Identification of Genes Required for Chronic Persistence of *Brucella abortus* in Mice

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The genetic basis for chronic persistence of *Brucella abortus* in lymphoid organs of mice, cows, and humans is currently unknown. We identified *B. abortus* genes involved in chronic infection, by assessing the ability of 178 signature-tagged mutants to establish and maintain persistent infection in mice. Each mutant was screened for its ability to colonize the spleens of mice at 2 and 8 weeks after inoculation. Comparison of the results from both time points identified two groups of mutants attenuated for chronic infection in mice. The first group was not recovered at either 2 or 8 weeks postinfection and was therefore defective in establishing infection. Mutants in this group carried transposon insertions in genes involved in lipopolysaccharide biosynthesis (*wbkA*), in aromatic amino acid biosynthesis, and in type IV secretion (*virB1* and *virB10*). The second group, which was recovered at wild-type levels 2 weeks postinfection but not 8 weeks postinfection was able to establish infection but was unable to maintain chronic infection. One mutant in this group carried a transposon insertion in a gene with homology to *gcvB* of *Mycobacterium tuberculosis*, encoding glycine dehydrogenase, an enzyme whose activity is increased during the state of nonreplicating persistence. These results suggest that some mechanisms for long-term persistence may be shared among chronic intracellular pathogens. Furthermore, identification of two groups of genes, those required for initiating infection and those required only for long-term persistence, suggests that *B. abortus* uses distinct sets of virulence determinants to establish and maintain chronic infection in mice.

Bacteria causing chronic infections, such as *Mycobacterium tuberculosis*, *Chlamydia trachomatis*, and *Brucella abortus* are able to evade the host’s immune system throughout the infection by colonizing an intracellular niche. This lifestyle may require adaptations other than the brief survival in phagocytic cells observed for well-characterized intracellular pathogens such as *Salmonella* serotypes which cause an acute infection. While *M. tuberculosis* and *C. trachomatis* are difficult to manipulate genetically, the genetic manipulation of the *Brucella* genome can be performed routinely, using tools such as plasmid vectors and systems for Tn5 mutagenesis (20–22). Thus, identification of the genes required for *B. abortus* to cause infection may reveal virulence mechanisms of chronic disease caused by other intracellular pathogens. A recent improvement in Tn5 mutagenesis, known as signature-tagged transposon mutagenesis (STM), has been developed for the in vivo selection of Tn5 mutants that are defective in colonization (18). This method uses experimentally infected animals to identify mutants that are attenuated in vivo from a large, mixed pool of mutants (29). Since Tn5 can be used in *B. abortus*, STM can be used to identify genes that are necessary for chronic intracellular infection.

Brucellosis is endemic in Mediterranean countries and Central and South America and is manifested as an undulant fever in humans that, if untreated, can develop into a chronic infection with symptoms persisting for several months (32). Chronic infections may result in infection of secondary tissues, including heart and brain, if the infection is left untreated. Symptoms may also recur years after the original infection. *B. abortus* infection is acquired by humans through contact with infected livestock and consumption of unpasteurized dairy products. Bacteria cause a systemic infection and localize preferentially to organs that are rich in elements of the reticuloendothelial system, such as liver, spleen, and lymph nodes, where they survive and multiply within host macrophages. *B. abortus* has been found to inhibit the bactericidal functions of phagocytes, including phagolysosomal fusion, neutrophil degranulation, and the oxidative burst (5); however, as with other intracellular pathogens which cause chronic infection, the genetic basis for the interaction of *Brucella* with phagocytic cells is still poorly understood. *B. abortus* virulence is conveniently studied in a mouse model that mimics the chronic infection observed in humans. Here bacteria are found intracellularly, within macrophages of infected organs (25). Experimental infection of BALB/c mice has shown that the infection has two phases: during the first 2 weeks, bacteria multiply rapidly. In the second phase, bacterial numbers stabilize over the next 5 to 6 weeks and then decrease slowly. Bacteria have been recovered from spleens of infected mice as late as 24 weeks postinfection (26, 28). The different phases of *B. abortus* infection in mice raise the question whether this pathogen uses different sets of virulence genes during the early and late stages of this disease.

To address this question, we have performed a random screen of the genome of *B. abortus* to identify genes required for infection at an early (2 weeks postinfection) and a late (8 weeks postinfection) time point postinoculation. Comparative analysis of these results provided new insight into the genetic basis for chronic intracellular infection and *B. abortus* pathogenesis. Furthermore, our results suggest that a better understanding of the mechanisms by which *B. abortus* is able to cause chronic intracellular infection may ultimately reveal strategies that are shared by other, less tractable pathogens.

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RESULTS

Generation of signature-tagged mutants of \textit{B. abortus}. \textit{B. abortus} 2308 was mutagenized with a pool of signature-tagged mini-Tn5Km2 derivatives carried on plasmid pUT by electroporation, and mutants were selected on TSA-Km. These mutants were screened for susceptibility to ampicillin to eliminate strains (fewer than 2\% of mutants) carrying cointegrates of the suicide vector pUT inserted in the chromosome. To confirm that mutants obtained from the same electroporation are not siblings but arise from independent transposition events, Southern hybridization was performed using the 1.3-kb EcoRI fragment of pUC4KSAAC, which contains the Tn903 Km\(^2\) gene (12). Using this probe, the hybridization profiles of EcoRI-digested chromosomal DNA from 10 randomly chosen mutants from a single electroporation were compared. Since EcoRI cuts only once within mini-Tn5Km2, a mutant with a single transposon insertion should have only one band hybridizing with the probe. All of the mutants examined had a single band of unique size hybridizing with the probe, indicating that each mutant contained a single, unique insertion of the transposon (data not shown).

Signature-tagged screen of \textit{B. abortus} mutants in mice. To identify mutants defective in establishing chronic infection, we used the BALB/c mouse model of chronic brucellosis. Mice infected i.p. with \textit{B. abortus} have been shown to harbor bacteria in the spleen for up to 24 weeks postinfection (5, 9). The persistence of \textit{B. abortus} in mice is similar to chronic infection observed in other host species, including humans.

It has not been shown whether attenuated \textit{B. abortus} mutants exhibit competitive infection defects in mice when coinfected with a virulent strain. We therefore performed a preliminary competitive infection experiment using \textit{B. abortus} 2308 and CA180, a Tn5 mutant defective in the synthesis of the O antigen of lipopolysaccharide (LPS), which was characterized previously (1). Three mice were infected i.p. with a mixture containing 6.4 \times 10^6 CFU of \textit{B. abortus} 2308 and 4.1 \times 10^6 CFU of CA180. At 1 week postinfection, no mutant \textit{B. abortus} could be recovered from the spleens of any infected mouse, while the number of wild-type \textit{B. abortus} ranged from 6 \times 10^4 to 2 \times 10^5 CFU/spleen. This result showed that a mutant with a defect in a known \textit{B. abortus} virulence factor, LPS, exhibits a competitive infection defect in the BALB/c mouse model of brucellosis. Furthermore, it suggested that STM, which utilizes competitive infection as the basis for screening pools of mutants, could be used to screen for mutants defective in chronic infection.

For the screen in mice, pools of 46 mutants were grown individually in tryptic soy broth (TSB) in 96-well plates for 48 h and then staped with a 48-prong replicator onto PIA plates and grown for 48 h. Bacteria were resuspended from PIA plates, and the concentration was adjusted to approximately 10^7 CFU/ml with PBS. Of this suspension, 0.1 ml was injected i.p. into each of six BALB/c mice. In the pooled inoculum, the dose of each individual mutant was therefore approximately 10^6 CFU. At 2 and 8 weeks postinfection, groups of three mice were sacrificed. At necropsy, the only sign of disease evident was enlargement of the spleen. To recover bacteria, spleens were homogenized in PES, and serial 10-fold dilutions were plated on TSA-Km. After incubation for 4 days, bacteria were resuspended from plates containing between 1,000 and 5,000 colonies, and chromosomal DNA was prepared from the recovered pool of mutants. Probes of input and output pools were generated by PCR amplification of tags from chromosomal DNA of pooled ST mutants as described before (18) and used for hybridization with dot blots containing individual mu-
tants in the corresponding pool. For each time point, a fresh input pool probe was prepared and a blot was hybridized for comparison with the output pools recovered from spleens of three mice at 8 weeks postinfection. We found that 39 mutants failed to give a reproducible hybridization signal when the input pool probe was prepared more than once from a chromosomal DNA preparation. These mutants were eliminated from the screen. Thus, a total of 178 mutants with consistently hybridizing tags were screened.

Mutants which gave weak or no hybridization signals on output pool blots from at least two of the three mice at each time point were identified as putatively attenuated by comparison with input pool blots (Fig. 1). At 2 weeks postinfection, 28 mutants exhibited a reduction in the hybridization signal, suggesting reduced recovery from the mouse spleens. Of these 28 mutants, 11 exhibited a reduced hybridization signal in output pools recovered at 8 weeks postinfection.

In addition to the 11 mutants reduced in colonization at both 2 and 8 weeks, we identified 16 mutants that were only defective for colonization at 8 weeks. While mutants identified from both the 2- and 8-week output pools may be unable to establish infection, those identified only from the 8-week output pools may be defective in sustaining chronic infection. The 17 mutants identified only from the 2-week output pools may represent mutants which are slow to colonize the spleen but are still able to persist. However, infection defects in these mutants were not characterized further, since these mutants were able to sustain chronic infection. Thus, two groups of mutants, those identified at both 2 and 8 weeks and those identified only...
at 8 weeks, were considered putatively attenuated for chronic infection and were chosen for further study (Table 1).

**Competitive infection of mice with mutants identified by STM.** Following our identification of mutants putatively attenuated for chronic infection, we performed a quantitative assay to confirm their colonization defect. To this end, each of the 27 mutants identified by the STM screen was inoculated i.p. at a total dose of approximately $10^7$ CFU to groups of four mice (Table 1). As a control, nonattenuated mutant were compared using a Student’s $t$ test.

### TABLE 1. Confirmation of competitive defects of STM mutants by competitive infection of mice with *B. abortus* S2308<sup>+</sup>

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Competitive index</th>
<th>Mutant</th>
<th>Competitive index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>In vivo</strong></td>
<td></td>
<td><strong>In vivo</strong></td>
</tr>
<tr>
<td></td>
<td>2 wk</td>
<td>8 wk</td>
<td></td>
</tr>
<tr>
<td>BA11</td>
<td>2.29*</td>
<td>1.86</td>
<td></td>
</tr>
<tr>
<td>BA41</td>
<td>4.74*</td>
<td>&gt;29.512***</td>
<td>0.97</td>
</tr>
<tr>
<td>BA73</td>
<td>3.36</td>
<td>147.9*</td>
<td>2.74</td>
</tr>
<tr>
<td>BA87</td>
<td>1.08</td>
<td>10.47**</td>
<td>0.79</td>
</tr>
<tr>
<td>BA100</td>
<td>20.89***</td>
<td>&gt;758.6***</td>
<td>4.70</td>
</tr>
<tr>
<td>BA102</td>
<td>1.47</td>
<td>93.3***</td>
<td>1.80</td>
</tr>
<tr>
<td>BA109</td>
<td>0.92</td>
<td>3.21</td>
<td></td>
</tr>
<tr>
<td>BA114</td>
<td>133.07**</td>
<td>&gt;1.047.1***</td>
<td>0.91</td>
</tr>
<tr>
<td>BA157</td>
<td>0.74</td>
<td>1.58</td>
<td></td>
</tr>
<tr>
<td>BA159</td>
<td>1.50</td>
<td>23.44*</td>
<td>2.20</td>
</tr>
<tr>
<td>BA184</td>
<td>1,306.8***</td>
<td>&gt;2,168.2***</td>
<td>0.57</td>
</tr>
</tbody>
</table>

*Competitive index was calculated as $([\text{CFU}_{2308}/\text{CFU}_{mutant}]$ inoculated/(CFU<sub>2308</sub>/CFU<sub>mutant</sub> inoculated). Significant differences between competitive indices of STM mutants and a control, nonattenuated mutant were compared using a Student’s $t$ test. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.005$.

Two additional mutants with defects in establishing persistent infection were identified: BA141 and BA114, each carrying a transposon insertion in homologues of *Brucella suis* virB<sup>1</sup> and virB<sup>10</sup>, respectively of the *Brucella suis* virB locus (27). *B. suis* mutants carrying disruptions at this locus show a reduced ability to multiply in vitro within macrophages and HeLa cells. Our data suggest that this locus is required for establishing chronic infection in organs of the mouse.

We could obtain no clone from DNA flanking the transposon insertion of the fourth mutant (BA100) defective in establishing chronic infection. However, BA100 was unable to grow on *Brucella* minimal medium unless supplemented with a combination of aromatic amino acids. These auxanography data indicated that this mutant was defective in an early step of the aromatic amino acid biosynthesis pathway.

Sequences obtained from the transposon insertion site in three mutants defective in sustaining chronic infection showed homology to metabolic genes. BA159 was interrupted at the *gluP* locus, encoding a putative transporter for glucose and galactose (14). Mutant BA152 carried a transposon insertion in a homologue of *Rhizobium etli* gltD, encoding the small subunit of glutamate synthase (10). The identification of these two mutants in the STM screen suggests that glucose, galactose, or glutamate may serve as carbon and/or nitrogen sources during growth of *B. abortus* in the host. BA102 carried an insertion in a putative glycine dehydrogenase or glycine cleavage system. Since BA102 did not have a competitive growth defect in TSA and was able to grow on minimal medium without glycine, the virulence defect of BA102 is likely not due to auxotrophy. Interestingly, the activity of glycine dehydrogenase has been shown to increase 10-fold upon entry of *M. tuberculosis* into a state of nonreplicating persistence (30, 31). Since *B. abortus*, like *M. tuberculosis*, is able to persist chronically in infected tissues, none of the mutants displayed a strong growth deficiency in laboratory medium.

**Identification of inactivated genes.** Genes inactivated by mini-Tn5 Km<sup>2</sup> insertions were identified by cloning transposon-flanking DNA, sequence determination, and comparison with the GenBank database using the BLAST2 search program at NCBI. Transposon-flanking DNA was cloned from eight mutants.

Mutants with defects in establishing chronic infection included BA184, which carried a transposon insertion in a *B. abortus* homologue of *Brucella melitensis* wkb<sup>A</sup>, encoding a mannose transferase that functions in the biosynthesis of O antigen (16). LPS biosynthesis is required for virulence in *Brucella* species, and it has recently been shown that strains of *B. abortus* and *B. melitensis* carrying defined mutations in genes required for LPS biosynthesis are unable to establish infection in mice (1, 16, 24). Identification of this known virulence factor thus validated the STM screen.

Two additional mutants with defects in establishing persistent infection, BA41 and BA114, carried transposon insertions in homologues (virB<sup>1</sup> and virB<sup>10</sup>, respectively) of the *Brucella suis* virB locus (27). *B. suis* mutants carrying disruptions at this locus show a reduced ability to multiply in vitro within macrophages and HeLa cells. Our data suggest that this locus is required for establishing chronic infection in organs of the mouse.

We could obtain no clone from DNA flanking the transposon insertion of the fourth mutant (BA100) defective in establishing chronic infection. However, BA100 was unable to grow on *Brucella* minimal medium unless supplemented with a combination of aromatic amino acids. These auxanography data indicated that this mutant was defective in an early step of the aromatic amino acid biosynthesis pathway.

Sequences obtained from the transposon insertion site in three mutants defective in sustaining chronic infection showed homology to metabolic genes. BA159 was interrupted at the *gluP* locus, encoding a putative transporter for glucose and galactose (14). Mutant BA152 carried a transposon insertion in a homologue of *Rhizobium etli* gltD, encoding the small subunit of glutamate synthase (10). The identification of these two mutants in the STM screen suggests that glucose, galactose, or glutamate may serve as carbon and/or nitrogen sources during growth of *B. abortus* in the host. BA102 carried an insertion in a putative glycine dehydrogenase or glycine cleavage system. Since BA102 did not have a competitive growth defect in TSA and was able to grow on minimal medium without glycine, the virulence defect of BA102 is likely not due to auxotrophy. Interestingly, the activity of glycine dehydrogenase has been shown to increase 10-fold upon entry of *M. tuberculosis* into a state of nonreplicating persistence (30, 31). Since *B. abortus*, like *M. tuberculosis*, is able to persist chronically in infected tissues, none of the mutants displayed a strong growth deficiency in laboratory medium.
hosts, glycine dehydrogenase may play a similar role in the entry of these pathogens into a latent state in the host.

Finally, DNA flanking two insertions in mutants defective for maintaining chronic infection showed either no homology to genes in the database (BA73) or homology to an open reading frame of unknown function (BA87) (Table 2).

**DISCUSSION**

The goal of the STM screen was to identify and compare *B. abortus* genes required for establishment and maintenance of chronic persistence in the mouse. Since fewer than 200 genes have been sequenced in *Brucella* species, we reasoned that screening a small number of mutants would be sufficient to identify new *B. abortus* genes required for chronic infection. The STM screen identified 27 mutants putatively attenuated for chronic infection. A statistically significant competitive-infection defect could be detected in 14 of these mutants. Thus, statistically significant evidence for attenuation was obtained for 8% (14 of 178) of the mutants screened. Assuming that our mutagenesis was random and that the coding density of the 3,200-kb *B. abortus* genome is similar to that of the *E. coli* genome, our data suggest that an estimated 257 genes may be required for establishing and maintaining chronic persistence in mice after i.p. infection. In contrast, i.p. infection of mice with an STM bank of *Salmonella enterica* Typhimurium revealed that only 5% of its 4,400-kb genome, or an estimated 153 genes, is required for the acute infection caused by this intracellular pathogen (18). The greater number of virulence genes required for chronic infection versus acute disease may reflect the requirement for additional adaptations to ensure long-term persistence, such as those which prevent clearance of *B. abortus* by the host immune system.

The working hypothesis of this study was that different sets of genes may be required for the initial steps or the establishment of chronic infection, which is characterized by rapid bacterial growth, than for maintenance of chronic infection, in which little or no growth is observed (5, 9). Indeed, the 14 attenuated mutants identified in this study fell into two classes (Fig. 2). Four mutants were unable to establish infection by either 2 or 8 weeks postinfection. In contrast, the remaining 10 mutants were able to establish infection at 2 weeks postinfection but displayed a defect in chronic persistence at 8 weeks postinfection. The first class included mutants with transposon insertions in genes required for O antigen biosynthesis (BA184), type IV secretion (BA41 and BA114), and biosynthesis of aromatic amino acids (BA100). The genes inactivated in these mutants are predicted to play a role early during infection. For example, BA184, a *wbkA* mutant, was the most highly attenuated and was outcompeted by the wild type by 1,000-fold at 2 weeks postinfection, suggesting that it was eliminated early in the infection process. Since rough mutants are sensitive to the bactericidal action of complement, it is possible that BA184 is cleared by complement-mediated lysis and may reach the spleen only in small numbers (1, 11, 13). The transposon insertion in BA100 rendered this mutant defective in the biosynthesis of aromatic amino acids, as determined by auxanography. This biosynthesis pathway is also required for the biosynthesis of 2,3-dihydroxybenzoic acid, the only siderophore known to be produced by *B. abortus* (23). However, since this siderophore has been shown to be dispensable for growth in mice (7), it is more likely that attenuation of this mutant is the result of its inability to acquire aromatic amino acids in the host. *Salmonella aro* mutants are unable to survive and replicate within macrophages and are attenuated for virulence (19). Thus, some of the virulence genes required in an early phase during chronic *B. abortus* infection may be similar to those used by intracellular pathogens, such as *S. enterica* serovar Typhimurium, which cause an acute infection. Two mutants (BA114 and BA41) defective for initiation of chronic infection carried insertions in a putative type IV secretion system of *B. abortus*, encoded by the *virB* genes (27). Mutant BA114 (*virB10*) displayed a greater competitive colonization defect in murine spleens at 2 weeks postinfection than BA41 (*virB1*) (Table 1). A similar effect has been described for the homologues of the *B. abortus* virB10 and virB1 genes present in the genome of the plant pathogen *Agrobacterium tumefaciens*. Inactivation of *virB1* in *A. tumefaciens* causes a lower degree of attenuation than a mutation in *virB10* (8). Mutations in the *virB* locus render *B. suis* unable to multiply in HeLa cells or macrophage cell lines in vitro (27). These data, together with our findings that *B. abortus* virB1 and virB10 mutants are unable to persist in mouse spleens after i.p. inoculation, suggest that attenuation in the animal model is due to an inability of these strains to grow intracellularly.

The second class of mutants, which were unable to maintain chronic infection, included strains defective in production of glutamate synthase (BA152), glycine cleavage (BA102), nutrient uptake (BA159), and several unknown functions (BA31, BA38, BA73, BA87, BA63, BA122, and BA142). While the inactivated genes in these mutants were not required for initiation of infection, they were required for chronic persistence. Mutant BA102 carried a transposon insertion in a gene with homology to *gcvB*, encoding glycine dehydrogenase, from *M. tuberculosis*. The activity of this enzyme has been found to increase 10-fold upon entry of *M. tuberculosis* into a state of nonrepli-cating persistence in vitro (31). The finding that glycine dehydrogenase is required for persistence of *B. abortus* in the mouse spleen suggests that *M. tuberculosis* and *B. abortus* may depend on similar metabolic pathways for chronic persistence in the host. This result underscores the potential for research on host pathogen interactions of *B. abortus* to elucidate mechanisms of intracellular persistence which are shared by other chronic intracellular pathogens.

**TABLE 2. Characterization of *B. abortus* genes identified by STM**

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Closest homologue (organism)</th>
<th>GenBank accession no.</th>
<th>Inferred function</th>
</tr>
</thead>
<tbody>
<tr>
<td>BA41</td>
<td>virB1 (<em>B. suis</em>)</td>
<td>AF141604</td>
<td>Type IV secretion</td>
</tr>
<tr>
<td>BA114</td>
<td>virB10 (<em>B. suis</em>)</td>
<td>AF141604</td>
<td>Type IV secretion</td>
</tr>
<tr>
<td>BA184</td>
<td><em>wbkA</em> (<em>B. melitensis</em>)</td>
<td>AF047478</td>
<td>O antigen biosynthesis</td>
</tr>
<tr>
<td>BA159</td>
<td><em>gldA</em> (<em>B. abortus</em>)</td>
<td>U34785</td>
<td>Uptake of glucose and galactose</td>
</tr>
<tr>
<td>BA102</td>
<td>P protein (<em>Synechocystis sp.</em>)</td>
<td>D90094</td>
<td>Glycine dehydrogenase</td>
</tr>
<tr>
<td>BA152</td>
<td><em>gldD</em> (<em>R. etli</em>)</td>
<td>Q50601</td>
<td>Glutamate synthase</td>
</tr>
<tr>
<td>BA87</td>
<td>HP1225 (<em>Helicobacter pylori</em>)</td>
<td>AF107264</td>
<td>Conserved inner membrane protein</td>
</tr>
<tr>
<td>BA73</td>
<td>No homology</td>
<td>AE000628</td>
<td>?</td>
</tr>
</tbody>
</table>

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