

The *Bartonella vinsonii* subsp. *arupensis* Immunodominant Surface Antigen BrpA Gene, Encoding a 382-Kilodalton Protein Composed of Repetitive Sequences, Is a Member of a Multigene Family Conserved among *Bartonella* Species

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***Bartonella* proteins that elicit an antibody response during an infection are poorly defined; therefore, to characterize antigens recognized by the host, a *Bartonella* genomic expression library was screened with serum from an infected mouse. This process led to the discovery of a *Bartonella vinsonii* subsp. *arupensis* gene encoding a 382-kDa protein, part of a gene family encoding large proteins, each containing multiple regions of repetitive segments. The genes were termed *brpA* to *-C* (bartonella repeat protein) and bore significant similarity to genes encoding the BadA adhesin protein and members of the variably expressed outer membrane protein family of proteins from *Bartonella henselae* and *Bartonella quintana*, respectively.**

Microbes belonging to the genus *Bartonella* are gram-negative, facultative intracellular, bacillus-shaped bacteria characterized by an ability to invade and parasitize erythrocytes and endothelial cells and are recognized as agents of emerging diseases of humans and animals (4, 11, 21). Important in human disease are *Bartonella bacilliformis*, the causative agent of Carrion's disease; *Bartonella henselae*, the causative agent of cat scratch disease and bacillary angiomatosis; and *Bartonella quintana*, the causative agent of trench fever and also associated with bacillary angiomatosis. Other *Bartonella* species that have been isolated from humans include *B. elizabethae* (17), *B. vinsonii* subsp. *arupensis* (49), *B. vinsonii* subsp. *berkhoffii* (41), and *B. washoensis* (27).

Bartonella infections are associated with arthropod vector transmission; e.g., *B. bacilliformis* is transmitted by sand flies, *B. henselae* by cat fleas, and *B. quintana* by the human body louse. There is recent evidence of *Bartonella* association with ticks, fleas, and flies (10, 14, 15, 46). Furthermore, several distinct *Bartonella* strains have been isolated from various rodent (5, 7, 8, 22, 23, 29) and ruminant (19, 6, 13) species throughout the world. Because of the ubiquitous nature of *Bartonella* and their association with arthropod vectors, this genus has been implicated as a potential causative agent in diverse disease manifestations seen in humans for which there have been no definitive clinical diagnoses. Our laboratory was interested in exploring whether the prevalence of *Bartonella* residing in wild-mammal populations may have had an association with unexplained human febrile illnesses in the North

American Southwest, as there was some serological evidence suggesting such a link (M. Y. Kosoy et al., Abstr. Am. Soc. Rickettsiol. Bartonella Emerg. Pathog. Group 2001 Joint Conf., abstr. 108, 2001; F. Koster et al., Abstr. Am. Soc. Rickettsiol. Bartonella Emerg. Pathog. Group 2001 Joint Conf., abstr. 133, 2001). Moreover, a *Bartonella* (*B. washoensis*) strain isolated from a Nevada patient with myocarditis was shown to be identical to strains cultured from ground squirrels in the same area (27). We wished to further investigate the putative role of rodent-associated *Bartonella* in human infections and, as an initial step, to identify *Bartonella* species-specific antigens that elicit antibody responses during infection. The goal was to initiate a primary analysis of immunodominant antigens related to rodent-borne *Bartonella* and to further develop a more detailed comparative analysis of immunogenic proteins from human *Bartonella* pathogens to define genus-specific antigens versus species-specific antigens. As a result of these aims, this report describes a novel immunodominant, surface-associated protein from *B. vinsonii* subsp. *arupensis* encoded by one of the largest bacterial genes yet identified. Additionally, at least two more related genes are present upstream, forming a multigene family. The genes in this family are characterized by multiple internal repetitive regions that are conserved within and among the individual genes, which we have therefore termed *brp* (bartonella repeat protein).

Identification of the *brp* gene complex. Mice were infected with live *Bartonella* strains isolated from rodent species to generate antibodies that could be used to clone and characterize genes encoding proteins associated with infection. Strains na19103nm (isolated from *Neotoma albigula* [wood rat] in New Mexico), Pm15590co and Pm136co (isolated from *Peromyscus maniculatus* [deer mouse] in Colorado), and Sb944nv (isolated from *Spermophilus beecheyi* [California ground squirrel] in Nevada) were obtained from Michael Kosoy, Division of Vector-Borne Infectious Diseases, Centers for Disease Control and

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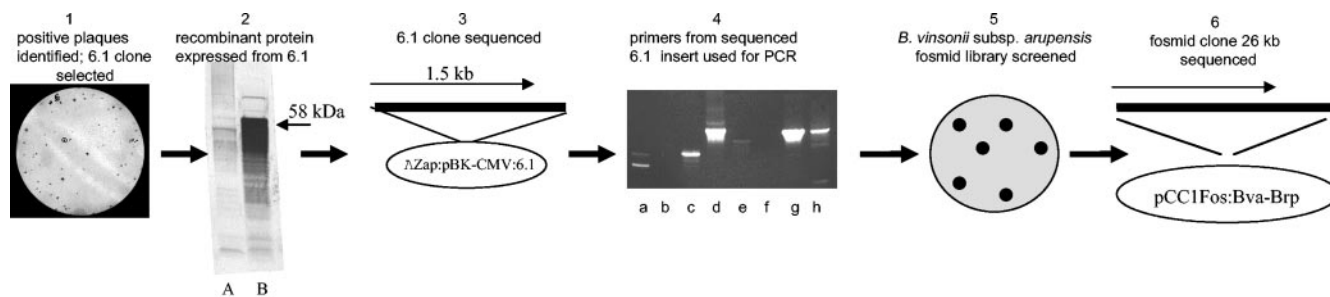


FIG. 1. Flow chart of steps describing the identification of the initial 6.1 clone through the DNA sequencing of the 26-kb Bva-Brp fosmid clone. (1) *Bartonella* strain na19103nm genomic lambda library screened with antiserum from a mouse infected with *Bartonella* strains isolated from *Peromyscus* and *Spermophilus* reveals several positive plaques. A positive clone, 6.1, was selected for further characterization. (2) Western blot of the 58-kDa recombinant protein expressed by clone 6.1 reacted with the antiserum used in the library screening in step 1. Lane A, *Escherichia coli* with expression vector with no insert; lane B, *E. coli* with the 6.1 cloned insert. (3) The 1.5-kb insert of 6.1 is sequenced. Repeat regions are recognized. (4) PCR primers are synthesized from the 6.1 termini and used for amplification from *Bartonella* species genomic DNA. A similar-sized amplicon is observed from *B. vinsonii* subsp. *arupensis* (lane d). Lanes: a, *B. quintana*; b, *B. henselae*; c, *B. vinsonii* subsp. *berkhoffii*; d, *B. vinsonii* subsp. *arupensis*; e, *B. elizabethae*; f, *B. bacilliformis*; g, strain na19103 nm; h, clone 6.1. (5) *B. vinsonii* subsp. *arupensis* fosmid library is hybridized with the clone 6.1 probe to identify large inserts harboring analogous region. Positive clones are selected for analysis. (6) A *B. vinsonii* subsp. *arupensis* fosmid clone containing a 26-kb insert, Bva-Brp, was sequenced and analyzed.

Prevention, Fort Collins, Colorado. *B. henselae*, *B. quintana*, *B. elizabethae*, *B. vinsonii* subsp. *berkhoffii*, *B. vinsonii* subsp. *arupensis*, and *B. bacilliformis* were purchased from the American Type Culture Collection, Manassas, Va. *Bartonella* strains were cultivated on Trypticase soy agar supplemented with 5% sheep blood. Plates were incubated at 37°C in 5% CO₂ until growth was visible, usually between 5 and 10 days. *B. bacilliformis* was incubated at 26°C under ambient atmosphere. Antiserum was generated from mice infected with *Bartonella* as follows. Female BALB/cJ mice (The Jackson Laboratory, Bar Harbor, Maine) were inoculated intraperitoneally with live *Bartonella* strain Pm15590co resuspended in 0.5 ml of phosphate-buffered saline (PBS) following harvesting from a plate culture. A booster injection with live Pm15590co was given 5 weeks after the primary injection, followed by a third booster 6 weeks later with strain Sb944nv. The mice were bled 3 weeks later and provided a source of anti-*Bartonella* polyclonal antiserum. Polyclonal antibodies against each of strains Sb944nv and Pm136co were generated in a similar fashion. The rodent-associated strains of *Bartonella* were used in this initial experiment because there was an interest in identifying specific antigens related to these types of *Bartonella* infections.

The progression from initially finding an immunoreactive clone from a rodent *Bartonella* strain genomic library to identifying the *brp* gene complex in *B. vinsonii* subsp. *arupensis* is summarized in Fig. 1. A positive expression clone, termed 6.1, was identified from the *Bartonella* strain na19203nm genomic library using the mouse antiserum described in the previous paragraph. Clone 6.1 contained a 1.5-kb insert and expressed a recombinant protein product of 58 kDa that reacted in an immunoblot with the same antibody used in the screening process (Fig. 1). DNA sequencing of the 6.1 insert revealed a truncated gene with no start or stop codon at the 5' or 3' end, respectively. To extend and obtain the DNA sequence of the 5' region of the 6.1 clone, probes specific to that area were used for hybridization to the genomic library, which yielded several new clones that were sequenced. It was found that these clones, although identical at the hybridizing ends, were entirely different from one another as the sequences extended. This

was the first evidence that this gene was composed of repeat regions and/or was part of a paralogous gene family.

A PCR primer pair was designed from sequences at each end of clone 6.1, and amplification of homologous regions present in other *Bartonella* spp. was attempted. Only *B. vinsonii* subsp. *arupensis* produced an amplicon of the same size as the rodent *Bartonella* clone 6.1 (Fig. 1). At this juncture, the decision was made to complete the molecular cloning of this gene using genomic DNA from *B. vinsonii* subsp. *arupensis* for four reasons: (i) PCR amplification using *B. vinsonii* subsp. *arupensis* DNA and primers generated from clone 6.1 produced an amplicon of the same size, indicating a gene relatedness; (ii) there is a close phylogenetic relationship between *B. vinsonii* and *Bartonella* rodent isolates (28, 30); (iii) subspecies of *B. vinsonii* have been shown to infect humans (41, 49); and (iv) the closely related genomes of *B. henselae* and *B. quintana* were being sequenced (and have since been released [2]); therefore, a duplication of effort was avoided.

Genomic DNA was purified from *Bartonella* strains for Southern blot analysis by phenol-chloroform extraction according to standard methods. Two μ g of genomic DNA was digested to completion with BamHI or XbaI restriction enzymes (New England Biolabs, Waverly, Mass.) and fractionated on a 0.8% Tris-acetate-EDTA agarose gel, whereby the gel was depurinated, denatured, and neutralized by respective soaking in 0.25 M HCl; 0.5 M NaOH, 1.5 M NaCl; and 0.5 M Tris, pH 7.5, 1.5 M NaCl. Hybridization was performed utilizing the AlkPhos direct labeling reagents (Amersham Biosciences, Little Chalfont, Buckinghamshire, England), and the hybridization probe was the 1.5-kb PCR amplicon amplified from *B. vinsonii* subsp. *arupensis* genomic DNA using primers designed from the 6.1 clone of strain na19103nm (Fig. 1). The forward primer, 6.1F, was 5' ATTGCCATTGGTAAGGAAA CAGGC 3'. The reverse primer, 6.1R, was 5' TTGTAAGGG CACTGACCTCTTCTG 3'. Southern blot analysis revealed orthologs of this gene present in various *Bartonella* species known to infect humans (Fig. 2). Hybridizing bands were present in *B. henselae*, *B. elizabethae*, *B. vinsonii* subsp. *arupensis*, and *B. vinsonii* subsp. *berkhoffii*, with less prominent hy-

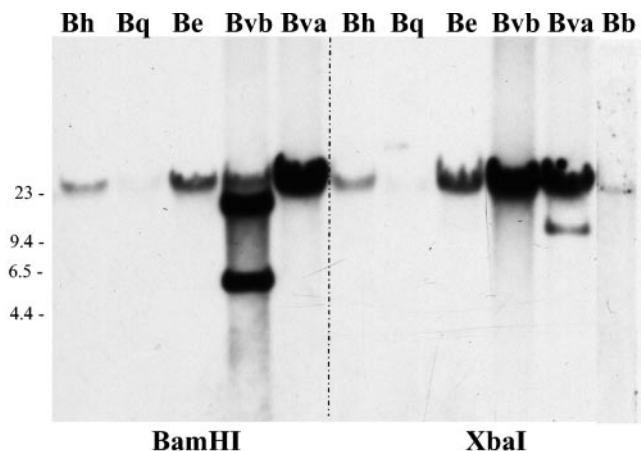


FIG. 2. Southern blot of *Bartonella* spp. genomic DNA. Genomic DNA samples were digested with either BamHI or XbaI as indicated and hybridized to the clone Bva-6.1 probe (for the location within the gene, see Fig. 3A). Bh = *B. henselae*, Bq = *B. quintana*, Be = *B. elizabethae*, Bvb = *B. vinsonii* subsp. *berkhoffii*, Bva = *B. vinsonii* subsp. *arupensis*, Bb = *B. bacilliformis*. Molecular weight markers in kb are noted on the left. A 23- to 25-kb weakly hybridizing band for *B. quintana* is present.

bridizing bands in *B. quintana* and *B. bacilliformis*. A single distinct *B. vinsonii* subsp. *arupensis* BamHI fragment of approximately 25 kb was observed in the Southern blot; therefore, a fosmid library was constructed with that genomic DNA to ensure a cloned insert large enough to harbor the entire gene plus the flanking regions. A *B. vinsonii* subsp. *arupensis* fosmid library was created with the CopyControl fosmid library production kit (Epicentre, Madison, Wis.). Purified genomic DNA was randomly sheared, and fragments were size selected from 25 kb to 40 kb. The sheared DNA was blunt ended and ligated into the fosmid vector, pCC1FOS, and packaged using MaxPlax lambda packaging extracts (Epicentre). Packaged phage particles were allowed to infect *E. coli* EPI300-T1 (Epicentre), with subsequent colony plating on chloramphenicol selection plates. Three positive fosmid clones were selected by colony hybridization, analyzed by BamHI restriction digestion, and rehybridized with the 1.5-kb probe to ensure the presence of the band seen in the Southern blot. Once confirmed, one of the clones containing a 26-kb insert, Bva-Brp (Fig. 1), was sequenced in its entirety. Purified Bva-Brp fosmid DNA was sequenced by primer walking on both strands to an average fourfold redundancy. Electrophoresis was performed using ABI (PE Biosystems, Foster City, Calif.) Big-Dye 3.1 dye chemistry and an ABI 3730XL automated DNA sequencer. Chromatogram data were analyzed using the program Phred (University of Washington) for base calling and quality assessment. Consensus sequence assembly was performed using the program Phrap, with manual editing being performed using the program Consed. Additional sequencing analyses were performed using SeqManII and MegAlign (DNASTAR, Madison, Wis.) for assembly, DNA and protein alignments, and dot plot matrix analyses of repetitive regions. Protein and DNA GenBank database search and alignments were performed using BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>).

DNA sequence of the *B. vinsonii* subsp. *arupensis* *brpA* gene

and flanking regions. The Bva-Brp insert was 26.4 kb and consisted of the complete gene encoding the immunoreactive protein (*brpA*), two large open reading frames located upstream of this gene (*brpB* and *brpC*), and four smaller genes located downstream (Fig. 3A). The largest gene, *brpA*, had a sequence of 10,863 bp encoding a protein of 3,619 amino acids with a deduced molecular mass of 382.5 kDa. *brpB*, upstream of *brpA*, had a sequence of 5,283 bp encoding a protein of 185 kDa. The Bva-Brp clone contained a truncated *brpC* gene upstream of *brpB* but nevertheless indicated a large gene, as the partial version is 4,263 bp long and encodes a truncated protein of 152 kDa. The number of contiguous genes belonging to this family remains to be determined, as the 26-kb fragment was not of sufficient size to contain the entire *brpC* gene plus others that may be located upstream. The single hybridizing band seen in the Southern blot suggested that there may be few or no more *brp*-like sequences residing elsewhere in the chromosome, although one cannot rule out the possibility of more *brp*-like genes or pseudogenes that did not hybridize with the probe.

Based on the DNA sequence analysis, the genes *brpA* and *brpB* appear to be independently transcribed genes and not part of a multicistronic locus or nontranscribed pseudogenes because of the spacing between the coding sequences and the characteristic promoter elements upstream of the coding sequence (Fig. 3A). *brpB* and *brpC* show inverted repeats characteristic of transcriptional terminators downstream of the stop codon; however, *brpA* may be cotranscribed with the hypothetical protein gene immediately downstream as there is only a 2-bp separation between these two coding sequences. The deduced amino acid sequences at the N termini of BrpA and BrpB have properties consistent with signal peptides cleavable by signal peptidase I (SPase I) (Fig. 3B) (38). These peptides begin with an arrangement of charged residues (lysine or arginine) at the N terminus, followed by a central region(s) of hydrophobic amino acids and the SPase I cleavage site (−3 [AGS], −1 [AGS]). BrpA and BrpB are predicted to have cleavable signal peptides by the SignalP program (Technical University of Denmark; <http://www.cbs.dtu.dk/services/SignalP/>). The presence of putative signal peptides thereby provide evidence that the Brp proteins are exported to the cell surface.

A search of the GenBank protein database with BrpA found the most significant match with portions of a *B. henselae* protein encoded by a gene termed *badA1*, submitted as part of the genome sequencing project recently reported by Alsmark et al. (2). The gene product, BadA, has recently been described as a 340-kDa surface protein adhesin of *B. henselae* that mediates proangiogenic responses in infected cells (40). The Clustal W alignment program of DNASTAR indicated there was a 35.9% amino acid identity between BadA and BrpA. Portions of BrpA also shared significant homology with a recently described *B. quintana* family of four variably expressed outer membrane proteins (Vomps), A to D (51). The *B. quintana* Vomps, like BadA, also function as adhesins and mediate autoaggregation. The Vomp gene family encodes proteins of approximately 100 kDa, smaller than the Brp family. Both the *B. quintana* Vomps and *B. henselae* BadA contain repeated regions and were shown to be immunogenic, consistent with properties observed with the *B. vinsonii* subsp. *arupensis* Brp proteins. The *B. henselae* *badA1* and the *B. quintana*

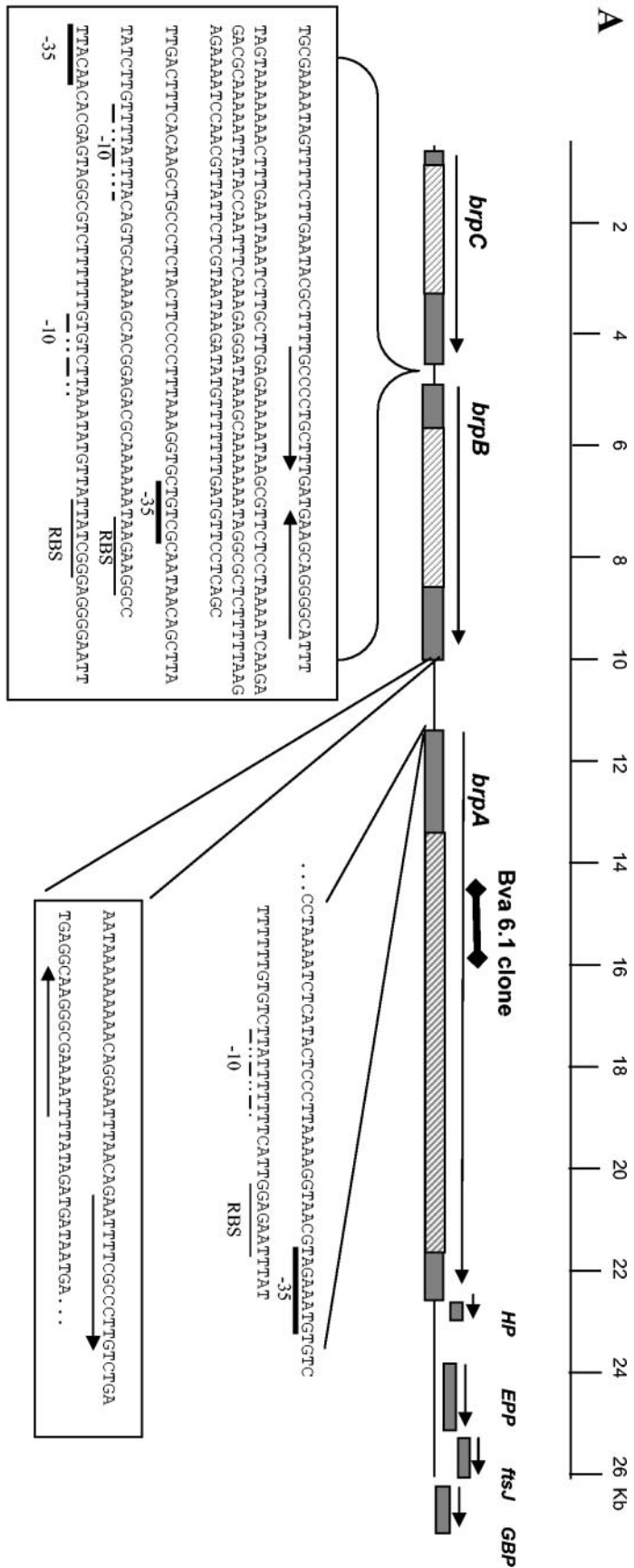


FIG. 3. Organization of genes on the 26-kb Bva-Brp clone of *B. vinsonii* subsp. *arupensis*. (A) *brpA*, *brpB*, and *brpC* coding sequences are represented by rectangular bars, with the arrows showing the direction of transcription. The hatched bars within the coding sequences denote portions of the genes composed of repetitive sequences. Genes downstream of *brpA* are abbreviated as HP (hypothetical protein), EPP (exopolysphatase), *ftsJ* (cell division protein), and GBP (GTP-binding protein). The 409-bp intervening sequence between the stop codon of *brpC* and the start codon of *brpB* is shown. The bidirectional arrows indicate inverted repeats characteristic of transcriptional termination sites for *brpC*. Two putative *brpB* promoter sites based on consensus sequences for the ribosome binding site (RBS) (underlined), the -10 site (underlined with a dot-and-dash line), and the -35 site (heavily underlined line) are denoted. The region immediately downstream of the stop codon for *brpB* is shown with inverted repeats illustrated with the bidirectional arrows. The immediate upstream noncoding region is shown for *brpA* with putative RBS, -10, and -35 sites. The location of the original truncated Bva-6.1 clone that generated the recombinant protein used in this study is shown above *brpA*. A scale in kbp is above the diagram. (B) Signal peptides deduced from the amino acid sequences for *brpA* and *brpB*. Charged residues are italicized, the hydrophobic central region is in bold, and putative S-Pase I cleavage sites are underlined. Cleavage sites predicted by the SignalP program follow the underlined VNA and VVA of BrpA and BrpB, respectively.

BrpA MKKL YAIQTTV RNLKNSHFV K MILLI AIMV AFLSNISPVNAGDV KPKSVK GNEKAKSSEVSSPAPVNEGAATSG LADAG...

BrpB MKKYSTPNLIK AVSLGVIATLLSSVSPVYAANLAITGQSVQSTNGTIDVSY P KSGHGSIVLAGDNDYCGVFNVIGRGMN...

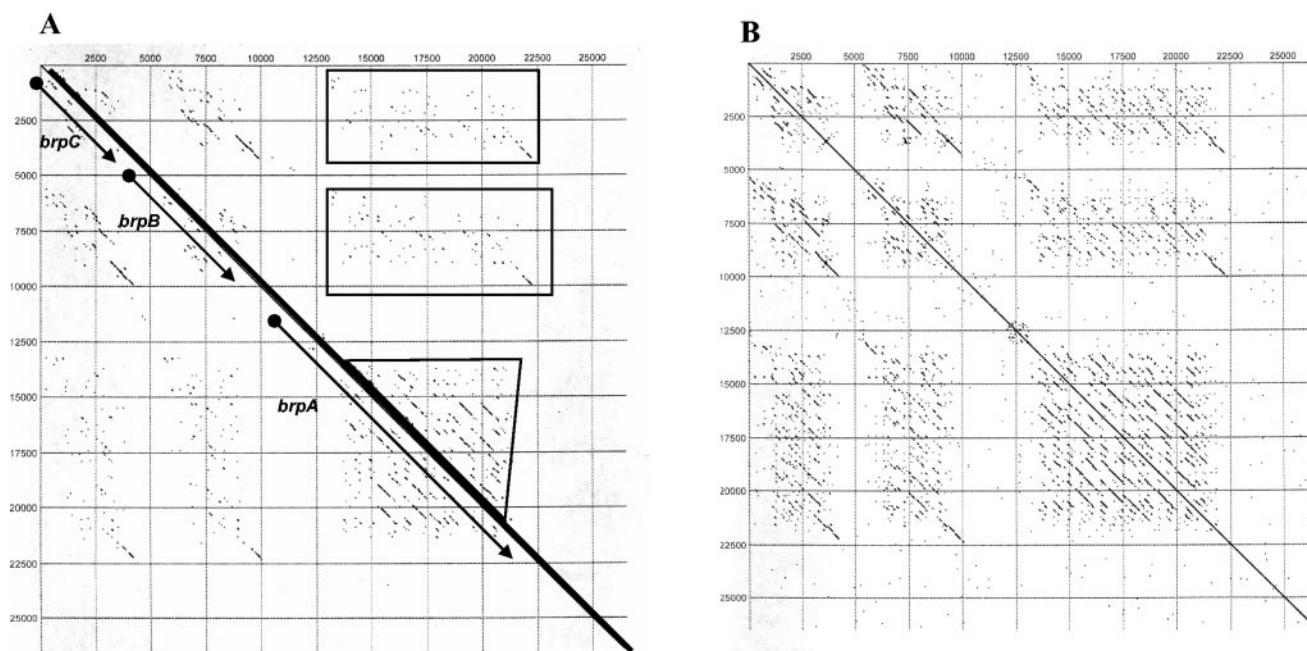


FIG. 4. Dot plot matrix analysis at the nucleotide level of the 26-kb Bva-Brp insert plotted against itself. (A) Analysis performed at an 80% homology level. Each line represents an area with a corresponding homologous area with an identity level of at least 80%. The bold diagonal in the center represents the 100% match of the sequence with itself. Areas illustrating the locations of *brpA*, *brpB*, and *brpC* are denoted along the diagonal. The boxed regions of the matrix indicate homologous segments to *brpA* within *brpA*, *brpB*, and *brpC*. (B) Same as panel A, except the analysis was performed at the 70% identity level, which illustrates the areas of homology within and among the genes more clearly. Numbers on the *x* and *y* axes denote the nucleotide positions of the 26-kb Bva-Brp cloned insert.

Vomp gene family members are likely to be orthologous companions of the *B. vinsonii* subsp. *arupensis* *brp* gene family, and it is not surprising that the comparably smaller sized Vomp proteins may be a result of the genome reduction of *B. quintana* as noted by the genome descriptions of Alsmark et al. (2).

There were four additional genes downstream of *brpA*, each showing similarity to database proteins by BLAST analysis. With the exception of the gene immediately downstream from *brpA*, the other genes were conserved and identified from analogous genes present in several other prokaryotic organisms. The gene downstream of *brpA* encoded a hypothetical protein of 22.3 kDa and has highly conserved orthologous partners in *B. henselae* and *B. quintana*, with amino acid identities of 82 and 81%, respectively. Next downstream was a gene encoding a 49-kDa protein described as an exopolyphosphatase, with 79% amino acid identity to the corresponding *B. henselae* gene. There was no BLAST match to this protein in *B. quintana*. Following the exopolyphosphatase gene were genes encoding the cell division protein *ftsJ* (also termed a 23S rRNA methyltransferase) and the GTP-binding protein. Both of these genes were present in *B. henselae* and *B. quintana*.

Repeat regions in *brp* genes. *brpA*, *brpB*, and *brpC* were each found to contain multiple extensive regions of internal repeats sometimes encoding long stretches of repetitive amino acid residues. These regions were not necessarily tandemly oriented but were separated by other repeats in no particular order or arrangement. Additionally, the repeats, although quite similar, were not identical. Figure 4 graphically illustrates the complexity of the repeat regions by dot plot matrix analysis of the *brp*

nucleotide sequences. The dot plot of the entire 26.4-kb insert plotted against itself clearly shows overlapping homologous areas within each gene and also the homology among genes. Dot plot analysis of the amino acid sequence of BrpA reveals the extensive repetitive nature of various lengths of sequence throughout the deduced protein sequence (Fig. 5A). For example, the longest repeats were seen at amino acid positions 1279 to 1504, 1800 to 2022, 2504 to 2738, and 2825 to 3046 (Fig. 5A). These particular repeats range from 221 to 234 amino acids and are aligned in Fig. 5B. The percentages of identity between each repeat ranged from 62.8% (amino acids 2504 to 2738 and 2825 to 3046) to 75.8% (amino acids 1800 to 2022 and 2504 to 2738). Several more smaller repetitive regions are interspersed throughout the BrpA coding region, as illustrated in the dot plot matrix. The apparent randomness of the repetitive arrangements, with some longer than 200 amino acids, are a unique characteristic of these genes. The larger *brpA* repeats are not tandemly oriented but are interspaced at various lengths, as are the many shorter repetitive regions. The repetitive regions of BrpA are limited to the internal majority of the protein, with the amino and carboxy ends of the protein having few or no repeats. The recent publication describing the genomes of *B. henselae* and *B. quintana* reported that these organisms contain repeated or partially repeated gene families, with *B. henselae* containing the higher proportion, with 78 repeat families of 2 to 14 members (2). The precise number of *brp* genes in *B. vinsonii* subsp. *arupensis* remains to be counted, and comparison of their organization and arrangements to the *badA1* and Vomp gene families within *Bartonella* species remains to be performed.

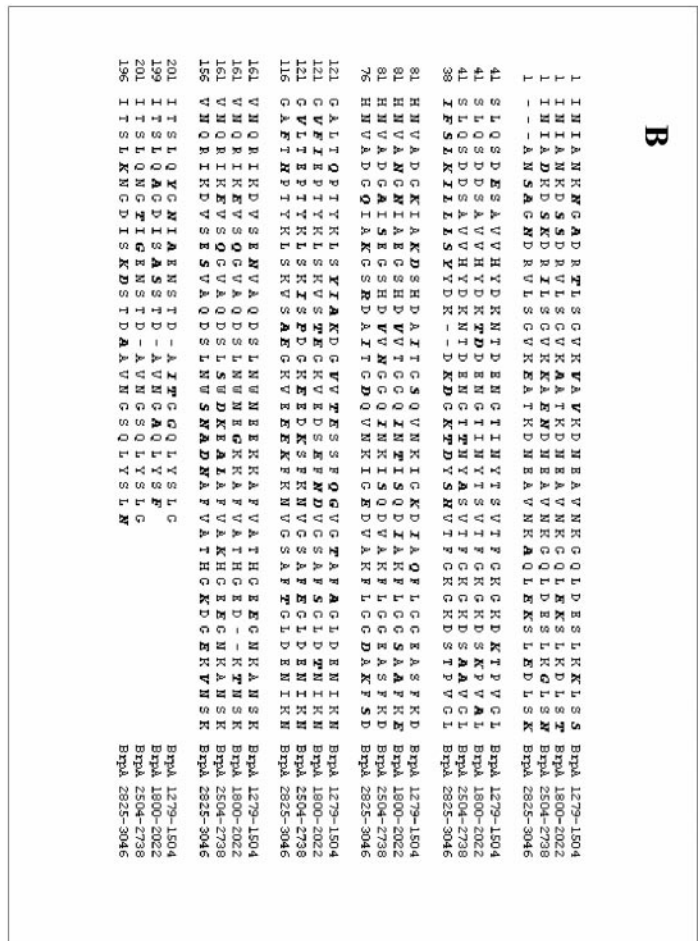
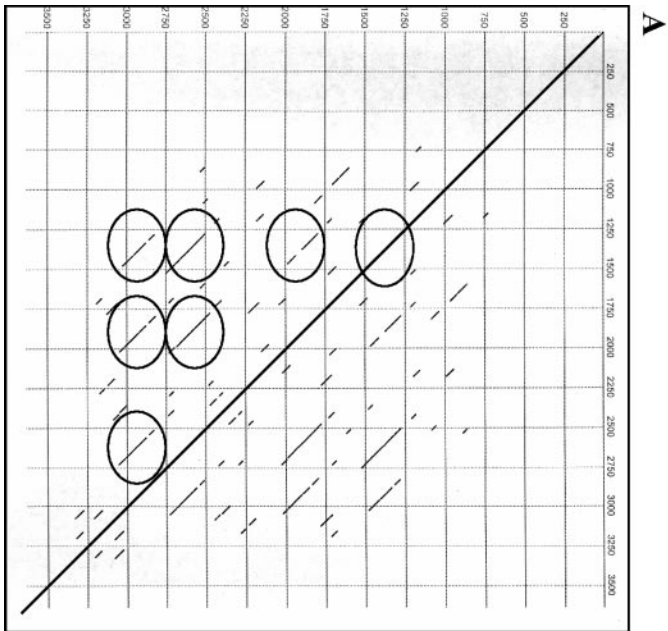


FIG. 5. (A) Dot plot analysis at the amino acid level of BrpA plotted against itself at the 70% identity level. The diagonal in the center indicates the 100% match of the sequence with itself. Circles surround the largest repeated regions corresponding to amino acid positions 1279 to 1504, 1800 to 2022, 2504 to 2738, and 2825 to 3046. Numbers on the x and y axes denote the amino acid positions for BrpA. (B) Amino acid alignment of repeats 1279 to 1504, 1800 to 2022, 2504 to 2738, and 2825 to 3046. Residues in bold italics represent divergence from the consensus.

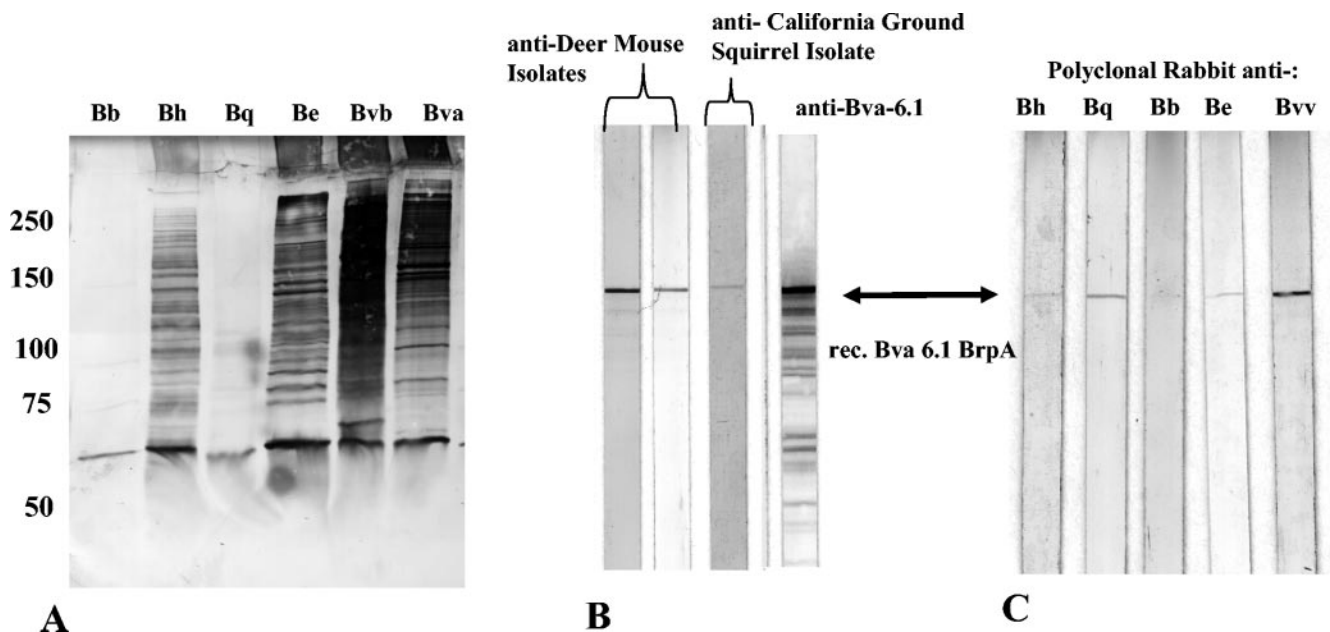


FIG. 6. (A) Immunoblot of whole-cell *Bartonella* lysates with anti-Bva 6.1. Anti-Bva-6.1 was used at a 1:1,000 dilution. Proteins were fractionated on a 7.5% polyacrylamide gel. Molecular size markers (in kilobases) are noted at the left. (B) Recombinant Bva-6.1 protein blotted against antisera from mice infected with rodent *Bartonella* strains. Sources of antibodies are indicated. Antibodies were used at a 1:500 dilution. The lower reacting bands in the anti-Bva-6.1 blot are breakdown products of recombinant BrpA. (C) Immunoblot of recombinant Bva-6.1 protein blotted with polyclonal antisera generated against *Bartonella* sp. whole-cell lysates. Antibodies were used at a 1:500 dilution. Bh = *B. henselae*, Bq = *B. quintana*, Bb = *B. bacilliformis*, Be = *B. elizabethae*, Bvv = *B. vinsonii* subsp. *vinsonii*.

Western blot and immunofluorescence assay analysis. Anti-recombinant Bva-6.1 (the original truncated protein; for the location, see Fig. 3A) was used in immunoblots against whole-cell lysates of various *Bartonella* species (Fig. 6A). A ladder or smear of several high-molecular-weight immunoreactive bands were apparent in *B. henselae*, *B. elizabethae*, *B. vinsonii* subsp. *berkhoffii*, and *B. vinsonii* subsp. *arupensis*. The reason for the high number of bands is not clear but may reflect polymeric, cleaved, or semidegraded forms of the proteins and/or several epitope-containing repeated regions within the *Bartonella* protein profiles. A common immunoreactive band of approximately 65 kDa was observed in all *Bartonella* lysates. *B. quintana* and *B. bacilliformis* did not exhibit seroreactivity to the high-molecular-weight bands with this antibody, with the exception of the 65-kDa band. This lack of reactivity is reflected in the Southern blot data which showed little homology to this region of the *brpA* gene. To determine whether the 65-kDa band may have been an artifactual cross-reactive antigen, the *Bartonella* cell lysates were blotted against polyclonal mouse preimmune serum and a polyclonal mouse serum against an unrelated protein. No cross-reactive immunoblot bands were observed (data not shown), suggesting that the 65-kDa band was specific to Brp-related proteins and probably not associated with bacterial common antigens, such as heat shock proteins or GroEL. The smaller 65-kDa band that was common among the assayed *Bartonella* strains may represent a cleaved portion of BrpA or an as-yet-undiscovered smaller version of BrpA that is conserved among species. Even though the antibody was directed against a relatively small segment of the BrpA protein, portions of that particular region are repeated throughout BrpA 18 times, and homologous regions are also

present in BrpB and BrpC, according to dot plot analysis at 70% amino acid homology, thereby allowing this antibody to be useful in detecting Brp proteins. Moreover, this region represents a highly conserved immunodominant domain, as it originated from the expression clone found using antiserum from a *Bartonella*-infected animal.

The purified 58-kDa recombinant Bva-6.1 protein was subjected to immunoblot analysis with antiserum from infected animals. Figure 6B demonstrates the seroreactivity of these antibodies against BrpA, indicating that a humoral response is generated during *Bartonella* infections. Polyclonal antibodies against whole-cell *Bartonella* spp. lysates reacted with the re-

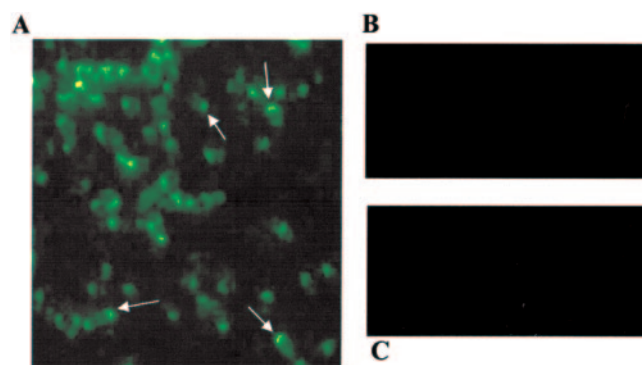


FIG. 7. Immunofluorescent staining of *Bartonella* cells. (A) *B. vinsonii* subsp. *arupensis* stained with anti-Bva 6.1; (B) *B. quintana* stained with anti-Bva 6.1; (C) *B. vinsonii* subsp. *arupensis* stained with pre-immunized mouse serum. Arrows in panel A point to the concentrated staining in the polar regions of the cells. Magnification, $\times 1,000$.

combinant Bva-6.1 protein (with the exception of *B. bacilliformis* antibody), demonstrating a degree of antigenic cross-reactivity among species to BrpA (Fig. 6C). Large-molecular-mass *Bartonella* immunoreactive bands of >200 kDa have been noted by other researchers in Western blots using antiserum from infected humans and animals, and they may represent orthologous Brp proteins (20, 32, 33). Work remains to be done to identify and characterize Brp-like proteins in other *Bartonella* species, determine whether infection with specific species or strains elicits an anti-Brp antibody response, and identify the immunodominant regions. There may be enough diversity in orthologous members of the Brp family in *Bartonella* for species-specific serological reactivity.

B. vinsonii subsp. *arupensis* and *B. quintana* cells were subjected to immunofluorescent staining using anti-Bva-6.1 protein (Fig. 7). *B. vinsonii* subsp. *arupensis* and *B. quintana* cells from a culture plate were separately mixed in 10 μ l sterile PBS on a microscope slide and allowed to air dry. The mouse antibody generated against the recombinant Bva-6.1 was incubated with the cells at a 1:50 dilution at 37°C for 1 h. Following five washes in PBS, the cells were incubated with a 1:50 dilution of fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G at 37°C for 1 h. After another series of PBS washes, the slide was air dried and viewed by epifluorescence microscopy. The staining showed strong fluorescent reactivity with *B. vinsonii* subsp. *arupensis* cells, providing evidence for an outer surface localization for at least the part of the protein to which the antibody was directed. The antibody reactivity was concentrated in discrete areas of the cell, usually at the ends, indicating a polarized location for BrpA following secretion or export to the outer surface (Fig. 7A). *B. quintana* cells stained with the same antibody did not demonstrate fluorescent activity (Fig. 7B), reflecting the result seen in the immunoblot. Epitopes in the Bva-6.1 segment may also be present in other Brp proteins; therefore, these could be detected on the surface as well.

Concluding remarks. During the course of our investigation of *Bartonella* immunogenic antigens, we discovered orthologous genes encoding large proteins from a rodent *Bartonella* isolate and from *B. vinsonii* subsp. *arupensis*. Taken together, the data describing their immunoreactive properties, probable surface localization, remarkable structure of repeats, and unusually large size reflect characteristics of gene products associated with pathogenic mechanisms of infection. Solid progress has been made in identifying *Bartonella*-related factors mediating host infection or invasion of host cells. Virulence factors that have been described include the VirB type IV secretion system, which mediates several processes involved in endothelial cell interactions (43, 44); GroEL as a mitogen to enhance the proliferation of endothelial cells (36); the *B. bacilliformis* invasion-associated locus (*ial*), which has a direct role in human erythrocyte parasitism (16, 37); flagella involved in host cell adherence (42, 48); deformin associated with cellular invasion (34); the *B. quintana* hemin binding protein family (12, 35); and the recently described adhesins BadA and Vomp A to D of *B. henselae* and *B. quintana*, respectively (40, 51). The function of the Brp family of proteins, other than the elicitation of antibodies following a *Bartonella* infection, is unknown, although given the homology with BadA and the Vomp proteins, it is not unrealistic to speculate that the Brp proteins may

share the same functional properties. In addition, the highly repetitive regions of the *brp* gene family suggest a gene rearrangement mechanism as a method for possible antigenic variation and immune evasion. Paralogous gene families and individual genes containing repeats have been documented to occur in many bacterial pathogens and have been shown to be essential in mediating infectious processes, such as bacterial adhesion to host cells and gene rearrangements leading to antigenic variation. Examples include the *vlsE* gene of *Borrelia burgdorferi* (50), the *vsp* and *vlp* families in *Borrelia hermsii* (24), the *ompA* gene of *Rickettsia rickettsii* and other spotted fever group rickettsiae (3), the *msh* gene family of *Anaplasma marginale* (1, 18), the *Ehrlichia risticii ssa* gene (9), *Listeria monocytogenes actA* (45), the *tpr* gene family of *Treponema pallidum* (47), *sof* and M protein genes of group A streptococci (25, 26, 39), and the alpha-like protein genes of group B streptococci (31).

BrpA is encoded by one of the largest prokaryotic genes thus far identified, and to our knowledge, the *brp* family is novel in having genes encoding such large proteins with the extensive and complex mosaic of repeated domains. The definition of the numbers of *brp* gene family members in *Bartonella* spp., outer membrane protein locations, kinetics of expression, secretion mechanisms, the role of the repeat regions, and the biological function of the Brps are some of the many issues awaiting future investigations.

Nucleotide sequence accession number. The GenBank accession number for the Bva-Brp 26-kb clone is AY730759.

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