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Immunodominant Epitope in the C-Terminus of a Variable Major Protein in *Borrelia duttonii*, an Agent of Tick-Borne Relapsing Fever

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Abstract: **Borrelia duttonii strain Ly was isolated from a child with tick-borne relapsing fever in Tanzania. *B. duttonii* produces variable major proteins (Vmps), which undergo antigenic variation. We previously reported transcription of the *vmpP* gene, which is one of the Vmp genes in strain Ly, detected *in vitro* cultivation. In the current study, we purified the recombinant non-lipidated VmpP protein by affinity chromatography and produced VmpP polyclonal antibodies. Antigenicity of VmpP was examined by Western immunoblot analysis and peptide-based enzyme-linked immunosorbent assays. Antigenic epitopes were shown to comprise five regions interspersed within the VmpP primary amino acid sequence. Synthetic peptides spanning residues of three of five regions, 232–237 (LASIVD), 280–285 (AGGIAL), and 350–355 (KAADQQ), reacted strongly with the VmpP-specific antibody and these residues were identified as epitopes. In particular, the C-terminal domain (KAADQQ) of this protein was immunoreactive. Further research based on our results will promote the development of a recombinant vaccine for *B. duttonii* infection.**

Key words: **Borrelia duttonii, Epitope, Infection, Variable major protein**

Tick-borne relapsing fever (TBRF) is characterized by cyclic spirochetemias with periodic fever, and occasionally with neurological symptoms (1). These repeated clinical manifestations are associated with antigenic changes of relapsing fever borreliae, which have been examined extensively in *Borrelia hermsii* (22, 29, 33). Antigenic variation of surface-exposed lipoproteins has been identified as an important immune evasion mechanism in relapsing fever borreliae (1–3). In a case of *B. hermsii* infection, periodic spirochetemia and fever occur when the spirochete alternates expression of at least 25 different lipoproteins that are called variable major proteins (Vmps) (34). *Borrelia burgdorferi*, an agent of Lyme disease, promotes extensive antigenic variation of outer surface Vmp-like sequences (Vls) to escape the host immune response (21, 45). The Vls locus is found on the 28-kb linear plasmid of *B. burgdorferi* strain B31 and shares some features with the antigenic variation system in *B. hermsii* (2). These proteins appear to play a pivotal role in the pathogenicity of borreliae.

In East Africa, TBRF is endemic and the causative agent, *Borrelia duttonii*, is transmitted by ticks of the genus *Ornithodoros*. Cutler et al. (9) have successfully cultivated *B. duttonii* strains in artificial BSKII medium from pediatric patients in Mvumi Hospital, Dodoma, Tanzania. The strain Ly, one of five such clinical isolates, contains a linear, 1-megabase chromosome and 12 linear plasmids of 11 to 200 kilobases in size (41). The size change of linear plasmid from 44 to 69-kb during *in vitro* cultivation of this strain caused loss of infectivity in experimental mice, leading us to believe that genes on this plasmid may be involved with infectivity in mammals. Therefore, we determined the entire nucleotide sequence of the plasmid, which had 21 *vmp* gene sequences (designated as *vmpA* to *U*) (40). To date, the complete genomic sequence of the Lyme disease spirochete *B. burgdorferi* strain B31 has revealed over 150

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Abbreviations: bp, base pair(s); BSKII, Barbour Stoenner Kelly II; DDBJ, DNA Data Bank of Japan; GST, glutathione-*S*-transferase; PCR, polymerase chain reaction.

putative lipoprotein genes on its chromosome and plasmids (7, 15). These lipoprotein genes include outer membrane proteins associated with antigenicity (4, 10, 26), bacterial adhesion (5), and colonization in vector ticks (30, 31). Outer surface protein (Osp) A of *B. burgdorferi* was used as protective vaccine for Lyme borreliosis in the U.S. from 1999 to 2002 (39), and new vaccine candidates are considered for targeting one or more Osps and newly identified antigens such as decorin-binding protein (DbpA) and fibronectin-binding protein (BBK32) (5). In contrast, no effective vaccine for relapsing fever currently exists due in part to the lack of finding common antigens among the agents, and also due to the temporal expression of surface Vmps on the bacterial surface while in the mammalian bloodstream.

Clinical and epidemiological studies have shown that TBRF causes significant morbidity in patients under 5 years old, perinatal mortality, and morbidity (42). Therefore, TBRF vaccine would be important to control infection of spirochetes. Our previous study demonstrated that *B. duttonii* strain Ly transcribes the *vmpP* gene during early infection in gerbils and also in culture media (unpublished data). To better understand the antigenicity of VmpP and to gain insights for possible vaccine development for relapsing fever borreliae, we determined the immunodominant epitopes on VmpP of *B. duttonii* strain Ly. In this study, five epitopes were identified with specific VmpP antibodies and one on the C-terminus was recognized strongly. The C-terminal domain may thus provide a target for the development of a vaccine against *B. duttonii* infection and our results may provide the basis for the design of a vaccine based on the Vmps.

Materials and Methods

Bacterial strains and growth media. *B. duttonii* strain Ly was isolated from a 2-year-old girl with TBRF in Tanzania (9) and used for all experimental work in this study. It has been passed in broth medium continuously at least 5 times, and stored at −80 C. *B. duttonii* strain Ly was grown in Barbour-Stoenner-Kelly's (BSK) II medium supplemented with 6% rabbit serum at 32 C as described previously (40).

Recombinant plasmid construction. Three different plasmids encoding N-terminal truncated VmpP and GST fusion protein were created by PCR using *Ex Taq* polymerase (TaKaRa Bio, Kyoto, Japan). The PCR was performed with each set of the following primers: forward primers, VmpP-C15: 5′- TCC GGA TCC TGT AAT AGT GGG GGA GTA AAG-3′, VmpP-S34: 5′-

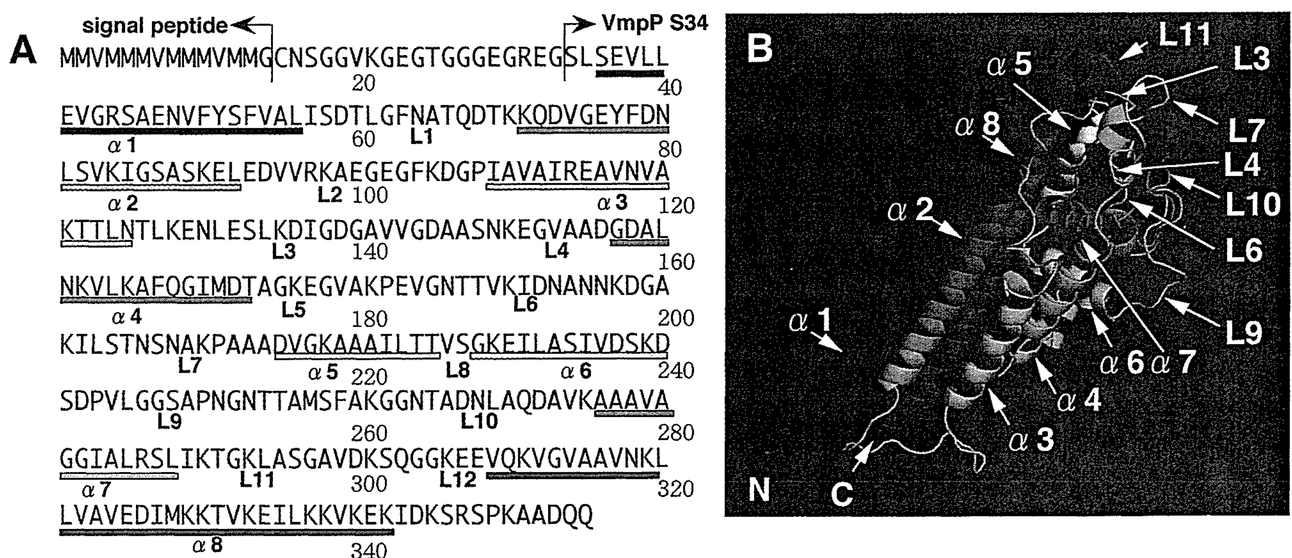

Fig. 1. Primary amino acid sequence and putative 3-D structure of VmpP. A: The primary amino acid sequence of VmpP is cited from DDBJ/EMBL/GenBank database (accession no. AB073701) (40). Amino acid number is indicated below the sequence, and putative signal sequence and VmpP-S34 regions are also shown. Presumed α-helices (α1 to α8) and short α-helices are indicated in blue characters, and loop regions (L1 to L12) in red. Color underlines for each α-helix (α1 to α8) correspond to those in the 3-D structure. B: Putative three-dimensional structure (from Gly 33 to Asp 344) of VmpP was constructed by using Swiss-Model server (http://swissmodel.expasy.org) based on the 3-D structure of *B. burgdorferi* OspC (11), and was depicted with Swiss Pdb Viewer (18). The N and C indicate the N-terminus and C-terminus of the amino acid sequence, respectively.

TCC GGA TCC AGT TTG AGT GAG GTA TTG CTG-3′, or VmpP-S58: 5′-TCC GGA TCC TCA GAT ACT TTG GGC TTT AAT-3′, reverse primer, VmpP-Rev: 5′-ATA GAG CTC *TTA* TTG CTG ATC CGC TGC CTT -3′. N-terminal amino acids of VmpP-C15, VmpP-S34, and VmpP-S58 indicate amino acid positions: Cys (position 15), Ser (position 34), and Ser (position 58), from putative initial methionine, respectively (Fig. 1A). *Bam*HI and *Sac*I recognition sequences were introduced into the forward and reverse primers, respectively (underlined sequences indicate restriction enzyme cleavage sites, and the stop codon in the reverse primer is italicized). DNA amplification using a Perkin-Elmer GeneAmp PCR System 9700 (Applied Biosystems Japan, Osaka, Japan) was performed in a thermal cycler with 25 cycles of denaturation (94 C, 30 sec), annealing (58 C, 30 sec), and extension (72 C, 1 min). The PCR product was cleaved and cloned into *Bam*HI and *Sac*I sites of pGEX-KG vector (17), and then *Escherichia coli* DH5α was transformed with the recombinant plasmid that contained the partial *vmpP* gene (pGEX-VmpP-C15 [nt 43–1070], pGEX-VmpP-S34 [nt 99–1070], and pGEX-VmpP-S58 [nt 172–1070]). Each recombinant plasmid sequence was confirmed with an automated DNA sequencer (ABI 3100 genetic analyzer; Applied Biosystems) by using a Big dye terminator cycle sequencing kit (Applied Biosystems).

Expression and purification of GST-VmpP fusion protein. Recombinant plasmids were purified from *E. coli* DH5α cells and then transformed into *E. coli* BL21 (DE3) cells. Transformants were grown overnight at 37 C in 2× YT medium (tryptone 8 g, yeast extracts 5 g, and NaCl 2.5 g in 500 ml) containing ampicillin (100 μg/ml). Fifty milliliters of the overnight culture was transferred into 500 ml of 2× YT broth and incubated for 3 hr at 37 C. Induction of the fusion proteins was performed by addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and incubation was continued for 3 hr at 25 C. The three fusion proteins were expressed and each protein was purified as described below. In brief, *E. coli* cells were collected by centrifugation and the fusion protein was released by sonication. Cell debris was removed by centrifugation, and supernatant was then applied to a Glutathione-Sepharose 4B column (Amersham Pharmacia Biotech, Uppsala, Sweden). After washing the column with PBS, the fusion proteins were digested with thrombin, and the truncated VmpP protein (aa 34–355) was eluted from the column. The purity and molecular size of the protein was verified by SDS-PAGE, and the N-terminal amino acid sequence of the protein was confirmed by using PPSQ-10 protein sequencer (Shimadzu, Tokyo).

Development of rabbit Anti-VmpP polyclonal antibody. Two Japanese White rabbits (2.0 kg) were used for development of antibodies. Primary immunizations were done with subcutaneous footpad injections of 400 μg of purified VmpP-S34 protein in Freund's complete adjuvant (1:1 in volume, Difco Laboratories, Detroit, Mich., U.S.A.). After 14 days, each animal received an intradermal boost with 200 μg of VmpP-S34 protein in Freund's incomplete adjuvant (1:1 in volume). Intradermal injection in the dorsal skin was done every 2 weeks until a clear arthus reaction became visible. Each rabbit was bled from the ear vein 7 days after the last injection. Blood was centrifuged and the serum was collected. Immunoglobulin G was purified from the serum by using an MAb Trap kit (Amersham Pharmacia Biotech) according to the manufacturer's recommendation. Antibody purities were examined by SDS-PAGE and antibodies were stored at −20 C.

Construction of plasmids containing vmpP *gene fragments.* Based on the *vmpP* gene sequence, the primers shown in Tables 1 and 2 were synthesized and used to amplify gene fragments encoding partial VmpP peptides. The reverse primer was designed with a stop codon upstream of the *Sac*I cleavage site. DNA amplifications were performed in a thermal cycler with 25 cycles of denaturation (94 C, 30 sec), annealing (58 C, 30 sec), and extension (72 C, 10 sec). Each DNA fragment was digested with *Bam*HI and *Sac*I enzymes and ligated into the pGEX-KG vector at the same restriction enzyme sites. The recombinant plasmid was introduced into *E. coli* DH5α cells and propagated. Plasmid DNA was then extracted, purified, and sequenced, and was then used to transform *E. coli* BL21(DE3) cells. Procedures for cultivation of bacterial cells, IPTG induction, and purification of the fusion protein were the same as described above.

SDS-PAGE and Western blotting. Cultured cell lysates (10 μg each) were subjected to SDS-PAGE according to Laemmli's method (25) using 12.5% acrylamide under reduced conditions. The gel was processed for Western blot analysis with antibody against VmpP according to standard procedures (43). The samples were boiled for 5 min with buffer (125 mM Tris-HCl, 3% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.01% bromophenol blue, pH 6.8) and resolved by gel electrophoresis. The gel was stained with Coomassie brilliant blue R-250, and proteins were transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, Calif., U.S.A.) for 2 hr at 2 mA/cm^2 by ElectroBlotter (ATTO, Osaka, Japan) using a transfer buffer (25 mM Tris, 200 mM glycine, 5% methanol). The membrane was blocked by incubation in TBST (20 mM Tris-HCl, 0.15 M NaCl, and 0.1% Tween 20, pH

Table 1. Primers for 30 amino acid fragments of VmpP employed in this study

Primer	Sequence (5′ to 3′)[a]	Fragment
BdVmpP C15	TCCGGATCCTGTAATAGTGGGGGAGTAAAG	f1
BdVmpP R1	ATAGAGCTC*TTA*TCTCCCTACTTCCAGCAA	
BdVmpP F2	TCCGGATCCCTGGAAGTAGGGAGAAGTGCA	f2
BdVmpP R2	ATAGAGCTC*TTA*TGTATCTTGAGTTGCATT	
BdVmpP F3	TCCGGATCCGCAACTCAAGATACAAAGAAG	f3
BdVmpP R3	ATAGAGCTC*TTA*ATCTTCTAACTCTTTTGA	
BdVmpP F4	TCCGGATCCAAAGAGTTAGAAGATGTAGTA	f4
BdVmpP R4	ATAGAGCTC*TTA*AACATTAACTGCTTCTCT	
BdVmpP F5	TCCGGATCCGAAGCAGTTAATGTTGCCAAG	f5
BdVmpP R5	ATAGAGCTC*TTA*ACCTACCACAGCTCCATC	
BdVmpP F6	TCCGGATCCGGAGCTGTGGTAGGTGATGCA	f6
BdVmpP R6	ATAGAGCTC*TTA*TCCTTGAAATGCTTTAAG	
BdVmpP F7	TCCGGATCCAAAGCATTTCAAGGAATAATG	f7
BdVmpP R7	ATAGAGCTC*TTA*TGCATTATCTATTTTTAC	
BdVmpP F8	TCCGGATCCAAAATAGATAATGCAAATAAT	f8
BdVmpP R8	ATAGAGCTC*TTA*TGCCTTTCCTACATCTGC	
BdVmpP F9	TCCGGATCCGATGTAGGAAAGGCAGCAGCT	f9
BdVmpP R9	ATAGAGCTC*TTA*TACAGGATCACTATCTTT	
BdVmpP F10	TCCGGATCCGATAGTGATCCTGTACTAGGA	f10
BdVmpP R10	ATAGAGCTC*TTA*TAAATTATCTGCAGTATT	
BdVmpP F11	TCCGGATCCACTGCAGATAATTTAGCACAA	f11
BdVmpP R11	ATAGAGCTC*TTA*TAATTTTCCTGTCTTAAT	
BdVmpP F12	TCCGGATCCAAGACAGGAAAATTAGCATCA	f12
BdVmpP R12	ATAGAGCTC*TTA*CTTATTTACTGCGGCTAC	
BdVmpP F13	TCCGGATCCGCCGCAGTAAATAAGCTATTA	f13
BdVmpP Rev	ATAGAGCTC*TTA*TTGCTGATCCGCTGCCTT	

[a] Underlines indicate the restriction enzyme sites (*Bam*HI and *Sac*I) and italics indicate stop codons.

8.0) with 10% skim milk, and was then incubated with anti-VmpP-S34 antibody (1:500 dilution) or anti-GST Tag™ monoclonal antibody (1:1,000 dilution) (Novagen, Darmstadt, Germany). Secondary antibodies were sheep anti-mouse IgG and donkey anti-rabbit IgG coupled to horseradish peroxidase (Amersham), and were used at a concentration of 1:10,000 dilution. The immunoreacting bands were detected using an enhanced chemiluminescence (ECL) detection kit (Amersham) or chemiluminescent peroxidase substrate (Sigma-Aldrich, St. Louis, Mo., U.S.A.). The protein concentration was determined using a Bradford protein assay kit (Bio-Rad Laboratories) and bovine serum albumin (BSA) was used as a standard. Densitometric analyses of protein bands were performed on a scanned digital gel image with an Interigent Quantifier (Bio Image, Relyon, Tokyo).

Synthetic peptides. Peptides spanning the entire immunoreactive regions (f3-b, f6-c, f9-b, f11-c, and f13-d fragments; each peptide consisted of 15 amino acids) of VmpP were synthesized as 8-mers or 7-mers overlapping each other by 4 amino acid residues. Lyophilized peptides (Sigma-Aldrich Corporation) were maintained as a concentrated stock (2–5 mg/ml) in 100% DMSO and diluted in the appropriate solution prior to immediate use.

Peptide-based enzyme-linked immunosorbent assay (ELISA). Each well of Maxsorp, 96-well ELISA plates (Nalge-Nunc, Roskilde, Denmark) was coated for 1 hr at 37 C by adding 100 μl of binding buffer (1 M sodium carbonate, pH 9.6) containing 10 μM of each peptide. The plate was washed four times with 300 μl of PBS supplemented with 1.0% BSA and was subsequently blocked by incubation with PBS-Tween (PBS-T) supplemented with 1.0% (wt/vol) BSA fraction V (Sigma) for 2 hr at room temperature. Polyclonal anti-VmpP rabbit IgG (diluted with 0.5% BSA containing 1 μg/ml PBS-T) was added to the wells, and the plate was then incubated for 2 hr at 37 C. Wells were washed four times with PBS-T and bound antibodies were detected by using *p*-nitrophenyl phosphate (1 mg/ml of diethanolamine buffer, pH 9.6) as a substrate and 10,000-fold diluted alkaline phosphatase-conjugated anti-rabbit total IgG (Sigma). The mixture was allowed to develop for 20 min and the reaction was stopped by addition of 50 μl of 0.5 N NaOH. The optical density at 405 nm (OD_{405}) was measured with a plate reader, ImmunoMini NJ-2300 (Nalge-Nunc). Results are

Table 2. Primers for 15 amino acid fragments of VmpP employed in this study

Primer	Sequence (5′ to 3′)[a]	Fragment
BdVmpP F3-1	TCCGGATCCGCAACTCAAGATACAAAGAAG	f3-a
BdVmpP R3-1	ATAGAGCTC*TTA*GTCAAAATATTCTCCTAC	
BdVmpP F3-2	TCCGGATCCGATGTAGGAGAATATTTTGAC	f3-b
BdVmpP R3-2	ATAGAGCTC*TTA*CGATCCAATCTTAACACT	
BdVmpP F3-3	TCCGGATCCAATTTAAGTGTTAAGATTGGA	f3-c
BdVmpP R3-3	ATAGAGCTC*TTA*ATCTTCTAACTCTTTTGA	
BdVmpP F6-1	TCCGGATCCGGAGCTGTGGTAGGTGATGCA	f6-a
BdVmpP R6-1	ATAGAGCTC*TTA*CGCTACTCCTTCTTTATT	
BdVmpP F6-2	TCCGGATCCAGTAATAAAGAAGGAGTAGCG	f6-b
BdVmpP R6-2	ATAGAGCTC*TTA*TTTATTTAATGCATCACC	
BdVmpP F6-3	TCCGGATCCGCAGATGGTGATGCATTAAAT	f6-c
BdVmpP R6-3	ATAGAGCTC*TTA*TCCTTGAAATGCTTTAAG	
BdVmpP F9-1	TCCGGATCCGATGTAGGAAAGGCAGCAGCT	f9-a
BdVmpP R9-1	ATAGAGCTC*TTA*TTTACCACTTACTGTTGT	
BdVmpP F9-2	TCCGGATCCCTAACAACAGTAAGTGGTAAA	f9-b
BdVmpP R9-2	ATAGAGCTC*TTA*GTCAACTATTGATGCTAA	
BdVmpP F9-3	TCCGGATCCGAAATATTAGCATCAATAGTT	f9-c
BdVmpP R9-3	ATAGAGCTC*TTA*TACAGGATCACTATCTTT	
BdVmpP F11-1	TCCGGATCCACTGCAGATAATTTAGCACAA	f11-a
BdVmpP R11-1	ATAGAGCTC*TTA*TACTGCCGCTGCTTTAAC	
BdVmpP F11-2	TCCGGATCCGCAGTTAAAGCAGCGGCAGTA	f11-b
BdVmpP R11-2	ATAGAGCTC*TTA*TGAACGTAGGGCTATTCC	
BdVmpP F11-3	TCCGGATCCGCAGGAGGAATAGCCCTACGT	f11-c
BdVmpP R11-3	ATAGAGCTC*TTA*TAATTTTCCTGTCTTAAT	
BdVmpP F13-1	TCCGGATCCGCCGCAGTAAATAAGCTATTA	f13-a
BdVmpP R13-1	ATAGAGCTC*TTA*TTTCATTATATCTTCTAC	
BdVmpP F13-2	TCCGGATCCGTAGAAGATATAATGAAAAAA	f13-b
BdVmpP R13-2	ATAGAGCTC*TTA*TTTTTTAAGTATTTCTTT	
BdVmpP F13-3	TCCGGATCCAAAGAAATACTTAAAAAAGTA	f13-c
BdVmpP R13-3	ATAGAGCTC*TTA*CCTTGATTTATCTATTTT	
BdVmpP F13-4	TCCGGATCCAAAATAGATAAATCAAGGTCT	f13-d
BdVmpP Rev	ATAGAGCTC*TTA*TTGCTGATCCGCTGCCTT	

[a] Underlines indicate the restriction enzyme sites (*Bam*HI and *Sac*I) and italics indicate stop codons.

expressed as means±standard deviations and the mean values of six independent experiments were calculated for each analysis. Statistical differences in OD_{405} values were assessed by one-factor ANOVA, followed by a post hoc test (Bonferroni/Dunn test). Statistical significance was defined as $P<0.05$ and $P<0.01$.

Nucleotide sequence accession numbers. The VmpP, VmpG, VmpL, and VmpK sequences of the *B. duttonii* strain Ly have been assigned DDBJ/EMBL/GenBank accession number AB073701 (40). The accession numbers of *B. hermsii* Vmp sequences published previously (6, 34) are as follows: Vmp7, X53926; Vmp18, U52149; Vmp21, M57256; Vmp25, L04787.

Results

Putative VmpP Structure

The structural model of VmpP was obtained by homology modeling based on *B. burgdorferi* OspC X-ray structure (PDB ID 1G5Z) (11) using Swiss-Model server (http://swissmodel.expasy.org) (18, 32, 38) (Fig. 1). Putative VmpP structure was similar to the OspC structure of *B. burgdorferi*, whereas the amino acid similarity was low (17.2%). VmpP is composed of 355 amino acids including the signal peptide, and the molecular weight of the mature protein was 34,766 (Fig. 1A). The putative 3-D structure of a single molecule has eight long α-helices (α1 to α8), five short α-helices (fewer than 7 amino acids each), and twelve connecting loop regions (L1 to L12). As shown in Fig. 1B, the eight α-helices are α1 (aa 36–56), α2 (aa 71–92), α3 (aa 109–125), α4 (aa 157–173), α5 (aa 215–225), α6 (aa 228–240), α7 (aa 276–288), and α8 (aa 309–342), and connecting loops.

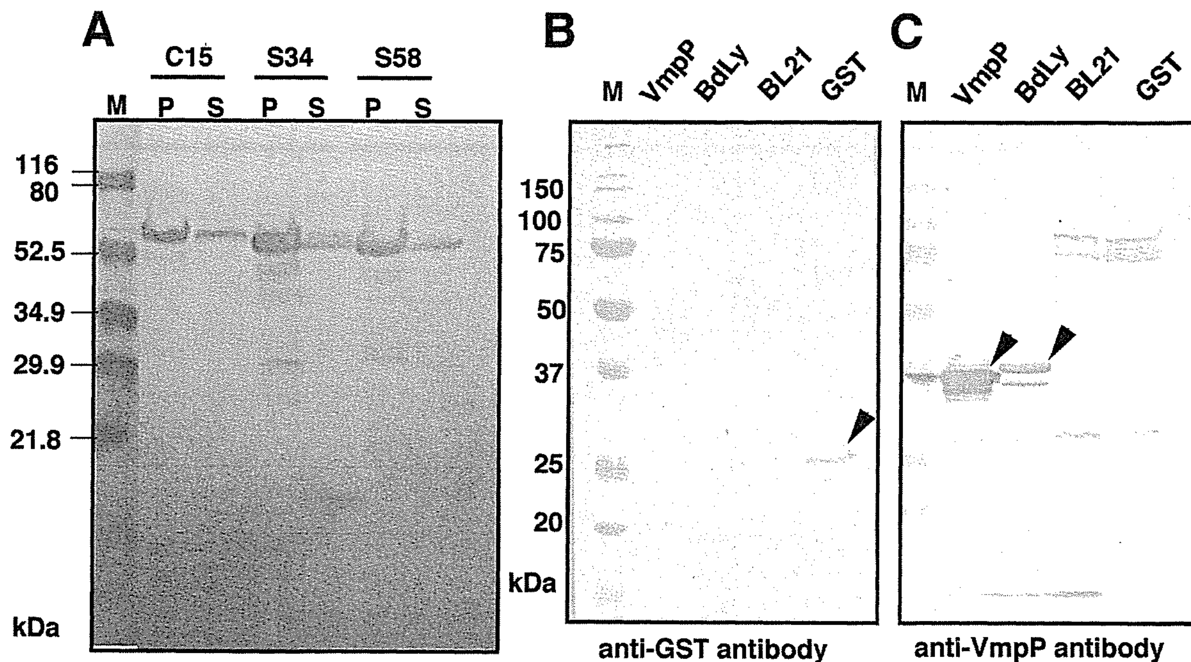

Fig. 2. Expression of GST-recombinant VmpP proteins and development of a specific polyclonal antibody for VmpP. A: Expression of serial N-terminal-truncated VmpP (GST-VmpP-C15, GST-VmpP-S34, GST-VmpP-S58) was analyzed by immunoblotting. After induction of fusion protein with IPTG, *E. coli* BL21 cells were sonicated, and the supernatant separated (S) from cell debris (precipitation, P) by centrifugation. Equal amounts (10 μg) of both fractions were applied on SDS-PAGE followed by Western blotting with monoclonal anti-GST antibody (1:1,000). Lanes: M, prestained SDS-PAGE standards low range marker (Bio-Rad Laboratories); P, precipitation; S, supernatant. GST-VmpP-S34 fusion protein was purified from supernatant (S34, lane S) and VmpP-S34 was eluted from the Glutathione-Sepharose 4B column with thrombin digestion. The antibody raised against VmpP-S34 was developed in rabbits. B and C: Purified VmpP-S34 protein, cells of *B. duttonii* strain Ly, *E. coli* BL21 cell lysate, and induced GST protein in *E. coli* BL21 were applied on SDS-PAGE and examined by Western immunoblotting analysis (B: anti-GST antibody, 1:1,000, C: anti-VmpP-S34 antibody 1:500). Lanes: M, precision plus protein™ dual color standards marker (Bio-Rad Laboratories); VmpP, purified VmpP-S34 (1 μg); BdLy, 10 μg protein of cultured *B. duttonii* strain Ly whole cell; BL21, *E. coli* BL21 without pGEX-KG vector (10 μg protein); GST, *E. coli* BL21 with pGEX-KG vector after induction of IPTG (10 μg protein). Arrowhead in Fig. B indicates GST (26-kDa) protein. Arrowheads shown in Fig. C indicate the purified VmpP-S34 (lane VmpP) and native VmpP (lane BdLy), respectively.

VmpP Fusion Protein

Western blot analysis using anti-GST antibody is shown in Fig. 2A. The molecular mass of each protein was 61-kDa (GST-VmpP-C15), 59-kDa (GST-VmpP-S34), and 57-kDa (GST-VmpP-S58). These mobilities corresponded to putative molecular weights. Most of the fusion protein was recovered in insoluble fraction (Fig. 2A, lanes P) and a part of the expressed protein was released to the soluble fraction. Fusion protein in soluble fraction was collected and purified by means of a GST-Sepharose column. Western blots using anti-GST antibody or anti-VmpP antibody are shown in Fig. 2B and 2C. As seen in Fig. 2C, anti-VmpP antibody reacted strongly with purified VmpP-S34 protein (lane VmpP) and the native VmpP protein band in whole cell lysate of *B. duttonii* strain Ly (lane BdLy). The molecular weight of VmpP-S34 was 33.2-kDa with a purity of over 95%, and the final yield was 5 mg per liter of culture (data not shown). The N-terminal amino acid sequence of this purified protein was SLSEVLLE, which was identical to the deduced sequence.

Reactivity of Anti-Recombinant VmpP Polyclonal Antibody with VmpP Fragments

Thirteen adjacent overlapping regions of VmpP were produced as GST fusion proteins. Each fusion protein has 30 amino acids of VmpP except the last (f13), which included 40 amino acids. The 33 amino acid amino-terminus that included the signal peptide was excluded from the analysis (Fig. 3A). All GST-fusion proteins were separated on SDS-PAGE gels, and examined with Western blot analysis. All proteins (f2 to f13) were recognized by a monoclonal antibody to GST, as indicated by the presence of bands at expected molecu-

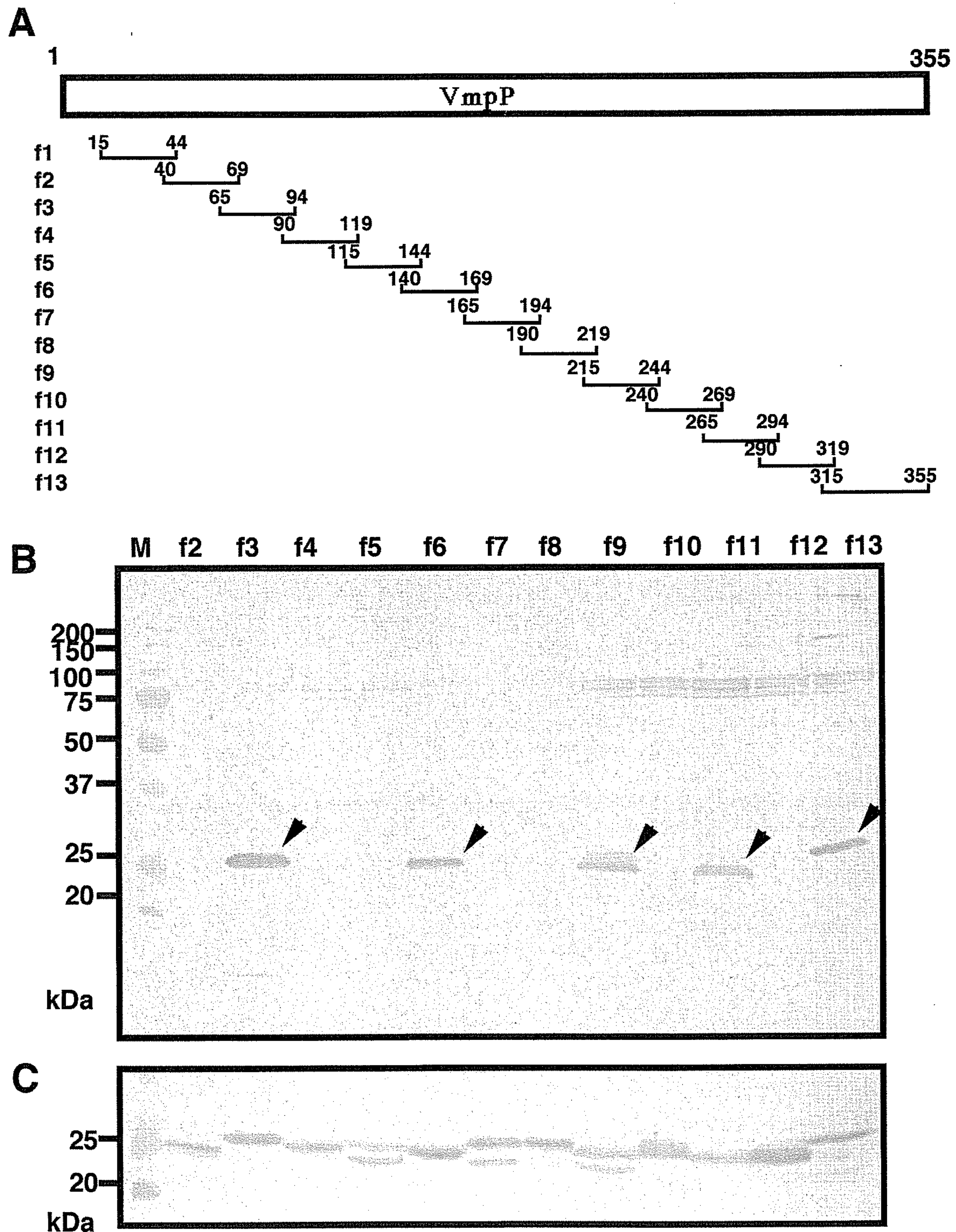

Fig. 3. Identification of antigenic regions of VmpP by immunoblot analysis. A: A schematic representation of the VmpP protein (355 amino acids) is shown. Each nucleotide fragment encoding 30 amino acids (f1 to f12; f13 has 41 aa) is designated and synthesized by PCR as described in "Materials and Methods." All fragments are designated to overlap 5 amino acid residues with each other. B: Each fragment was inserted into the vector and the GST fusion protein was expressed in *E. coli* BL21 cells. Fusion proteins were separated by SDS-PAGE, transferred onto nitrocellulose membranes, and incubated with rabbit anti-VmpP antibody (1:500). Arrowheads (lanes f3, f6, f9, f11, and f13) indicate the anti-VmpP antibody-reacting peptides. Lanes: M, precision plus protein™ dual color standards marker (Bio-Rad Laboratories); f2 to f13, whole-cell lysate of *E. coli* BL21 cells expressing each fragment. C: Fusion proteins were also analyzed by Western blotting with mouse monoclonal anti-GST antibody (1:1,000). Lanes are the same as in Fig. 4B.

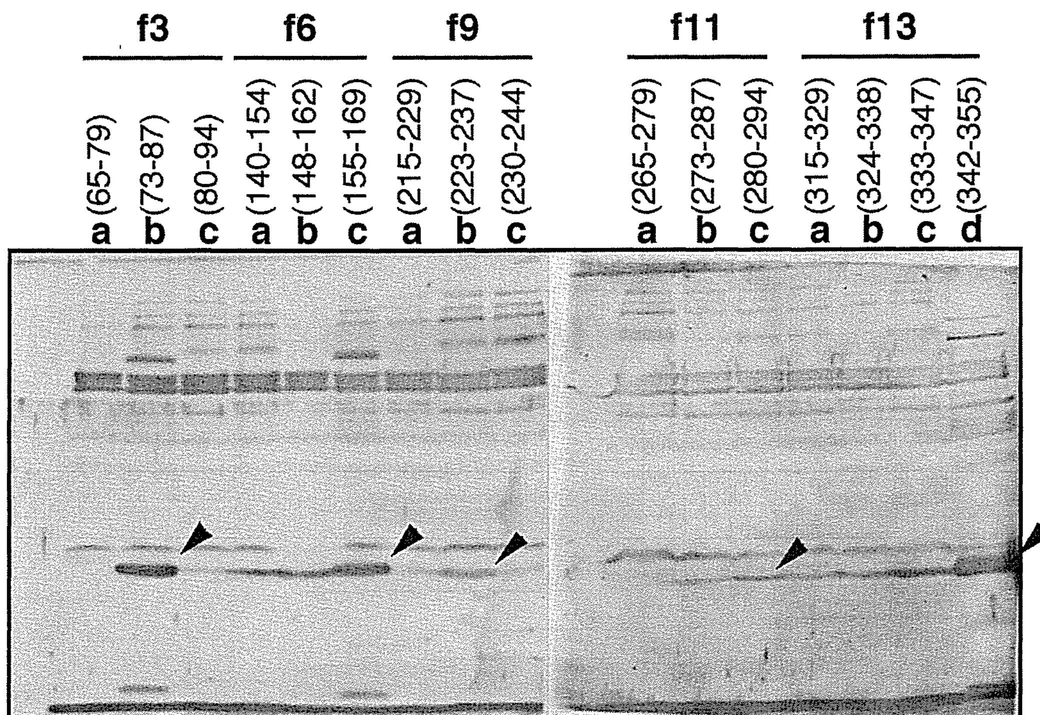

Fig. 4. Western blot analysis of epitope regions of VmpP with specific antibody. The fragments (f3, f6, f9, f11, and f13) are divided into 3 or 4 fragments encoding 15 amino acids overlapping seven to eight amino acids (f13 fragments overlapped six amino acid residues each) and were expressed in *E. coli* BL21. Fusion proteins were separated by SDS-PAGE, transferred onto nitrocellulose membranes, and incubated with rabbit anti-VmpP antibody (1:500). Arrowheads indicate the immunodominant regions (f3-b, f6-c, f9-b, f11-c, and f13-d).

lar masses (Fig. 3C). However, anti-VmpP-S34 antibodies reacted with five VmpP fragments, f3 (aa 65–94), f6 (aa 140–169), f9 (aa 215–244), f11 (aa 265–294), and f13 (aa 315–355) (Fig. 3B). Moreover, we constructed three DNA fragments (four fragments for f13) encoding 15 amino acid residues encompassing each antibody-reacting fragment. GST fusion proteins were produced and analyzed with Western blotting as explained previously. VmpP antibody reacted intensively with f3-b (aa 73–87), f6-c (aa 155–169), f9-b (aa 223–237), f11-c (aa 280–294), and f13-d (aa 342–355) (Fig. 4). Weak signals were detected on f6-b and f13-c, indicating that the epitope of f6 and f13 extended over these two fragments.

Identification of Immunodominant Epitopes of VmpP Using Peptide-Based ELISA

The epitopes recognized by anti-VmpP-S34 antibody were composed of five main regions (f3-b, f6-c, f9-b, f11-c, and f13-d). In order to define the core of the epitope, 15 peptides encompassing the five anti-VmpP antibody-reacting fragments were synthesized, and their reactivities examined with anti-VmpP antibody. Quantitative peptide-based ELISA data are shown in Fig. 5. Statistical significance was achieved in the peptides f9-b, f11-c, and f13-d. Significant differences ($P<0.05$) between f9-bM (0.254 ± 0.069) and f9-bR (0.398 ± 0.107), and between f11-cL (0.424 ± 0.117) and f11-cM (0.272 ± 0.088), were observed. At $P<0.01$, f13-dR (0.523 ± 0.132) demonstrated significant absorbance compared to f13-dM (0.323 ± 0.017). These results indicate that antigenic VmpP epitopes consist of hexapeptides: LASIVD (f9-bR, aa 232 to 237), AGGIAL (f11-cL, aa 280 to 285), and KAADQQ (f13-dR, aa 350 to 355).

The deduced amino acid sequences of Vmps from *B. duttonii* strain Ly and *B. hermsii* were aligned and identified five epitopes (Fig. 6). The overall amino acid sequence similarities ranged from 43.7 to 74.4% except the VmpL by using DNASTAR (DNASTAR, Inc., Madison, Wis., U.S.A.). Epitopes (hexapeptides) in the f3-bR, f6-cL, and f13-dR were located on variable regions, while those of f9-bR and f11-cL were on relatively conserved regions. The C-terminus region (f13-dR, KAADQQ) of VmpP was variable and predominantly epitope.

Discussion

In this study, we analyzed the reactivities of poly-

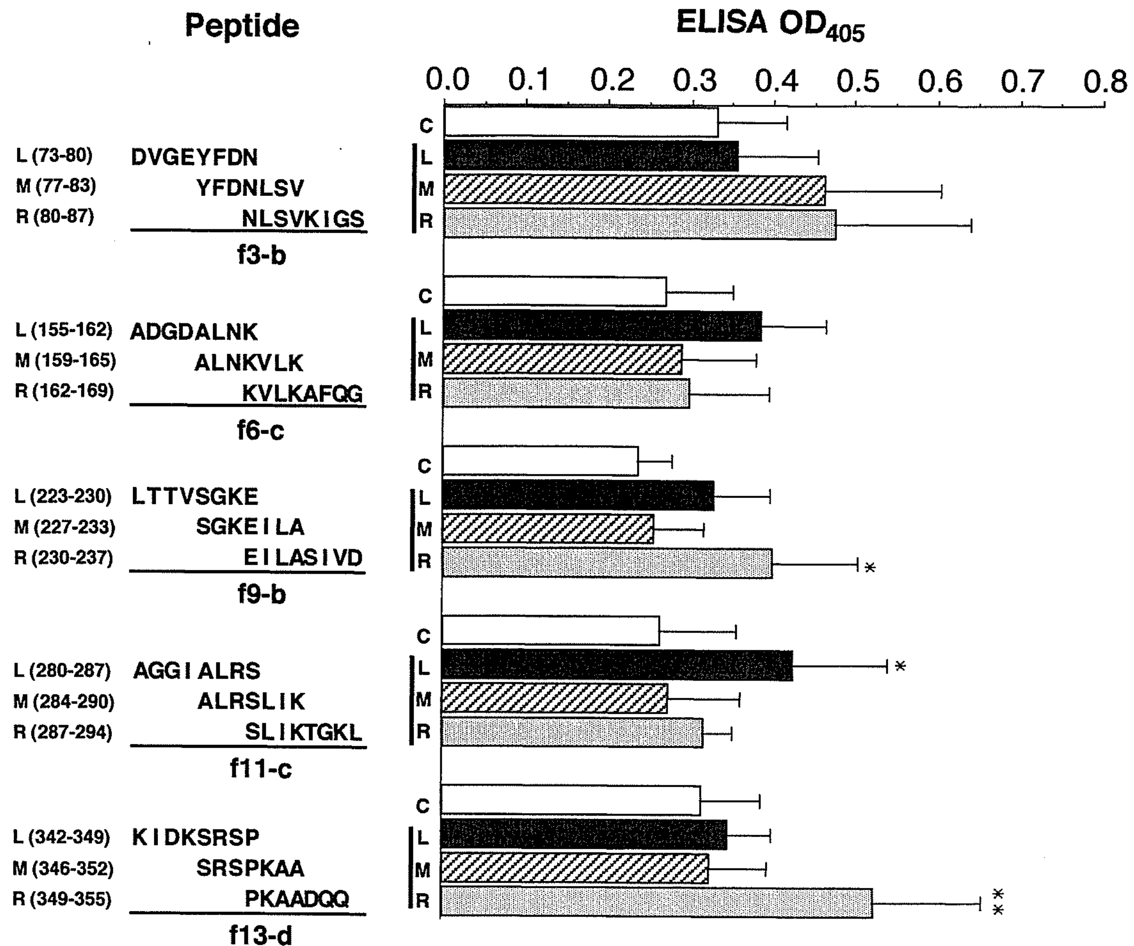

Fig. 5. Synthetic peptides reacted with anti-VmpP antibody as analyzed by ELISA. Synthesized peptides are 8-mers or 7-mers overlapping each other by 4 amino acids. Using these peptides covering five epitope regions, quantitative peptide-based ELISA was performed with anti-VmpP polyclonal antibody (1:500). The OD_{405} values of the six independent experiments were analyzed by one-factor ANOVA, followed by a post hoc test (Bonferroni/Dunn test). Control bar (C) at each peptide group indicates the six independent values by using pre-immune rabbit serum (1:500). Each value represents the mean±S.D. Statistical significance was defined as $*P<0.05$ and $**P<0.01$.

clonal VmpP antibodies and epitopes of the protein. We identified five epitopes that were comprised of at least six amino acid residues. Antigenic epitopes on VmpP were scattered throughout the primary sequence. In particular, the C-terminus region was strongly reactive with polyclonal VmpP antibody and may have an immunodominant epitope. These antigenic epitopes of VmpP were assessed with the Hopp-Wood hydrophilicity algorithm (19). The VmpP fragment which contained the antigenic determinant for the specific antibody was located in a highly hydrophilic region of VmpP and was thus suitable as an epitope for B-cells (19). The average hydrophilicity values for the hexapeptides within the C-terminal amino acid residues (positions 340 to 355) ranged from 0.967 to 1.967. The C-terminal region of VmpP would therefore be conformationally flexible and extruded from the surface of the spirochete.

B. burgdorferi produces abundant amounts of OspA and OspB in unfed ticks, and rapidly down-regulates these proteins during feeding. In contrast, OspC expression is up-regulated during mammalian infection (37). Recently, Mathiesen et al. (27, 28) reported that the OspC of the Lyme disease spirochete *Borrelia garinii* had one single major immunodominant epitope of the C-terminal amino acid sequence PVVAESPKKP which is accessible to antibodies in Lyme disease patients. The C-terminus of native OspC was flexible and extruded from the outer surface of the spirochetes (24). Moreover, by using epitope mapping, Sears et al. (35) reported that the C-terminal of *B. burgdorferi* OspA become an important protective epitope. This region is structurally distinct from the rest of the OspA. The three-dimensional structure shows that OspA of *B.*

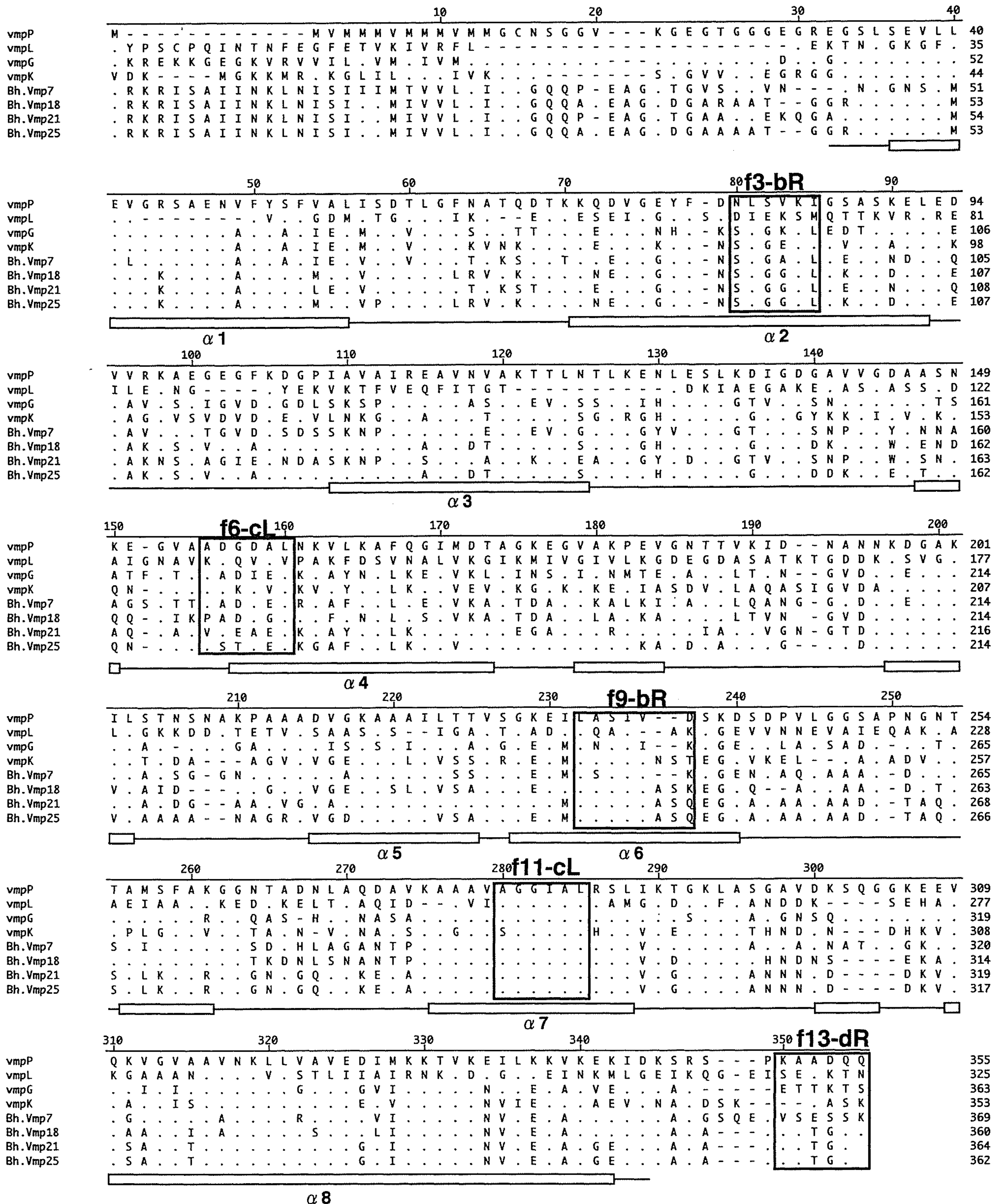

Fig. 6. Comparison of Vmp amino acid sequences for *B. duttonii* and *B. hermsii*. The sequences used in this comparison were as follows: deduced amino acid sequence of the vmpP, vmpL, vmpG, and vmpK of *B. duttonii* strain Ly (40) and *B. hermsii* HS1 (Bh.) vmp7, vmp18, vmp21, and vmp25 (6, 34). Boxes indicate epitope regions. Dots represent identical amino acids and dashes correspond to deleted amino acids. Putative secondary structure of VmpP is indicated under the sequence alignments. The amino acid sequence alignments were performed with the DNASTAR program (DNASTAR, Inc.).

burgdorferi strain B31 is folded into 21 consecutive anti-parallel β-strands connected by turns or short loops, followed by a single surface-exposed C-terminal α-helix (26). The C-terminus of OspA is also recognized by two murine monoclonal antibodies LA-2 and C3.78, which have complement-independent bactericidal activity (20, 26). Furthermore, the bactericidal antigen binding fragments (Fabs) H6831 and monoclonal antibody CB2 recognize the C-terminal fragment of *B. burgdorferi* OspB (4, 8, 12). Thus, the C-termini of the lipoproteins of Lyme disease borreliae Osps may stimulate targeting antibodies in the hosts (13, 14, 16, 36). It is considered that the C-termini of the Vmp protein family of relapsing fever borreliae may be predominant B-cell epitopes for antibodies on these regions. Therefore, VmpP might also have a C-terminal immunodominant epitope that induces bactericidal activity.

Prophylactic vaccines are currently being developed (44), one of which is already commercially available (39). The OspA C-terminal fragment has been incorporated into a second-generation vaccine by Koide et al. (23). Moreover, a recent study indicates the OspA function of the *B. burgdorferi* which colonizes the tick midgut via tick receptor for OspA (TROSPA), the *Ixodes scapularis* tick midgut receptor; this receptor is also being considered for a new vaccine target (31). The development of a relapsing fever vaccine is hampered by antigenical complexities of these spirochetes. Mice immunized with recombinant VmpP in our preliminary experiments were only partially protected when challenged with spirochetes (data not shown). These reasons for incomplete protection are being considered now.

The three-dimensional structure of VmpP is currently unknown, but evidence does implicate it as an important virulence factor in the mammalian host. The sequence conservation of the f9-bR and f11-cL regions across Vmps within the organism and *B. hermsii* vmps indicates that these regions are important in some aspect of the physiology of *B. duttonii* (Fig. 6). Therefore, one would not expect these sequences to be antigenic in hosts during a relapsing fever borrelia infection. These regions may be inaccessible to antibodies, either because of their conformation or subsurface location. Moreover, antigenicity of epitopes was supported by the predicted structure of VmpP (Figs. 1 and 6). Our results showed that the four immunogenic regions of f3-bR, f6-cL, f9-bR and f11-cL, but not f13-dR, are included on a part of the α-helices (α2, α4, α6, and α7), but not the distal loop regions from the borrelial outer membrane. Therefore, the C-terminus (f13-dR) of VmpP might become a predominant epitope.

In this study, we identified the antigenic epitopes of VmpP at the molecular level. The immunogenic regions are confined to a small portion of the protein. Common antigenic determinants from VmpP of *B. duttonii* may be useful in the production of a synthetic vaccine and in the determination of three-dimensional structures of VmpP and other Vmp proteins of spirochetemias. To develop a recombinant vaccine for *B. duttonii* infection, we need further experiments about the reactivity for VmpP to anti-sera of other experimental animals and relapsing fever patients with *B. duttonii* infection in Tanzania.

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