**Brucella abortus** HtrA Functions as an Authentic Stress Response Protease but Is Not Required for Wild-Type Virulence in BALB/c Mice

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Received 20 November 2000/Returned for modification 15 February 2001/Accepted 6 June 2001

A second mutation has recently been identified in the previously described **Brucella abortus** htrA mutant PHE1. As a result of this finding, a new **B. abortus** htrA mutant, designated RWP11, was constructed to evaluate the biological function of the **Brucella** HtrA protease. RWP11 is more sensitive to oxidative killing in vitro and less resistant to killing by cultured murine neutrophils and macrophages than the virulent parental strain 2308 but is not attenuated in BALB/c mice through 4 weeks postinfection. The in vitro phenotype of **B. abortus** RWP11 is consistent with the proposed function of bacterial HtrA proteases as components of a secondary line of defense against oxidative damage. The in vivo phenotype of this mutant, however, indicates that, unlike the corresponding **Salmonella** and **Yersinia** proteins, **Brucella** HtrA does not play a critical role in virulence in the mouse model.

Homologs of the high-temperaturerequirement A (HtrA) protein, an ATP-independent serine protease, have now been identified in numerous bacterial species (16). Based on biochemical and genetic studies, HtrA is generally thought to serve as a stress response protease in the periplasmic space, degrading damaged proteins resulting from exposure to a variety of environmental stresses, including elevated temperatures and exposure to reactive oxygen intermediates (3, 10, 13, 23). The HtrA proteases of the intracellular pathogens **Salmonella** spp. (8) and **Yersinia** spp. (11, 25) have been shown to contribute to virulence. **Salmonella** and **Yersinia** htrA mutants show decreased ability to survive within macrophages and attenuation in mice. The necessity for HtrA in virulence is believed to be related to its ability to protect cells from the products of the oxidative burst of host macrophages (1, 11, 25). Correspondingly, **Salmonella** and **Yersinia** htrA mutants have been shown to be sensitive to killing by hydrogen peroxide and superoxide in vitro (8, 11, 25).

**Brucella** htrA mutants have been described as being temperature-sensitive, sensitive to oxidative killing in vitro, sensitive to killing by cultured murine neutrophils and macrophages, and attenuated in both mice and ruminants (4, 5, 18, 19, 20, 24). These characteristics are consistent with the proposed function of the HtrA protease and similar to those described for htrA mutants of **Escherichia coli**, **Salmonella enterica** serovar Typhimurium, **Yersinia enterocolitica**, and **Legionella pneumophila** (1, 8, 11, 13, 17, 23, 25). However, we have recently identified a second mutation in the previously described **Brucella abortus** htrA mutant PHE1 (4) that was inadvertently introduced during the construction of this strain. Just upstream of **B. abortus** htrA and overlapping the htrA promoter resides a set of genes showing significant homology to the cycK and cycL genes of **Rhizobium meliloti** (9), which are involved in the biosynthesis of cytochrome c. The gene deletion strategy employed for the construction of **B. abortus** PHE1 removed not only a significant portion of the 5’ end of the htrA gene, but also a portion of the 3’ end of the **B. abortus** cycL homolog. Consequently, it became necessary to construct an authentic **B. abortus** htrA mutant to evaluate the function of **Brucella** HtrA and its possible role in virulence.

Using previously described procedures (4), the **B. abortus** htrA mutant RWP11 was constructed from virulent strain 2308 by replacing an approximately 400-bp **S<sub>n</sub>H<sub>1</sub>** fragment internal to the htrA coding region with the kanamycin resistance gene from **TrpH<sub>α**A (14). The genotype of RWP11 was confirmed by Southern blot analysis using htrA-, cycL-, vector-, and kanamycin resistance gene-specific probes (data not shown). Western blot analysis using **Brucella** HtrA-specific antibodies was also employed to show either the presence or the absence of the HtrA protein in strains 2308 and RWP11, respectively. **B. abortus** 2308 and RWP11 both displayed cytochrome c activity, as evidenced by their ability to reduce the indicator P-aminomethylidene line in the oxidase test (data not shown).

The **B. abortus** htrA mutant RWP11 displayed a stress response-defective phenotype in vitro, consistent with the proposed function of the corresponding gene product (21). For instance, RWP11 was more sensitive to H<sub>2</sub>O<sub>2</sub> and the antibiotic puromycin, an inhibitor of protein translation that leads to the production of truncated peptides (15), than the parental strain 2308 when these two strains were examined in a previously described disk diffusion assay (Table 1). Wild-type resistance to H<sub>2</sub>O<sub>2</sub> and puromycin was restored in RWP11 when the **Brucella** htrA gene was supplied in trans on pRIE1 (4). Sensitivity to killing by H<sub>2</sub>O<sub>2</sub> or O<sub>2</sub><sup>-</sup> in vitro has also been shown for htrA mutants of **Salmonella**, **Yersinia**, and **Pseudomonas** spp. (1, 2, 8, 11, 25) and is consistent with the role of HtrA as a secondary defense mechanism against oxidative stress, as proposed by Davies and Lin (3). The sensitivity of RWP11 to puromycin suggests that the htrA mutation leads to a decrease in proteolysis of puromycin-containing peptides, which is consistent with the predicted protease function of the
**Brucella HtrA** (21). RWP11 also produced much smaller colonies following 72 h of growth at 41°C on Schaedler agar plates supplemented with 5% defibrinated bovine blood (SBA) than did the parental 2308 strain. When the htrA gene was supplied in trans on plasmid pRIE1 (4), however, RWP11(pRIE1) formed colonies equivalent in size to those formed by 2308 at 41°C. The growth restriction displayed by RWP11 at elevated temperature is similar to that described for *E. coli* and *Yersinia htrA* mutants (11, 13, 25), but not as dramatic.

Decreased resistance to killing by cultured murine macrophages is a characteristic of *Salmonella* and *Yersinia htrA* mutants (1, 11, 25). To determine if the increased sensitivity of the *B. abortus htrA* mutant RWP11 to oxidative killing in vitro corresponds to a decreased resistance to killing by host macrophages, previously described procedures (5) were used to evaluate the survival of strains 2308 and RWP11 in cultured murine resident peritoneal macrophages. Briefly, following euthanasia, cells were harvested by lavage from the peritoneal cavities of 9-week-old female BALB/c mice with 8 ml of Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% fetal calf serum (FCS) and 5 U of heparin per ml. 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% CO₂. Cell cultures were enriched for macrophages by washing away nonadherent cells after overnight incubation. *B. abortus* cells opsonized with a subagglutinating dilution (1:2000) of hyperimmune BALB/c mouse serum in DMEM plus 5% FCS were added to the macrophages at a ratio of approximately 100 bacteria per macrophage. 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. 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 number of surviving intracellular brucellae was 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 present at time zero and multiplying by 100. The one-tailed Student *t* test (22) was used for a statistical comparison of the results obtained with 2308 and RWP11, and *P* values of <0.05 were considered significant.

When examined for its capacity to resist killing by cultured murine macrophages (Fig. 1), RWP11 showed a decrease in its ability to survive within these cells at both 24 and 48 h after infection compared to parental strain 2308. However, between 24 and 48 h, 2308 and RWP11 both replicated in these phagocytes (Fig. 1). These findings suggest that a functional HtrA is important for adapting to the intracellular environment of host macrophages but that once this niche is established, a functional HtrA is not essential. The increased sensitivity of the *B. abortus htrA* mutant to killing by cultured macrophages during the first 24 h following ingestion by these cells corresponds to the time that the oxidative burst should be active as well as the time that scavengers of oxygen radicals protect intracellular brucellae in cultured macrophages (7). The brucellae were opsonized with immunoglobulin G (IgG) prior to phagocytosis, which should enhance the oxidative burst of the cultured macrophages following ingestion of 2308 and RWP11. Consequently, based on the proposed function of HtrA, the most likely basis for the decreased resistance of the *B. abortus htrA* mutant to killing by cultured murine macrophages is its increased sensitivity to reactive oxygen intermediates compared to the parental strain. However, this relationship was not confirmed experimentally.

*BALB/c* mice experimentally infected with strains 2308 and RWP11 were sacrificed over a 4-week period to determine if the increased sensitivity of the *B. abortus htrA* mutant to killing by cultured murine macrophages affects the virulence of this strain in the mouse model. Female *BALB/c* mice (Harlan Sprague Dawley, Indianapolis, Ind.), 7 to 8 weeks of age, were infected via the intraperitoneal route with approximately 5 × 10^6 CFU of *B. abortus* 2308 or RWP11 by previously described procedures (5). At 1, 2, and 4 weeks postinfection, five mice from each group were euthanized by halothane overdose, their spleens were removed and homogenized, and the number of brucellae per spleen was determined by serial dilution and plating on SBA. Statistical comparisons between 2308 and RWP11 at selected time points were performed by the one-tailed Student *t* test (22), and *P* values of <0.05 were considered significant.

The *B. abortus* mutant RWP11 displayed a spleen colonization profile in *BALB/c* mice equivalent to that of 2308 through 4 weeks postinfection (Fig. 2). The wild-type virulence of the *B. abortus htrA* mutant RWP11 stands in stark contrast to the

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**TABLE 1. Sensitivity of *B. abortus* 2308, RWP11 (2308 *htrA*), and RWP11(pRIE1) to killing by hydrogen peroxide and puromycin in a disk sensitivity assay**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mean size of zone of inhibition (mm) ± SD</th>
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<tr>
<td></td>
<td>25% H₂O₂</td>
</tr>
<tr>
<td><em>B. abortus</em> 2308</td>
<td>49.88 ± 2.76</td>
</tr>
<tr>
<td><em>B. abortus</em> RWP11</td>
<td>62.66 ± 2.97*</td>
</tr>
<tr>
<td><em>B. abortus</em> RWP11(pRIE1)</td>
<td>48.16 ± 3.34</td>
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</tbody>
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*P < 0.01 compared with 2308 and RWP11(pRIE1).*

*P < 0.05 compared with 2308 and RWP11(pRIE1).*
FIG. 2. Spleen colonization of BALB/c mice by B. abortus strains 2308 and RWP11 (htrA) after intraperitoneal infection.

dramatic attenuation reported for Salmonella and Yersinia htrA mutants (8, 11, 25). However, Salmonella and Yersinia infections in mice are generally lethal and are characterized by large net increases in bacterial numbers within host tissues. This large net increase in bacterial numbers in vivo coincides with repeated infection of new host macrophages (12). On the contrary, Brucella infections in mice are chronic and are characterized by limited net replication and long-term persistence within host tissues. This suggests that in vivo, the brucellae are undergoing limited rounds of macrophage infection. Indeed, such a style in the murine host is consistent with the lack of attenuation of the B. abortus htrA mutant. If the brucellae were repeatedly infecting new macrophages, then the incremental killing of the B. abortus htrA mutant would be expected to result in accelerated clearance of this strain from mice compared to 2308 due to progressive rounds of macrophage infection. This would be especially true after the induction of Brucella-specific IgG type antibodies in these animals. Indeed, given the increased sensitivity of RWP11 to killing by murine macrophages in vitro compared to 2308 following opsonization with IgG, it is likely that the absence of Brucella-specific antibodies during the early stages of infection in mice allowed the B. abortus htrA mutant to establish an intracellular niche before specific opsonins arose which would have increased the bactericidal activity of host macrophages. In this regard, it is notable that over a 20-week period, neither Brucella abortus nor Brucella melitensis htrA cycL double mutants show accelerated clearance relative to their parental strains in the BALB/c mouse model (5, 18). Recent experiments performed with Brucella suis suggest that the brucellae can prevent the programmed cell death of infected macrophages (6). This ability may allow the brucellae to survive within individual macrophages for an extended period of time and prevent the need for continual reinfection of new host cells. It may also protect the brucellae from repeated exposure to the antibacterial processes of phagocytosis.

This work was supported by a grant (96-35204-3558) from the U.S. Department of Agriculture’s National Research Initiative Competitive Grants Program.

REFERENCES


