Brucella abortus is a facultative intracellular pathogen able to invade and replicate within macrophages. It will induce a chronic infection which can result in abortion and infertility in animals, as well as undulant fever in humans.[1] Several mechanisms have been postulated to explain the survival of *B. abortus* within macrophages, including blockage of phagosome–lysosome fusion through the production of guanosine monophosphate and adenine as observed in neutrophils.[2] The presence of two types of superoxide dismutases (SOD) has been demonstrated in *B. abortus.*[3–5] *Brucella abortus* produces a copper/zinc SOD and a second type of SOD, presumably a manganese SOD based on its insensitivity to KCN.[5] Since SOD activities are high in *B. abortus,*[5] they can protect against oxygen toxicity by catalysing the dismutation of oxygen radicals.[6] The presence of SOD has been demonstrated to be associated with

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virulence in a number of bacterial species.[7,8] Thus, it is attractive to postulate that the presence of SOD in *B. abortus* is important for the pathogen to survive within macrophages which produce free oxygen radicals. Recent information on the presence of Cu/Zn SOD in *B. abortus* strains of relatively low virulence[5,9] would indicate that Cu/Zn SOD by itself is not a major virulence factor. In order to clarify the role of Cu/Zn SOD in virulence, we produced a Cu/Zn SOD deficient *B. abortus* strain 2308 and tested its virulence in BALB/c mice.

Results

Cloning of the Cu/Zn SOD gene

Utilizing goat hyperimmune serum against *B. abortus*[9] to screen the genomic library cloned as *Sau*3A fragments in pUC9 and expressed in *Escherichia coli*, several positive colonies were identified. Fifteen of these colonies were further analysed by native gel electrophoresis for specific SOD activity. One particular *E. coli* clone was shown to have a plasmid, designated pBA23, containing a 3.2 kb *Brucella* insert and producing Cu/Zn SOD activity. Furthermore, this plasmid complemented *E. coli sodA, sodB*[10] and produced a 20 kDa protein which reacted specifically with antiserum to Cu/Zn SOD (data not shown).

Production of a Cu/Zn SOD mutant

Based on the nucleotide sequence of the Cu/Zn SOD gene (personal communication, J. Mayfield, Iowa State University), the location of the Cu/Zn SOD gene was determined on the plasmid pBA23 (Fig. 1) by restriction mapping. A kanamycin resistance gene was ligated into the unique *Sma*I site (Fig. 2) within the Cu/Zn SOD

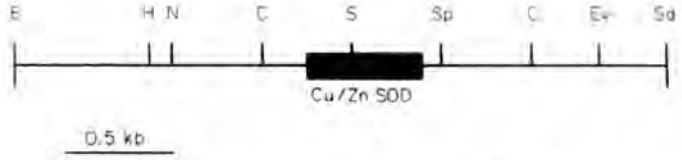

Fig. 1. pBA23 containing the *B. abortus* Cu/Zn SOD gene.

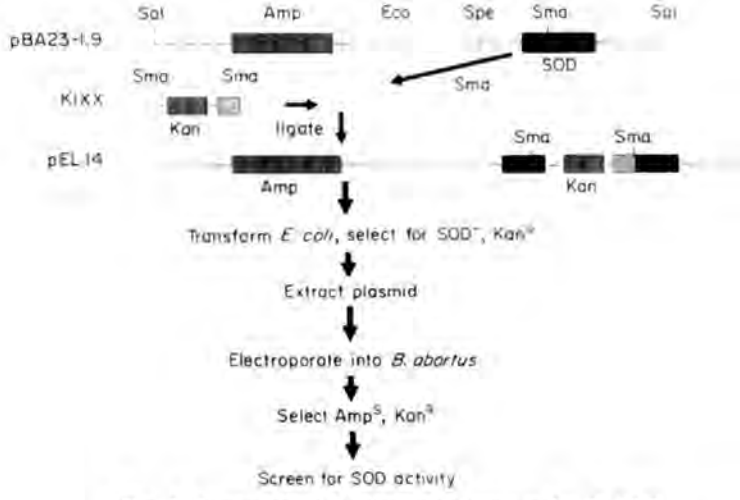

Fig. 2. Production of *B. abortus* deficient in Cu/Zn SOD.

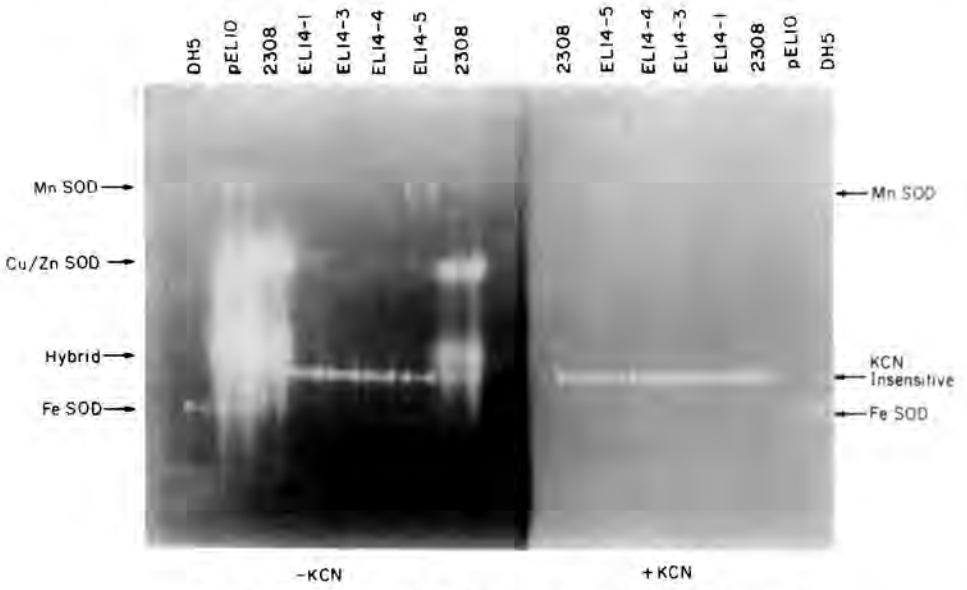

Fig. 3. SOD activities of *B. abortus* mutants and *E. coli* extracts.

gene to disrupt Cu/Zn SOD expression. *Escherichia coli* transformed with pEL14 did not produce Cu/Zn SOD activity or antigenic SOD (data not shown). pEL14 was electroporated into *B. abortus* and the cells were plated on TSB-kanamycin. Colonies which were kanamycin-resistant were patched to TSB-ampicillin to check for plasmid replication (KanR, AmpR) or recombination between the inactivated Cu/Zn SOD gene on the plasmid and the normal Cu/Zn SOD on the genome (KanR, AmpS). Ten *B. abortus* colonies which were selected for on TSB/Kan media were also ampicillin-sensitive (AmpS). Four of these KanR and AmpS clones were further tested and found to be deficient in Cu/Zn SOD activity (Fig. 3, Table 1) but still possessed the KCN insensitive form of SOD as expected.

To further confirm that the Cu/Zn SOD deficient clones were the result of an allelic exchange (i.e. a double reciprocal crossover between the disrupted Cu/Zn SOD gene on the plasmid and the Cu/Zn SOD on the genome), a Southern analysis was performed utilizing probes for both KanR and Cu/Zn SOD (Fig. 4). Since both the KanR and Cu/Zn SOD probes are hybridizing to the same band of genomic DNA, it appears that all four *B. abortus* strains have had their normal Cu/Zn SOD gene replaced by the plasmid-born disrupted Cu/Zn SOD gene, via a double reciprocal crossover. We

Table 1 SOD activities in extracts of bacterial strains

	Units/mg[a]				
Strain	Total activity	KCN insensitive	(%)	KCN sensitive	(%)
2308	89.7	37.8	42.1	51.9	57.9
EL14-1	54.0	49.6	91.7	4.6	8.3
EL14-3	54.9	49.6	96.7	1.8	3.3
EL14-4	46.6	46.6	96.8	1.6	3.2
EL14-5	49.8	46.8	94.0	3.0	6.0
E. coli	66.7	69.6	100	0	0

[a] Unit = 50% inhibition of epinephrine auto-oxidation; activities represent the average of three separate determinations performed in duplicate.

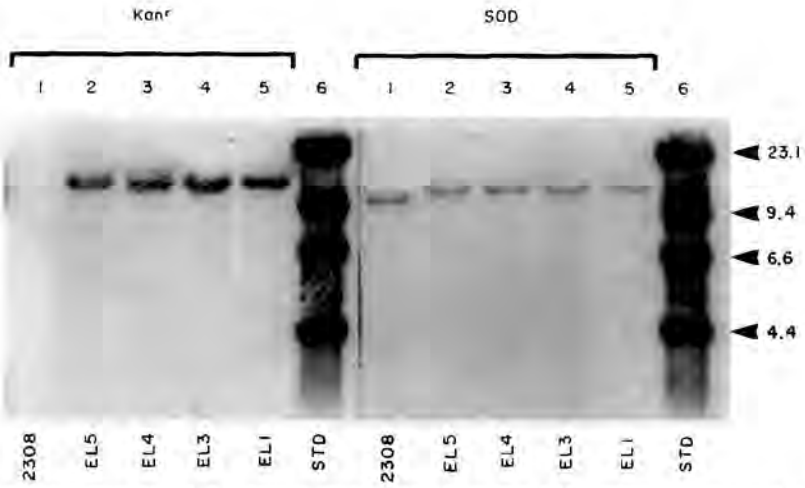

Fig. 4. Southern analysis of *B. abortus* strains. Chromosomal DNA extracted, digested with *Eco*RI, electrophoresed on a 0.7% agarose gel, transferred to nitrocellulose, and hybridized to either lambda DNA and Cu/Zn SOD or KanR probe. Lanes: (1) lambda DNA; *B. abortus* strains: (2) 3A5 (Tn5$^+$, SOD$^+$); (3) 2308 (wild-type); (4) EL5; (5) EL4; (6) EL3; (7) EL1 (EL clones are Tn5$^+$, SOD$^-$).

selected one of the *B. abortus* Cu/Zn SOD mutants, designated EL14-4, for further work. Although not shown here, we have found that following electroporation, some of our *B. abortus* clones which are KanR and AmpR are the result of a single cross-over recombination. This conclusion is based on demonstrating in a Southern analysis that an AmpR probe hybridizes to genomic fragments recognized by either Cu/Zn SOD or a KanR probe.

O-side chain of Cu/Zn SOD mutants

Monoclonal antibody, BRU-38 specific for the O-side chain in *B. abortus*,[11] reacted similarly with extracts of both *B. abortus* EL14-4 and control strain 2308, as observed by Western blot analysis (Fig. 5), indicating that both strains are smooth with similar or identical O-side chains. In addition, the staining ability of 100 colonies of either strain 2308 or EL14-4 with crystal violet[12] and their reactivity in a rapid identification test with monoclonal antibody BRU38,[13] indicated that the populations of these strains were 100% smooth. Analysis of both strains and *S. typhimurium* pYA 248 and pYA 23-3 by Western blot with rabbit anti-Cu/Zn Brucella SOD (Fig. 5) indicated that *B. abortus* EL-14-4 did not express any antigenically active Cu/Zn SOD.

Virulence study in BALB/c mice

Groups of BALB/c mice infected i.p. with either *B. abortus* 2308 or EL14-4 and killed at 7 and 28 days p.i., did not reveal significant differences in the level of splenic infections (Table 2). *Brucella* isolated from any of the groups had O-side chain as revealed by Western blot analysis of whole bacteria with monoclonal antibody BRU-38.[9]

Brucella isolated from the EL14-4 infected mice remained Cu/Zn SOD deficient as determined by Western blot analysis and enzyme activity assay. All the mice were serologically negative by STA and Western blot analysis before infection. At 7 days p.i., all mice had developed an STA titer which varied from 1:25 to 1:50; Western blot

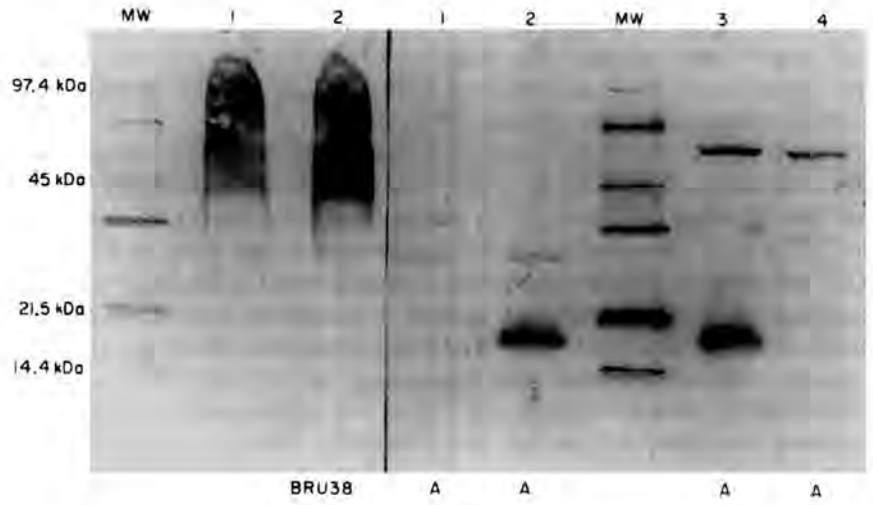

Fig. 5. Western blot analysis of monoclonal antibody BRU38 and rabbit anti-Cu/Zn SOD antiserum reacted with whole cell extracts of *B. abortus* and *S. typhimurium* 4072 strains. Lanes: (1) *B. abortus* EL14-4 (SOD^-); (2) *B. abortus* 2308 (SOD^+); (3) *S. typhimurium* pYA 23-3 (SOD^+); (4) *S. typhimurium* pYA 248 (SOD^-). Antiserum: A = polyclonal rabbit anti-Cu/Zn SOD; BRU38 = monoclonal anti-O side chain. MW = molecular standards in kilodaltons.

Table 2 Quantitation of *B. abortus* from spleens of infected mice

Group	Strain	Days p.i.	Mean (log_{10}/spleen ± SD)	
I	2308	7	5.91	0.19
II	EL14-4	7	5.45	0.34
III	2308	28	5.26	0.41
IV	EL14-4	28	5.34	0.11

analysis was not performed on these sera. At 28 days post-infection, all mice had developed titers of 1:200 to 1:400. The titers were randomly distributed among the groups and Western blot analyses of all sera indicated a good response to the O-side chain by both strain 2308 and EL14-4 infected mice. No antibodies against the Cu/Zn SOD were detected in any of the mice when their sera were reacted with *B. abortus* strains 2308, EL14-4, RB51 and *S. typhimurium* pYA 23-3 (data not shown).

Discussion

The O-side chain of *B. abortus* is considered to be a major virulence factor, since rough strains are less virulent *in vivo*.[9,14–16] Analysis of the virulence of rough and transposon mutant strains of *B. abortus* in bovine mammary gland macrophages indicate that although smoothness is probably the most important virulence factor, other undefined factors also contribute to virulence.[17,18] Therefore, if one is analysing the contribution of an SOD to virulence, one should insure that comparisons are carried out among strains of similar or identical 'O' side chain characteristics. Both *B. abortus* strains, 2308 (Cu/Zn SOD^+) and EL14-4 (Cu/Zn SOD^-) used in this study, did not stain with crystal violet. Also, rapid identification test[13] and Western blot analysis[9] with monoclonal anti-O-side chain antibody BRU38,[18] did not reveal any differences, indicating that both strains were clearly smooth and probably identical in their O-side chain

composition. Also, post-immunization serum obtained from mice infected with either of the two strains demonstrated the presence of similar levels of anti-O-side chain antibodies (detected by SAT and Western blot analysis) indicating that both strains were clearly expressing antigenic O-side chain.

Mice infected with either of the two strains demonstrated similar levels of *B. abortus* spleen colonization at 7 and 28 days p.i., indicating that virulence *in vivo* was not altered by the absence of Cu/Zn SOD. All isolates from the EL14-4 infected mice remained Cu/Zn SOD^- demonstrating that survival in the host was not due to reversion to an SOD^+ phenotype. This data strongly suggests that Cu/Zn SOD is not a crucial virulence factor for *B. abortus* smooth strains. It is possible that in the presence of a major virulence factor like the O-side chain, the virulence role of the Cu/Zn SOD is overshadowed and not detected. The production of a Cu/Zn SOD^- mutant of *B. abortus* strain RB51, which is rough and highly attenuated and also produces Cu/Zn SOD,[5] appears necessary to resolve this possibility. Such a rough mutant would eliminate the virulence contribution of the O-side chain and allow detection of virulence effects due to the production of Cu/Zn SOD. Experiments designed to construct and test such an RB51 Cu/Zn SOD mutant are in progress. The experiments described in this study only address the question of the role of Cu/Zn SOD in virulence but do not resolve if the KCN insensitive SOD found in *B. abortus*[5] plays a crucial role as a virulence factor. It is possible that the quantity of KCN insensitive SOD produced after the elimination of the Cu/Zn SOD is still sufficient to neutralize oxygen toxicity within the macrophages, or that *B. abortus* will compensate the loss of Cu/Zn SOD production by increasing the output of the KCN insensitive SOD. In Table 1, the Cu/Zn SOD mutants exhibit approximately 30% greater KCN insensitive SOD activity than wild-type *Brucella*. The construction of a double SOD mutant would allow the question of the virulence contribution of the KCN insensitive SOD to be addressed.

We were unable to detect antibodies against the *Brucella* Cu/Zn SOD in either of the infected groups of mice. Tabatabai *et al.*[19] have reported the presence of such antibodies in some cattle infected with *B. abortus*. It is possible that the length of the mouse experiment reported here was not sufficient to allow for the production of these antibodies. Since there is some homology between the *B. abortus* Cu/Zn SOD and eukaryotic host Cu/Zn SOD,[20] it is possible that a detectable humoral immune response to a partially homologous antigen will take longer than the response to a foreign antigen.

Materials and methods

Strains and Plasmids. See Table 3.

Cloning of Cu/Zn SOD. B. abortus 2308 genomic DNA was partially digested with *Sau*3A fragments in the 1–22 kb range were ligated into *Bam*H1-digested pUC9. *E. coli* DH5α were transformed with this mixture and plated onto Luria-Bertani agar (LB) plates with ampicillin (100 μg/ml) and Bluogal (Bethesda Research Laboratories). The resulting colonies were screened for antigenic reactivity by blotting them onto nitrocellulose, lysing with chloroform, and reacting them with goat anti-*B. abortus* RB51 hyperimmune serum;[9,13] the serum was adsorbed three times with whole *E. coli* DH5α and once with lysed *E. coli* DH5α to remove cross-reactive antibodies. The goat anti-RB51 serum detects a variety of *B. abortus* antigens including the Cu/Zn SOD. Colonies producing Cu/Zn SOD were selected from the goat anti-RB51 positive colonies by detection of the SOD activity on native gels.[5]

SOD native gels. Cell-free extracts were prepared by growing cells at 37°C in LB (*E. coli*) or Trypticase Soy Broth (TSB) (*B. abortus*) to mid-log phase, harvesting and washing twice with 0.1 M phosphate buffered saline (PBS). The cells were lysed either by sonication (2 min at

Table 3 Bacterial strains and plasmids

Strain/plasmid	Description	Source
Strains		
E. coli DH5α	F⁻ phi80, ΔlacΔM15, relA1, recA1, endA1, HsdR17, supE44, Thi-1, gyrA96	Bethesda Research Laboratories
B. abortus 2308	Wild type	G. G. Schurig
B. abortus 3A5	Tn5 induced rough, KanR	This study
B. abortus RB51	Rough strain, rifampin R	21
B. abortus EL14-4	KanR, Cu/Zn SOD⁻	This study
S. typhimurium 4072	pStSr100⁺ gyrA1816 Δcya-1 Δcrp-1 ΔasdA1 Δ[zhf-4::Tn10]	22
Plasmids		
pBA23	pUC9 with a 3 kb *Sau*3A fragment of *B. abortus* DNA; expresses Cu/Zn SOD	This study
pBA23-1.9	1.9 kb *Eco*RV–Hind III from pBA23 ligated into pUC18; expresses Cu/Zn SOD	This study
pUC4-KIXX	Source of KanR gene	Promega Corp.
pEL14-4	pBA23-1.9 with a KanR gene ligated into *Sma*I site of Cu/Zn SOD gene	This study
pYA248	3.0 kb plasmid with the *asd* gene of *Streptococcus mutans* and a MCS containing *Eco*R I	22
pYA23-3	A 3.0 kb fragment from pBA23 cloned into the *Eco*R I site of pYA248	This study

30% of maximum output on ice, Fisher sonicator) or with mechanical breakage (Mini Beadbeater, 0.1 mm glass beads, 2 min) in a final volume of 1.0 ml PBS. Protein concentrations were determined by the Bradford method.[23] Twenty μg of protein from each sample were loaded onto duplicate acrylamide gels (10% running and 3.5% stacking). The gels were run at 17 mA through the stacking gel and 25 mA through the running gel. One gel was soaked in 2 mM KCN for 15 min to inactivate Cu/Zn SOD activity and then both gels were stained with Nitro-Blue Tetrazolium (0.05 M phosphate, 0.1 mM EDTA, 0.02% NBT, 3.3×10^{-2} mM riboflavin, and 0.25% TEMED) for 15 min.[5] The gels were exposed to fluorescent light until the gel background was stained purple. The reaction was stopped with 7% acetic acid and photographs taken.

SOD activity. Cell-free extracts prepared using a Mini-bead beater were assayed for SOD using the epinephrine assay of Misra and Fridovich.[24] One unit of SOD activity is defined as the amount of SOD required to inhibit the rate of auto-oxidation of epinephrine by 50%. The amount of Cu/Zn SOD was determined by assaying in the presence of 2 mM KCN.[5]

Production of Cu/Zn SOD mutant of B. abortus. The 1.9 kb *Eco*RV–*Hin*dIII fragment of pBA23 was blunt ended and ligated into the *Sma*I site of pUC18 to produce pBA23-1.9 (Fig. 2). The kanamycin resistance gene of pUC4-KIXX was prepared by *Sma*I digestion and separated by electrophoresis in low melt agarose. The kanamycin resistance gene was ligated into the *Sma*I site of the Cu/Zn SOD gene in PBA23-1.9 to produce pEL14. The presence or absence of SOD activity produced by *E. coli* transformed with either pBA23-1.9 or pEL14 was verified by native gel electrophoresis. One μg of pEL14 was electroporated into *B. abortus* 2308 using the electroporation conditions outlined by Lai *et al.*[25]

Western blots. Extracts of the putative *Brucella* SOD mutants, *Salmonella* strains and *B. abortus* 2308 were prepared by pelleting cells, resuspending in 10 mM Tris–HCl, pH 8.0 to 10% T at OD_{525}, centrifuging 1 ml and resuspending in 200 μl 10 mM Tris and 400 μl 2×Laemmli sample buffer.[26] Samples were boiled for 5 min, centrifuged, and 15 μl of the supernatant electrophoresed in a 12.5% SDS–PAGE gel as described.[9] Following electrophoresis, the preparations were transferred to nitrocellulose membranes using the procedure of Towbin *et al.*[27] Transfer was carried out for 2 h with 125 V at 4°C in a Transphor unit (Hoefer Scientific Instruments). Molecular weight markers were stained with Ponceau S stain (0.5% Ponceau S, 1% glacial acetic acid). Blots were blocked with 2% BSA in TBS, incubated with primary antibody, washed in TBS-Tween 20, incubated with secondary antibody labeled with horseradish

peroxidase, washed in TBS-Tween 20, and developed in 0.06% 4-chloro-naphthol, 10% methanol, 0.6% hydrogen peroxide, in TBS as described.[9] To identify the presence of antigenically active SOD, the primary antibody used was a polyclonal rabbit anti-*B. abortus* Cu/Zn SOD serum kindly provided by L. Tabatabai (NADC, Ames, Iowa). In order to confirm the presence of the O-side chain on the putative SOD mutant, monoclonal antibody BRU38 specific for the O-side chain[11] was used as the primary antibody. Secondary antibodies were goat anti-rabbit IgG and rabbit anti-rat IgG respectively, obtained from Cappel Research Products (Organon Teknica, Durham, NC). Representative individual colonies were also analysed for their ability to express the O-side chain by the rapid identification test described by Roop *et al.*[13] using monoclonal antibody BRU38.

Southern blots. Genomic DNA was prepared with the CTAB method[28] except that 2-day growth of four 100×15 mm TSB plates were used instead of the liquid culture. DNA was digested overnight with *Eco*R1 and run on a 0.7% agarose gel at 50 V for several hours. The gels were blotted to Nytran (Schleicher and Schuell) by the method of Southern[29] and developed with a digoxigenin/anti-digoxigenin system (Genius kit, Boehringer-Mannheim). The SOD probe was made by digesting pBAII-3 (Fig. 1) with *Taq*I and purifying the 0.5 kb fragment on a Spin-Bind column (FMC Corporation). The pUC4-KIXX plasmid was used as a source of the kanamycin probe. Lambda digested with *Hin*d III were used as markers. All probes were labeled with digoxigenin.

Infection protocol. Four groups of five female BALB/c mice, each 6 weeks of age, were used. Two groups were inoculated intraperitoneally (i.p.) with 5×10^4 colony forming units (cfu) of *B. abortus* strain 2308 and two groups with 4.6×10^4 cfu of strain EL14-4. One group of mice inoculated with each strain was killed at 7 days post-inoculation (p.i.) and the remaining two groups were killed at 28 days p.i. Spleens were cultured for the presence of *B. abortus*[9] and cfu per spleen were determined. Statistical analysis of the number of cfu was performed using one way analysis of variance followed by Duncan's multiple range test.[28]

Serology. Mice were bled retro-orbitally before inoculation and at the time of death by cardiac puncture. All serum samples were tested in the standard tube agglutination (STA) test starting at a 1:25 dilution as described by Alton *et al.*[12] All pre-immunization and 28 day p.i. sera were tested by Western blot analysis using whole cells of both *B. abortus* strains 2308 and RB51 and *Yersinia enterocolitica* O:9 LPS as antigens. *Y. enterocolitica* O:9 LPS contains an O-antigen which is nearly identical to the A-antigen (O-side chain) of *B. abortus*;[9] positive reactions with this LPS indicate the presence of anti-Brucella O-side chain antibodies.

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