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Induction of *dnaK* through Its Native Heat Shock Promoter Is Necessary for Intramacrophagic Replication of *Brucella suis*

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The heat shock protein DnaK is essential for intramacrophagic replication of *Brucella suis*. The replacement of the stress-inducible, native *dnaK* promoter of *B. suis* by the promoter of the constitutively expressed *bla* gene resulted in temperature-independent synthesis of DnaK. In contrast to a *dnaK* null mutant, this strain grew at 37°C, with a thermal cutoff at 39°C. However, the constitutive *dnaK* mutant, which showed high sensitivity to H_2O_2-mediated stress, failed to multiply in murine macrophage-like cells and was rapidly eliminated in a mouse model of infection, adding strong arguments to our hypothesis that stress-mediated and heat shock promoter-dependent induction of *dnaK* is a crucial event in the intracellular replication of *B. suis*.

We have described the important role of the heat shock protein and molecular chaperone DnaK in intramacrophagic growth of *Brucella suis* and its acid-induced expression (12). *Brucella* spp., facultative intracellular, gram-negative bacteria which are the causative agents of brucellosis in humans and animals (6), resist intracellular killing and replicate within the phagosome of macrophages (3, 11, 16). It has been observed recently that this phagosome is acidic (pH 4.0 to 4.5), suggesting a stressful environment, and that early acidification is essential for intracellular multiplication of *B. suis* (19). Work on the identification of proteins induced under stress conditions in *Brucella abortus* and *Brucella melitensis* confirmed our results (17, 20, 23). From previous data (12), we concluded that DnaK from *B. suis* may play an essential role as part of protein repair systems in protecting the bacteria from the environment encountered in the phagosome. Another hypothesis is that DnaK may be directly involved in folding and proper localization of virulence factors, as intracellular multiplication is abolished in the null mutant.

The earlier work was done with a *dnaK* null mutant, allowing us to conclude only that the chaperone participated in intracellular multiplication of *B. suis* (12). On the other hand, *dnaJ*, which is located downstream of *dnaK* and forms an operon with the latter, is not involved in resistance to acid stress and intracellular multiplication of the pathogen (12). As a *dnaJ* knockout mutant of *B. suis* behaves like the wild-type strain with respect to these properties, we concluded that *dnaJ* was of no relevance in the context of the work presented here. Western blot analysis showed induction of *dnaK* during heat and acid shock and under intramacrophagic growth conditions (12). We therefore hypothesized that low-level, constitutive expression of *dnaK* was not sufficient for *B. suis* to resist macrophage attack. To verify this hypothesis, we replaced the original *dnaK* promoter on the *B. suis* chromosome by the constitutive promoter of the β-lactamase gene bla_{TEM} (P_{bla}) from pUC18, and we studied the phenotype of the mutant obtained. P_{bla} was chosen, as we and others (13, 14) have observed that *bla* is expressed in brucellae, conferring ampicillin resistance to strains bearing this gene on a plasmid. Furthermore, *bla* and its promoter region are well characterized, in contrast to promoters of constitutively expressed *Brucella* genes.

Replacement of the native heat shock promoter of *dnaK* by the β-lactamase promoter. To perform promoter exchange, three distinct sets of cloning steps were performed in *Escherichia coli* DH5α (Life Technologies, Cergy Pontoise, France). (i) A 580-bp DNA fragment containing the promoter region and the sequence coding for the N-terminal 36 amino acids of the *bla* protein was isolated from pUC18 (24) following digestion by *Sau*3AI, and cloned into the promoter selection vector pKK232-8 (Amersham Pharmacia Biotech, Orsay, France). Activation of the reporter gene *cat*, leading to chloramphenicol resistance, determined the orientation of the *bla* promoter. We then inserted a blunted kanamycin resistance gene isolated from pUC4K (Amersham Pharmacia Biotech, Orsay, France) in the *Sma*I site upstream of the *bla* promoter and in the opposite direction of transcription. (ii) Based on the sequence from *B. ovis* described earlier (4), a 1-kb DNA fragment containing 330 bp upstream of the start codon of *dnaK* from *B. suis* and the first 670 bp of the same gene was obtained by PCR with the primers 5′-GCGGGGTGAAAATGTGCCGC-3′ (positions 108 to 127), and 5′-ACCGCCAAGGAACGTGTCG C-3′ (1113 to 1094) and cloned blunt-ended into pUC18 (Amersham Pharmacia Biotech). This DNA was then digested by the restriction enzymes *Age*I and *Nde*I, resulting in the excision of a 60-bp fragment from positions 330 to 390 of the published sequence carrying the promoter region of the *dnaK*/*dnaJ* operon (4, 21). (iii) In a final step, the *Age*I-*Nde*I gap was blunted and filled with a 1.8-kb DNA fragment containing the kanamycin resistance gene and the *bla* promoter from step 1 in the proper orientation, i.e., the *bla* promoter supporting transcription of *dnaK*. For allelic exchange between this modified 5′ region of *dnaK* and the wild-type gene of *B. suis*, we recloned the final construct from pUC18 into the brucella suicide vector

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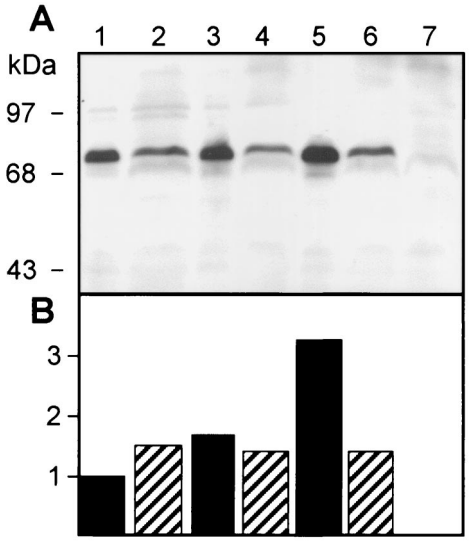

FIG. 1. (A) Western blot analysis of *dnaK* expression in total cell lysates of wild-type *B. suis* (lanes 1, 3, and 5) and the $dnaK_{mut}$ strain (lanes 2, 4, and 6) with monoclonal anti-DnaK antibody. Growth temperatures were 30°C (lanes 1, 2, and 7), 37°C (lanes 3 and 4), and 46°C (lanes 5 and 6). The *dnaK* null mutant was used as a control (lane 7). (B) Quantification of DnaK in panel A, using SigmaGel software, relative to the amount of DnaK present in the wild-type lysate at 30°C (lane 1).

pCVD442 and performed electroporation of *B. suis* 1330 and selection for homologous recombination on sucrose-containing tryptic soy (TS) agar plates as described previously (7). Southern blot analysis (22) of the resulting $dnaK_{mut}$ mutant with a *dnaK* probe revealed the correct insertion of the Kan^r-P_{bla} sequence into the expected site of the chromosomal *dnaK* gene (data not shown).

Constitutive, temperature-independent expression of *dnaK* and growth of *B. suis* at various temperatures. The expression of *dnaK* in the mutant strain at 30, 37, and 46°C compared to that in wild-type *B. suis* 1330 was analyzed by Western blotting with monoclonal anti-DnaK antibody as described previously (5, 12). Briefly, strains were grown to an optical density at 600 nm (OD_{600}) of 0.5 at 30°C, concentrated fourfold in preheated TS broth, and incubated for 30 min at the appropriate temperatures. Bacteria were immediately heat killed at 65°C for 45 min, washed once in phosphate-buffered saline (PBS), and resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer. Total cell lysates equivalent to 5×10^8 bacteria per lane were loaded onto a sodium dodecyl sulfate–12.5% polyacrylamide gel. Total proteins were visualized by Coomassie staining after transfer onto a PolyScreen membrane (NEN) prior to antibody incubation and detection of DnaK by enhanced chemiluminescence (Amersham). As determined with SigmaGel software (SPSS Science, Chicago, Ill.), *B. suis* DnaK was induced approximately 1.7-fold between 30 and 37°C and another 2-fold between 37 and 46°C in the wild type, whereas the amounts of DnaK produced by the mutant containing the *bla* promoter remained constant (Fig. 1). The *dnaK* null mutant, obtained by gene knockout (12), did not produce any DnaK (Fig. 1A, lane 7). This result provided evidence that promoter replacement led to the noninducible expression of *dnaK* in this *B. suis* mutant.

The absence of DnaK in the null mutant of *B. suis* described earlier resulted in temperature sensitivity for growth at 37°C and above (12). We compared this observation to the behavior of the $dnaK_{mut}$ strain at different temperatures: As we observed that the strain grew well on agar plates at 37 but not at 42°C (data not shown), we performed growth curve experiments in TS broth at 37, 38, and 39°C. *B. suis* 1330 (wild type) and the $dnaK_{mut}$ strain were grown to stationary phase, diluted to an OD_{600} of 0.03, and grown at the appropriate temperatures for 56 h. We observed lower growth rates for the mutant than for the wild-type at 37 and 38°C. At both temperatures, wild-type *B. suis* entered stationary phase at about 15 h postdilution, whereas the $dnaK_{mut}$ strain reached a similar OD_{600} only at 56 h (Fig. 2). The *dnaK* null mutant (12), in contrast, did not show any growth at 37°C. The temperature limit for normal growth of *B. suis* $dnaK_{mut}$ was 39°C. Constitutive, low-level production of DnaK therefore appeared to be sufficient for growth at temperatures of up to 38°C. In contrast, heat stress conditions such as growth at 39°C and above necessitate higher-level synthesis of DnaK, which is not possible in the $dnaK_{mut}$ strain (Fig. 1, lane 6).

Sensitivity of the $dnaK_{mut}$ strain and of the *dnaK* null mutant to acid pH and oxidative stress. Additional phenotypic characteristics that might be linked to a modification of *dnaK* expression, such as sensitivity to acid pH and to oxidative stress, were investigated according to previously published protocols (8, 12). Briefly, for pH sensitivity experiments *B. suis* 1330 and the $dnaK_{mut}$ strain were grown to stationary phase (OD_{600} = 1.5) in TS broth, and the number of CFU per milliliter was determined prior to dilution 1:50 in TS broth adjusted to pH 4.5. At 0, 6, 24, 48, and 54 h postdilution, culture samples were diluted appropriately and plated on TS agar for colony enumeration. In contrast to the very high sensitivity of the *dnaK* null mutant to acid pH, as expressed by a 5,000-fold reduction in viable bacteria after 24 h of incubation

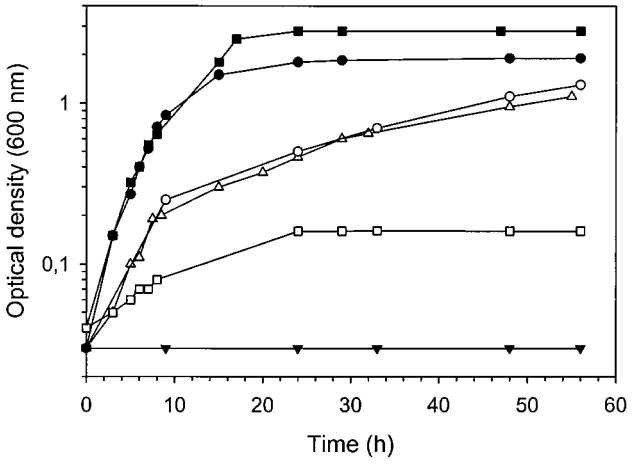

FIG. 2. Growth rates in TS broth of the *B. suis* wild-type strain at 37°C (●) and 39°C (■) and of the $dnaK_{mut}$ strain at 37°C (○), 38°C (△), and 39°C (□). The absence of growth of the *dnaK* null mutant at 37°C is indicated (▼). Growth curves are from one representative experiment out of three performed under identical conditions.

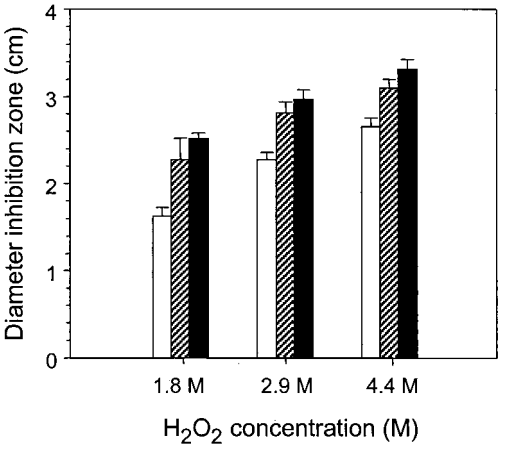

FIG. 3. Sensitivities of wild-type *B. suis* 1330 (open bars), the *dnaK*$_{mut}$ strain (hatched bars), and the *dnaK* null mutant (solid bars) to various concentrations of H_2O_2. Six disk assays were performed per strain per concentration. Error bars indicate standard deviations.

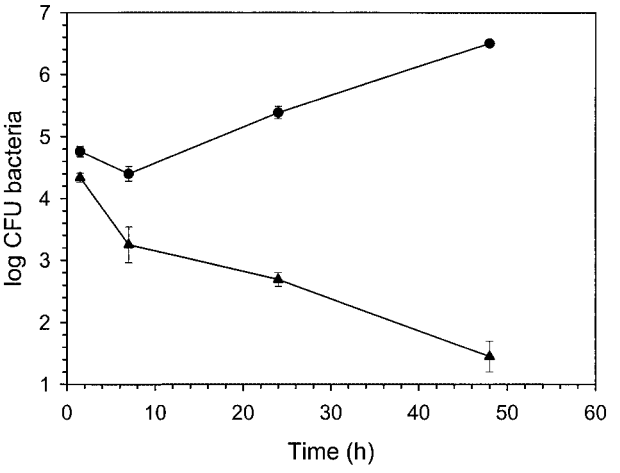

FIG. 4. Intracellular growth of *B. suis* in J774 murine macrophage-like cells. Adherent cells were infected with the wild-type strain (●) or the *dnaK*$_{mut}$ strain (▲). Experiments were performed in triplicate. Error bars indicate standard deviations.

(12), viability of the *dnaK*$_{mut}$ strain decreased only 1.8-fold over the same period compared to the wild-type and 5-fold following incubation for 54 h. The mutant constitutively expressing low levels of DnaK therefore resisted acid pH significantly better than the null mutant but did not reach the wild-type level of resistance. We concluded from these data that an acid pH stress could not by itself account for the rapid elimination of the *dnaK*$_{mut}$ strain in the macrophage.

To investigate the sensitivity of the different strains to oxidative killing, strains were grown to stationary phase and rediluted to an OD_{600} of 0.25 each. Each strain (75 μl) was plated on TS agar, and sterile paper disks saturated with 10 μl of H_2O_2 at concentrations of 1.8, 2.9, and 4.4 M were layered on top prior to incubation at 30°C for 2 days and measurement of inhibition zone diameters (8). Interestingly, both the *dnaK* null mutant and the *dnaK*$_{mut}$ strain were significantly more sensitive to the three concentrations of H_2O_2 than the parental strain (Fig. 3) ($P < 0.001$). The sensitivities of both mutants were comparable, with inhibition zone diameters that were not significantly different from one another. The results therefore showed (i) that DnaK participated in the resistance of *B. suis* to oxidative stress and (ii) that noninducible *dnaK* expression was not sufficient for protection from oxidative stress.

The *dnaK*$_{mut}$ strain is rapidly eliminated in J774 murine macrophages and in BALB/c mice. In order to assess the capacity of the *dnaK*$_{mut}$ strain of *B. suis* to survive within the host cell, we infected J774 murine macrophage-like cells with the mutant and the wild-type strain. Bacteria were added to adherent cells at a multiplicity of infection of 20 and incubated for 30 min at 37°C in the presence of 5% CO_2, followed by three washes with PBS and incubation of the cells in RPMI medium supplemented with 10% fetal calf serum and gentamicin at a concentration of 30 μg/ml for at least 1 h. At 1.5, 7, 24, and 48 h postinfection, cells were washed once with PBS and lysed in 0.2% Triton X-100. CFU were enumerated by plating of serial dilutions on TS agar plates and incubation at 37°C. Low-level, noninducible expression of *dnaK* resulted in rapid elimination of the mutant strain in J774 cells, whereas the wild-type strain showed a profile typical of intracellular multiplication (Fig. 4). This is in contrast to the mutant's extracellular growth capacities at 37°C. These results therefore made it clear that production of DnaK necessary and sufficient for *B. suis* growth at 37°C in culture medium is not sufficient for intracellular replication of the pathogen, and they added support for our previous hypothesis that induction of *dnaK* is crucial for intramacrophagic replication of *B. suis* (12). The rapid elimination of *B. suis dnaK*$_{mut}$ in macrophages could not easily be explained by an eventual sensitivity of the strain to low pH, encountered in the phagosome at least during the first hours of infection, as there was no obvious correlation between its 40-fold reduction during the first 24 h of infection and the only 2-fold loss in viability at acid pH in vitro during the same period. The high sensitivity of the *dnaK*$_{mut}$ strain to oxidative stress, in contrast, suggested that this type of stress may par-

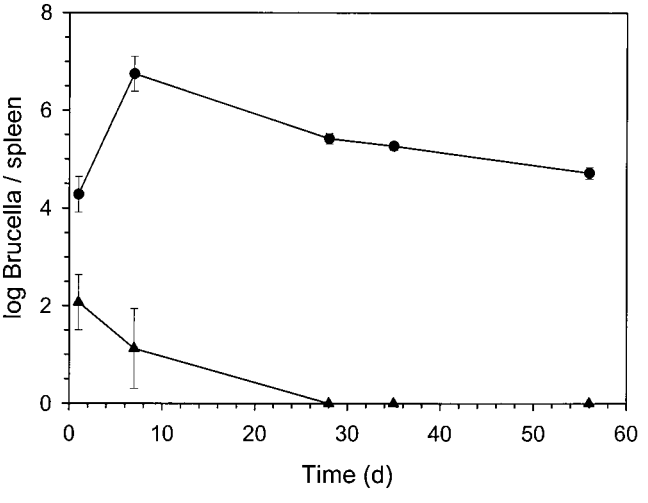

FIG. 5. Course of infection in BALB/c mice of wild-type *B. suis* (●) and the *dnaK*$_{mut}$ strain (▲). Growth of the bacteria in spleens was determined at various time points for 8 weeks. Data are means and standard deviations for four mice.

ticipate in the antimicrobial defense mechanisms of murine macrophages against brucellae.

With the well-established murine model of infection (9, 10), the fate of the bacteria was monitored in vivo by the enumeration of *B. suis* 1330 and the *dnaK*$_{mut}$ strain in the spleens of BALB/c mice at various times postinfection. An infectious dose of 5×10^4 bacteria was injected intraperitoneally, and residual virulence of the strains was evaluated following killing of four mice per point and strain at 1 day and 1, 4, 5, and 8 weeks postinoculation, homogenization of the spleens, and plating of serial dilutions on TS agar plates. Virulent *B. suis* multiplied rapidly in the spleen, and numbers reached a maximum at 1 week postinoculation before slightly declining until the end of the experiment (Fig. 5). In contrast, the *dnaK*$_{mut}$ strain was very quickly eliminated from the spleen, and only a few bacteria were recovered at 1 week in only one mouse out of four (Fig. 5). The 2-log difference observed at 1 day postinfection in intrasplenic survival of the wild-type versus the *dnaK*$_{mut}$ strain was in agreement with the 100-fold reduction of the intramacrophagic survival of the mutant 24 h after infection of J774 cells (Fig. 4). We therefore concluded that the effect observed in the complex in vivo model could be attributed mainly to the murine macrophage. In analogy to what we observed in J774 macrophage-like cells, these results confirmed the necessity of the presence of the native, inducible promoter of *dnaK* for survival of *B. suis* in mice.

Conclusions. It was observed previously that intracellular pathogens such as *Salmonella enterica* serovar Typhimurium and *Legionella pneumophila* have adapted to the hostile environment of the macrophage by selective induction of a multitude of proteins, among which are many stress proteins (1, 2, 15). Virulence gene transcription in salmonellae is activated after phagosome acidification, illustrating the importance of a coordinated gene activation in response to the microenvironment encountered by the bacterium (18). Based on the previous finding that a *dnaK* null mutant lost its capacity for intramacrophagic multiplication (12), our aim in this work on the pathogen *B. suis* was to determine whether basic levels of DnaK were sufficient for intracellular resistance. We concluded that a constitutive but noninducible expression of *dnaK* in *B. suis* was not sufficient to allow survival in a macrophage model of infection and in a host organism, despite extracellular growth of the mutant at 37 and 38°C. The increased sensitivity to oxidative stress of the strain producing constitutively low levels of DnaK therefore suggested that the induction of the gene via its native heat shock promoter was an essential event in the resistance of the pathogen not only to high temperature but also to the stressful environment of the macrophage in the host animal.

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REFERENCES

1. **Abshire, K. Z., and F. C. Neidhardt.** 1993. Analysis of proteins synthesized by *Salmonella typhimurium* during growth within a host macrophage. J. Bacteriol. **175:**3734–3743.
2. **Buchmeier, N. A., and F. Heffron.** 1990. Induction of *Salmonella* stress proteins upon infection of macrophages. Science **248:**730–732.
3. **Caron, E., J. P. Liautard, and S. Köhler.** 1994. Differentiated U937 cells exhibit increased bactericidal activity upon LPS activation and discriminate between virulent and avirulent *Listeria* and *Brucella* species. J. Leukoc. Biol. **56:**174–181.
4. **Cellier, M. F., J. Teyssier, M. Nicolas, J. P. Liautard, J. Marti, and J. Sri Widada.** 1992. Cloning and characterization of the *Brucella ovis* heat shock protein DnaK functionally expressed in *Escherichia coli*. J. Bacteriol. **174:** 8036–8042.
5. **Cloeckaert, A., O. Grépinet, H. Salih-Alj Debbarh, and M. S. Zygmunt.** 1996. Overproduction of the *Brucella melitensis* heat shock protein DnaK in *Escherichia coli* and its localization by use of specific monoclonal antibodies in *B. melitensis* cells and fractions. Res. Microbiol. **147:**145–157.
6. **Corbel, M. J.** 1990. Brucella, p. 339–353. *In* M. T. Parker and L. H. Collier (ed.), Principles of bacteriology, virology, and immunity, vol. 2. E. Arnold, London, United Kingdom.
7. **Ekaza, E., L. Guilloteau, J. Teyssier, J. P. Liautard, and S. Köhler.** 2000. Functional analysis of the ClpATPase ClpA of *Brucella suis*, and persistence of a knockout mutant in BALB/c mice. Microbiology **146:**1605–1616.
8. **Ekaza, E., J. Teyssier, S. Ouahrani-Bettache, J. P. Liautard, and S. Köhler.** 2001. Characterization of *Brucella suis clpB* and *clpAB* mutants and participation of the genes in stress responses. J. Bacteriol. **183:**2677–2681.
9. **Endley, S., D. McMurray, and T. A. Ficht.** 2001. Interruption of the *cydB* locus in *Brucella abortus* attenuates intracellular survival and virulence in the mouse model of infection. J. Bacteriol. **183:**2454–2462.
10. **Godfroid, F., B. Taminiau, I. Danese, P. Denoel, A. Tibor, V. Weynants, A. Cloeckaert, J. Godfroid, and J. J. Letesson.** 1998. Identification of the perosamine synthetase gene of *Brucella melitensis* 16M and involvement of lipopolysaccharide O side chain in *Brucella* survival in mice and in macrophages. Infect. Immun. **66:**5485–5493.
11. **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.
12. **Köhler, S., J. Teyssier, A. Cloeckaert, B. Rouot, and J. P. Liautard.** 1996. Participation of the molecular chaperone DnaK in intracellular growth of *Brucella suis* within U937-derived phagocytes. Mol. Microbiol. **20:**701–712.
13. **Kovach, M. E., P. H. Elzer, D. S. Hill, G. T. Robertson, M. A. Farris, R. M. Roop II, 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.
14. **Kovach, M. E., R. W. Phillips, P. H. Elzer, R. M. Roop II, and K. M. Peterson.** 1994. pBBR1MCS: a broad-host-range cloning vector. BioTechniques **16:**800–802.
15. **Kwaik, Y. A., B. I. Eisenstein, and N. C. Engleberg.** 1993. Phenotypic modulation by *Legionella pneumophila* upon infection of macrophages. Infect. Immun. **61:**1320–1329.
16. **Liautard, J. P., A. Gross, J. Dornand, and S. Köhler.** 1996. Interactions between professional phagocytes and *Brucella* spp. Microbiol. SEM **12:**197–206.
17. **Lin, J., and T. A. Ficht.** 1995. Protein synthesis in *Brucella abortus* induced during macrophage infection. Infect. Immun. **63:**1409–1414.
18. **Miller, J. F., J. J. Mekalanos, and S. Falkow.** 1989. Coordinate regulation and sensory transduction in the control of bacterial virulence. Science **243:** 916–922.
19. **Porte, F., J. P. Liautard, and S. Köhler.** 1999. Early acidification of phagosomes containing *Brucella suis* is essential for intracellular survival in murine macrophages. Infect. Immun. **67:**4041–4047.
20. **Rafie-Kolpin, M., R. C. Essenberg, and J. H. Wyckoff III.** 1996. Identification and comparison of macrophage-induced proteins and proteins induced under various stress conditions in *Brucella abortus*. Infect. Immun. **64:**5274–5283.
21. **Robertson, G. T., M. E. Kovach, C. A. Allen, T. A. Ficht, and R. M. Roop II.** 2000. The *Brucella abortus* Lon functions as a generalized stress response protease and is required for wild-type virulence in BALB/c mice. Mol. Microbiol. **35:**577–588.
22. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **Teixeira-Gomes, A. P., A. Cloeckaert, and M. S. Zygmunt.** 2000. Characterization of heat, oxidative, and acid stress responses in *Brucella melitensis*. Infect. Immun. **68:**2954–2961.
24. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.

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