INFECTION AND IMMUNITY, Mar. 2001, p. 1409–1419 Vol. 69, No. 3
0019-9567/01/$04.00+0 DOI: 10.1128/IAI.69.3.1409–1419.2001

VraA (BBI16) Protein of *Borrelia burgdorferi* Is a Surface-Exposed Antigen with a Repetitive Motif That Confers Partial Protection against Experimental Lyme Borreliosis

MARIA LABANDEIRA-REY, ELIZABETH A. BAKER, AND JONATHAN T. SKARE*

Department of Medical Microbiology and Immunology, The Texas A&M University System Health Science Center, College Station, Texas 77843-1114

Received 19 September 2000/Returned for modification 26 October 2000/Accepted 4 December 2000

We have previously described the expression cloning of nine *Borrelia burgdorferi* antigens, using rabbit serum enriched for antibodies specific for infection-associated antigens, and determined that seven of these antigens were associated with infectious *B. burgdorferi* strain B31. One of these infection-associated antigens encoded a 451-amino-acid putative lipoprotein containing 21 consecutive and invariant 9-amino-acid repeat sequences near the amino terminus that we have designated VraA for virulent strain-associated repetitive antigen A. The *vraA* locus (designated BBI16 by The Institute for Genomic Research) maps to one of the 28-kb linear plasmids (designated lp28-4) that is not present in noninfectious strain B31 isolates. Subsequent PCR analysis of clonal isolates of *B. burgdorferi* B31 from infected mouse skin revealed a clone that lacked only lp28-4. Southern blot and Western blot analyses indicated that the lp28-4 and VraA proteins, respectively, were missing from this clone. We have also determined that VraA is a surface-exposed protein based on protease accessibility assays of intact whole cells. Furthermore, *vraA* expression is modestly derepressed when cells are grown at 37°C relative to cells grown at 32°C, suggesting that VraA is, in part, a temperature-inducible antigen. Homologues cross-reactive to *B. burgdorferi* B31 VraA, most with different molecular masses, were identified in several *B. burgdorferi* sensu lato isolates, including *B. andersonii*, suggesting that the immunogenic epitope(s) present in strain B31 VraA is conserved between *Borrelia* spp. In protection studies, only 8.3% of mice (1 of 12) immunized with full-length recombinant VraA fused to glutathione *S*-transferase (GST) were susceptible to infectious challenge with 10^2 *B. burgdorferi* strain B31, whereas naive mice or mice immunized with GST alone were infected 40% or 63 to 67% (depending on tissues assayed) of the time, respectively. As such, the partial protection elicited by VraA immunization provides an additional testable vaccine candidate to help protect against Lyme borreliosis.

Infection by the *Borrelia burgdorferi* sensu lato complex is spread via the bite of infected ticks and manifests initially as a flu-like illness that, if untreated, can develop into a chronic state consisting of arthritic and neurological complications (25, 41–43). In the United States, the prevailing isolate found in areas where the disease is endemic is *B. burgdorferi* sensu stricto, although other sensu lato isolates, such as *B. andersonii* (7, 26), have recently been identified in Missouri and in several Southern states, along with other *Borrelia* spp. identified in Europe, Eurasia, and Japan (9, 30, 46). The worldwide distribution of the Lyme disease spirochete and its associated morbidity has provided the impetus to develop a vaccine to combat this disease. A great amount of work has been devoted toward testing the efficacy of various *B. burgdorferi* lipoproteins as protective immunogens; these proteins include OspA, OspB, OspC, OspD, OspE, and OspF and, more recently, DbpA and DbpB (2, 11, 12, 15, 18, 19, 29, 47). OspA was initially tested as a vaccine candidate based on its surface exposure and abundance on in vitro-cultivated *B. burgdorferi* sensu stricto (3, 12). In the laboratory, tick-infected animals do not generate a significant antibody titer against OspA, suggesting that *ospA* is not expressed in vivo, thereby implying that OspA vaccination may not protect against natural *B. burgdorferi* infection (11, 27, 34). Consistent with this finding, humans infected with *B. burgdorferi* do not exhibit a high titer antibody response to OspA early in infection (5). More recently, OspA has been purported as an arthropod-specific vaccine (11); that is, antibody against OspA binds and kills *B. burgdorferi* within the midgut of the tick during the blood meal of the immunized host. OspA has been evaluated as a protective immunogen in human trials, and the results indicate that the vaccine has marked efficacy (36, 44). However, the vaccine requires multiple boosts since anti-OspA titers may decrease over time. Because *ospA* is not expressed in the infected host, *B. burgdorferi* infection does not function as an immunological boost; as such, a preexisting high titer to OspA is essential for clearance of the spirochetes from the midgut of the tick. More recently, cross-reactivity between OspA and human leukocyte function-associated antigen 1 (hLFA-1) suggests that immunization with OspA may contribute to an autoimmune disorder (17).

The limitations of the OspA vaccine highlight the need to evaluate additional protective antigens, particularly those expressed in mammals, to protect against *B. burgdorferi* infection. Along these lines, we have identified nine genetic loci of *B. burgdorferi* that are preferentially recognized by rabbits that have developed infection-derived immunity to low-passage, virulent *B. burgdorferi* B31 (38). It is conceivable that subsets of

* Corresponding author. Mailing address: 407 Reynolds Medical Bldg., Department of Medical Microbiology and Immunology, The Texas A&M University System Health Science Center, College Station, TX 77843-1114. Phone: (979) 845-1376. Fax: (979) 845-3479. E-mail: jskare@tamu.edu.

TABLE 1. Borrelial strains used in this study

Strain	Relevant characteristics	Source or reference
B. burgdorferi sensu stricto		
MSK5 B31, low passage	Passage 2 infectious clonal isolate; obtained from infected mouse skin	24
MSK7 B31, low passage	Passage 2 attenuated clonal isolate; obtained from infected mouse skin and determined to be lp28-4$^-$	24
B31, high passage	Passaged several hundred times; nonclonal and noninfectious	13
297, low passage	Passage 3 nonclonal isolate; obtained from infected rabbit skin biopsy	D. Foley et al.[a]
CA-2-87, low passage	Passage 3 nonclonal isolate; obtained from infected rabbit skin biopsy	D. Foley et al.
ECM-NY-86, low passage	Passage 3 nonclonal isolate; obtained from infected rabbit skin biopsy	D. Foley et al.
JD-1, low passage	Passage 3 nonclonal isolate; obtained from infected rabbit skin biopsy	D. Foley et al.
NT-1, low passage	Passage 3 nonclonal isolate; obtained from infected rabbit skin biopsy	D. Foley et al.
2872-2	Low-passage nonclonal isolate	V. Sambri[b]
B. afzelii ACA-1, low passage	Infectious nonclonal isolate	S. Norris[c]
B. garinii Ip90, low passage	Infectious nonclonal isolate	S. Norris
B. andersonii		
MOD-3, low passage	Infectious nonclonal isolate	D. Foley et al.
MOD-5, low passage	Infectious nonclonal isolate	D. Foley et al.
MOD-6, low passage	Infectious nonclonal isolate	D. Foley et al.

[a] D. Foley, J. Miller, and M. Lovett, University of California, Los Angeles.
[b] V. Sambri, University of Bologna, Bologna, Italy.
[c] S. Norris, University of Texas Health Science Center, Houston.

these antigens are targets for borreliacidal antibodies and, as such, may function as protective immunogens against *B. burgdorferi*. Consistent with this idea, one of the clones identified encoded *dbpA*, which has recently been shown to function as a protective immunogen (18, 19). Yet another of these clones encodes a 451-amino-acid protein antigen that contains 21 consecutive and invariant 9-amino-acid repeats near its amino terminus that we have designated VraA for virulent strain-associated repetitive antigen A (designated BBI16 by The Institute for Genomic Research [TIGR]; see the TIGR website at http://www.tigr.org/tdb/CMR/gbb/htmls/SplashPage.html). In this study we report that VraA is a surface exposed antigen that is slightly induced in *B. burgdorferi* B31 at 37°C relative to 32°C. We also demonstrate that antibodies directed against either full-length or the amino-terminal half of VraA recognize antigens of various molecular masses in several *B. burgdorferi* sensu lato isolates. Furthermore, we demonstrate that VraA functions as a partially protective immunogen in the mouse model of Lyme borreliosis.

MATERIALS AND METHODS

Bacteria and plasmids. *B. burgdorferi* sensu stricto strain B31 was used in the majority of studies presented in this report unless otherwise indicated. All *B. burgdorferi* sensu stricto and sensu lato isolates used in this study are listed in Table 1. Most strains used were passaged no more than three times in vitro and were subsequently isolated from infected rabbits or mice to determine their infective phenotype unless otherwise indicated. *B. burgdorferi* sensu lato strains were cultured in BSK II media supplemented with 6% normal rabbit serum (Pel-Freez Biologicals, Rogers, Ark.) at 32°C in a 1% CO_2 atmosphere as previously outlined (38). Clonal isolates of *B. burgdorferi* were obtained by plating diluted cultures into agarose overlays as previously described (24). Two clones, designated MSK5 and MSK7, were obtained after intradermal inoculation of mice with 10^3 *B. burgdorferi* strain B31 passage 3 and isolation of infected skin at 2 weeks postinfection, followed by plating in BSK II agarose. The entire plasmid profile of both MSK5 and MSK7 was determined by designing primer pairs that were specific for each individual plasmid followed by resolution of the amplimers by agarose gel electrophoresis (24).

Escherichia coli strains used in this study were DH5α (Gibco-BRL, Bethesda, Md.), TOP10F′ (Invitrogen Corp., Carlsbad, Calif.), and BL21(DE3)pLysE (Novagen Corp., Madison, Wis.). All *E. coli* strains were grown in Luria-Bertani (LB) broth at 37°C with aeration or on LB agar at 37°C. *E. coli* was grown with appropriate antibiotics at the following concentrations: ampicillin at 100 μg/ml, kanamycin at 50 μg/ml, and chloramphenicol at 50 μg/ml. All plasmids used and constructed in this report are listed in Table 2.

PCR. PCR was conducted essentially as described elsewhere (24), and the oligonucleotide primers used are listed in Table 2. Briefly, the template was prepared by pelleting infectious *B. burgdorferi* B31 (fewer than four in vitro passages) at 5,800 × *g* for 10 min and washing it in an equal volume of phosphate-buffered saline (PBS), followed by centrifugation and resuspension in sterile water to yield a final concentration of 5×10^6 *B. burgdorferi* per μl. The sample was then boiled for 5 min, the insoluble material was pelleted by centrifugation (16,000 × *g*) for 2 min, and the supernatant was placed in a new tube. A 1-μl volume (or a 5×10^6 cell equivalence) was then added to the PCR. In some instances individual colonies of infectious *B. burgdorferi* were instead added to the PCR. Each appropriate primer set, in a 1 μl volume and at a final concentration of 0.2 μM per primer, was incubated with 1 μl of the appropriate *B. burgdorferi* template and brought to a final volume of 20 μl by adding 2 μl of sterile water and 16 μl of Gibco-BRL SuperMix (Gaithersburg, Md.) which contains buffer, all deoxynucleotides, and *Taq* polymerase. Samples were then denatured at 94°C for 1 min, followed by 35 cycles of the following: 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min. Incubation at 72°C for 6 min served as the final extension step in the reaction. Products were resolved on 0.8 to 1% agarose gels buffered in Tris-acetate-EDTA containing 0.5 μg of ethidium bromide per ml.

Construction of the GST-vraA fusion proteins. Three separate glutathione *S*-transferase (GST)-VraA fusion proteins were constructed by using the oligonucleotide primers listed in Table 3. A plasmid encoding a fusion of GST to full-length, mature VraA that lacked the VraA leader peptide and the initial cysteine residue was constructed by PCR amplification using the primers Sal/GST-FL and Not/GST-FL in conjunction with *B. burgdorferi* template DNA as indicated above (Sal and Not refer to *Sal*I and *Not*I restriction enzyme sites engineered at the 5′ ends of the oligonucleotides; see Table 3). The Sal/GST-FL and Not/GST-FL primers amplified a 1,302-bp *vraA* fragment, encoding the mature VraA protein (i.e., minus the leader peptide), and this fragment was subsequently cloned into the pCR2.1-TOPO vector (Invitrogen) and designated pCRL3-FL. The ligated product was transformed into TOP10F′ cells (Invitrogen), and transformants were selected for on LB agar containing kanamycin and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) according to the manufacturer's instructions. White colonies (putative positives) were grown in LB broth, and DNA was purified from these cells by alkaline lysis to screen for those containing the desired insert.

Similar constructs were made using either the oligonucleotides Sal/GST-FL

TABLE 2. Plasmids used in this study

Plasmid	Relevant characteristics	Source
pCR2.1-TOPO	TA cloning vector; Ap^r Kan^r	Invitrogen Corp., Carlsbad, Calif.
pGEX-4T-1	Vector used to make GST fusions to VraA; Ap^r	Amersham Pharmacia Biotech Inc., Piscataway, N.J.
pCRL3-FL	1,302-bp fragment, from PCR amplification using oligonucleotide primers Sal/GST-FL and Not/GST-FL, cloned into pCR2.1-TOPO	This study
pCRL3-NT	729-bp fragment, from PCR amplification using oligonucleotide primers Sal/GST-FL and Not/GST-NT, cloned into pCR2.1-TOPO	This study
pCRL3-CT	594-bp fragment, from PCR amplification using oligonucleotide primers Sal/GST-CT and Not/GST-FL, cloned into pCR2.1-TOPO	This study
pL3-FL10	Full-length VraA construct residues 18 to 451 of VraA fused to GST (minus leader peptide and initial cysteine residue; nucleotides 50 to 1352[a] of VraA fused in-frame to GST sequence on pGEX-4T-1)	This study
pL3-NT3	Amino-terminal VraA construct residues 18 to 260 of VraA fused to GST (minus leader peptide and initial cysteine residue; nucleotides 50 to 779[b] of VraA fused in-frame to GST sequence on pGEX-4T-1)	This study
pL3-CT7	Carboxy-terminal VraA construct residues 253 to 451 of VraA fused to GST (minus leader peptide and initial cysteine residue; nucleotides 758 to 1352[c] of VraA fused in-frame to GST sequence on pGEX-4T-1)	This study

[a] See Table 3 for The oligonucleotides used; Sal/GST-FL and Not/GST-FL amplify full-length VraA.
[b] As in footnote *a* except oligonucleotides Sal/GST-FL and Not/GST-NT were used to amplify the amino-terminal half of VraA
[c] As in footnote *a* except oligonucleotides Sal/GST-FL and Not/GST-CT were used to amplify the carboxy-terminal half of VraA.

and Not/GST-NT or the oligonucleotides Sal/GST-CT and Not/GST-FL to PCR amplify the amino-terminal and carboxy-terminal *vraA* domains, respectively. The resulting PCR amplimers, 729 and 594 bp in size, were cloned into the pCR2.1-TOPO vector and named pCRL3-NT (amino-terminal clone) and pCRL3-CT (carboxy-terminal clone) accordingly (Table 2). Following digestion with *Sal*I and *Not*I, the various fragments were gel purified via phenol-chloroform extraction, precipitated, and ligated to plasmid pGEX-4T-1 restriction digested with *Sal*I and *Not*I. Ligated DNA was then transformed into DH5α (Gibco-BRL) using standard methodologies. Transformants were screened for inserts by subjecting clones to alkaline lysis followed by digestion with appropriate restriction enzymes.

Purification of GST-VraA fusion proteins. A representative clone of the three GST-VraA fusions, i.e., full-length VraA (residues 18 to 451 of VraA fused to GST), the amino-terminal half (residues 18 to 260 of VraA fused to GST), and the carboxy-terminal half (residues 253 to 451 of VraA fused to GST) were transformed into BL21(DE3)pLysE (Novagen), and 2 liters of cells (with appropriate antibiotic selection) was grown to early log phase (optical density at 600 nm of 0.3 to 0.4). The same methodology was applied to each recombinant GST-VraA fusion protein separately as outlined below. The appropriate GST-VraA fusion protein was induced by the addition of 1 mM IPTG and subsequent incubation for 2 h at 37°C. The resulting cells were pelleted by centrifugation at 6,000 × *g*, resuspended in 40 ml of PBS (pH 7.4), and subjected to one cycle of freezing and thawing to convert the cells to spheroplasts. The cells were then subjected to two passages through the French pressure cell at 16,000 lb/in^2. The clarified supernatant was centrifuged at 3,000 × *g* for 10 min to pellet the unlysed *E. coli*, and the resulting supernatant was recentrifuged at 40,000 × *g* for 20 min to pellet the total membrane component of *E. coli*. After we determined that all three forms of the GST-VraA fusion proteins were soluble upon induction, the supernatants (i.e., soluble protein) were then separately added to glutathione-Sepharose 4B beads and rocked gently overnight at 4°C to promote binding of the GST-VraA fusion proteins to immobilized glutathione. After the overnight incubation, the beads were pelleted at 500 × *g*, the supernatant was removed, and the beads were washed with 25 ml of PBS (pH 7.4). The wash step was repeated and, following resuspension with PBS, the resulting suspension was poured into a column and the column was washed with 25 ml of PBS. Following the wash step, 5 mM reduced glutathione in PBS was added, and 1-ml column fractions were collected. Fractions containing the various GST-VraA proteins were identified by subjecting 2.5 μl of each fraction to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by staining with Coomassie blue and destaining. Fractions containing the different recombinant GST-VraA samples were further purified from low-molecular-weight contaminants by preparative SDS-PAGE. Briefly, 1 mg of the given GST-VraA fusion protein was resolved on a 16-cm-by-20-cm SDS-polyacrylamide gel (Bio-Rad, Richmond, Calif.) with a 13-cm wide preparative gel comb, and the gel was stained with Coomassie blue in double-distilled, sterile water. The band corresponding to the appropriate GST-VraA molecule was excised from the gel and electroeluted using the S&S Elutrap Electro-separation System from Schleicher & Schuell, Inc. (Keene, N.H.) as outlined by the manufacturer.

Antibody production. Three separate rabbits were prebled and subsequently immunized with each of the three affinity-purified GST-VraA fusion proteins obtained. In all cases, 100 μg of the GST-VraA protein, in complete Freund adjuvant, was injected subcutaneously (four sites, 25 μg per site). After 6 weeks, the rabbits were boosted with 100 μg of the same GST-VraA protein in incomplete Freund's adjuvant. Two weeks later the rabbits were bled to obtain serum specific for either full-length VraA fused to GST, the amino-terminal half of VraA fused to GST, or the carboxy-terminal half of VraA fused to GST. Each serum was tested qualitatively by Western blotting to determine the specificity of the antiserum for both homologous and heterologous forms of recombinant GST-VraA, as well as native VraA (see below).

Protease accessibility. Protease accessibility studies were conducted as previously described (4) with the following modifications. *B. burgdorferi* B31 passage 7 cells (approximately 2 × 10^8) were centrifuged at 4,300 × *g* for 15 min at 4°C, the supernatant was removed, and the pellet was resuspended in an equal volume of PBS (pH 7.4)–5 mM $MgCl_2$–50 mM sucrose. The wash was repeated again,

TABLE 3. Oligonucleotides used to PCR amplify *vraA* and *vraA* derivatives

Primer	Sequence (5′ to 3′)[a]	Position of *vraA*[b]
Sal/GST-FL	ACGCGTCGACGCAGGCCGGATTTTAATATCGATC	50→73
Not/GST-FL	ACGCGCGGCCGCAGTTTATATTTTGACACTATAAGC	1352→1329
Not/GST-NT	ACGCGCGGCCGCTCATTAGATAGCGTATTTTTTAG	779→757
Sal/GST-CT	ACGCGTCGACTAAAAAATACGCTATCTAATGAT	758→780

[a] Underlined sequence indicates the *Sal*I or *Not*I site; see primer name for restriction site used.
[b] Numbers refer to the TIGR designation of the BBI16 open reading frame; the arrow indicates the orientation of the oligonucleotide relative to the sense and antisense strands of the *vraA* sequence. The repetitive domain of VraA is encoded by nucleotides 157 to 723 of *vraA*.

and the samples were split into equal volumes. Either 50 μl of sterile water or proteinase K (to a final concentration of 200 μg/ml) was added to each sample, and both were incubated at 20°C for 40 min. After 40 min, phenylmethylsulfonyl fluoride (PMSF) was added to a final concentration of 1 mM, and the samples were examined by dark-field microscopy to assess their motility (i.e., as an indirect test of viability). The samples were then centrifuged and washed again as described above except that the solution also contained 1 mM PMSF. The final pellets were resuspended in Laemmli sample buffer, and the proteins were separated by SDS-PAGE and analyzed by immunoblotting as described below.

Growth phase induction of *vraA*. *B. burgdorferi* B31 MSK5 (passage 2) was inoculated at an initial cell density of 5×10^5 per ml in BSK II media and was grown in separate cultures at 32 and 37°C. Aliquots at different time points were removed at the following cell densities in a volume equal to a final count of 10^8 *B. burgdorferi* cells (the numbers listed are approximate per-milliliter values): 5×10^6, 1×10^7, 2.5×10^7, 5×10^7, 1×10^8, 2×10^8, or 3×10^8 organisms. Each sample was pelleted at 5,800 $\times$ *g* for 20 min, washed in PBS (pH 7.4), and resuspended in Laemmli sample buffer. Equivalent amounts of *B. burgdorferi* whole-cell lysates per time point were resolved by SDS-PAGE, immunoblotted, and probed with anti-VraA serum as described below.

pH induction of *vraA*. Experiments to determine whether *vraA* was pH inducible were conducted as described by Carroll et al. (8).

SDS-PAGE and Western blotting. SDS-PAGE and Western blotting with the different anti-GST-VraA sera was done essentially as described previously (38, 40). The dilution of anti-VraA serum used was 1:5,000 regardless of the GST-VraA immunogen used to immunize rabbits. Rabbit anti-p66 serum was generously provided by Sven Bergström, Umeå University, and was diluted 1:5,000. Mouse monoclonal antibody directed against *B. burgdorferi* endoflagella was kindly provided by Alan Barbour, University of California at Irvine, and was used at a 1:50 dilution. Protein A, conjugated to horseradish peroxidase, and diluted 1:1,000, was used to detect all immobilized immune complexes. Blots were developed using Amersham's enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, Piscataway, N.J.).

Mouse immunization with VraA. Two groups of 24 8-week-old female C3H/HeN mice (Charles River Laboratories, Inc., Wilmington, Mass.) were each immunized with 25 μg of either GST or full-length VraA fused to GST (GST-VraA FL). In addition, 24 naive mice served as controls for *B. burgdorferi* infection. After 10 weeks, the mice were immunized with the same amount of protein (with the exception of the untreated controls). Representative mice (four were randomly chosen per group) were bled on day 15 postboost, and individual sera were used in a Western blot assay to determine qualitatively whether the animals had a humoral response to recombinant VraA. Since all sera from immunized mice showed the appropriate reactivity to either GST or GST-VraA at day 17 postimmunization, 12 mice per group were challenged with either 10^2 or 10^4 infectious *B. burgdorferi* B31 isolate MSK5 which contains all of the known borrelial plasmids (as determined by PCR amplification [24]). At 2 weeks after challenge, the mice were sacrificed and their abdomen skins, spleens, and bladders were aseptically removed and cultured at 32°C in 10 ml of BSK II medium supplemented with 6% normal rabbit serum. After 4 days, 0.5 ml of the culture was passaged blindly into 10 ml of BSK II medium, and the samples incubated at 32°C. Protection was assessed by the absence of cultured *B. burgdorferi* from each tissue sample (both the initial culture and the blindly passaged sample) out to a 6-week period.

Statistical analysis. A 3×2 contingency table and χ^2 test of independence were used to test the null hypothesis, "Protection against *B. burgdorferi* challenge is independent of the vaccinogen used." The null hypothesis was rejected when *P* values of <0.05 were obtained, indicating statistical significance.

RESULTS

Sequence analysis of *vraA*. Previously we had identified nine distinct proteins that were antigenic in infection-immune rabbits (38). One of these antigens showed extensive redundancy at the nucleotide level. The complete nucleotide sequence indicated that this locus contained a hydrophobic core sequence at the amino terminus connected to the putative leader peptidase II cleavage sequence FLAC, suggesting that this gene encoded a lipoprotein antigen. Further assessment of this gene indicated that the mature domain contained a 27-bp invariant, repetitive sequence, as shown in the Pustell DNA matrix plot in Fig. 1A, that encodes the following primary

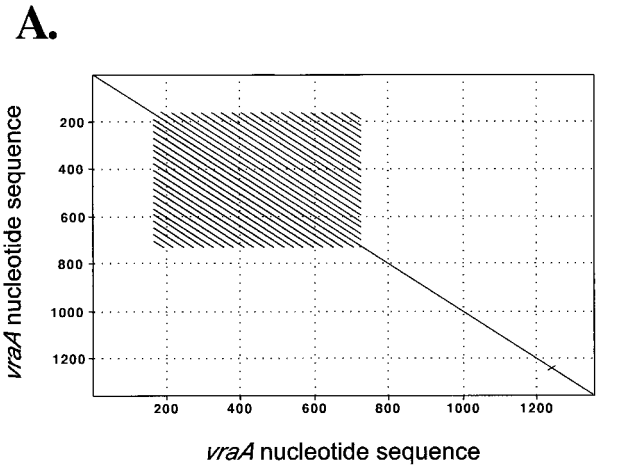

FIG. 1. Sequence analysis of *vraA* repetitive domain. (A) Pustell DNA matrix analysis of *vraA* aligned against itself. Lines framing the main diagonal show the location of the repetitive domain from nucleotides 157 through 723 of *vraA* encoding the 21 consecutive 9-amino-acid repeat EEELKKKQQ. The number of lines above or below the diagonal indicates the number of repeat units in *vraA*. The absence of any breaks within the upper and lower diagonals demonstrates that the repeats are consecutive and invariant. The short perpendicular line in the lower right corner indicates a short imperfect inverted repeat sequence located at nucleotides 1222 to 1240 of *vraA*. (B) Comparison between the single region of homology of BBI28 and the *vraA* repeat unit. Underlined nucleotides and amino acid indicate differences in the *vraA* and BBI28 sequences, respectively.

sequence: Glu-Glu-Glu-Leu-Lys-Lys-Lys-Gln-Gln. Elucidation of the entire *B. burgdorferi* genome sequence by TIGR corroborated our nucleotide sequence and indicated that this genetic locus, named BBI16 by TIGR (see TIGR website and also reference 14), was located on linear plasmid 28-4 (lp28-4). Because of these repetitive domains, we have designated this gene *vraA* for virulent strain-associated repetitive antigen A. Although the predicted molecular mass of mature VraA (i.e., minus the putative amino-terminal leader peptide) is 52,375 Da, VraA migrates at an apparent molecular mass of approximately 70 kDa, presumably due to the conformation of the repetitive domain. This type of anomalous migration has been observed previously in other repetitive proteins (33, 48). The *vraA* locus (BBI16) is a member of a *B. burgdorferi* paralogous family containing 17 genes (family 60; see TIGR website); however, the homology between the paralogues resides predominantly within the predicted carboxy-terminal domain (i.e., no other paralogue contains a repetitive domain like that observed in *vraA*). One of the paralogues, encoded by BBI28, contains one imperfect copy of the repetitive domain observed

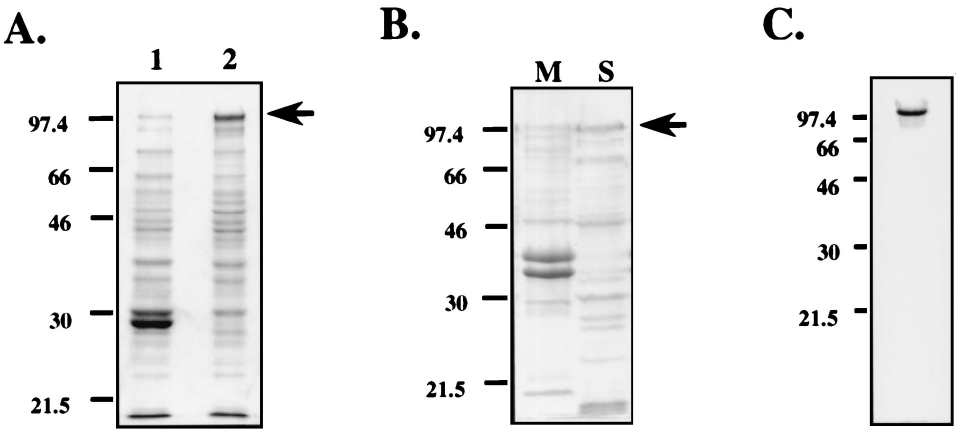

FIG. 2. Overproduction and purification of full-length VraA fused to GST. (A) Coomassie blue stain of an SDS–10% polyacrylamide gel showing the overproduction of GST alone (lane 1) and full-length VraA fused to GST [GST-VraA(FL); lane 2, indicated by an arrow] following IPTG (isopropyl-β-D-thiogalactopyranoside) induction as outlined in Materials and Methods. (B) Solubility of recombinant GST-VraA(FL). After induction, *E. coli* cells synthesizing GST-VraA(FL) were subjected to breakage with a French pressure cell and the insoluble component cleared by centrifugation as indicated in the Methods section. The resulting membrane material (M) and soluble component (S) were resolved by SDS-PAGE, and the gel was stained with Coomassie blue. Note the appearance of an approximately 100-kDa species in the lane containing the soluble fraction (lane S, indicated by an arrow). (C) Purified GST-VraA(FL). GST-VraA(FL) was affinity purified with glutathione-Sepharose beads, electroeluted from an unfixed SDS-polyacrylamide gel to eliminate lower-molecular-mass contaminants, reseparated by SDS-PAGE, and stained with Coomassie blue. Purified GST-VraA(FL) was then used to immunize both rabbits and mice to generate anti-VraA serum and in protection studies, respectively. Numbers on the left refer to the molecular mass of protein markers (in kilodaltons).

in *vraA* with 88% identity at the nucleotide level, resulting in a single amino acid substitution relative to the repetitive domain in VraA (Fig. 1B). With the noted exception of the single small domain in BBI28, no homology is observed at the nucleotide or amino acid level within the repetitive domains of *vraA* with any other sequence in the database.

Production of antiserum specific for VraA. In order to obtain sufficient amounts of recombinant VraA for immunization purposes, we constructed a translational fusion of the gene encoding GST to *vraA* using the pGEX-4T-1 vector as described in the Materials and Methods section. After induction of the fusion protein, the soluble component, containing the GST fused to full-length VraA recombinant protein [GST-VraA (FL)], was affinity purified using a glutathione-Sepharose column (Fig. 2). The fusion protein shown in Fig. 2 represents a carboxy-terminal fusion to GST of residues 18 to 451 of VraA. Panel A shows the overproduction of VraA (lane 2) relative to the induction of GST in the vector only control (lane 1). We then assessed the solubility of recombinant VraA and found that it was associated essentially with the soluble component (Fig. 2B, lane S) relative to the membrane fraction (Fig. 2, lane M) when produced in *E. coli*. Affinity purification resulted in a single pure fraction containing recombinant VraA that we used for subsequent immunization and vaccination studies (see below). We also made similar separate constructs that joined residues 18 to 260 of VraA and residues 253 to 451 of VraA to GST, designated amino-terminal VraA and carboxy-terminal VraA, respectively, and purified these fusion proteins to homogeneity (data not shown). Purified full-length, amino-terminal, and carboxy-terminal VraA (all fused to GST) were used to immunize rabbits separately, and the polyclonal antiserum obtained was tested to confirm that these antibody reagents were specific for VraA. Clonal isolates of *B. burgdorferi* B31, derived from infected C3H/HeN mouse skin, designated MSK5 and MSK7, were analyzed by SDS-PAGE and Western blot with all three sera obtained. We have previously determined that MSK5 contains all known plasmids, whereas MSK7 is lacking lp28-4, the linear plasmid that encodes *vraA* (24). As predicted, the MSK7 sample showed no immunoreactivity to the 70-kDa full-length VraA species when probed either with serum against full-length VraA (Fig. 3B, lane 2) or with serum directed against the amino- or carboxy-terminal constructs (data not shown). In contrast, a 70-kDa antigen and a probable 50-kDa degradation product was observed in MSK5 when probed with antiserum generated against rabbits immunized with either full-length (Fig. 3B, lane 1) or amino-terminal (data not shown) VraA. However, no reactivity was observed when serum from rabbits immunized with the carboxy-terminal half of VraA was used as the primary antibody (data not shown). In all instances, sera directed against the three different GST-VraA proteins contained antibodies reactive with each homologous recombinant protein (data not shown). This indicated that the rabbits immunized with GST fused to either full-length, amino-terminal, or carboxy-terminal VraA generated a humoral response specific for the appropriate VraA construct. Since serum directed against the carboxy-terminal half of VraA did not react with native VraA from *B. burgdorferi* B31 and only weakly reacted with full-length and amino-terminal recombinant VraA fused to GST, it is most likely that the immunoreactivity observed is restricted to the GST moiety (data not shown). As controls, we also determined that neither rabbit preimmune serum nor antiserum to GST recognized any *B. burgdorferi* proteins, indicating that the immunoreactivity observed was restricted to VraA (data not shown). Furthermore, infection derived rabbit serum recognized full-length and amino-terminal recombinant VraA fused to GST, but not carboxy-terminal VraA fused to GST (data not shown). These results, taken together, suggest that

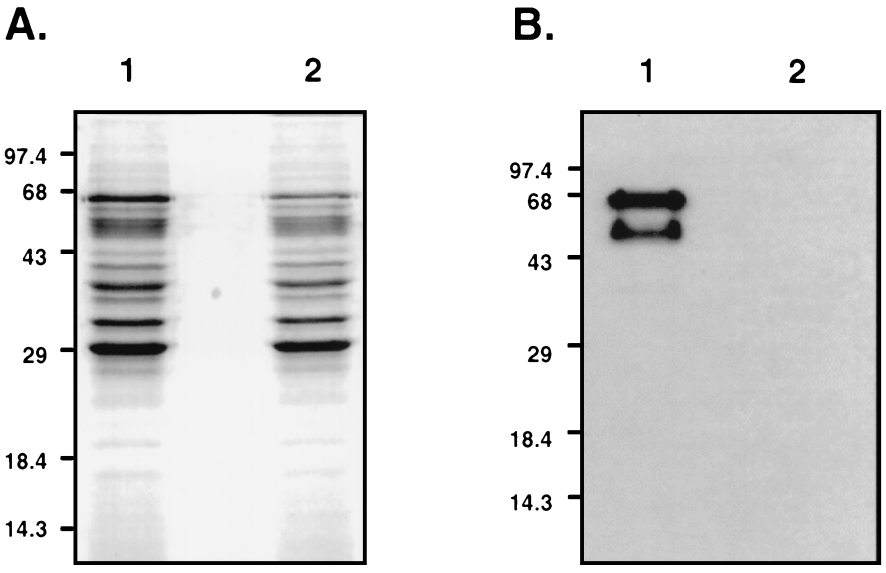

FIG. 3. Specificity of antiserum directed against full-length VraA. Whole-cell lysates of protein derived from *B. burgdorferi* B31 MSK5 (wild type) and MSK7 (lp28-4$^-$) were separated by SDS-PAGE and stained with Coomassie blue (A) or immunoblotted and probed with rabbit serum directed against full-length VraA fused to GST (B). Lane 1, MSK5; lane 2, MSK7. The numbers on the left refer to the molecular masses of the protein markers (in kilodaltons).

the repetitive domain is the predominant immunogenic component of VraA.

Surface localization of VraA. To determine whether VraA was a surface exposed antigen in *B. burgdorferi* B31, we made changes to the protease accessibility methodology previously published (4) inasmuch as *B. burgdorferi* began to clump and were nonmotile after incubation in PBS (pH 7.4)–5 mM $MgCl_2$. Based on our previous experience with outer membrane protein localization and outer membrane purification studies (37, 39, 40), we hypothesized that the loss of motility was due to the loss of the structural integrity of the *B. burgdorferi* outer membrane. *B. burgdorferi* cells were therefore incubated in several different buffers, and their viability was assessed as a function of motility. We found that *B. burgdorferi* resuspended in PBS (pH 7.4)–5 mM $MgCl_2$ containing 50 mM sucrose was active and motile for periods of time exceeding 1 h with or without added proteinase K. Under these conditions, addition of proteinase K resulted in a near-complete reduction of VraA (Fig. 4A), modified the surface exposed protein P66 (Oms66) (39) (Fig. 4B), but did not alter the levels of endoflagella (Fig. 4C), a known subsurface marker of spirochetal bacteria (4). This result implies that VraA is a surface-exposed protein and that the *B. burgdorferi* cells were structurally intact, respectively.

Temperature- and growth-phase-dependent induction of *vraA*. We initially evaluated whether *vraA* was induced at 23, 32, and 37°C and found that VraA was overproduced at 37°C (data not shown). However, subsequent studies to repeat this observation yielded variable results. This led us to surmise that *vraA* may be subjected to growth phase regulation, pH regulation, or both. To assess this, cultures of infectious *B. burgdorferi* were inoculated at a density of 5×10^5 per ml, and

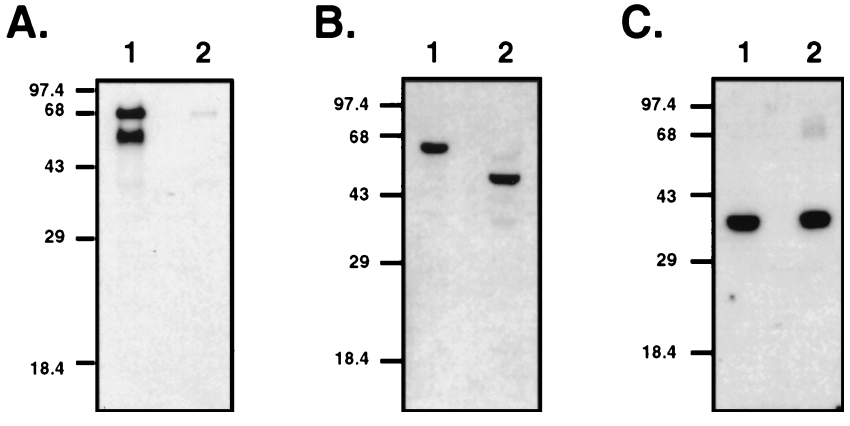

FIG. 4. Surface Localization of VraA. Intact *B. burgdorferi* B31 MSK5 either left untreated (lane 1) or treated with proteinase K (lane 2), resolved by SDS-PAGE, and immunoblotted. Identical blots were then probed with either anti-VraA (full-length VraA fused to GST) (A), anti-p66 (B), or anti-endoflagellum (C) serum. The numbers on the left refer to the molecular mass of protein markers (in kilodaltons).

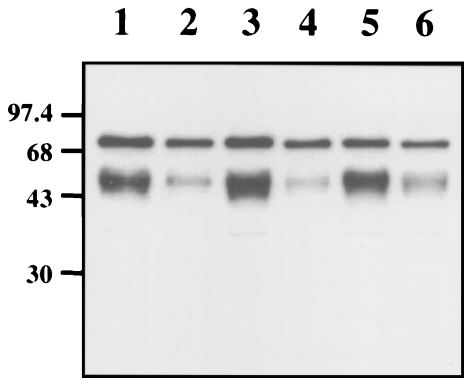

FIG. 5. *vraA* induction at 37°C relative to 32°C. *B. burgdorferi* B31 MSK5 (passage 2) was inoculated at an initial cell density of 5×10^5 per ml in BSK II medium and cultivated as outlined in Materials and Methods at both 32 and 37°C. At various phases of growth, samples were removed and protein from whole-cell lysates was subjected to SDS-PAGE and immunoblotting with anti-VraA serum. Lane 1, 1.25×10^7 *B. burgdorferi* grown at 37°C; lane 2, 1.9×10^7 *B. burgdorferi* grown at 32°C; lane 3, 1.22×10^8 *B. burgdorferi* grown at 37°C; lane 4, 1.37×10^8 *B. burgdorferi* grown at 32°C; lane 5, 3.14×10^8 *B. burgdorferi* grown at 37°C; lane 6, 3.1×10^8 *B. burgdorferi* grown at 32°C. The numbers on the left refer to the molecular mass of protein markers (in kilodaltons).

TABLE 4. Protection of C3H/HeN mice immunized with recombinant VraA[a]

Immunogen[b]	Challenge dose[c] (CFU)	No. of culture-positive samples/ total no. of samples tested[d] in C3H/HeN tissues		
		Skin	Bladder	Spleen
None or naive	10^4	11/12	12/12	12/12
GST	10^4	11/12	11/12	11/12
GST-VraA	10^4	10/12	10/11	11/12
None or naive	10^2	4/10	4/10	4/10
GST	10^2	8/12	8/12	7/11
GST-VraA	10^2	1/12[e]	1/12[e]	1/11[f]

[a] The data reflect results from two independent immunization experiments.
[b] Mice were immunized with 25 μg of either GST or GST-VraA and boosted 4 weeks later with an equivalent amount of protein. After 2 weeks the mice were bled and tested for antibody response against the appropriate antigen. Animals were then challenged with the inoculum indicated.
[c] Mice were challenged intradermally with *B. burgdorferi* B31 MSK5 of the high-infectivity phenotype at the inoculum size indicated.
[d] Equal numbers of mice were used for each group. Values of less than 12 indicate liquid culture contamination that did not support *B. burgdorferi* growth.
[e] χ^2 analysis indicates a significant difference from naive and GST immunized mice ($0.01 < P < 0.025$).
[f] χ^2 analysis indicates a difference from naive and GST immunized mice ($0.05 < P < 0.1$).

samples were taken at various time points commensurate with early-, mid-, late-, and stationary-phase growth at both 32 and 37°C using a previously published methodology (21, 31). Identical amounts of protein for each time point were resolved by SDS-PAGE and either stained with Coomassie blue (data not shown) or immunoblotted to ensure that equivalent levels of *B. burgdorferi* protein were present and to assess levels of VraA synthesized at each time point, respectively. Our results, after four independent experiments, indicate that VraA is only slightly overproduced when cells are grown at 37°C and that *vraA* expression is not affected by the growth phase of the culture (Fig. 5). Additionally, VraA appears to be proteolytically degraded more efficiently at 37°C as seen by the increase in the approximate 50-kDa degradation product, perhaps due to the induction of a temperature-dependent protease (Fig. 5). Alternatively, it is possible that the 50-kDa degradation product accumulates at 37°C due to decreased proteolysis of this lower-molecular-weight species independent of full-length VraA. Only three samples, at various growth phases, ranging from approximately 1×10^7 (Fig. 5, lanes 1 and 2) to 3×10^8 cells (Fig. 5, lanes 5 and 6), are shown in Fig. 5; however, other intermediate samples at different growth phases were also evaluated and yielded identical immunoblot profiles at 32 and 37°C relative to the samples shown in Fig. 5 (data not shown). These results, taken together, indicate that *vraA* expression is not regulated by growth phase.

We also determined whether *vraA* was pH inducible inasmuch as the levels of VraA produced could be increased under conditions where the BSK II culture medium would be acidified. Using the protocol outlined by Carroll et al. (8), we did not see induction of *vraA* when cells were incubated at pH 6.0 relative to cells cultivated at pH 7.0 or pH 8.0 (data not shown), indicating that pH has no direct or indirect effect on *vraA* expression.

Although we were not able to demonstrate induction of *vraA* in these various analyses, these results do not preclude the possibility that *vraA* is derepressed in either infected mammals or arthropod vectors (or both) in response to host-specific signals that have not been effectively simulated in our in vitro based studies.

VraA confers partial protection in mice. To test whether VraA could function as a protective immunogen, we immunized 24 C3H/HeN mice with recombinant full-length VraA fused to GST, i.e., GST-VraA(FL) (FL represents full length) as described in Materials and Methods. The controls used included 24 mice immunized with GST alone and 24 naive mice. Prior to the challenge, we collected serum from several randomly chosen mice to qualitatively test whether a humoral response had been generated against the appropriate immunogen [GST or GST-VraA(FL)]. In all cases, mice immunized with GST or GST-VraA(FL) generated antibodies to the appropriate antigen, respectively (data not shown). Twelve mice from each set were needle challenged with either 10^2 or 10^4 of a clonal isolate of *B. burgdorferi* B31 MSK5 passage 3 that contains all known *B. burgdorferi* plasmids (24). The results indicated that nearly all of the mice challenged with 10^4 infectious *B. burgdorferi* were susceptible regardless of the immunogen given (Table 4). In contrast, only 8.3% (1 of 12) of the mice immunized with GST-VraA(FL) were infected when challenged with 10^2 *B. burgdorferi* (Table 4), whereas the naive and GST-immunized controls were infected 40% and 63 to 67% of the time (depending on the infected tissue assessed), respectively (Table 4). To assess statistical significance, a 3×2 contingency table was used to test the null hypothesis "Protection against *B. burgdorferi* challenge is independent of the vaccinogen used." Values corresponding to skin samples obtained using a 10^2 challenge inoculum were used in this analysis. The calculated χ^2 of 8.67 exceeded that of $\chi^2_{0.025,2}$ of 7.378, so the null hypothesis was rejected ($0.01 < P < 0.025$; see Table 4), indicating that the protection observed in the VraA immunized mice was statistically significant. Similar results were obtained

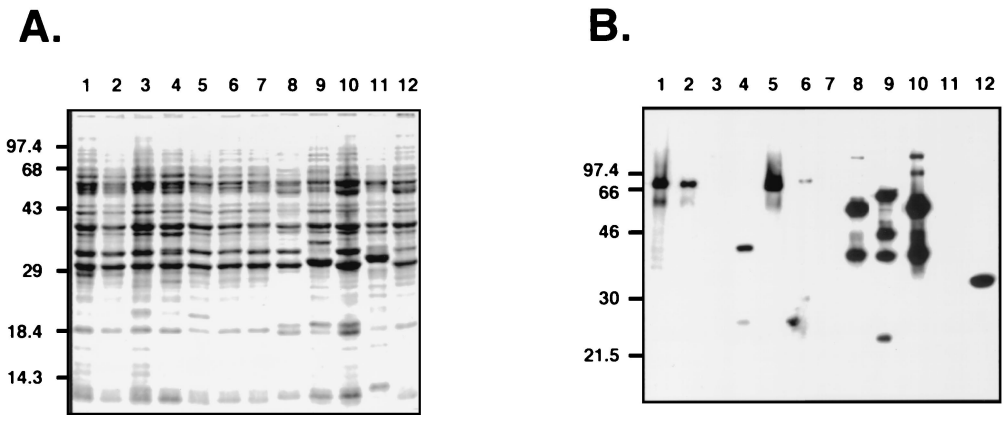

FIG. 6. Presence of VraA or VraA homologues in various *B. burgdorferi* sensu lato isolates. Whole-cell lysates from *B. burgdorferi* sensu stricto strain B31 passage 4 (lane 1), strain B31 passage 47 (lane 2), strain 297 (lane 3), strain CA-2-87 (lane 4), strain ECM-NY-86 (lane 5), strain JD-1 (lane 6), strain NT-1 (lane 7), and strain 2872-2 (lane 12) were resolved by SDS-PAGE along with *B. andersonii* strains MOD3, MOD5, and MOD6 (lanes 8 to 10) and *B. garinii* strain IP-90 (lane 11). All isolates with the exception of the strain B31 isolates were passaged two times in vitro. The resulting gels were either stained with Coomassie blue (A) or immunoblotted and probed with anti-VraA serum (B). The numbers on the left refer to the molecular mass of protein markers (in kilodaltons).

with bladder tissue and, to a lesser extent, spleen tissue (see Table 4).

Cross-reactivity of VraA antiserum against *B. burgdorferi* sensu lato isolates. To determine whether other *Borrelia* spp. encoded a homologue of VraA, we tested protein lysates for reactivity to antiserum directed against full-length strain B31 VraA in a Western blot assay. All *B. burgdorferi* sensu stricto isolates encoded a cross-reactive VraA-like antigen; however, only strain ECM-NY-86 encoded an antigen of the same molecular mass as that of strain B31 VraA (Fig. 6; note a similar immunoreactive band in both lanes 1 and 5). In contrast, strains 297, JD-1, and NT-1 (Fig. 6, lanes 3, 6, and 7, respectively) encoded antigens that were weakly reactive (only visible after long exposures; not seen in Fig. 6) and larger in molecular mass relative to strain B31 VraA, whereas strains CA-2-87 and 2872-2 (Fig. 6, lanes 4 and 12, respectively) produced strongly reactive homologues that were smaller in molecular mass. Some *B. burgdorferi* sensu lato isolates such as *B. garinii* strain Ip90, did not encode a VraA-like molecule, whereas others (Fig. 6, lane 11), including *B. afzelii* ACA1 (data not shown) and *B. andersonii* strain MOD-3, MOD-5, and MOD-6, did synthesize reactive homologues (Fig. 6, lanes 8 to 10, respectively). Interestingly, *B. andersonii* encoded multiple, highly reactive antigens. These strains were obtained from infected rabbit skin samples and represent a polyclonal population. Therefore, the profile observed for these samples may reflect multiple genetic loci that encode VraA-like molecules that are either present in each individual bacterium or a subset of individual clones within the polyclonal pool tested. The reactivity observed for antiserum against full-length VraA was identical to that seen for reactivity against the amino-terminal half of VraA, which is composed mostly of the repetitive domain. In contrast, little or no reactivity was seen for the antiserum generated against the carboxy-terminal half of VraA, implying that the cross-reactivity between the VraA homologues is mediated via the antigenicity of the repetitive motifs (data not shown).

DISCUSSION

In this report we describe the characterization of a surface-exposed repetitive motif containing antigen, designated VraA, that we originally identified as an infection associated antigen in infection immune rabbits from *B. burgdorferi* sensu stricto strain B31 (13, 38). The most striking feature of the *vraA* locus was the number of completely conserved 27-bp repeats encoding an invariant 9-amino-acid repetitive motif (see Fig. 1). Unlike other antigens, such as the Bdr proteins of *Borrelia* spp. (32, 51), the Vlp repetitive antigens of *Mycoplasma* spp. (10, 33), and various proteins from *Plasmodium* spp. (16, 22, 23), that contain repetitive domains, the repetition in *vraA* is absolute at the nucleotide level, suggesting that this conservation is important in maintaining some as-yet-unknown function essential for maximal infectivity. Alternatively, the repeat units may represent a site for recombination resulting in antigenic variation or slipped strand mispairing resulting in either antigenic or phase variants. By analogy to other prokaryotic systems, notably the Vlp proteins of *Mycoplasma* spp. (33, 48) and the Opa antigens of *Neisseria* spp. (6, 28), VraA may change its antigenicity by varying its length via slipped-strand mispairing. This change in length has been shown previously to change the antigenicity of *Mycoplasma* Vlp antigens (33, 48) and cell-wall-associated proteins from gram-positive bacteria (20). This antigenic variation could aid in immune evasion resulting in persistent *B. burgdorferi* infection. Specifically, the repeat region of *vraA* contains a stretch of six consecutive adenine nucleotides (within a region containing adenines at 15 of 18 locations). A single mismatch within this mononucleotide repetitive domain would yield an antigen with either a modified repetitive primary sequence that would be truncated soon after the repeats or a variant that was abbreviated almost immediately (i.e., a stop codon introduced within a repeat unit). In either case, the resulting modified forms might exhibit reduced or nullified antigenicity. The location of this change, i.e., which repeat unit is

modified, would dictate whether any of the repeats in native VraA would be synthesized and to what degree the truncated variants might cross-react with antibodies directed against full-length VraA. This could, in part, explain the resistance to infectious challenge with 100 low-passage *B. burgdorferi* observed in 1 of the 12 mice immunized with recombinant VraA (Table 4). That is, if a subpopulation of our inoculum changed such that VraA was either antigenically modified by the increased or decreased length in the number of repeats or by frameshift mutations within the repeat units that resulted in truncated antigenic variants, then *B. burgdorferi* encoding these proteins may not be killed by antibody directed against "full-length" recombinant VraA. To date, no evidence of such changes in native VraA have been observed in *B. burgdorferi* B31 inasmuch as the frequency of these changes may be similar to that seen in other systems (approximately 10^{-3} to 10^{-4} [6, 28]). However, we have recently determined that variants of *vraA* are present in in vitro-cultivated *B. burgdorferi* B31 that have additional repetitive domains (i.e., greater than 21 in number; K. L. Swingle and J. T. Skare, unpublished data), suggesting that VraA may be an antigenically variable protein. Additionally, as seen in Fig. 6, other sensu stricto isolates synthesize putative homologues relative to strain B31 that may represent putative variants of *B. burgdorferi* sensu stricto VraA. The changes predicted for these variants may occur more frequently in vivo (i.e., within an infected animal) not unlike what has been shown for both the *vlsE* (49, 50) and *ospEF*-related (*erp*) loci (45), whose changes occur exclusively during infection. A more detailed analysis of the strain B31 *vraA* variants and its requisite homologues should indicate whether this hypothesis is valid.

An important consideration related to protective immunity in *B. burgdorferi* is the form in which the challenge inoculum is delivered. Recently, it has become apparent that host-adapted *B. burgdorferi* repress or derepress several genes, notably, *ospA* or *ospC*, respectively (34), in the disparate environments that *B. burgdorferi* occupies (i.e., ticks versus mammals), as well as under experimental ex vivo conditions (27). *B. burgdorferi* is present at very low levels during mammalian infection; therefore the ability to evaluate gene expression in vivo has required the implementation of indirect methodologies (38). To circumvent this dilemma, Akins et al. have demonstrated recently that *B. burgdorferi* synthesizes a subset of proteins and/or antigens in dialysis membrane chambers that are implanted within the peritoneal cavity of rats (1). Although temperature certainly upregulates some *B. burgdorferi* genes, clearly other host signals were required for expression of some of the gene products observed in this model system (1). Our results indicate that a temperature shift in vitro results in the modest induction of VraA (Fig. 5). Previous studies indicated that the growth phase is also an important consideration for assessing gene regulation in *B. burgdorferi*. Specifically, OspC and BmpD are regulated in the transition from logarithmic-phase growth to stationary-phase growth (21, 31). However, in contrast, our data indicate that VraA is not subject to growth phase regulation (Fig. 5 and data not shown). It is conceivable that *vraA* may be expressed at high levels in vivo (i.e., within the arthropod vector or mammalian hosts) where nutrients and other important cofactors may be limiting. Whether *vraA* is subject to such regulation remains to be determined.

Shang et al. recently demonstrated that rabbits immunized with outer membrane vesicles containing surface proteins were protected only when challenged with in vitro cultivated *B. burgdorferi*; rabbits challenged with skin biopsies containing host-adapted *B. burgdorferi* were infected in a manner indistinguishable from naive controls (35). One explanation could be alternative gene expression; that is, the expression of new antigens within the infected mammal that are not expressed during in vitro cultivation. Another viable possibility is that *B. burgdorferi* changes antigens expressed during in vitro cultivation (i.e., antigenic variation) or uses other mechanisms, including phase variation, to alter their antigenic profile. Once inside the host, this effect could manifest in a polyclonal manner such that each *B. burgdorferi* cell may express a different form of a given antigen, such as VlsE (49, 50) or OspEF (45) or different antigens in various combinations. This hypothesis may, in part, explain the presence of numerous paralogous gene families. Such genetic paralogues may therefore be involved in immune evasion independent of function. The *vraA* locus belongs to one such paralogous family (family 60; see TIGR website). However, in contrast to the other family members, *vraA* is the only family member that contains the aforementioned repeat units with the notable exception of the protein encoded by BBI28, which contains a similar but nonidentical single repeat unit relative to VraA (see Fig. 1).

The absolute nature of the repetitive domains at both the nucleotide and the amino acid level suggests an important role for this motif. One possible explanation could be related to an increased avidity of VraA for a target ligand. In this regard, we have no indication that VraA functions as an adhesin in either arthropod or mammalian structures. Alternatively, these repeats may serve as an immunological "smokescreen," as has been proposed for several *Plasmodium* antigens (22). If so, then the epitope(s) defined by these repeat units would theoretically mediate a robust yet nonprotective response. Our immunization results, indicating partial protection to needle challenge, argue against this; however, the possibility that the repeats of strain B31 VraA change in length in a manner analogous to that observed in other *B. burgdorferi* sensu lato isolates (see Fig. 6), resulting in antigenic variation, suggest that a strong immune response to a single form of VraA may not protect in all cases. Heterologous challenge against animals immunized with *B. burgdorferi* B31 VraA could decipher this; these studies and others to determine whether VraA is subject to phase and/or antigenic variation are in progress.

ACKNOWLEDGMENTS

M.L.-R. and E.A.B. contributed equally to this report.

We thank Kristen L. Swingle and J. Seshu for critical evaluation of the manuscript and Deanna C. Moore for excellent technical assistance.

This work was supported by a Scientist Development Grant from the American Heart Association and United States Public Health Service grant R01-AI42345 from the National Institute of Allergy and Infectious Diseases (both to J.T.S.).

REFERENCES

1. **Akins, D. R., K. W. Bourell, M. J. Caimano, M. V. Norgard, and J. D. Radolf.** 1998. A new animal model for studying Lyme disease spirochetes in a mammalian host-adapted state. J. Clin. Investing. **101:**2240–2250.
2. **Barbour, A. G.** 1989. The molecular biology of *Borrelia*. Rev. Infect. Dis. **11**(Suppl. 6)**:**S1470–S1474.
3. **Barbour, A. G., R. A. Heiland, and T. R. Howe.** 1985. Heterogeneity of major proteins in Lyme disease borreliae: a molecular analysis of North American and European isolates. J. Infect. Dis. **152:**478–484.
4. **Barbour, A. G., S. L. Tessier, and S. F. Hayes.** 1984. Variation in a major surface protein of Lyme disease spirochetes. Infect. Immun. **45:**94–100.
5. **Barthold, S. W., E. Fikrig, L. K. Bockenstedt, and D. H. Persing.** 1995. Circumvention of outer surface protein A immunity by host-adapted *Borrelia burgdorferi*. Infect. Immun. **63:**2255–2261.
6. **Belland, R. J., S. G. Morrison, P. van der Ley, and J. Swanson.** 1989. Expression and phase variation of gonococcal P.II genes in *Escherichia coli* involves ribosomal frameshifting and slipped-strand mispairing. Mol. Microbiol. **3:**777–786.
7. **Campbell, G. L., W. S. Paul, M. E. Schriefer, R. B. Craven, K. E. Robbins, and D. T. Dennis.** 1995. Epidemiologic and diagnostic studies of patients with suspected early Lyme disease, Missouri, 1990–1993. J. Infect. Dis. **172:** 470–480.
8. **Carroll, J. A., C. F. Garon, and T. G. Schwan.** 1999. Effects of environmental pH on membrane proteins in *Borrelia burgdorferi*. Infect. Immun. **67:**3181–3187.
9. **Casjens, S., N. Palmer, R. van Vugt, W. M. Huang, B. Stevenson, P. Rosa, R. Lathigra, G. Sutton, J. Peterson, R. J. Dodson, D. Haft, E. Hickey, M. Gwinn, O. White, and C. M. Fraser.** 2000. A bacterial genome in flux: the twelve linear and nine circular extrachromosomal DNAs in an infectious isolate of the Lyme disease spirochete *Borrelia burgdorferi*. Mol. Microbiol. **35:**490–516.
10. **Citti, C., R. Watson-McKown, M. Droesse, and K. S. Wise.** 2000. Gene families encoding phase- and size-variable surface lipoproteins of *Mycoplasma hyorhinis*. J. Bacteriol. **182:**1356–1363.
11. **de Silva, A. M., S. R. r. Telford, L. R. Brunet, S. W. Barthold, and E. Fikrig.** 1996. *Borrelia burgdorferi* OspA is an arthropod-specific transmission-blocking Lyme disease vaccine. J. Exp. Med. **183:**271–275.
12. **Fikrig, E., S. W. Barthold, F. S. Kantor, and R. A. Flavell.** 1990. Protection of mice against the Lyme disease agent by immunizing with recombinant OspA. Science **250:**553–556.
13. **Foley, D. M., R. J. Gayek, J. T. Skare, E. A. Wagar, C. I. Champion, D. R. Blanco, M. A. Lovett, and J. N. Miller.** 1995. Rabbit model of Lyme borreliosis: erythema migrans, infection-derived immunity, and identification of *Borrelia burgdorferi* proteins associated with virulence and protective immunity. J. Clin. Investing. **96:**965–975.
14. **Fraser, C. M., S. Casjens, W. M. Huang, G. G. Sutton, R. Clayton, R. Lathigra, O. White, K. A. Ketchum, R. Dodson, E. K. Hickey, M. Gwinn, B. Dougherty, J. F. Tomb, R. D. Fleischmann, D. Richardson, J. Peterson, A. R. Kerlavage, J. Quackenbush, S. Salzberg, M. Hanson, R. van Vugt, N. Palmer, M. D. Adams, J. Gocayne, and J. C. Venter.** 1997. Genomic sequence of a Lyme disease spirochaete, *Borrelia burgdorferi*. Nature **390:**580–586.
15. **Gilmore, R. D., Jr., K. J. Kappel, M. C. Dolan, T. R. Burkot, and B. J. Johnson.** 1996. Outer surface protein C (OspC), but not P39, is a protective immunogen against a tick-transmitted *Borrelia burgdorferi* challenge: evidence for a conformational protective epitope in OspC. Infect. Immun. **64:**2234–2239.
16. **Godson, G. N., J. Ellis, P. Svec, D. H. Schlesinger, and V. Nussenzweig.** 1983. Identification and chemical synthesis of a tandemly repeated immunogenic region of *Plasmodium knowlesi* circumsporozoite protein. Nature **305:**29–33.
17. **Gross, D. M., T. Forsthuber, M. Tary-Lehmann, C. Etling, K. Ito, Z. A. Nagy, J. A. Field, A. C. Steere, and B. T. Huber.** 1998. Identification of LFA-1 as a candidate autoantigen in treatment-resistant Lyme arthritis. Science **281:** 703–706.
18. **Hagman, K. E., P. Lahdenne, T. G. Popova, S. F. Porcella, D. R. Akins, J. D. Radolf, and M. V. Norgard.** 1998. Decorin-binding protein of *Borrelia burgdorferi* is encoded within a two-gene operon and is protective in the murine model of Lyme borreliosis. Infect. Immun. **66:**2674–2683.
19. **Hanson, M. S., D. R. Cassatt, B. P. Guo, N. K. Patel, M. P. McCarthy, D. W. Dorward, and M. Höök.** 1998. Active and passive immunity against *Borrelia burgdorferi* decorin binding protein A (DbpA) protects against infection. Infect. Immun. **66:**2143–2153.
20. **Hartford, O., P. Francois, P. Vaudaux, and T. J. Foster.** 1997. The dipeptide repeat region of the fibrinogen-binding protein (clumping factor) is required for functional expression of the fibrinogen-binding domain on the *Staphylococcus aureus* cell surface. Mol. Microbiol. **25:**1065–1076.
21. **Indest, K. J., R. Ramamoorthy, M. Sole, R. D. Gilmore, B. J. Johnson, and M. T. Philipp.** 1997. Cell-density-dependent expression of *Borrelia burgdorferi* lipoproteins in vitro. Infect. Immun. **65:**1165–1171.
22. **Kemp, D. J., R. L. Coppel, and R. F. Anders.** 1987. Repetitive proteins and genes of malaria. Annu. Rev. Microbiol. **41:**181–208.
23. **Koenen, M., A. Scherf, O. Mercereau, G. Langsley, L. Sibilli, P. Dubois, L. Pereira da Silva, and B. Muller-Hill.** 1984. Human antisera detect a *Plasmodium falciparum* genomic clone encoding a nonapeptide repeat. Nature **311:**382–385.
24. **Labandeira-Rey, M., and J. T. Skare.** 2001. Decreased infectivity in *Borrelia burgdorferi* strain B31 is associated with loss of linear plasmid 25 or 28-1. Infect. Immun. **69:**446–455.
25. **Logigian, E. L., R. F. Kaplan, and A. C. Steere.** 1990. Chronic neurologic manifestations of Lyme disease. N. Engl. J. Med. **323:**1438–1444.
26. **Masters, E. J., and H. D. Donnell.** 1995. Lyme and/or Lyme-like disease in Missouri. Missouri Med. **92:**346–353.
27. **Montgomery, R. R., S. E. Malawista, K. J. Feen, and L. K. Bockenstedt.** 1996. Direct demonstration of antigenic substitution of *Borrelia burgdorferi* ex vivo: exploration of the paradox of the early immune response to outer surface proteins A and C in Lyme disease. J. Exp. Med. **183:**261–269.
28. **Murphy, G. L., T. D. Connell, D. S. Barritt, M. Koomey, and J. G. Cannon.** 1989. Phase variation of gonococcal protein II: regulation of gene expression by slipped-strand mispairing of a repetitive DNA sequence. Cell **56:**539–547.
29. **Nguyen, T. P., T. T. Lam, S. W. Barthold, S. R. r. Telford, R. A. Flavell, and E. Fikrig.** 1994. Partial destruction of *Borrelia burgdorferi* within ticks that engorged on OspE- or OspF-immunized mice. Infect. Immun. **62:**2079–2084.
30. **Postic, D., N. M. Ras, R. S. Lane, M. Hendson, and G. Baranton.** 1998. Expanded diversity among Californian borrelia isolates and description of *Borrelia bissettii* sp. nov. (formerly *Borrelia* group DN127). J. Clin. Microbiol. **36:**3497–3504.
31. **Ramamoorthy, R., and M. T. Philipp.** 1998. Differential expression of *Borrelia burgdorferi* proteins during growth in vitro. Infect. Immun. **66:**5119–5124.
32. **Roberts, D. M., J. A. Carlyon, M. Theisen, and R. T. Marconi.** 2000. The *bdr* gene families of the Lyme disease and relapsing fever spirochetes: potential influence on biology, pathogenesis, and evolution. Emerg. Infect. Dis. **6:**110–122.
33. **Rosengarten, R., and K. S. Wise.** 1990. Phenotypic switching in mycoplasmas: phase variation of diverse surface lipoproteins. Science **247:**315–318.
34. **Schwan, T. G., J. Piesman, W. T. Golde, M. C. Dolan, and P. A. Rosa.** 1995. Induction of an outer surface protein on *Borrelia burgdorferi* during tick feeding. Proc. Natl. Acad. Sci. USA **92:**2909–2193.
35. **Shang, E. S., C. I. Champion, X. Y. Wu, J. T. Skare, D. R. Blanco, J. N. Miller, and M. A. Lovett.** 2000. Comparison of protection in rabbits against host-adapted and cultivated *Borrelia burgdorferi* following infection-derived immunity or immunization with outer membrane vesicles or outer surface protein A. Infect. Immun. **68:**4189–4199.
36. **Sigal, L. H., J. M. Zahradnik, P. Lavin, S. J. Patella, G. Bryant, R. Haselby, E. Hilton, M. Kunkel, D. Adler-Klein, T. Doherty, J. Evans, P. J. Molloy, A. L. Seidner, J. R. Sabetta, H. J. Simon, M. S. Klempner, J. Mays, D. Marks, and S. E. Malawista.** 1998. A vaccine consisting of recombinant *Borrelia burgdorferi* outer-surface protein A to prevent Lyme disease. Recombinant Outer-Surface Protein A Lyme Disease Vaccine Study Consortium. N. Engl. J. Med. **339:**216–222.
37. **Skare, J. T., C. I. Champion, T. A. Mirzabekov, E. S. Shang, D. R. Blanco, H. Erdjument-Bromage, P. Tempst, B. L. Kagan, J. N. Miller, and M. A. Lovett.** 1996. Porin activity of the native and recombinant outer membrane protein Oms28 of *Borrelia burgdorferi*. J. Bacteriol. **178:**4909–4918.
38. **Skare, J. T., D. M. Foley, S. R. Hernandez, D. C. Moore, D. R. Blanco, J. N. Miller, and M. A. Lovett.** 1999. Cloning and molecular characterization of plasmid-encoded antigens of *Borrelia burgdorferi*. Infect. Immun. **67:**4407–4417.
39. **Skare, J. T., T. A. Mirzabekov, E. S. Shang, D. R. Blanco, H. Erdjument-Bromage, J. Bunikis, S. Bergstrom, P. Tempst, B. L. Kagan, J. N. Miller, and M. A. Lovett.** 1997. The Oms66 (p66) protein is a *Borrelia burgdorferi* porin. Infect. Immun. **65:**3654–3661.
40. **Skare, J. T., E. S. Shang, D. M. Foley, D. R. Blanco, C. I. Champion, T. Mirzabekov, Y. Sokolov, B. L. Kagan, J. N. Miller, and M. A. Lovett.** 1995. Virulent strain associated outer membrane proteins of *Borrelia burgdorferi*. J. Clin. Investig. **96:**2380–2392.
41. **Steere, A. C.** 1989. Lyme disease. N. Eng. J. Med. **321:**586–596.
42. **Steere, A. C.** 1995. Musculoskeletal manifestations of Lyme disease. Am. J. Med. **98:**44S–51S.
43. **Steere, A. C., A. Gibofsky, M. E. Patarroyo, R. J. Winchester, J. A. Hardin, and S. E. Malawista.** 1979. Chronic Lyme arthritis. Clinical and immunogenetic differentiation from rheumatoid arthritis. Ann. Intern. Med. **90:**896–901.
44. **Steere, A. C., V. K. Sikand, F. Meurice, D. L. Parenti, E. Fikrig, R. T. Schoen, J. Nowakowski, C. H. Schmid, S. Laukamp, C. Buscarino, and D. S. Krause.** 1998. Vaccination against Lyme disease with recombinant *Borrelia burgdorferi* outer-surface lipoprotein A with adjuvant. Lyme Disease Vaccine Study Group. N. Engl. J. Med. **339:**209–215.
45. **Sung, S. Y., J. V. McDowell, J. A. Carlyon, and R. T. Marconi.** 2000. Mutation and recombination in the upstream homology box-flanked *ospE*-related

genes of the Lyme disease spirochetes result in the development of new antigenic variants during infection. Infect. Immun. **68:**1319–1327.
46. **Wang, G., A. P. van Dam, I. Schwartz, and J. Dankert.** 1999. Molecular typing of *Borrelia burgdorferi* sensu lato: taxonomic, epidemiological, and clinical implications. Clin. Microbiol. Rev. **12:**633–653.
47. **Wormser, G. P.** 1996. Lyme disease vaccine. Infection **24:**203–207.
48. **Yogev, D., R. Rosengarten, R. Watson-McKown, and K. S. Wise.** 1991. Molecular basis of *Mycoplasma* surface antigenic variation: a novel set of divergent genes undergo spontaneous mutation of periodic coding regions and 5′ regulatory sequences. EMBO J. **10:**4069–4079.
49. **Zhang, J. R., J. M. Hardham, A. G. Barbour, and S. J. Norris.** 1997. Antigenic variation in Lyme disease borreliae by promiscuous recombination of VMP-like sequence cassettes. Cell **89:**275–285.
50. **Zhang, J. R., and S. J. Norris.** 1998. Kinetics and in vivo induction of genetic variation of *vlsE* in *Borrelia burgdorferi*. Infect. Immun. **66:**3689–3697.
51. **Zückert, W. R., J. Meyer, and A. G. Barbour.** 1999. Comparative analysis and immunological characterization of the *Borrelia* Bdr protein family. Infect. Immun. **67:**3257–3266.

Editor: D. L. Burns