INFECTION AND IMMUNITY, Nov. 1996, p. 4838–4841 Vol. 64, No. 11
0019-9567/96/$04.00+0

The HtrA Stress Response Protease Contributes to Resistance of *Brucella abortus* to Killing by Murine Phagocytes

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Received 5 April 1996/Returned for modification 15 May 1996/Accepted 9 August 1996

Compared with virulent *Brucella abortus* 2308, the isogenic *htrA* mutant PHE1 shows decreased resistance to killing by cultured murine neutrophils and macrophages and significant attenuation during the early stages of infection in the BALB/c mouse model. These findings further define the contributions of the *htrA* gene product to the pathogenesis of *B. abortus* infections.

Brucella spp. are zoonotic bacterial pathogens which produce abortion and infertility in animals and a chronic debilitating disease in humans known as undulant fever (23). Prolonged survival and replication in host macrophages are essential for disease production by *Brucella* spp. (2, 6, 12). Since oxidative killing appears to be the primary means by which host neutrophils (25) and macrophages (16) can potentially eliminate brucellae, the mechanisms by which these successful intracellular pathogens resist reactive oxygen intermediate (ROI)-mediated killing by host phagocytes represent important virulence determinants. Biochemical and genetic studies indicate that bacterial stress response proteases of the high temperature requirement A (HtrA) family represent important components of cellular defense against oxidative killing (3, 4, 8, 9, 17, 20). Furthermore, recent studies in our laboratory have shown that both *Brucella abortus* (11, 26) and *Brucella melitensis* (24) *htrA* mutants demonstrate increased sensitivity to oxidative killing in vitro and significant attenuation at 1 week postinfection in BALB/c mice. The purpose of the study reported here was to better define a role for the HtrA protease in the pathogenesis of *Brucella* infections by examining the capacity of the *B. abortus htrA* mutant PHE1 (11) to resist killing by cultured murine neutrophils and macrophages and to produce chronic spleen infection in BALB/c mice.

To determine if the increased sensitivity of the *B. abortus htrA* mutant to oxidative killing observed in vitro (11) corresponds to a decreased resistance to killing by host phagocytes, the survival of strains PHE1 and 2308 in the presence of cultured murine neutrophils and macrophages was evaluated. Adaptations of the methods of Kreutzer et al. (19) and Morrison et al. (22) were used for bactericidal assays employing cultured murine neutrophils. Briefly, 1-ml portions of thioglycolate broth were injected into the peritoneal cavities of four 9-week-old BALB/c mice, and 4 h later, the mice were euthanized via halothane overdose. Cells were collected from the peritoneal cavities of these mice by lavage with 8-ml portions of Dulbecco's modified Eagle medium (DMEM) supplemented with 5% fetal calf serum (FCS) and 5 U of heparin per ml. Microscopic observation of these cells following treatment with Wright's Giemsa stain determined that the preparation contained 89% neutrophils. Pooled neutrophils were added to 96-well plates at a concentration of 2×10^5 cells in 40 μl of DMEM plus 5% FCS per well. Immediately following the addition of the cells, 160 μl of *B. abortus* (approximately 2×10^7 CFU/ml) opsonized with 10% normal BALB/c mouse serum (complement preserved) was added to each well, and the plates were incubated at 37°C with 5% CO_2. After 10-, 30-, 60-, and 120-min incubations, the neutrophils were lysed by the addition of 50 μl of 0.5% deoxycholate (0.1% final concentration per well), and the number of viable brucellae per well was determined by serial dilution and plating on Schaedler agar supplemented with 5% defibrinated bovine blood (SBA). Five replicate wells for each strain were evaluated at each time point. The results obtained were expressed as percent survival, determined by dividing the number of brucellae present at a particular sampling time by the number of brucellae added to the neutrophils at time zero and multiplying by 100. Statistical comparisons between experimental groups were performed by the one-tailed Student *t* test (29), and *P* values of <0.05 were considered significant.

The results of a representative bactericidal experiment employing cultured neutrophils are shown in Fig. 1. By 10 min postinfection, 47% of the PHE1 strain added to the neutrophils had been killed, and by 120 min postinfection, $<0.01\%$ of the bacterial inoculum could be recovered. In contrast, substantial killing of 2308 by cultured neutrophils was not observed until 30 min postinfection, and $>20\%$ of the inoculum was still viable after 120 min of exposure to cultured neutrophils. This experiment was repeated four times, and although there was variation in the percent survival observed for PHE1 and 2308 between experiments, PHE1 was always killed more quickly and to a significantly greater extent ($P < 0.05$) than 2308 in each individual experiment (data not shown).

Modifications of the procedures described by Halling et al. (14) and Jiang and Baldwin (15) were used to evaluate the survival of strains 2308 and PHE1 in cultured murine resident peritoneal macrophages. Briefly, following euthanasia, cells were harvested by lavage from the peritoneal cavities of four 9-week-old BALB/c mice with 8 ml of DMEM plus 5% FCS supplemented with 5 U of heparin per ml, and pooled peritoneal cells were cultivated in 96-well plates at a concentration of 1.5×10^5 per well in 200 μl of DMEM plus 5% FCS at 37°C with 5% CO_2. Cell cultures were enriched for macrophages by washing away nonadherent cells after overnight incubation.

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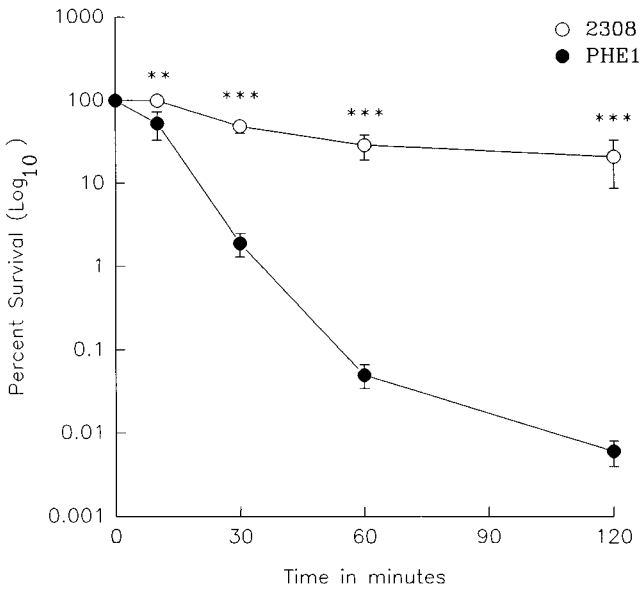

FIG. 1. Killing of *B. abortus* 2308 and PHE1 (2308 *htrA*) by murine neutrophils in culture. The data presented are the results of a representative experiment, and five individual wells containing bacteria and cultured phagocytes were assayed at each time point. Symbols: vertical bars, standard deviations; asterisks, significance values (**, $P < 0.01$; ***, $P < 0.001$).

B. abortus opsonized with a subagglutinating dilution (1:2,000) of hyperimmune BALB/c mouse serum in DMEM plus 5% FCS was added to the macrophages at a ratio of approximately 100 bacteria per macrophage (10). Phagocytosis was allowed to proceed for 2 h at 37°C. At this point, the culture medium was replaced with 200 μl of DMEM plus 5% FCS containing 50 μg of gentamicin per ml, and the culture was incubated for 1 h at 37°C to kill the extracellular brucellae. After 1 h, the medium was removed and replaced with 200 μl of DMEM plus 5% FCS containing 12.5 μg of gentamicin per ml (10). At 0, 24, and 48 h after the addition of 12.5 μg of gentamicin per ml, the cultures were washed and lysed with 0.1% deoxycholate, and the numbers of surviving intracellular brucellae were determined by serial dilution and plating on SBA. Growth medium was changed every 24 h. Five replicate wells for each strain were evaluated at each time point. Results obtained were expressed as percent survival, which was determined by dividing the number of brucellae present at a particular sampling time by the number of brucellae present at time zero and multiplying by 100. Statistical comparisons between experimental groups were performed by the one-tailed Student *t* test (29), and *P* values of <0.05 were considered significant.

The results of a representative macrophage killing experiment are shown in Fig. 2. In this particular experiment, an approximate 10-fold reduction in intracellular survival was observed for PHE1 compared with 2308 at 24 h postinfection (2% for PHE1 versus 20% for 2308 [$P < 0.01$]). Interestingly, both strains appeared to replicate at similar rates in cultured macrophages between 24 and 48 h postinfection (Fig. 2). The macrophage killing experiments were repeated six times, and as with the neutrophil killing experiments, variations in percent survival were noted for both PHE1 and 2308 in individual experiments. However, significantly increased killing of PHE1 ($P < 0.01$) was always observed relative to that of 2308 at 24 h postinfection in cultured macrophages, and both strains always resumed replication in these phagocytes by 48 h postinfections in individual experiments (data not shown).

The decreased capacity of the *B. abortus htrA* mutant to withstand killing by murine neutrophils and macrophages in culture is consistent with the previously reported ROI-sensitive nature of this strain in vitro (11). Moreover, reintroduction of the *B. abortus htrA* on pRIE1 (11) restored the resistance of PHE1 to killing by cultured murine macrophages to wild-type levels (data not shown), directly demonstrating the connection between the increased sensitivity of PHE1 to killing by phagocytes and the *htrA* mutation. These results are similar to those previously reported for *Salmonella typhimurium htrA* mutants (3) and support our hypothesis that the *Brucella htrA* gene product contributes to resistance to killing by host phagocytes (11, 27). The observation that PHE1 is much more sensitive to killing by neutrophils than it is to macrophages suggests that there is a direct correlation between ROI production (18) and the capacity of cultured phagocytes to show enhanced killing of PHE1 relative to that of 2308. This relationship is further supported by the observation that differential killing of PHE1 in cultured macrophages appears to be limited to the first 24 h after phagocytosis, when the majority of the brucellacidal activity of these cells is thought to be mediated by products of the oxidative burst (16).

BALB/c mice experimentally infected with strains 2308 and PHE1 were sacrificed over a 20-week period to determine if the increased sensitivity of PHE1 to killing by murine neutrophils and macrophages affected the capacity of this strain to produce a chronic infection in the murine model. Female BALB/c mice (Harlan Sprague Dawley, Indianapolis, Ind.) seven to eight weeks of age were infected intravenously with approximately 5×10^4 CFU of *B. abortus* 2308 or the isogenic *htrA* mutant PHE1 in 100 μl of phosphate-buffered saline by previously described procedures (11, 21). At selected times after infection, five mice from each group were euthanized by halothane overdose, their spleens were removed and homogenized, and the numbers of brucellae per spleen were determined by serial dilution and plating on SBA. Statistical com-

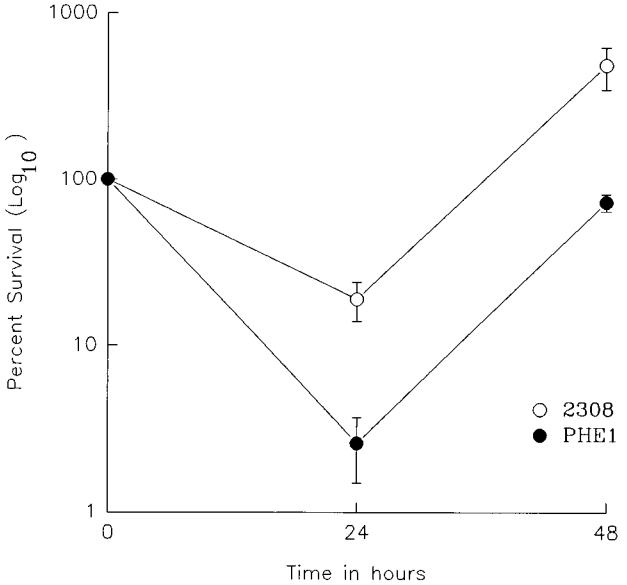

FIG. 2. Killing of *B. abortus* 2308 and PHE1 (2308 *htrA*) opsonized with hyperimmune murine serum by resident peritoneal macrophages in culture. The data presented are the results of a representative experiment, and five individual wells containing bacteria and cultured phagocytes were assayed at each time point. Vertical bars indicate standard deviations. The values for 2308 and PHE1 at 24 and 48 h postinfection were significantly different ($P < 0.01$).

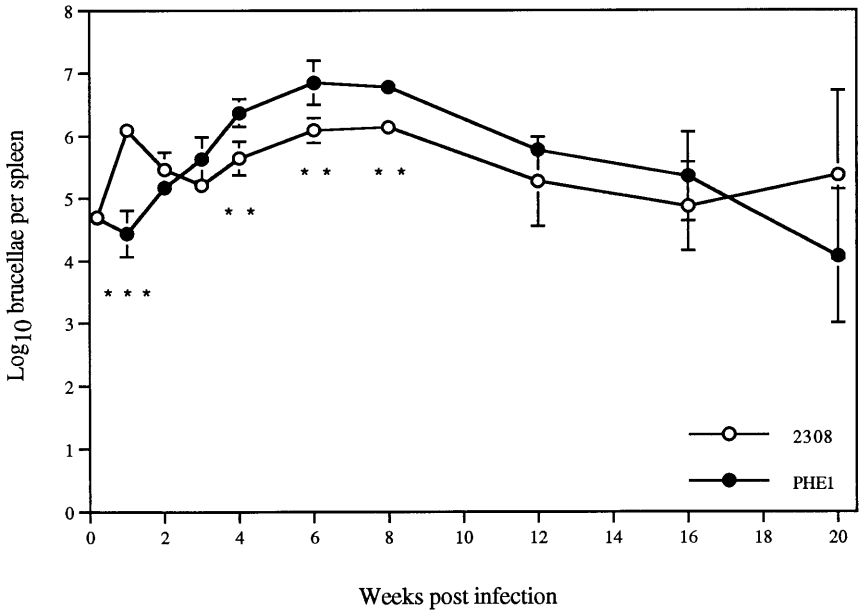

FIG. 3. Spleen colonization of BALB/c mice by *B. abortus* 2308 and PHE1 (2308 *htrA*). Five mice per group were sacrificed at each time point, and the CFUs were determined by serial dilution and plating on SBA. Symbols: vertical bars, standard deviations; asterisks, significance values (**, $P < 0.01$; ***, $P < 0.001$).

parisons between experimental groups were performed by the one-tailed Student *t* test (29), and *P* values of <0.05 were considered significant. As expected on the basis of a previous study (11), significantly fewer brucellae ($P < 0.001$) were isolated from the spleens (Fig. 3) of mice infected with PHE1 than from spleens obtained from mice infected with 2308 at 1 week postinfection. However, by 2 weeks postinfection, the bacterial loads were similar in the spleens of mice infected with PHE1 and 2308, and beginning at 4 and continuing through 8 weeks postinfection, significantly greater numbers of brucellae ($P < 0.010$) were recovered from the spleens of mice infected with PHE1 than from those infected with 2308 (Fig. 3).

Isolates of PHE1 obtained from the spleens of BALB/c mice at 8 (PHE1.8), 12 (PHE1.12), and 20 (PHE1.20) weeks postinfection were evaluated for selected, relevant in vitro and in vivo phenotypes (11) to evaluate the possibility that a stable genetic change resulting from mouse passage was the basis for the apparent recovery of this strain in mice. Like PHE1, PHE1.8, PHE1.12, and PHE1.20 failed to form isolated colonies at 40°C on Schaedler agar plates. These reisolates also showed sensitivity to killing by H_2O_2 equivalent to that of PHE1 in disk assays (zone sizes [in millimeters] for isolates, 2308, 42.9 ± 1.52; PHE1, 63.1 ± 1.19; PHE1.8, 62 ± 1.58; PHE1.20, 61.8 ± 0.84). Furthermore, no significant differences were observed between the spleen colonization profiles of PHE1 and PHE1.8 in BALB/c mice at 1 and 4 weeks postinfection (data not shown). The genotypes of PHE1.8, PHE1.12, and PHE1.20 were confirmed by using *htrA*-, kanamycin resistance-, and pUC-specific probes in Southern blot analysis as previously described (11). Failure of these isolates to produce HtrA was verified by Western blot (immunoblot) analysis employing HtrA-specific antiserum (11).

Differential sensitivity to killing by neutrophils and the potential for recovery in murine resident macrophages offer one potential explanation for why the attenuation of PHE1 is limited to the early stages of infection, i.e., when neutrophils are the predominant phagocyte present. This same pattern of early attenuation followed by recovery in the BALB/c mouse model has also been observed for *htrA* mutants constructed from virulent *B. melitensis* 16M (24) and the *B. abortus* vaccine strain S19 (26), as well as for *htrA* mutants constructed in *B. abortus* 2308 in another laboratory (30). PHE1 and 2308 show comparable growth rates in a complex medium (11), suggesting that a slower growth rate is unlikely to be the basis for the unusual growth pattern of PHE1 in mice. The *htrA* mutant and parental strain also show equivalent survival in serum bactericidal assays (data not shown), indicating that increased sensitivity to complement-mediated killing does not likely contribute to the colonization profile observed for PHE1 in mice. Passive transfer experiments employing T lymphocytes obtained from *B. abortus* 2308-infected BALB/c mice have shown that protective cellular immune responses are induced in infected mice by 4 to 6 weeks postinfection (1). Thus, on the basis of the ROI-sensitive nature of PHE1 in vitro, it is puzzling that enhanced clearance of this mutant relative to 2308 was not observed beginning at this time, when ROI production by activated macrophages should be optimal. It is also unclear why PHE1 colonized the spleens of BALB/c mice in greater numbers than 2308 from 4 to 8 weeks postinfection. These observations suggest that some form of adaptation may be occurring in PHE1 which increases its fitness for survival in the murine host. Evaluation of reisolates of PHE1 obtained from BALB/c mice at various times postinfection indicates that their relevant phenotypic characteristics are unchanged upon mouse passage; therefore, the acquisition of suppressor mutations or other stable genetic changes resulting from prolonged residence in the murine host does not appear to be the basis for this adaptation. If indeed PHE1 is actively adapting to the host environment, it appears more likely that this adaptive response is inducible and reversible. Candidates for this type of adaptive response would include global upregulation of oxidative defenses (i.e., *oxyR*- [7] or *soxRS*-like [13] responses) or the induction of specific genes such as those encoding ROI quenchers like catalase (28) or superoxide dismutase (5) or those encoding heterologous stress response proteases also capable of degrading oxidatively damaged proteins (8, 9).

In summary, the experimental evidence presented here and elsewhere (11, 24, 26) confirms our earlier hypothesis that the *Brucella htrA* gene product protects these intracellular pathogens from oxidative damage and contributes to their resistance to killing by host phagocytes. We are presently evaluating the interactions between *Brucella htrA* mutants and cultured murine and ruminant phagocytes more thoroughly. The results obtained from these studies should not only help us better understand and appreciate the contribution of the *htrA* gene product to the resistance of brucellae to oxidative killing by host phagocytes but also allow us to gain a better overall perspective on how the brucellae are able to successfully survive and replicate for prolonged periods in host macrophages.

This study was supported by Public Health Service grant AI-28867 from the National Institute of Allergy and Infectious Disease, a grant from the LSUMC Center for Excellence in Cancer Research, Treatment and Education, and contract DAMD17-94-C-4054 (contribution 2005) from the United States Army Medical Research and Materiel Command.

REFERENCES

1. **Araya, L. N., P. H. Elzer, G. E. Rowe, F. M. Enright, and A. J. Winter.** 1989. Temporal development of protective cell-mediated and humoral immunity in BALB/c mice infected with *Brucella abortus*. J. Immunol. **143:**3330–3337.
2. **Baldwin, C. L., and A. J. Winter.** 1994. Macrophages and *Brucella*, p. 363–380. *In* B. S. Zwilling and T. K. Eisenstein (ed.), Macrophage-pathogen interactions. Marcel Dekker, New York.
3. **Bäumler, A. J., J. G. Kusters, I. Stojiljkovick, and F. Heffron.** 1994. *Salmonella typhimurium* loci involved in survival within macrophages. Infect. Immun. **62:**1623–1630.
4. **Boucher, J. C., J. Martinez-Salazar, M. J. Schurr, M. H. Mudd, H. Yu, and V. Deretic.** 1996. Two distinct loci affecting conversion to mucoidy in *Pseudomonas aeruginosa* in cystic fibrosis encode homologs of the serine protease HtrA. J. Bacteriol. **178:**511–523.
5. **Bricker, B. J., L. B. Tabatabai, B. A. Judge, B. L. Deyoe, and J. E. Mayfield.** 1990. Cloning, expression, and occurrence of the *Brucella* Cu-Zn superoxide dismutase. Infect. Immun. **58:**2935–2939.
6. **Canning, P. C.** 1990. Phagocyte function in resistance to brucellosis, p. 151–163. *In* L. G. Adams (ed.), Advances in brucellosis research. Texas A&M University Press, College Station.
7. **Christman, M. F., R. W. Morgan, F. S. Jacobson, and B. N. Ames.** 1985. Positive control of a regulon of defenses against oxidative stress and some heat shock proteins in *Salmonella typhimurium*. Cell **41:**753–762.
8. **Davies, K. J. A., and S. W. Lin.** 1988. Degradation of oxidatively denatured proteins in *Escherichia coli*. Free Radical Biol. Med. **5:**215–223.
9. **Davies, K. J. A., and S. W. Lin.** 1988. Oxidatively denatured proteins are degraded by an ATP-independent proteolytic pathway in *Escherichia coli*. Free Radical Biol. Med. **5:**225–236.
10. **Elzer, P. H., R. H. Jacobson, S. M. Jones, K. H. Nielsen, J. T. Douglas, and A. J. Winter.** 1994. Antibody-mediated protection against *Brucella abortus* in BALB/c mice at successive periods after infection: variation between virulent strain 2308 and attenuated vaccine strain 19. Immunology **82:**651–658.
11. **Elzer, P. H., R. W. Phillips, M. E. Kovach, K. M. Peterson, and R. M. Roop II.** 1994. Characterization and genetic complementation of a *Brucella abortus* high-temperature-requirement A (*htrA*) deletion mutant. Infect. Immun. **62:**4135–4139.
12. **Enright, F. M.** 1990. The pathogenesis and pathobiology of *Brucella* infections in domestic animals, p. 301–320. *In* K. H. Nielsen and J. R. Duncan (ed.), Animal brucellosis. CRC Press, Boca Raton, Fla.
13. **Greenberg, J. T., P. Monach, J. H. Chou, P. D. Josephy, and B. Demple.** 1990. Positive control of a global antioxidant defense regulon activated by superoxide-generating agents in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **87:**6181–6185.
14. **Halling, S. M., P. G. Detilleux, F. M. Tatum, B. A. Judge, and J. E. Mayfield.** 1991. Deletion of the BCSP31 gene of *Brucella abortus* by replacement. Infect. Immun. **59:**3863–3868.
15. **Jiang, X., and C. L. Baldwin.** 1993. Effects of cytokines on intracellular growth of *Brucella abortus*. Infect. Immun. **61:**124–134.
16. **Jiang, X., B. Leonard, R. Benson, and C. L. Baldwin.** 1993. Macrophage control of *Brucella abortus*: role of reactive oxygen intermediates and nitric oxide. Cell. Immunol. **151:**309–319.
17. **Johnson, K., I. Charles, G. Dougan, D. Pickard, P. O'Gaora, G. Costa, T. Ali, I. Miller, and C. Hormaeche.** 1991. The role of a stress-response protein in *Salmonella typhimurium* virulence. Mol. Microbiol. **5:**401–407.
18. **Klebanoff, S. J.** 1992. Oxygen metabolites from phagocytes, p. 541–588. *In* J. I. Gallin, I. M. Goldstein, and R. Snyderman (ed.), Inflammation: basic principles and clinical correlates. Raven Press, New York.
19. **Kreutzer, D. L., L. A. Dreyfus, and D. C. Robertson.** 1979. Interaction of polymorphonuclear leukocytes with smooth and rough strains of *Brucella abortus*. Infect. Immun. **23:**737–742.
20. **Li, S.-R., N. Dorrell, P. H. Everest, G. Dougan, and B. W. Wren.** 1996. Construction and characterization of a *Yersinia enterocolitica* O:8 high-temperature requirement A (*htrA*) isogenic mutant. Infect. Immun. **64:**2088–2094.
21. **Montaraz, J. A., and A. J. Winter.** 1986. Comparison of living and nonliving vaccine for *Brucella abortus* in BALB/c mice. Infect. Immun. **53:**245–251.
22. **Morrison, C. J., R. A. Isenberg, and D. Stevens.** 1988. Enhanced oxidative mechanisms in immunologically activated versus elicited polymorphonuclear neutrophils: correlations with fungicidal activity. J. Med. Microbiol. **25:**115–121.
23. **Nicoletti, P. L.** 1989. Relationship between animal and human disease, p. 41–52. *In* E. J. Young and M. J. Corbel (ed.), Brucellosis: clinical and laboratory aspects. CRC Press, Boca Raton, Fla.
24. **Phillips, R. W., P. H. Elzer, and R. M. Roop II.** 1995. A *Brucella melitensis* high temperature requirement A (*htrA*) deletion mutant demonstrates a stress response defective phenotype *in vitro* and transient attenuation in the BALB/c mouse model. Microb. Pathog. **19:**277–284.
25. **Riley, L. K., and D. C. Robertson.** 1984. Brucellacidal activity of human and bovine polymorphonuclear leukocyte granule extracts against smooth and rough strains of *Brucella abortus*. Infect. Immun. **46:**231–236.
26. **Robertson, G. T., P. H. Elzer, and R. M. Roop II.** 1996. *In vitro* and *in vivo* phenotypes resulting from deletion of the high temperature requirement A (*htrA*) gene from the bovine vaccine strain *Brucella abortus* S19. Vet. Microbiol. **49:**197–207.
27. **Roop, R. M., II, T. W. Fletcher, N. M. Sriranganathan, S. M. Boyle, and G. G. Schurig.** 1994. Identification of an immunoreactive *Brucella abortus* HtrA stress response protein homolog. Infect. Immun. **62:**1000–1007.
28. **Sha, Z., T. J. Stabel, and J. E. Mayfield.** 1994. *Brucella abortus* catalase is a periplasmic protein lacking a standard signal sequence. J. Bacteriol. **176:**7375–7377.
29. **Snedecor, G. W., and W. G. Cochran.** 1985. Statistical methods. Iowa State University Press, Ames.
30. **Tatum, F. M., N. F. Cheville, and D. Morfitt.** 1994. Cloning, characterization and construction of *htrA* and *htrA*-like mutants of *Brucella abortus* and their survival in BALB/c mice. Microb. Pathog. **17:**23–36.

Editor: P. E. Orndorff