A SacB Mutagenesis Strategy Reveals that the *Bartonella quintana* Variably Expressed Outer Membrane Proteins Are Required for Bloodstream Infection of the Host

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*Bartonella* bacteria adhere to erythrocytes and persistently infect the mammalian bloodstream. We previously identified four highly conserved *Bartonella quintana* adhesin genes that undergo phase variation during prolonged bloodstream infection. The variably expressed outer membrane proteins (Vomp) encoded by these genes are members of the trimeric autotransporter adhesin family. Each *B. quintana* Vomp appears to contribute a different adhesion phenotype, likely mediated by the major variable region at the adhesive tip of each Vomp. Although studies document that the Vomp adhesins confer virulence phenotypes in vitro, little is known about in vivo virulence strategies of *Bartonella*. We sought to determine whether the *B. quintana* Vomp adhesins are necessary for infection in vivo by using a *vomp* null mutant. It first was necessary to develop a system to generate in-frame deletions of defined genes by allelic exchange in a wild-type *Bartonella* background, which had not been achieved previously. We utilized sacB negative selection to generate a targeted, in-frame, markerless deletion of the entire *vomp* locus in *B. quintana*. We also recently developed the first animal model for *B. quintana* infection, and using this model, we demonstrate here that the deletion of the entire *vomp* locus, but not the deletion of two *vomp* genes, results in a null mutant strain that is incapable of establishing bloodstream infection in vivo. The Vomp adhesins therefore represent critical virulence factors in vivo, warranting further study. Finally, our allelic exchange strategy provides an important advance in the genetic manipulation of all *Bartonella* species and, combined with the animal model that recapitulates human disease, will facilitate pathogenesis studies of *B. quintana*.

*Bartonella* species are fastidious, gram-negative bacteria that persistently infect the bloodstream of many mammals. The three major *Bartonella* pathogens infecting humans are *Bartonella quintana*, *B. henselae*, and *B. bacilliformis*. *B. quintana* is transmitted by the human body louse and causes relapsing fever (“trench fever”), endocarditis, and the highly vascular lesions of bacillary angiomatosis (13). Bacteremia can persist for months, and unsuspected bloodstream infection with *B. quintana* can be detected in 5 to 14% of asymptomatic individuals in certain geographic regions (6, 27). *B. quintana* can cause debilitating, even fatal, illness in immunocompromised individuals with cancer, transplanted organs, or AIDS.

Phase and antigenic variation are immune response-evading virulence strategies exploited by microbial pathogens to persist in a host (3). We identified a family of *B. quintana* proteins that appears to undergo phase variation (28). These surface-localized adhesins, designated Vomp (variably expressed outer membrane proteins), are variably expressed over the course of prolonged bloodstream infection in vivo and are encoded by four highly conserved, tandemly arranged genes (28). These genes, *vompD*, *vompA*, *vompB*, and *vompC*, are located on a 12.8-kb region of the *B. quintana* genome. VompA, VompB, and VompC are highly conserved except in the major variable region, located in the N-terminal half of these three Vomp.

The Vomp adhesins are members of a newly recognized group of afimbrial adhesins of gram-negative bacteria known as trimeric autotransporter adhesins (TAA) (9, 15). TAA transport utilizes the type V secretion system, and the most extensively studied TAA is the *YadA* adhesin of *Yersinia enterocolitica*. *YadA* is a multifunctional virulence factor involved in autoaggregation, as well as adherence to epithelial cells, phagocytes, and extracellular matrix proteins, including collagen. We have shown previously that, similar to *YadA*, VompA is necessary and sufficient to mediate *B. quintana* autoaggregation and that the heterologous expression of either VompA or VompC in *Escherichia coli* is sufficient to effect collagen binding (28). Each Vomp appears to contribute a different phenotype: VompA is the major determinant of the autoaggregation phenotype, and VompC contributes most significantly to collagen binding (28). This specificity is likely mediated by the major variable region at the adhesive tip of each Vomp.

Although in vitro studies have documented that *YadA*, VompA, and VompC confer virulence phenotypes and, in vivo, a *Y. enterocolitica* *yadA* mutant is highly attenuated in a mouse infection model (22), little is known about in vivo virulence strategies of *Bartonella* species. We sought to determine whether the *Bartonella* Vomp adhesins are necessary for infection in vivo by using a *vomp* null mutant. First, however, it was necessary to develop a system to generate in-frame deletions of target genes by allelic exchange in a wild-type *Bartonella* back-
To amplify 800 bp of 5′ vomp locus (with prJM02); BamHI site underlined

PrJM05

GCGGGATCCGATCGCAAATTGTGACGTTTTT

To amplify 3′ flanking region of vomp locus; fully complementary to prJM03

To amplify 800 bp of 3′ flanking region of vomp locus (with prJM03); BamHI site underlined

Table 2. Oligonucleotide primers

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>SacBS-F</td>
<td>GGGGAGCTCGATCCTTTTTA</td>
<td>To amplify sacB from pJEN34; SacI site underlined</td>
</tr>
<tr>
<td>SacBS-R</td>
<td>GGGGAGCTCAGAAAGTTAATAGGCGTAAAGGCAGGTTAAGCC</td>
<td>To amplify sacB from pJEN34; SacI site underlined</td>
</tr>
<tr>
<td>prJM02</td>
<td>TTGGATAAGGACCATCAGTGC</td>
<td>To amplify 5′ flanking region of vomp locus; fully complementary to prJM03</td>
</tr>
<tr>
<td>prJM03</td>
<td>GCACTGATGCTCCTTATGCCCAAGGCTAGTGCAAGGTGCTATT</td>
<td>To amplify 3′ flanking region of vomp locus; half of primer is complementary to prJM02</td>
</tr>
<tr>
<td>prJM05</td>
<td>GCGGGATCCGATCGCAAATTGTGACGTTTTT</td>
<td>To amplify 800 bp of 3′ flanking region of vomp locus (with prJM03); BamHI site underlined</td>
</tr>
<tr>
<td>b′consF1</td>
<td>GCATGACCGCGAGAAGAGACGG</td>
<td>To amplify conserved region present in 3′ region of each vomp gene for Southern probe (Fig. 1B)</td>
</tr>
<tr>
<td>b′consR1</td>
<td>CGACCCAAAGGCGAATGCACCC</td>
<td>Same as that of b′consF1</td>
</tr>
<tr>
<td>VompD-up1</td>
<td>TCAATCTCGTTATACGCTTTTT</td>
<td>To amplify noncoding 5′ region of vompD for Southern probe (Fig. 1B)</td>
</tr>
<tr>
<td>VompD-up2</td>
<td>GCAGCGAAACAGGAGAGAC</td>
<td>Same as that of VompD-up1</td>
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grown separately in Luria-Bertani media containing kanamycin and chloramphenicol, respectively. The following morning, each of these *E. coli* cultures was diluted 1:10 and grown to mid-log phase and then centrifuged, washed three times with M199 medium supplemented with 20% fetal calf serum, 2 mM glutamine, and 110 μg of sodium pyruvate/ml (M199S) (21), and resuspended at a final optical density at 600 nm (OD 600) of 1.0. Two confluent platefuls of *B. quintana* cultures were resuspended in 1 ml of M199S, washed once, and resuspended at a final OD<sub>600</sub> of 3.0 to 5.0. Fifty microliters of each of the two *E. coli* parental strains and of the resuspended *B. quintana* culture were combined and gently mixed on the center of a nonselective chocolate agar plate, and the plate was incubated at 35°C for 5 to 6 h. Transconjugants were then selected on chocolate plates supplemented with kanamycin. The kanamycin-resistant (Kan<sup>R</sup>) transconjugants resulted from the homologous recombination of the pJM06 suicide plasmid into the *B. quintana* chromosome at one of the *vomp*-flanking sequences.

FIG. 1. Wild-type *B. quintana* vomp locus and plasmids utilized in construction of the vomp locus null mutant. (A) Plasmids utilized in the construction of the mutagenic plasmid pJM06, derived from pRS14, are shown. Note that the origin of replication for these plasmids in *B. quintana* is not functional and, thus, pJM06 in *B. quintana* is a suicide plasmid. Plasmid loci shown are as follows: oriT, origin of transfer for conjugation; Kan<sup>R</sup> gene, gene conferring kanamycin resistance; lacI<sup>q</sup>, gene encoding lac repressor; P<sub>taclac</sub>, IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible promoter; rpsL, gene encoding ribosomal S12 protein of the 30S ribosome from *B. henselae*; sacB, gene encoding levansucrase from *Bacillus subtilis*; gfp, gene encoding green fluorescent protein; T<sub>rrnB</sub> to rrnB transcriptional terminator. (B) vomp gene loci of the JK31 wild-type strain and the isogenic vomp null mutant strain. EcoRV sites, Southern blot probes, and primers used in generating and confirming the in-frame deletion of the vomp locus are indicated. Numbers are nucleotide positions.
regions. To verify integration, colony PCR was performed using the primers prJM05 and prJM06 (Fig. 1B).

The Kan' transconjugants from the single-crossover event also contained the sucB gene encoding levansucrase, which is lethal for many gram-negative bacteria when they are grown in the presence of sucrose. This genotype allowed for a two-step mutagenesis strategy for the bloodstream and establish persistent infection in vivo, we inoculated two naı¨ve animals intradermally, one with a total dose of 3.1 × 10⁹ CFU and the other with 5.7 × 10⁹ CFU (mean total dose, 4.4 × 10⁹ CFU) of this null mutant strain, divided among six different inoculation sites on each animal. Blood samples from the animals inoculated with the vomp null mutant strain were cultured using the same methods employed for the animals inoculated with the wild-type strain. The culture results from the two vomp null mutant inoculations were compared statistically with those from the eight wild-type JK31 strain inoculations. We used two-sided Fisher's exact test to assess the statistical significance of the difference in infection rates between B. quintana wild-type and vomp null mutant strains.

In addition, to determine if two vomp genes are sufficient for infection, we inoculated a macaque with strain BQ2-D70, in which only the vompD and vompC genes are present. This BQ2-D70 strain was originally isolated from macaque no. BQ2, which was infected with the wild-type JK31 strain during a previous study. The last culture-positive blood sample from this animal was documented at day 70 postinoculation. During the prolonged, 70-day bloodstream infection, the vompA and vompB genes from the JK31 inoculum strain were deleted in vivo, yielding the strain BQ2-D70. Although no VompA or VompB protein expression in BQ2-D70 can be identified (28), vompC mRNA is detectable by reverse transcriptase PCR (P. Zhang and J. E. Koehler, unpublished data).

RESULTS

A negative selection strategy generates a markerless, non-polar, in-frame deletion of the vomp locus in a wild-type B. quintana background. To study the contribution of the Vomp adhesins to B. quintana pathogenesis, i.e., the ability to invade the bloodstream and establish persistent infection in vivo, we developed a two-step mutagenesis strategy for B. quintana by using SacB negative selection (11) to generate a vomp locus null mutant. The expression of the Bacillus subtilis sacB gene in gram-negative bacteria grown in the presence of sucrose is lethal due to the synthesis of levansucrase, thus permitting a two-step selection for the loss of the integrated plasmid containing sacB. Successful deletion of the vomp locus was confirmed by sequencing of the insert and the plasmid-insert junctions in plJM06 prior to conjugative transfer into B. quintana. The deletion of the vomp locus was then confirmed by PCR and sequencing after transfer into the JK31 wild-type strain. Finally, Southern blotting and immunoblotting confirmed the absence of vomp genes and their protein products, respectively, in the vomp null mutant.

Southern blotting with genomic DNA from the vomp null mutant demonstrates the deletion of the vomp genes. The in-frame vomp deletion was confirmed by Southern blot analysis of EcoRV-digested DNA using two probes, the vompD
The deletion of the vomp genes resulted in the loss of EcoRV restriction endonuclease sites, generating a larger product visualized with the probe for the noncoding 5’ region of the VompD gene (~6.9 kb for the mutant, compared with ~1.8 kb for the wild type). The probe for the conserved region recognized all four vomp genes in JK31 (on fragments of ~2.1, 3.0, 3.3, and 8.8 kb), but these sequences were deleted in the vomp null mutant except for 32 bp of the conserved sequence remaining at the 5’ end of vompD, generating a faint band at ~6.9 kb.

vompD probe  vomp conserved probe

Kb
9.4
6.5
4.3
2.3
2.1
JK31 wild type  JK31 vomp null mutant  JK31 wild type  JK31 vomp null mutant

FIG. 2. The in-frame deletion of the B. quintana vomp locus was confirmed by Southern blotting. The deletion of the vomp locus resulted in the loss of EcoRV restriction endonuclease sites, generating a larger product visualized with the probe for the noncoding 5’ region of the VompD gene (~6.9 kb for the mutant, compared with ~1.8 kb for the wild type). The probe for the conserved region recognized all four vomp genes in JK31 (on fragments of ~2.1, 3.0, 3.3, and 8.8 kb), but these sequences were deleted in the vomp null mutant except for 32 bp of the conserved sequence remaining at the 5’ end of vompD, generating a faint band at ~6.9 kb.

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2D SDS-PAGE and immunoblotting document the absence of Vomp expression in the isogenic vomp null mutant. 2D SDS-PAGE analysis of the vomp null mutant TOMP fraction revealed that the three proteins previously identified as Vomp adhesins by N-terminal sequencing (28) (Fig. 3A), in addition to their smaller degradation products, were absent in the vomp null mutant (Fig. 3B). Immunoblotting of whole-cell lysates from the JK31 wild type and the isogenic vomp null mutant by using antibody to an N-terminal conserved region of VompA, VompB, and VompC confirmed that these proteins were not detectable in the vomp null mutant (Fig. 3C).

The Vomp adhesins are required for autoaggregation. We have shown previously that Vomp expression in wild-type B. quintana mediates autoaggregation (28). In addition, the heterologous expression of VompA, but not VompC, in E. coli is sufficient to confer an autoaggregative phenotype on nonaggregative E. coli (28). We compared the autoaggregative phenotypes of the wild type JK31 and the isogenic vomp null mutant by using tube suspension assays, each performed in triplicate. The vomp null mutant did not autoaggregate, in contrast to the JK31 wild type (Fig. 4). The OD600 of the wild-type JK31 B. quintana strain was significantly lower than that of the mutant strain (P < 0.001) at the final time point (9 h) by Student’s t test.

The presence of all or part of the vomp locus is required to establish B. quintana bloodstream infection in vivo. The homology between the Vomp adhesins and TAA of other gram-negative bacterial pathogens suggested that the Vomp adhesins have an important role in establishing bloodstream

FIG. 3. Vomp expression was not detected in the B. quintana vomp null mutant. (A) VompA, VompB, and VompC proteins were present, each at a mass of ~100 kDa, in the TOMP fraction of the JK31 wild type (bracket and arrow) after separation by 2D SDS-PAGE. VompD was not detectably expressed in the JK31 wild type under these conditions, as shown schematically in Fig. 1B. The conserved probe recognized all four vomp genes in JK31 (on fragments of ~2.1, 3.0, 3.3, and 8.8 kb), but these sequences were deleted in the mutant except for 32 bp of the conserved sequence remaining at the 5’ end of vompD, generating a faint band at ~6.9 kb.

Silver stained 2D SDS-PAGE of TOMP fractions

Immunoblot with anti-Vomp antibody

JK-31 wild type  vomp null mutant  JK31 wild type  vomp null mutant

Increasing pl

Increasing pl

43
83
46
210
111
83
46
210
111

C

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invasion by and persistent infection with *B. quintana*. To test this hypothesis, we utilized our recently developed animal model of *B. quintana* infection in the macaque (28). For the present study, it was first necessary to determine the usual course of infection with wild-type *B. quintana* by inoculating eight animals. Figure 5 shows the mean number of *B. quintana* CFU isolated per milliliter of blood from the eight animals inoculated with wild-type JK31 *B. quintana*. Every animal inoculated with wild-type *B. quintana* became infected and developed detectable bloodstream infection by day 14, with a mean peak bacterial burden of \(1.62 \times 10^3\) CFU/ml and a mean bacteremia duration of 59 days. In contrast, no colonies were recovered from the blood of two naïve animals inoculated with the isogenic *B. quintana* vomp null mutant at any time point in the 7 weeks following inoculation (Fig. 5). The difference in infection rates (0 of 2 animals for the *vomp* null mutant versus 8 of 8 for the wild-type strain) has a \(P\) value of 0.022 by Fisher’s exact test.

To determine if two *vomp* genes are sufficient for infection, we inoculated a naïve macaque with strain BQ2-D70 (derived in vivo from JK31), in which only the *vompD* and *vompC* genes are present in the *vomp* locus but in which no VompD or VompD protein expression can be identified (28). Unlike the *vomp* null mutant, which was incapable of invading and establishing infection in the bloodstream, BQ2-D70 inoculation resulted in bloodstream infection, although the peak number of CFU per milliliter was lower than the mean peak number of wild-type JK31 CFU per milliliter and the time to reach this peak was longer (Fig. 5). Thus, although infection was abrogated by the deletion of the entire *vomp* locus, the presence of only the *vompD* and *vompC* genes was sufficient for bloodstream infection with *B. quintana* in vivo.

**DISCUSSION**

*B. quintana* is an emerging pathogen that has only recently been studied in the laboratory. Few *B. quintana* virulence factors have been identified or described, and there are few techniques for genetic manipulation. The Vomp adhesins are the first *B. quintana* virulence factors identified in vivo, and as members of the TAA family, they are expressed on the surfaces of *B. quintana* cells (28). In most gram-negative bacteria, the TAA is encoded by only one gene (e.g., the *Y. enterocolitica* yadA gene), and the monomer expressed by this gene then forms homotrimers on the bacterial surface. However, *B. quintana* Vomp adhesins are encoded by four *vomp* paralogs, and each adhesin appears to have unique binding specificity. The capacity of each Vomp to bind a different substrate could have evolved through *vomp* gene duplication to permit *B. quintana* binding to host cells in the disparate niches that *B. quintana* must occupy: binding to erythrocytes and endothelial cells in the bloodstream of the mammalian reservoir host and to collagen in the cutaneous lesions of bacillary angiomatosis, as well as to the gastrointestinal epithelium of the body louse vector.

The importance of the TAA in other pathogens, the binding heterogeneity required by *B. quintana*, and the presence of
FIG. 5. The *B. quintana* vomp null mutant was unable to establish bloodstream infection in a rhesus macaque animal model. Eight animals were infected with the *B. quintana* JK31 wild type, and all developed detectable bloodstream infections by day 14, with a mean peak bacterial burden of $1.62 \times 10^7$ CFU/ml, in contrast to those animals infected with the vomp null mutant, from which no colonies were isolated. A naturally occurring *B. quintana* strain derived from JK31 during in vivo infection and containing only two vomp genes (strain BQ2-D70, with only vompD and vompC present) was able to infect a macaque. Thus, the vomp locus is necessary for *B. quintana* infection in vivo, and the vompD and vompC genes are sufficient for infection. Error bars for the eight animals infected with JK31 represent the standard errors of the means. Statistical comparison of BQ2-D70 infection to wild-type infection was not possible because we had only one observation of BQ2-D70 infection.

Four Vomp adhesins led us to hypothesize that the Vomp adhesins are necessary for host infection. Surface proteins that are critical virulence factors are frequently targeted by the host immune system early after infection; in response, the bacterium often modifies the expression of these surface proteins by using phase variation. As additional evidence that the Vomp adhesins are important in vivo, these adhesins demonstrate both these properties: the Vomp adhesins are among the most antigenic of the *B. quintana* outer membrane proteins (5), and the Vomp adhesins undergo phase variation during prolonged host bloodstream infection (28). To test our hypothesis that the Vomp adhesins are necessary for infection in vivo, we used a macaque animal model of infection (28) and quantified the course of infection for eight animals inoculated with *B. quintana* wild-type strain JK31. All eight animals became bacteremic for a prolonged period (Fig. 5).

Next, we developed a two-step mutagenesis strategy for *B. quintana* to create a nonpolar, in-frame, markerless mutation in a fully virulent wild-type background. Such a strategy for * Bartonella* had never before been achieved, and it overcomes a substantial obstacle to *B. quintana* research and will facilitate the genetic manipulation of * Bartonella* species in general. Other * Bartonella* researchers have used transposon insertion (10) or gene disruption from a single-crossover event (4, 8) to generate mutations in *Bartonella* species. However, polar effects and reversion to a wild-type genotype (especially in vivo, with selective pressure against the mutation) limit these approaches. Another mutagenesis method employed a spontaneous streptomycin-resistant (StrR) *Bartonella* isolate (with resistance due to an rpsL mutation) (25, 26), which was used as the parental strain for a two-step targeted allelic replacement.

Although this method permitted in-frame deletion, the resulting mutagenized strain retains the parental StrR rpsL mutant genotype. Unfortunately, every spontaneously occurring *B. quintana* StrR rpsL mutant strain we isolated to use as the parent strain was either avirulent or highly attenuated in our animal model (Zhang and Koehler, unpublished), as has been noted previously for other gram-negative pathogens, e.g., *Salmonella* spp. (16). It was thus necessary to develop a different approach for generating the vomp null mutant.

We successfully constructed the vomp null mutant by using sacB negative selection (11); the strategy we describe provides an important advance in the genetic manipulation of all * Bartonella* species. The resulting isogenic vomp null mutant, in a wild-type background, was used to inoculate two naïve macaques. No infection with the vomp null mutant was detectable (Fig. 5), in contrast to infection with the wild-type *B. quintana* strain. The fact that both vomp null mutant inoculations did not produce infection is unlikely to be due to chance alone ($P = 0.022$). These data strongly suggest that the vomp null mutant is avirulent or that its ability to infect is severely attenuated in vivo, below the threshold of detection by blood culture.

To determine if two vomp genes are sufficient for infection, we inoculated a macaque with strain BQ2-D70, in which only the vompD and vompC genes are present and no VompC or VompD protein expression can be identified (although vompC mRNA is detectable by reverse transcriptase PCR). Interestingly, we previously found that this BQ2-D70 strain is autoaggregation deficient (28), as we have shown here for the vomp null mutant as well. However, unlike the vomp null mutant, which was avirulent in vivo, we found that BQ2-D70 was vir-
ulent in vivo, although the peak number of CFU per milliliter was lower than that of the wild type JK31 and the time to reach this peak was substantially longer (Fig. 5). This result suggests that vompD and/or vompC gene expression was upregulated after animal inoculation or that very low levels of VompD and/or VompC (undetectable by immunoblotting) were sufficient to establish infection with B. quintana. Thus, although infection was prevented by the deletion of the entire vomp locus, the presence of vompD and vompC was sufficient to achieve bloodstream infection.

The function(s) of the Vomp adhesins required for establishing infection in vivo is unknown but likely includes the critical adhesion events observed during B. quintana infection, i.e., binding to erythrocytes and endothelial cells. For Y. enterocolitica, the inactivation of yadA results in a severely attenuated strain that can invade the intestine but cannot disseminate or survive host defenses in a mouse model (22). Furthermore, a Y. enterocolitica mutant with the replacement of two histidyl residues in the adhesin head domain of YadA can be translocated from the intestinal lumen to Peyer’s patches but cannot disseminate to the spleen or survive and multiply in extraintestinal tissues (23). The related TAA from Neisseria meningitidis, NadA, is present in three of the four known hypervirulent lineages, suggesting a role for NadA in invasive meningococcal disease, as well (7). The Vomp adhesins appear to be even more critical for virulence than TAA of other pathogens, because they are necessary for the invasion of the normally sterile bloodstream, where the Bartonella can persist in association with erythrocytes. The four paralogous Vomp adhesins could have evolved to provide the great diversity in adhesin specificity that is essential for B. quintana infection and persistence, enabling adhesion to erythrocytes, endothelial cells, and collagen. The identification of the binding specificity and the environmental signals regulating the expression of each Vomp will provide insight into the pathogenicity of the unusual and persistent pathogen B. quintana.

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2. Reference deleted.

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