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Similar Requirements of a Plant Symbiont and a Mammalian Pathogen for Prolonged Intracellular Survival

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***Brucella abortus*, a mammalian pathogen, and *Rhizobium meliloti*, a phylogenetically related plant symbiont, establish chronic infections in their respective hosts. Here a highly conserved *B. abortus* homolog of the *R. meliloti bacA* gene, which encodes a putative cytoplasmic membrane transport protein required for symbiosis, was identified. An isogenic *B. abortus bacA* mutant exhibited decreased survival in macrophages and greatly accelerated clearance from experimentally infected mice compared to the virulent parental strain. Thus, the *bacA* gene product is critical for the maintenance of two very diverse host-bacterial relationships.**

Rhizobia establish agriculturally important symbioses with leguminous plants (*1*), whereas brucellae are highly infectious pathogens of animals and cause the human disease brucellosis (*2*, *3*). Despite leading to exceedingly different outcomes in interactions with their respective eukaryotic hosts, the in-host life-styles of these closely phylogenetically related bacteria (*4*) show striking parallels. In the establishment of chronic infection, both rhizobia and brucellae are endocytosed by host cells, where they then undergo adaptive changes and ultimately live for prolonged periods in intracellular, acidic, host-membrane–bound compartments (*1–3*, *5*, *6*).

Rhizobium meliloti bacA mutants invade alfalfa nodules like wild-type bacteria, but lyse upon release into plant cells before they can differentiate and establish a chronic host infection (*7*). BacA is predicted to be a cytoplasmic membrane transport protein with seven transmembrane domains (*7*, *8*). BacA is 64% identical to, and functionally interchangeable with, the *Escherichia coli* SbmA protein inferred to be a transporter of bleomycin and microcins B17 and J25 (*9*). *R. meliloti bacA* mutants also have increased resistance to bleomycin (*9*).

We identified a *B. abortus* DNA fragment that included a monocistronic 1248-nucleotide open reading frame (ORF) (GenBank AF244996) (*10*) that encodes a predicted protein of 47.3 kD with 68.2% identity to *R. meliloti* BacA. In both *B. abortus* and *R. meliloti*, *bacA* is flanked by an upstream gene for a putative transporter and a downstream gene, transcribed in the opposite direction, that has similarity to a putative bacterial secreted protein. We constructed an allele (*bacA1*) of the *bacA* gene in which 41% of the *bacA* ORF was replaced with a drug resistance cassette (strain KL7) (*11*). As with *R. meliloti*, disruption of *bacA* function resulted in increased resistance to bleomycin (Fig. 1) (*12*).

The capacity of the brucellae to survive and replicate in host macrophages is critical to their ability to produce disease (*2*). The *bacA1* mutant and its wild-type parent were opsonized and used to infect cultured murine macrophages (*13*). During the first 24 hours post infection (p.i.), both strains showed net intracellular killing by the phagocytes (Fig. 2). The virulent wild-type strain showed characteristic net replication in macrophages at 36 hours p.i. and beyond, but the *bacA1* mutant did not appear to recover after the initial period of killing.

To determine if this defect in intracellular replication in macrophages correlated with an inability to establish a chronic infection in the host, we experimentally infected BALB/c mice with the *bacA1* mutant and its wild-type parent (*14*). BALB/c mice represent the classic model for chronic *B. abortus* infection in the host (*15*): Substantial numbers of brucellae can be recovered from the spleens and livers of mice infected with virulent strains

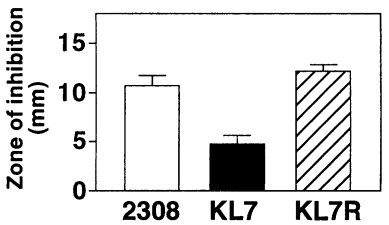

Fig. 1. Increased resistance of the *B. abortus bacA* mutant to killing by bleomycin. Strains 2308 (wild-type), KL7 (Δ*bacA*), and KL7R (reconstructed Δ*bacA*) were spread onto Schaedler agar plates and overlayed with filter paper disks containing bleomycin. The diameter of cleared zones of *B. abortus* growth inhibition in response to the drug was measured after 72 hours. Data are presented as the mean ± SD (n = 5).

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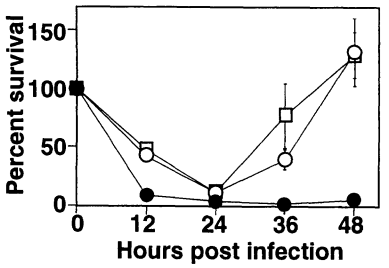

Fig. 2. Decreased intracellular survival of the *B. abortus bacA* mutant in cultured murine macrophages. Isolated macrophages were infected with opsonized *B. abortus* strains 2308 (○), KL7 (●), and KL7R (□). Percent survival of bacteria in macrophages was determined at various times after infection. Data are presented as the mean ± SD (n = 5).

for beyond 20 weeks p.i. For the first 2 weeks p.i., the *bacA1* mutant and its wild-type parent colonized the spleens and livers of mice at virtually equivalent levels (Fig. 3). However, at 3 weeks p.i. and beyond, the *bacA1* mutant was recovered from spleens and livers of the mice at markedly lower levels than the isogenic parental strain. Thus, the onset of clearance of the *bacA1* mutant seems to parallel the induction of *Brucella*-specific humoral and cellular immune responses, which lead to enhanced brucellacidal activity of host macrophages (*2*). By 8 weeks p.i., the mutant strain was present in spleens at levels five orders of magnitude lower than in the wild-type strain. Thus, the *B. abortus* BacA protein is critically required for the maintenance of chronic infection in this mouse model, and the basis for the attenuation of the *bacA1* mutant is likely to be its defect in intracellular survival in host macrophages. Mice infected with the *bacA1* mutant and the wild-type parent exhibited equivalent delayed-type hypersensitivity and serologic responses to antigens derived from the parental strain (*16*). Thus, the *bacA1* mutant was able to induce *Brucella*-specific cellular and humoral immune responses, making it an interesting vaccine candidate; currently, there is no safe and effective vaccine for human brucellosis (*2*, *3*).

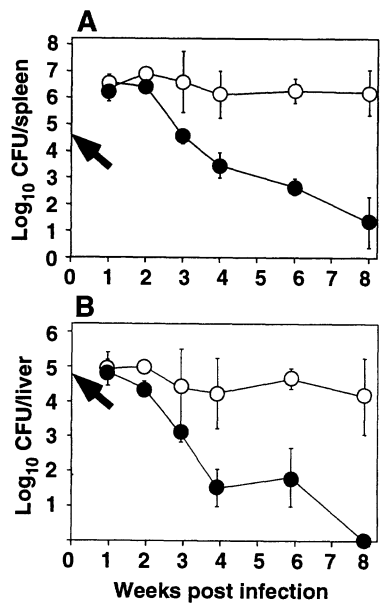

Fig. 3. Accelerated clearance of the *B. abortus bacA* mutant from experimentally infected BALB/c mice. (**A**) Spleen colonization. *B. abortus* strains 2308 (○) and KL7 (●) were injected intraperitoneally into BALB/c mice. At each time point, mice were killed, spleens dissected, and total number of brucellae per organ determined. Arrows denote level of inocula. Data are presented as the mean ± SD (n = 5). (**B**) Liver colonization. Counts were determined as in (A), except that left lateral lobes of the liver were analyzed. KL7R was present in tissues at levels equivalent to those in 2308 at all time points (*22*).

Because elements of the host innate immune response are conserved among mammals, insects, and plants (*17*), all of which share common antimicrobial defense strategies (*18*), BacA function may be required to overcome a host defense response necessary for the prevention of chronic infection, irrespective of whether that results in a pathogenic or symbiotic relationship. It has previously been shown that pathogens that can infect widely differing hosts require common virulence functions for these interactions (*19*).

Numerous studies with *Brucella* have failed to identify virulence factors typically found in pathogenic bacteria (e.g., exotoxins and adhesins). Thus, it is notable that we were able to identify a gene crucial for *B. abortus* pathogenesis on the basis of knowledge gained by genetic analysis of a bacterial-plant symbiosis. Recently, a *B. abortus* two-component regulatory system necessary for pathogenicity was found to be closely related to a symbiotically important *R. meliloti* two-component system (*20*). It will be interesting to learn whether the downstream targets of these regulatory systems are also conserved and are mechanistically required for chronic host infection, as BacA appears to be.

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10. The complete *R. meliloti bacA* ORF (GenBank X73522) was used to probe a *B. abortus* 2308 Lambda ZAP II library (Stratagene) and a 2308 genomic Southern blot.
11. *B. abortus* 2308, a virulent laboratory strain, was used for the construction of a *bacA* mutant. All protocols using live brucellae were performed in a Biosafety Level 3 containment facility in accordance with U.S. Centers for Disease Control and Prevention–NIH guidelines [U.S. Department of Health and Human Services (USDHHS), 1993]. Brucellae were grown at 37°C in Albimi broth or on Schaedler agar (SA; both Difco); plates were incubated in 5% CO_2. SBA plates were supplemented with 5% bovine blood. Ampicillin was used at 100 μg/ml and kanamycin at 45 μg/ml. A 2845-nucleotide (nt) *bacA*-containing fragment of *B. abortus* 2308 DNA was cloned into pNEB193 (New England Biolabs) and designated pKL1. A fragment removing 41% of *bacA* was replaced by a kanamycin resistance cassette [V. de Lorenzo, M. Herrero, U. Jakubzik, K. N. Timmis, *J. Bacteriol.* **172**, 6568 (1990)]. The knockout construct pKL2 was electroporated into strain 2308, and mutant strain KL7 was used for further study. Because $bacA^+$ carried on a multicopy number plasmid was not tolerated in *B. abortus* strains grown in host tissues (R. W. Phillips and K. LeVier, unpublished data), a 2845-nt *bacA*-containing fragment was ligated into pEX100T [H. P. Schweizer and T. T. Hoang, *Gene* **158**, 15 (1995)], a *sacB*-containing vector, in the opposite orientation from the *lacZ* promoter (pKL4). pKL4 was electroporated into KL7, and $bacA^+$ reconstructed strains were selected by standard methods. Strain KL7R, the resulting restored wild-type strain, was used for further studies.
12. Saturated *B. abortus* cultures grown with antibiotics were adjusted to an optical density at 600 nm of 0.2, and a 100-μl sample was spread onto SA plates with antibiotics. Filter paper disks (10-mm diameter) soaked with 10 μl of 3 U/ml bleomycin sulfate (Sigma) in dimethylsulfoxide were placed on the plates and zones of growth inhibition measured.
13. *B. abortus* strains opsonized with hyperimmune serum were added to resident peritoneal macrophages isolated from 8- to 10-week-old female BALB/c mice (Harlan Sprague Dawley) at an approximate ratio of 50 bacteria per macrophage in a gentimycin protection assay (*21*).
14. Seven- to 8-week-old female BALB/c mice were infected intraperitoneally with ~5×10^4 colony-forming units of *B. abortus* strains (*21*). At selected time points, five mice from each experimental group were killed and the spleens and left lateral lobes of the livers cultured. The total number of brucellae per organ was determined by serial dilution and plating on SBA.
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