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

Joanna K. MacKichan,† Helen L. Gerns, Yu-Ting Chen, Peng Zhang, and Jane E. Koehler*

Microbial Pathogenesis and Host Defense Program and Division of Infectious Diseases, Department of Medicine, University of California at San Francisco, San Francisco, California 94143-0654

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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 *v*ariably expressed *o*uter *m*embrane *p*roteins (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 *Bartonella* 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 (*v*ariably expressed *o*uter *m*embrane *p*roteins), 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-

* Corresponding author. Mailing address: Division of Infectious Diseases, 513 Parnassus Ave., Room S-380, University of California at San Francisco, San Francisco, CA 94143-0654. Phone: (415) 476-3536. Fax: (415) 476-9364. E-mail: jkoehler@medicine.ucsf.edu.

† Present address: Institute of Environmental Science and Research, P.O. Box 50-348, Porirua 5240, New Zealand.

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TABLE 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Description	Source or reference
Strains		
B. quintana strain JK31	Isolated from a bacillary angiomatosis lesion of an AIDS patient	28
B. quintana vomp null mutant	JK31 background with in-frame, markerless deletion of the entire *vomp* locus	This study
B. quintana BQ2-D70	Isolated from animal bloodstream 70 days following experimental inoculation with JK31	28
E. coli strain TOP 10	Cloning strain for TOPO	Invitrogen
E. coli strain DH12S	Cloning strain	Invitrogen
Plasmids		
pCR 2.1-TOPO	TOPO cloning vector; Kan^r Amp^r	Invitrogen
pRS14	Vector for *virB4* mutagenesis; Kan^r *rpsL*; carries inducible *gfp*	26
pJM02	Derivative of pRS14 with the *rpsL* gene and *virB4*-flanking sequences deleted	This study (Fig. 1A)
pJM05	Derivative of pJM02 with *sacB* inserted into SacI sites	This study (Fig. 1A)
pJM06	Derivative of pJM05 with *vomp*-flanking sequences inserted	This study (Fig. 1A)
pJEN34	Contains *sacB* gene; used as a template for PCR	12

ground, which had not been achieved previously. As reported here, we developed a *Bartonella* mutagenesis approach using *sacB* negative selection to generate an in-frame, markerless deletion of the entire *vomp* locus. This strategy created the first targeted, defined deletion mutation in *B. quintana*. Using a rhesus macaque (*Macaca mulatto*) animal model (28), we demonstrated that the deletion of the entire *vomp* locus, but not the deletion of two *vomp* genes, resulted in an avirulent *B. quintana* null mutant strain that was incapable of establishing bloodstream infection in the macaque.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *B. quintana* wild-type strain JK31 (with the entire, four-gene *vomp* locus intact) was isolated from the blood of an AIDS patient with bacillary angiomatosis. Colonies were isolated directly from the blood of this patient and were frozen after one or two agar passages. All subsequent experiments used *B. quintana* JK31 streaked from these one- or two-passage frozen stocks. *B. quintana* strain BQ2-D70 was isolated 70 days postinoculation from the bloodstream of a macaque that was experimentally infected with JK31 (28). *B. quintana* strains were streaked onto chocolate agar plates, the plates were incubated at 37°C in candle extinction jars, and the strains were harvested after 5 to 7 days (21). *E. coli* strains were grown in Luria-Bertani medium at 37°C. When required, kanamycin, chloramphenicol, nalidixic acid, or cefazolin was added at a concentration of 50, 35, 20, or 2 μg/ml, respectively. Bacterial strains and plasmids are listed in Table 1.

Generation of a nonpolar, in-frame deletion mutation of the *vomp* locus in *B. quintana*. (i) Plasmid pJM06 construction. We employed a two-step mutagenesis method using *sacB* negative selection of wild-type *Bartonella* for the first time (11). Primers used for this strategy are listed in Table 2. Plasmids used to generate the mutagenic plasmid pJM06 are shown in Fig. 1A. pJM06 was derived from plasmid pRS14, kindly supplied by C. Dehio (26). The *rpsL* gene and the sequence homologous to the *virB4*-flanking regions were excised from pRS14 to generate plasmid pJM02. A 2.6-kb fragment containing *sacB* from *Bacillus subtilis* was amplified with primers SacBS-F and SacBS-R from plasmid pJEN34 (generously provided by J. Engel) (12). This PCR product was cloned into TOPO TA (Invitrogen, Carlsbad, CA) and sequenced, and the construct was assayed for SacB function. The *sacB* fragment was then removed from TOPO TA and cloned into pJM02, creating pJM05 (Fig. 1A).

Plasmid pJM06 was derived by the insertion of a 1.6-kb fragment of the sequences flanking the *vomp* locus into pJM05. This insert was constructed by PCR "megapriming" from two PCR products amplified from JK31 genomic DNA, similar to the method described by Schulein and Dehio (26). The two PCR products amplified together to create this insert were PCR product 1 (0.85 kb; amplified with primers prJM02 and prJM05 [Fig. 1B] and including the first 159 bp of the *vompD* gene at the 5′ end) and PCR product 2 (0.83 kb; generated with primers prJM03 and prJM06 [Fig. 1B] and including 136 bp of the *vompC* gene 3′ end). The 1.6-kb PCR product was gel purified and cloned into TOPO TA, sequenced, and cloned into the BamHI site of plasmid pJM05, resulting in pJM06 (Fig. 1A).

(ii) Two-step selection strategy for *vomp* locus deletion in *B. quintana*. Plasmid pJM06 was transferred into wild-type *B. quintana* JK31 by triparental conjugation. Overnight cultures of two different *E. coli* parental strains, one containing plasmid pJM06 and the other containing the helper plasmid pRK600, were

TABLE 2. Oligonucleotide primers

Name	Sequence	Function
SacBS-F	GGGGAGCTCGATCCTTTTTAACCCATCACATATAC	To amplify *sacB* from pJEN34; SacI site underlined
SacBS-R	GGGGAGCTCAAAAGGTTAGGAATACGGTTAGCC	To amplify *sacB* from pJEN34; SacI site underlined
prJM02	TTGGATAAGGACCATCAGTGC	To amplify 5′ flanking region of *vomp* locus; fully complementary to prJM03
prJM03	GCACTGATGGTCCTTATCCAAGGCGTAGTCAAGGTGCATTT	To amplify 3′ flanking region of *vomp* locus; half of primer is complementary to prJM02
prJM05	GCGGGATCCGATCGCAAATTGTGACGTTTTT	To amplify 800 bp of 5′ flanking region of *vomp* locus (with prJM02); BamHI site underlined
prJM06	GCGGGATCCCATTTTGCTAAATCATTTCTCTGA	To amplify 800 bp of 3′ flanking region of *vomp* locus (with prJM03); BamHI site underlined
b'consF1	GCTATGACCGAGATGAGCATGGG	To amplify conserved region present in 3′ region of each *vomp* gene for Southern probe (Fig. 1B)
b'consR1	CGCACCAAAGGCAAATGCACC	Same as that of b'consF1
VompD-up1	TCATTCTGCTTATGCGCTTT	To amplify noncoding 5′ region of *vompD* for Southern probe
VompD-up2	GCAGCGAAAACAGGAGAGAC	Same as that of VompD-up1

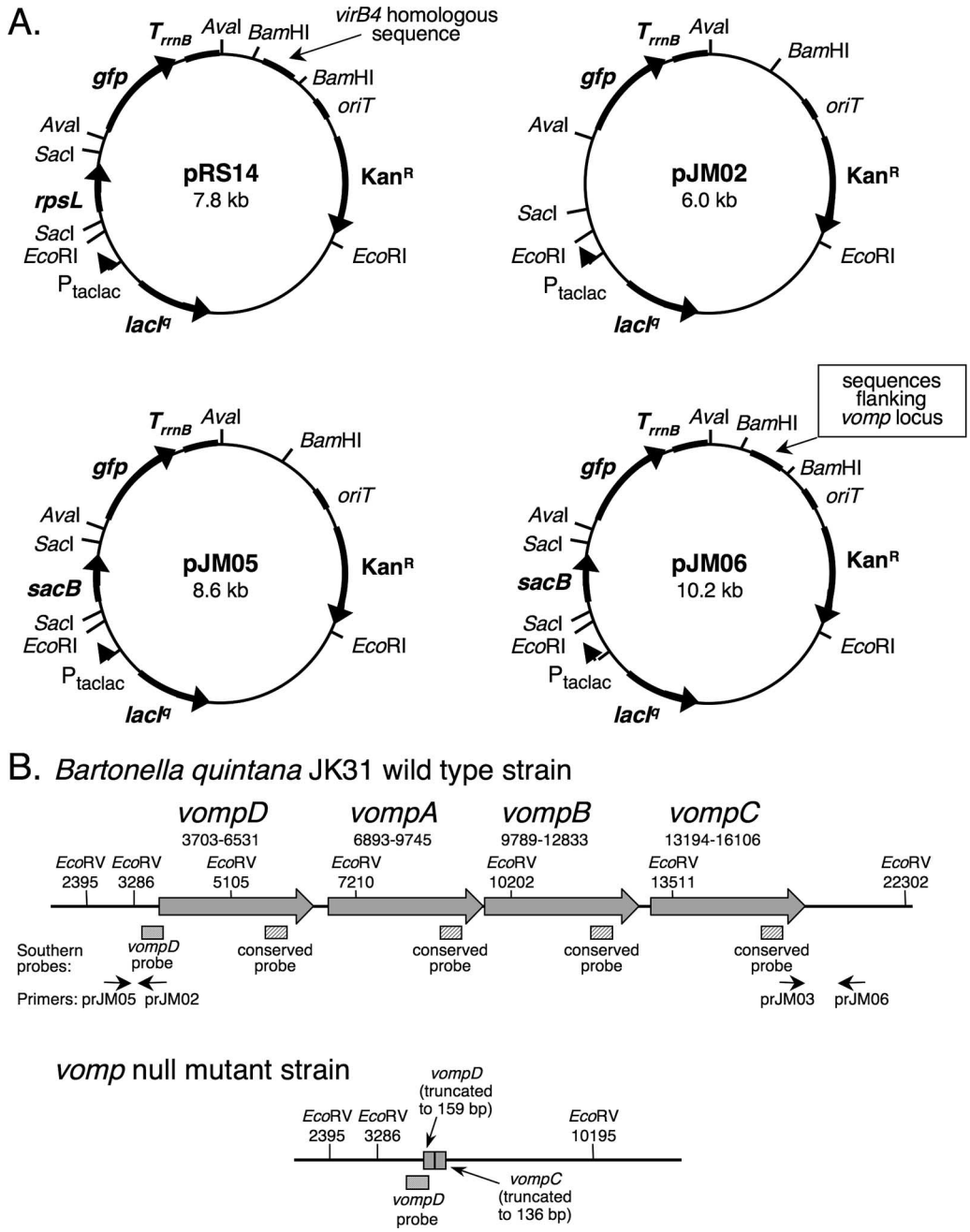

FIG. 1. Wild-type *B. quintana vomp* locus and plasmids utilized in construction of the *vomp* locus null mutant. (A) Plasmids utilized in the construction of the mutagenic plasmid pJM06, derived from pRS14, are shown. Note that the origin of replication for these plasmids in *B. quintana* is not functional and, thus, pJM06 in *B. quintana* is a suicide plasmid. Plasmid loci shown are as follows: *oriT*, origin of transfer for conjugation; Kan^r gene, gene conferring kanamycin resistance; *lacI*^{q}, gene encoding *lac* repressor; p_{taclac}, 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_{rrnB}, *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.

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_{600} 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^r) transconjugants resulted from the homologous recombination of the pJM06 suicide plasmid into the *B. quintana* chromosome at one of the *vomp*-flanking

regions. To verify integration, colony PCR was performed using the primers prJM05 and prJM06 (Fig. 1B).

The Kan^r transconjugants from the single-crossover event also contained the *sacB* 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 subsequent negative selection step, i.e., selection for the loss of the integrated plasmid containing *sacB*. Transconjugants were grown on chocolate agar containing 5% sucrose to select for the loss of the integrated plasmid through a second homologous crossover event. Colonies that lost the integrated plasmid became sucrose resistant (Suc^r) and kanamycin sensitive (Kan^s), whereas colonies that had undergone spontaneous *sacB* inactivation became Suc^r and Kan^r. To distinguish between these two outcomes, colonies were replica plated onto kanamycin plates to exclude colonies that had become Suc^r through spontaneous *sacB* inactivation. Kan^s Suc^r colonies carried either the wild-type allele (reconstitution) or an in-frame deletion of the *vomp* locus (gene replacement). Colony PCR, using primers prJM05 and prJM06, allowed discrimination between these different outcomes, and one *vomp* null mutant colony was chosen for further verification and experimental evaluation.

Southern blotting with genomic DNA from the JK31 wild-type and *vomp* null mutant *B. quintana* strains. Genomic DNA from JK31 or the *vomp* null mutant was digested with EcoRV and subjected to Southern blotting using conventional methods (24), except that probes were labeled with the AlkPhos direct labeling system (Amersham, Piscataway, NJ). Two PCR-amplified probes were used (Fig. 1B): the conserved probe, consisting of a 446-bp fragment conserved in the 3′ region of all *vomp* genes (constructed with primers b'consF1 and b'consR1), and the *vompD* probe, consisting of a 446-bp fragment from the 5′ noncoding region upstream of *vompD* (constructed with primers VompD_up1 and VompD_up2).

Two-dimensional (2D) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separation of the *B. quintana* TOMP fraction. Subcellular protein fractions of *B. quintana* strains were prepared as previously described (17), with the following modifications. Bacteria were harvested from chocolate agar plates, washed twice with phosphate-buffered saline, and pelleted in a microcentrifuge for 2 to 3 min at 4°C. The final pellet was resuspended in 10 mM HEPES buffer. Protease inhibitor cocktail {20 mM AEBSF [4-(2-aminoethyl)-benzenesulfonyl fluoride; Calbiochem]; 1 mg of leupeptin/ml, 0.36 mg of E-64/ml, and 5.6 mg of benzamidine/ml (all from Sigma); and 50 mM EDTA} was added, after which the suspension was incubated on ice for 10 min. Bacterial cells were sonicated with five 1-min bursts while cooling on ice. Cellular debris was pelleted at 4,300 × *g* for 30 min at 4°C. The supernatant was then centrifuged at 100,000 × *g* with a Beckman L8-M ultracentrifuge for 1.5 h at 4°C, and the supernatant was discarded. The pellet, comprising the total outer membrane protein (TOMP) fraction, was resuspended in 10 mM HEPES and treated with nuclease (50 mM $MgCl_2$, 100 mM Tris [pH 7.0], 500 μg of RNase [Sigma]/ml, and 1 mg of DNase [Sigma]/ml). The TOMP fraction was then washed twice in 10 mM HEPES and pelleted at 40,000 × *g* for 30 min at 4°C. The protein concentration was determined, and fractions were frozen at −80°C until use.

2D SDS-PAGE was performed according to the method of O'Farrell (20) (Kendrick Labs, Inc., Madison, WI), as follows: isoelectric focusing was carried out in glass tubes with an inner diameter of 2.0 mm by using 2% ampholines, pH 4 to 8 (BDH; Hoefer Scientific Instruments, San Francisco, CA), for 9,600 V · h. After equilibration for 10 min in buffer O (10% glycerol, 50 mM dithiothreitol, 2.3% SDS, and 0.0625 M Tris, pH 6.8), tube gels were laid on top of 10% acrylamide slab gels (0.75 mm thick) and SDS slab gel electrophoresis was carried out for 4 h at 12.5 mA/gel. Gels were stained with silver stain (19).

Immunoblotting with antibody to the conserved N-terminal domain of *B. quintana* VompA, VompB, and VompC. For immunoblotting, whole-cell lysates were prepared as described for subcellular preparations (up to the ultracentrifugation step) and the lysate was separated using SDS–PAGE with 12% acrylamide. Proteins were transferred onto 0.45-μm-pore-size Protran nitrocellulose (Schleicher & Schuell, Keene, NH) and probed with rabbit polyclonal antibody to the N-terminal conserved region in VompA, VompB, and VompC, followed by alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (Zymed, South San Francisco, CA).

Autoaggregation assay of *B. quintana* JK31 wild-type and isogenic *vomp* null mutant strains. Autoaggregation was assayed using the method described by Laird and Cavanaugh (14), with the following modifications. *B. quintana* was harvested, washed, and resuspended at an OD_{600} of 1.0 in M199S. A 3-ml aliquot of each *B. quintana* suspension was added to plastic test tubes, and the tubes were incubated in a CO_2-enriched atmosphere at 35°C. To quantify autoaggregation, a 50-μl sample from the top of each culture tube was removed after 1, 2, 6, and 9 h and the OD_{600} was measured immediately. Samples were assayed in triplicate, and statistical analyses were performed using Student's *t* test.

Inoculation of rhesus macaques with *B. quintana* strains to assess the ability of *vomp* mutants to establish bloodstream infection. We recently developed an animal model of *B. quintana* infection in rhesus macaques (*Macaca mulatto*) that reproduces the prolonged, high-titer bacteremia observed in humans infected with *B. quintana* (28). No other mammalian host (except humans) is permissive for infection with *B. quintana*. The animal work was performed at the California National Primate Research Center at the University of California, Davis, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. The housing and handling of the animals were in accordance with the *Guide for the Care and Use of Laboratory Animals* (18) and the Animal Welfare Act. The experimental protocol was approved by the University of California, Davis, Animal Use and Care Administrative Advisory Committee.

For this study, the macaque animal model enabled us first to establish the course of infection with wild-type *B. quintana* in eight animals. Each of the eight macaques was inoculated intradermally with a total dose of the wild-type JK31 strain ranging from 3.5×10^6 to 1.78×10^9 CFU (the mean total dose for the eight animals was 2.5×10^8 CFU). The total dose inoculated into each animal was divided among six different inoculation sites on the animal. Blood from each animal was cultured prior to inoculation and then twice weekly for 4 to 6 weeks after inoculation and weekly for another 2 to 4 months, as previously described (28).

To assess the ability of the isogenic *B. quintana vomp* null mutant to infect a permissive host, we inoculated two naïve animals intradermally, one with a total of 3.1×10^9 CFU and the other with 5.7×10^9 CFU (mean total dose, 4.4×10^9 CFU) of this null mutant strain, divided among six different 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, and 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 VompC or VompD 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, nonpolar, 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 pJM06 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*

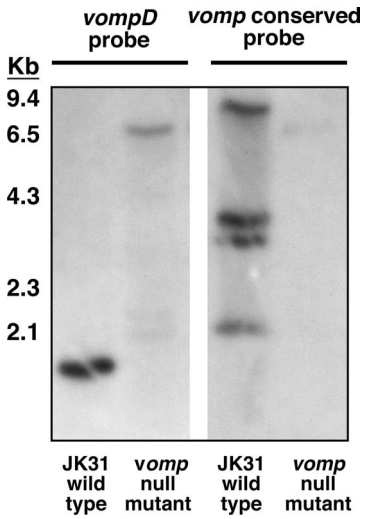

FIG. 2. The in-frame deletion of the *B. quintana vomp* locus was confirmed by Southern blotting. The deletion of the *vomp* locus resulted in the loss of EcoRV restriction endonuclease sites, generating a larger product visualized with the probe for the noncoding 5′ region of the VompD gene (~6.9 kb 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.

probe and the conserved probe (Fig. 2). The deletion of the *vomp* genes resulted in the loss of EcoRV restriction endonuclease sites, generating a larger product in the isogenic *vomp* null mutant identified with the *vompD* probe (~6.9 kb for the mutant, compared with ~1.8 kb for the wild type), 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 also present in the conserved probe sequence, producing only a faint band at ~6.9 kb. Note that the location of the most 3′ EcoRV site was identified using the published genome sequence of the *B. quintana* Toulouse strain (1); the precise location of this EcoRV site in the *B. quintana* strain JK31 genome has not yet been determined but is likely to be conserved.

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 OD_{600} 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

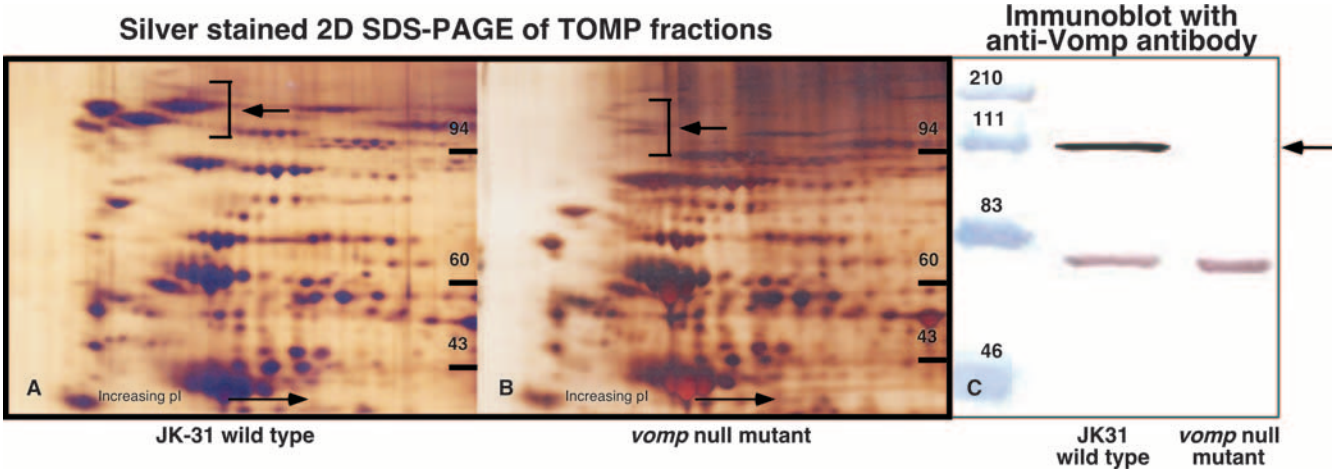

FIG. 3. Vomp expression was not detected in the *B. quintana vomp* null mutant. (A) VompA, VompB, and VompC proteins were present, each at a mass of ~100 kDa, in the TOMP fraction of the JK31 wild type (bracket and arrow) after separation by 2D SDS-PAGE. VompD was not detectably expressed in the JK31 wild type under these conditions. (B) Vomp adhesins were absent in the *vomp* null mutant TOMP fraction separated by 2D SDS-PAGE (bracket and arrow). (C) Immunoblotting of whole-cell lysates from the JK31 wild type and the *vomp* null mutant with an antibody to an N-terminal conserved region present in VompA, VompB, and VompC revealed that the anti-Vomp antibody binds to a band with a mass of ~100 kDa in the JK31 wild-type strain. However, no Vomp adhesins were detectable in the *vomp* null mutant strain (arrow at right).

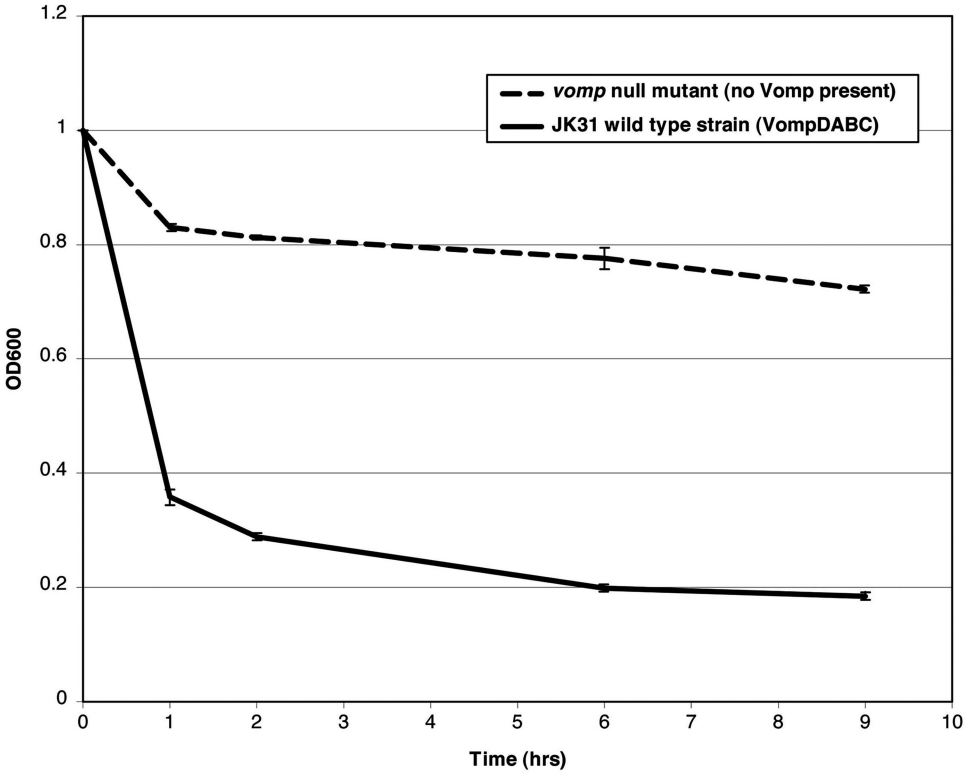

FIG. 4. The *B. quintana* Vomp adhesins mediate autoaggregation. Although the JK31 wild-type strain autoaggregated, the *vomp* null mutant did not autoaggregate. Each experiment was performed in triplicate, and representative results from one experiment are shown. Error bars represent the standard errors of the means.

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 1.62×10^3 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 VompC 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

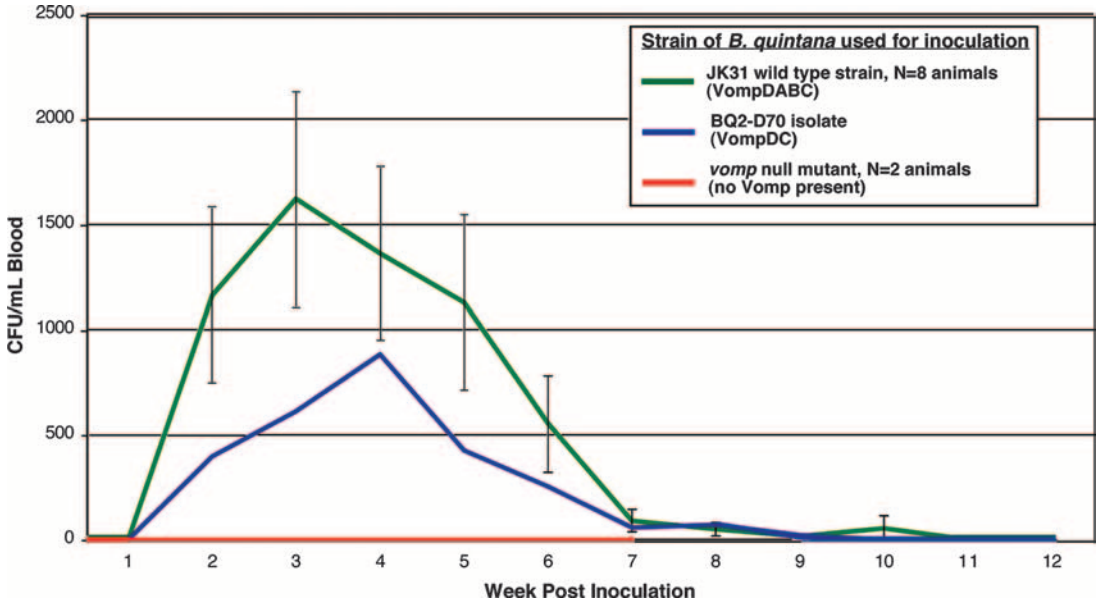

FIG. 5. The *B. quintana vomp* null mutant was unable to establish bloodstream infection in a rhesus macaque animal model. Eight animals were infected with the *B. quintana* JK31 wild type, and all developed detectable bloodstream infections by day 14, with a mean peak bacterial burden of 1.62×10^3 CFU/ml, 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 (Str^r) *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 Str^r *rpsL* mutant genotype. Unfortunately, every spontaneously occurring *B. quintana* Str^r *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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