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Antigenic Variation in Lyme Disease Borreliae by Promiscuous Recombination of VMP-like Sequence Cassettes

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Summary

We have identified and characterized an elaborate genetic system in the Lyme disease spirochete Borrelia burgdorferi that promotes extensive antigenic variation of a surface-exposed lipoprotein, VlsE. A 28 kb linear plasmid of B. burgdorferi B31 (lp28–1) was found to contain a *vmp*-like sequence (*vls*) locus that closely resembles the variable major protein (*vmp*) system for antigenic variation of relapsing fever organisms. Portions of several of the 15 nonexpressed (silent) *vls* cassette sequences located upstream of *vlsE* recombined into the central *vlsE* cassette region during infection of C3H/HeN mice, resulting in antigenic variation of the expressed lipoprotein. This combinatorial variation could potentially produce millions of antigenic variants in the mammalian host.

Introduction

B. burgdorferi, the etiologic agent of Lyme disease, is able to persist for years in patients or animals despite the presence of an active immune response (Steere, 1989; Schutzer, 1992). Antigenic variation has been postulated previously as a mechanism whereby B. burgdorferi evades the immune response in the mammalian host (Schwan et al., 1991; Wilske et al., 1992). Antigenic variation has been defined as changes in the structure or expression of antigenic proteins that occur during infection at a frequency greater than the usual mutation rate (Seifert and So, 1988). Previous studies have provided little direct evidence for the occurrence of antigenic variation in Lyme disease borreliae. Genetic heterogeneity in the genes encoding the membrane lipoproteins OspA, OspB, OspC, and OspD has been well documented among strains of Lyme disease borreliae (Marconi et al., 1993, 1994; Livey et al., 1995). In addition, mutations in *ospA* and *ospB* have been shown to occur in vitro (Rosa et al., 1992; Sadziene et al., 1992). However, no significant antigenic change (Barthold, 1993) or gross genetic alteration (Persing et al., 1994; Stevenson et al., 1994) was detected in B. burgdorferi N40 isolates from chronically infected BALB/c and C3H mice, other than the loss of the 38 kb plasmid encoding OspD. Therefore, the heterogeneity in Osp proteins observed among B. burgdorferi sensu lato isolates appears to represent evolutionary divergence, or "antigenic drift," rather than antigenic variation.

A complex antigenic variation mechanism has been well characterized in Borrelia hermsii, a relative of B. burgdorferi that causes relapsing fever (Barbour, 1993). Surface-exposed lipoproteins called variable major proteins (Vmps) are encoded by homologous genes located in 28–32 kb linear plasmids with covalently closed telomeres (Kitten and Barbour, 1990). Each organism contains at least 26 *vmp* genes, most of which are located in an unexpressed (silent) form in the so-called storage plasmids (Barbour, 1993). Only one *vmp* gene located near one of the telomeres of a different plasmid, i.e., the "expression plasmid," is expressed in each organism (Kitten and Barbour, 1990; Barbour et al., 1991a). Antigenic variation occurs when the expressed *vmp* is replaced completely or partially by one of the silent *vmp* genes at the telomeric expression site through interplasmic recombination (Plasterk et al., 1985; Barbour et al., 1991b), intraplasmic recombination (Restrepo et al., 1994), and postswitch rearrangement (Restrepo and Barbour, 1994). The antigenic switch occurs spontaneously at a frequency of 10^{-3} to 10^{-4} per generation (Stoenner et al., 1982).

In this study, we have identified and characterized a genetic locus called *vmp*-like sequence (*vls*) in B. burgdorferi that closely resembles the *vmp* system of B. hermsii. A *vls* expression site (*vlsE*) and 15 additional silent *vls* cassettes were identified on a 28 kb linear plasmid (designated lp28–1). The presence of lp28–1 correlates with the high infectivity phenotype in B. burgdorferi sensu lato strains tested. *vlsE*, located near a telomere of lp28–1, encodes a surface-exposed lipoprotein. Examination of ear and blood isolates from C3H/HeN mice infected 4 weeks previously with B31 clone 5A3 demonstrated the occurrence of promiscuous recombination at the *vlsE* site. The resultant VlsE variants exhibited a decreased reactivity to antiserum directed against the parental Vls1 cassette region. It therefore appears that this elaborate genetic system permits combinatorial antigenic variation of *vlsE* in the mammalian host and thereby may contribute to evasion of the immune response and long-term survival in the mammalian host.

Results

Identification of the 28 kb Linear Plasmid, lp28–1

B. burgdorferi strains generally exhibit loss of infectivity following 10–17 in vitro passages (Schwan et al., 1988; Norris et al., 1995), coinciding with the loss of plasmids (Xu et al., 1996). We hypothesized that the decreased infectivity occurring during in vitro passage of Lyme disease borreliae is due to loss of genetic content, specifically plasmids encoding virulence factors. One of the complications involved in studying B. burgdorferi plasmids is that many plasmids are in the 20 to 40 kb

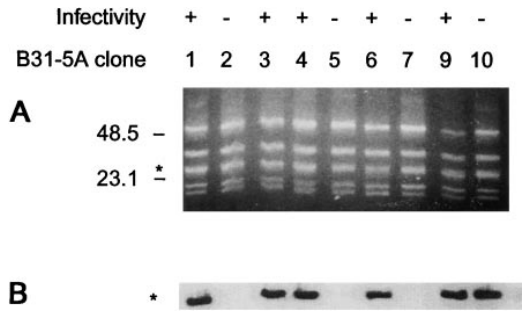

Figure 1. Correlation of Infectivity of B. burgdorferi B31 Clones 5A1–10 with Presence of a 28 kb Linear Plasmid (lp28–1)

(A) Plasmid profiles of low (−) and high (+) infectivity B31 clones as determined by pulse-field gel electrophoresis and ethidium bromide staining.

(B) Hybridization of a DNA blot of the gel shown in (A) with the pJRZ53 probe. Molecular sizes of the standards are indicated in kilobases, and an asterisk marks the location of lp28–1.

size range (Xu et al., 1996), making it difficult to resolve plasmids with similar sizes by standard electrophoretic techniques. In addition, mutagenic techniques and other genetic manipulation tools are in an early stage of development in B. burgdorferi (Samuels et al., 1994; Rosa et al., 1996), thereby limiting the ability to examine the importance of these plasmids in pathogenesis by direct genetic approaches.

To overcome these limitations, we utilized a simple subtractive hybridization technique (Seal et al., 1992) to enrich and eventually to identify sequences present only in high infectivity organisms. One of the eight resultant clones, designated pJRZ53, contained a 562 bp Sau3AI fragment with a single contiguous open reading frame. The predicted amino acid sequence of this open reading frame shared a high degree of similarity to Vmps of B. hermsii, particularly Vmp17 (27.2% identity and 56.8% similarity). Based on this sequence similarity, the pJRZ53 insert was called a *vmp*-like sequence (*vls*). The *vls* sequence was localized to a 28 kb linear plasmid (see Figure 1B) by two-dimensional agarose gel electrophoresis and hybridization using the pJRZ53 insert as a probe (data not shown). This *vls*-containing plasmid of B. burgdorferi B31 was designated lp28–1.

Correlation between lp28–1 and Infectivity

In previous studies (Norris et al., 1995), clones of low passage B. burgdorferi strains B31 and Sh-2–82 exhibited two distinct high and low infectivity phenotypes when tested in C3H/HeN mice. To test whether presence of the *vls*-containing plasmid lp28–1 is correlated with the high infectivity phenotype, the pJRZ53 probe was hybridized with total DNA from both high and low infectivity B. burgdorferi clones. All nine B31 clones tested had a plasmid banding pattern almost identical to each other when visualized by ethidium bromide staining (Figure 1A). However, hybridization of pJRZ53 with the blot made from the same gel revealed that all five high infectivity B31 clones possessed the *vls*-containing lp28–1 plasmid, whereas only one of four low infectivity clones (B31–5A10) had this plasmid (Figure 1B). Preliminary evidence indicates that the low infectivity B31–5A10 clone is lacking a different plasmid that correlates with infectivity (unpublished data). These results indicated a strong correlation between the presence of lp28–1 and the high infectivity phenotype in clonal populations of B. burgdorferi B31.

An lp28–1 homolog was also detected in seven high infectivity clones but not in three low infectivity clones of strain Sh-2–82 (data not shown). Similar experiments revealed the presence of a single *vls*-containing plasmid ~28 kb in size in infectious B. burgdorferi N40, B. afzelii ACA-1, and B. garinii IP-90 strains (data not shown). In contrast to the multiple *vmp*-containing linear plasmids in B. hermsii, only one *vls*-containing plasmid was found in each of the Lyme disease isolates tested under our hybridization conditions.

Characterization of the *vls* Locus

In subsequent studies, we utilized the high infectivity B31 clone 5A3 (B31–5A3) to minimize clonal variation. A 14 kb EcoRI fragment was cloned into λDASH II to permit a detailed analysis of this region. An internal EcoRI site was shown to divide the insert of a resultant lambda clone (designated λDASH-Bb12) into two smaller 4 and 10 kb fragments. The physical linkage of the 4 and 10 kb EcoRI fragments in the native B. burgdorferi plasmid lp28–1 was confirmed by PCR analysis (data not shown). Nearly 10 kb of the λDASH-Bb12 insert was sequenced using a random-cloning "shotgun" strategy. A total of 80 random clones were sequenced, and the assembled sequence had an average 5-fold redundancy.

The sequence data revealed an extensive *vls* locus within the 10 kb EcoRI fragment, consisting of an expression site (designated *vlsE*) and 15 *vls* cassettes that are highly homologous to the central portion of *vlsE* (Figure 2A). The presence of the EcoRI linker sequence between the insert DNA and the vector sequence defined the location of the right telomeric end. *vlsE* is located 82 bp from the right telomere of lp28–1. It possesses two unique sequences at each of the 5′ and 3′ regions and a 570 bp *vls* cassette in the middle, which was designated *vls1* (Figure 2B). The *vls1* cassette is flanked at either end by a 17 bp direct repeat sequence (Figure 2C). An array of 15 *vls* cassettes begins approximately 500 bp upstream of *vlsE* on the same plasmid (Figure 2A). The *vls1* cassette and the other *vls* cassettes (*vls2* through *vls16*) share 90.0%–96.1% nucleotide sequence identity and 76.9%–91.4% predicted amino acid sequence identity.

vlsE of B. burgdorferi B31–5A3 is predicted to encode a 356 amino acid protein with an M_r of 35,986 (Figure 2C). VlsE contains a putative lipoprotein leader sequence with an apparent signal peptidase II cleavage site (FINC) (Wu and Tokunaga, 1986). Cleavage of the 18 amino acid signal peptide would result in a mature polypeptide with a calculated M_r of 33,956 and an isoelectric point (pI) of 7.3. Except for the leader peptide, VlsE is predominantly hydrophilic. *vlsE* is highly homologous to *vmp17* of B. hermsii at both the nucleotide (58.8% identity) and deduced amino acid (37.4% identity and 57.8% similarity) sequence levels (Figure 3A). The particular *vlsE* allele contained in B. burgdorferi B31–5A3

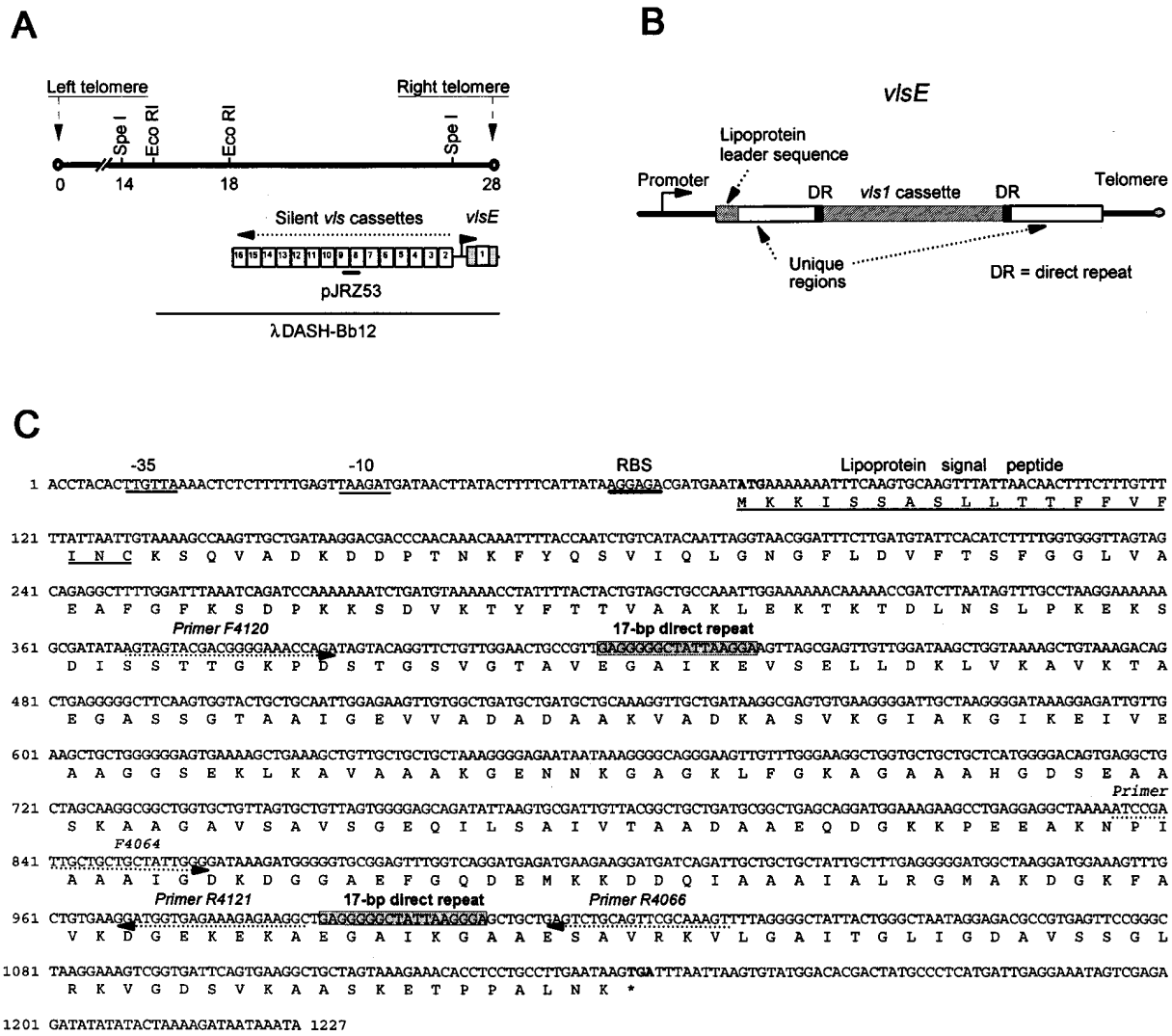

Figure 2. Structure of the *vls* Locus of B. burgdorferi Clone B31–5A3

(A) Diagrammatic illustration of the overall arrangement of the *vls* locus in B. burgdorferi plasmid lp28–1. Distances from the left telomere are indicated in kilobases, and the locations of the subtractive hybridization clone pJRZ53 and the λDASH-Bb12 insert are shown.

(B) Structure of *vlsE*.

(C) Nucleotide and predicted amino acid sequences of the allele *vlsE1* of the B. burgdorferi B31–5A3 *vlsE* gene. The predicted −10 and −35 promoter sequences, the putative ribosome-binding site (RBS), the 17 bp direct repeat, and primers used for PCR and RT–PCR are marked.

has been designated *vlsE1*, to distinguish it from variant *vlsE* alleles (see below).

An additional 15 *vls* cassettes (474–594 bp in length) are oriented in the opposite direction to *vlsE* and are arranged in a head-to-tail fashion in a nearly contiguous open reading frame interrupted only by a stop codon in cassette *vls11* and two frame shifts in cassettes *vls14* and *vls16* (Figures 2A and 3B). None of these *vls* cassettes has recognizable ribosome-binding sites or promoter sequences; therefore, they are thought to be nonexpressed or silent. The ends of the *vls* cassettes were defined by alignment with the *vls1* cassette (Figure 3B). In general, the *vls* cassettes have the same 17 bp direct repeat at either end. One exception is the joint region between *vls9* and *vls10*, where only 10 identical nucleotides were identified. The 562 bp insert of the subtractive hybridization clone pJRZ53 was localized to the joining region between *vls8* and *vls9* by sequence comparison.

The *vls* cassettes contain six highly conserved regions that are interspersed by six variable regions (VR), which differ at both the nucleotide and amino acid levels (Figure 3B). Except for the occasional codon changes and the deletions mentioned previously, the conserved regions are almost identical in all cassettes. However, the *vls* cassettes are distinguished from each other by considerable sequence variations limited predominantly to the six variable regions (VR-I through VR-VI). With the exception of an insertion of a TAG stop codon in *vls11* and TG insertions in *vls14* and *vls16* resulting in frameshifts, all deletions and insertions are nucleotide triplets, indicating preservation of the open reading frame. The sequence variations at most polymorphic positions result

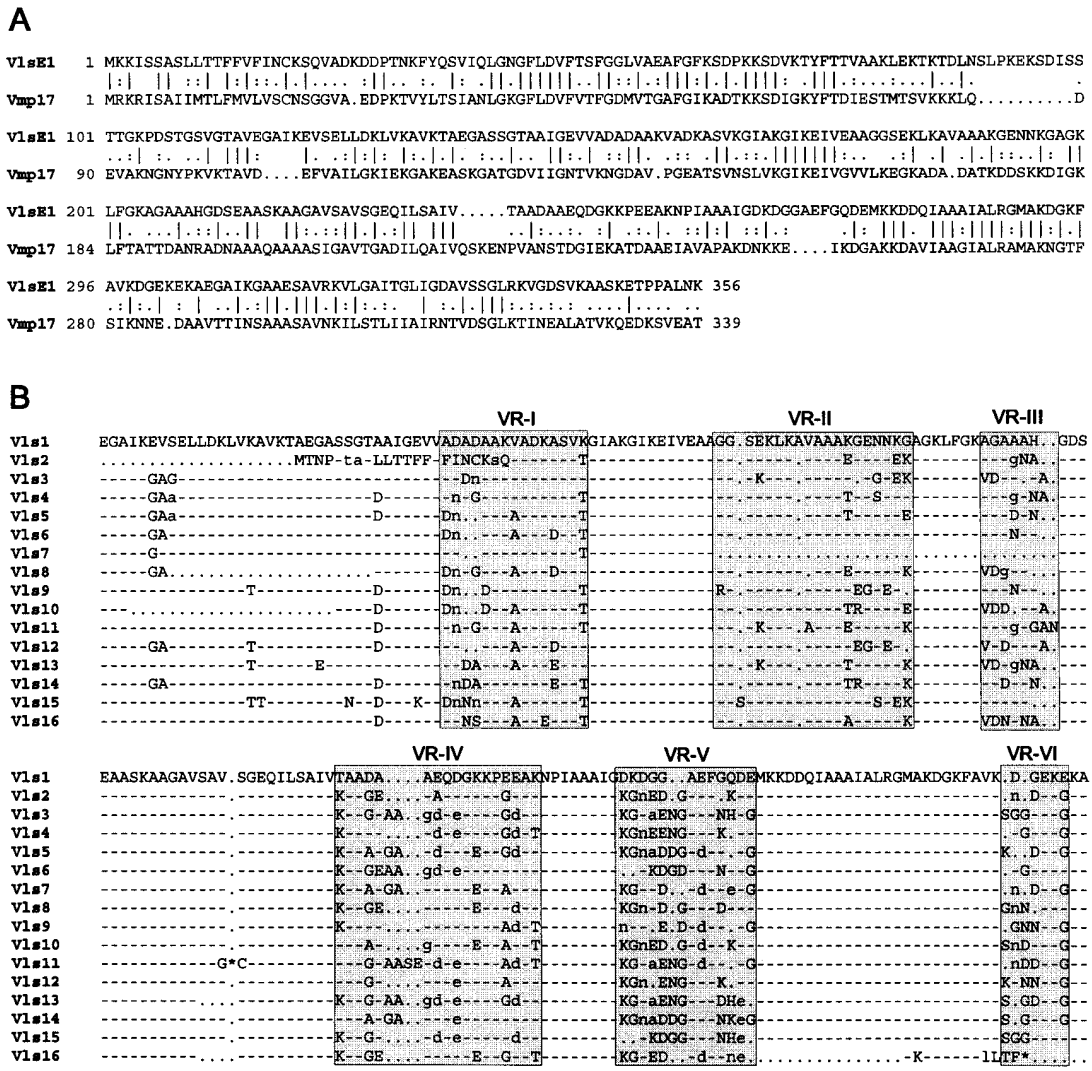

Figure 3. Sequence Similarity of the Predicted VlsE Sequence (Allele *vlsE1*) with the Vmps of B. hermsii and with the Predicted Amino Acid Sequences of the Silent *vls* Cassettes

(A) Alignment of the predicted amino acid sequence of VlsE (allele *vlsE1*) with that of Vmp17 (GenBank entry L04788). Identical amino acid residues are indicated by vertical lines, and similar residues are marked with colons and periods.

(B) Alignment of the deduced peptide sequences of 16 *vls* cassettes. Residues identical to the VlsE cassette region (Vls1) of B31–5A3 are marked as dashes; similar amino acids are shown in lower case. Gaps and the predicted stop codons are indicated by dots and asterisks, respectively. Variable regions VR-I through VR-VI are shaded.

in conservative amino acid changes, suggesting that certain amino acids are required at these positions for function. Even within the six variable regions, there is obvious sequence conservation (Figure 3B).

Expression of *vlsE*

To determine transcription of *vlsE*, we utilized reverse transcription–polymerase chain reaction (RT–PCR) to amplify a 3′ region of *vlsE* from total RNA of in vitro-cultured B31–5A3. Three independently derived recombinant plasmids contained DNA sequences identical to the corresponding region of *vlsE*, demonstrating that *vlsE* is transcribed in vitro. No RT–PCR products were observed in the agarose gel if reverse transcriptase was omitted from the reaction, confirming that the RT–PCR products were derived from mRNA, not DNA (data not shown). Consistent with the RT–PCR results, the protein product of *vlsE* was identified in B31–5A3 by immunoblot analysis (see Figure 6B). The M_r of VlsE expressed by B31–5A3 (45,000) is larger than the predicted molecular mass of 34 kDa. The reason for this altered mobility is not known, although it may be related to an unusual structural conformation or posttranslational modification (including lipidation).

Surface Localization of VlsE

The presence of a putative lipoprotein leader peptide and the overall hydrophilic nature of VlsE raised the possibility that VlsE is attached to the bacterial membrane via a lipid anchor. To test this possibility, B. burgdorferi B31–5A3 was incubated in the presence of [^{3}H]palmitate as described previously (Norris et al.,

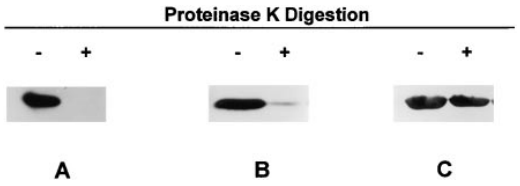

Figure 4. Surface Localization of VlsE as Indicated by Treatment of Intact B. burgdorferi with Proteinase K

Freshly cultured B. burgdorferi B31 clone 5A3 cells were incubated with (+) or without (−) proteinase K at room temperature for 10 min. The proteins of the washed organisms were then separated by SDS–PAGE. The protein blots were reacted with (A) antiserum against the GST-Vls1 fusion protein; (B) antiserum against B. burgdorferi B31 OspD; and (C) monoclonal antibody H9724 against the B. burgdorferi flagellin (Fla).

1992). Radioimmunoprecipitation results showed that VlsE was radiolabeled by [^{3}H]palmitate (data not shown), indicating that VlsE is a lipoprotein.

Exposure of viable B. burgdorferi B31–5A3 to proteinase K produced results consistent with the surface localization of VlsE. VlsE was degraded by proteinase K in as little as 10 min (Figure 4A), even though the organisms appeared intact by dark-field microscopy. Consistent with a previous study (Norris et al., 1992), B. burgdorferi OspD protein was also removed by proteinase K treatment (Figure 4B). In contrast, the Fla subunit of the periplasmic flagella was not affected by proteinase K (Figure 4C), providing evidence that the outer membranes of the organisms remained intact during the proteinase K treatment.

Genetic Variation at the *vlsE* Site

The similarity of the *vls* locus to the *vmp* system of B. hermsii prompted us to examine whether genetic recombination between the expressed and silent *vls* cassettes could be demonstrated in the mammalian host, as outlined in Figure 5A. The *vlsE* cassette regions of clonal populations from 11 different mouse isolates were amplified by PCR and sequenced. The B. burgdorferi clones and associated *vlsE* allele sequences derived from the 4-week isolates were designated by a combination of mouse number (m1 to m8), tissue source ("e" for ear and "b" for blood), week postinfection (4), and a clone designation (A to P) for the 16 clones from each isolate.

When compared with the parental *vlsE* of the clone B31–5A3 (allele *vlsE1*) used for inoculation, the 11 reisolated clones from 8 C3H/HeN mice contained multiple base substitutions, deletions, and insertions within the *vls* cassette region of *vlsE*, making each allele unique. These changes resulted in numerous differences in the predicted amino acid sequences (Figure 5B). As found in the silent *vls* cassettes (Figure 3B), these changes were primarily confined within the six variable regions. The variable sequences at almost all positions in the 11 *vlsE* alleles could be found in the corresponding regions of the silent *vls* cassettes. For example, the *m1e4A* and *m5e4A* alleles have VR-I and VR-II identical to *vls4*, whereas the VR-I and VR-III regions of *m6b4A* are identical to the same regions of *vls10* (Figure 5B). In addition, the clonal populations from a single tissue site also exhibited similar sequence variations (Figure 5C).

None of the *vlsE* alleles tested thus far contained *vls* cassette sequences entirely identical to the 15 silent *vls* cassettes (Figure 3B). Instead, each of these clones contained a unique combination of sequences identical to portions of several silent *vls* cassettes. These observations thus suggest that segments, but not entire regions, of the silent *vls* cassettes are recombined into the *vlsE* site. Comparison to the silent cassette sequences at the nucleotide level suggests that 6–11 separate and apparently random recombination events have occurred in each of the clones isolated from mice 4 weeks postinoculation. This combinatorial form of recombination could potentially result in millions of different *vlsE* alleles.

As controls, the *vlsE* cassette regions of the original B31–5A3 frozen stock and of organisms that had undergone 2 consecutive 7-day passages in vitro were also amplified and sequenced. Two sets of PCR products and four independently derived recombinant plasmids containing the PCR products all had sequences identical to the initial *vlsE* sequence (data not shown). These results indicate that the *vlsE* sequence variations do not occur at high frequency under standard in vitro culture conditions.

Antigenic Variation of the VlsE Variants

The promiscuous genetic recombination at the *vlsE* site suggested that sequence variations in the *vlsE* alleles may result in changes in antigenicity. Nine clonal populations carrying unique *vlsE* alleles (see Figure 5B) were subjected to immunoblot analysis. Although similar amounts of total protein were loaded into each lane, as indicated by reactivity to antibody against the B. burgdorferi flagellin protein (Figure 6A), the VlsE variants exhibited much less immunoreactivity to the antiserum against a GST-Vls1 cassette fusion protein than did the B31–5A3 parent expressing the *vls1* allele (Figure 6B). The mouse isolates containing *m1b4A* and *m3b4A* alleles had weakly reactive bands (Figure 6B, lanes 2 and 5). The other clones examined exhibited faint bands that were visible only with longer chemiluminescent exposures of the membrane (data not shown). These VlsE variants migrated at lower M_rs than VlsE expressed by the parental clone B31–5A3, indicative of changes in either size or conformation. No reactive bands were observed in clone B31–5A2, which lacks the lp28–1 plasmid. The decreased reactivity of mouse isolates with antiserum against the parental Vls1 cassette region indicates that the sequence differences in these VlsE variants (Figure 5B) resulted in changes in important cassette region epitopes and hence antigenic variation.

Sera from the mice in the experiment outlined in Figure 5A were used to determine whether VlsE is expressed and immunogenic in vivo. Although the prebleed sera had no detectable reactivity (data not shown), the serum sample collected from the same mice 4 weeks after initial infection with B. burgdorferi B31–5A3 reacted strongly with the VlsE protein of B. burgdorferi B31–5A3 and with the GST-Vls1 fusion protein, but not with GST alone (Figure 6C), indicating the expression of VlsE in

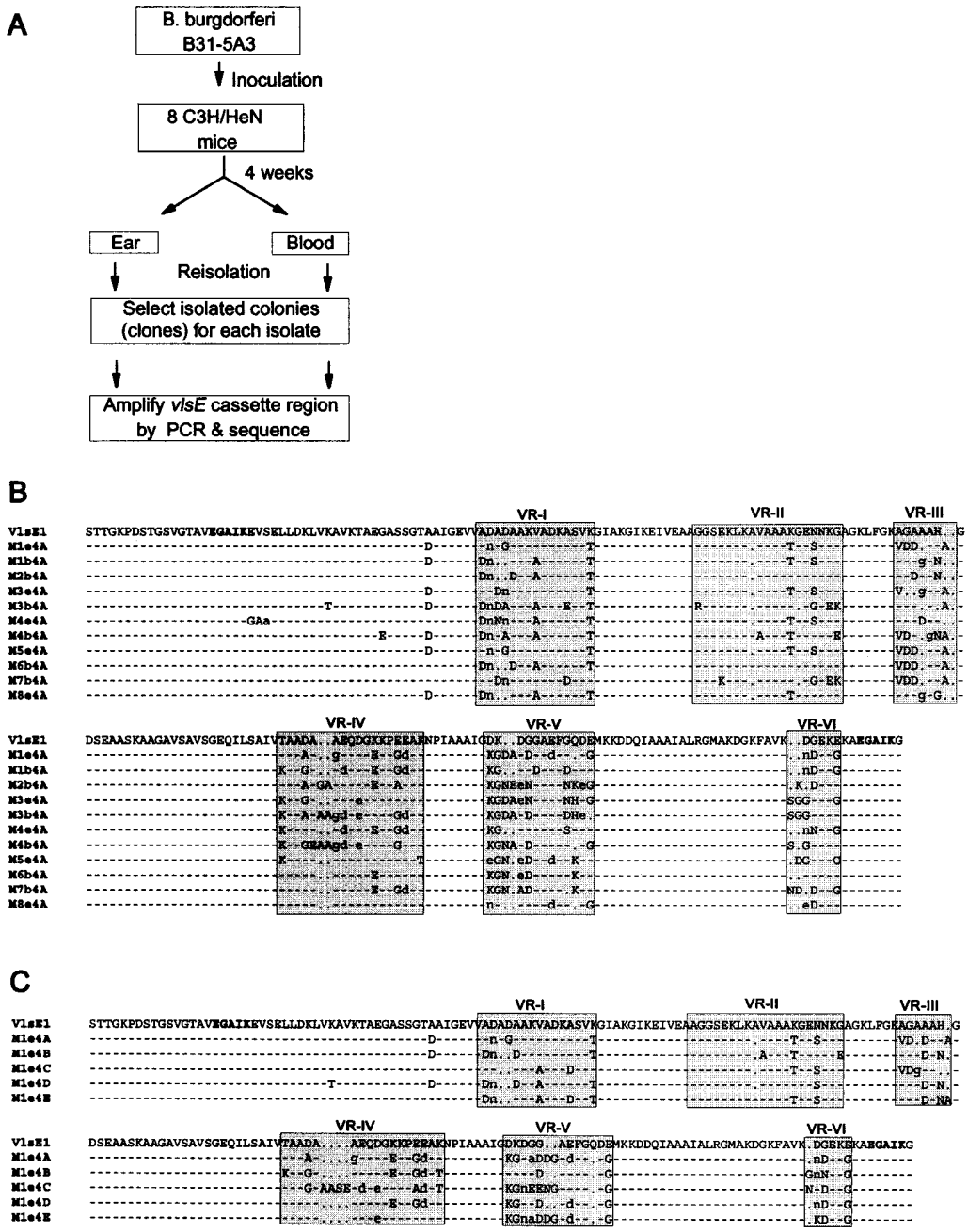

Figure 5. Changes in Deduced Amino Acid Sequences of VlsE Occurring during Infection of C3H/HeN Mice with B. burgdorferi B31–5A3

(A) Flow chart of the overall experimental design.

(B) Amino acid sequence alignment of the *vlsE* alleles in one clonal population from each of 11 different isolates.

(C) Amino acid sequence alignment of the *vlsE* alleles in 5 clonal populations from a single ear isolate.

In (B) and (C), the deduced amino acid sequences of the mouse isolates were compared with those of the inoculating clone (VlsE1); similarity to this sequence is depicted as described in Figure 3B. Amino acid residues (EGAIK) encoded by the 17 bp direct repeat are highlighted to indicate the boundaries of the *vls* cassette.

the mammalian host. In contrast, the VlsE variant M1e4A exhibited decreased reactivity when reacted with the same mouse serum (Figure 6C).

Consistent with the above results, sera from a representative white-footed mouse (Peromyscus leucopus) infected with B. burgdorferi B31 via tick bite (Figure 6D) and from a human Lyme disease patient (Figure 6E) were also reactive to the VlsE protein of B. burgdorferi B31–5A3 and GST-Vls1 fusion protein. Similar to the serum from the C3H/HeN mouse (Figure 6C), the sera from the Peromyscus mouse (Figure 6D) and the Lyme disease patient (Figure 6E) had little reactivity to the VlsE variant M1e4A. These results further indicate that VlsE is expressed during infection and is highly immunogenic in the mammalian host, but that genetic variation may generate unique VlsE variants that are no longer

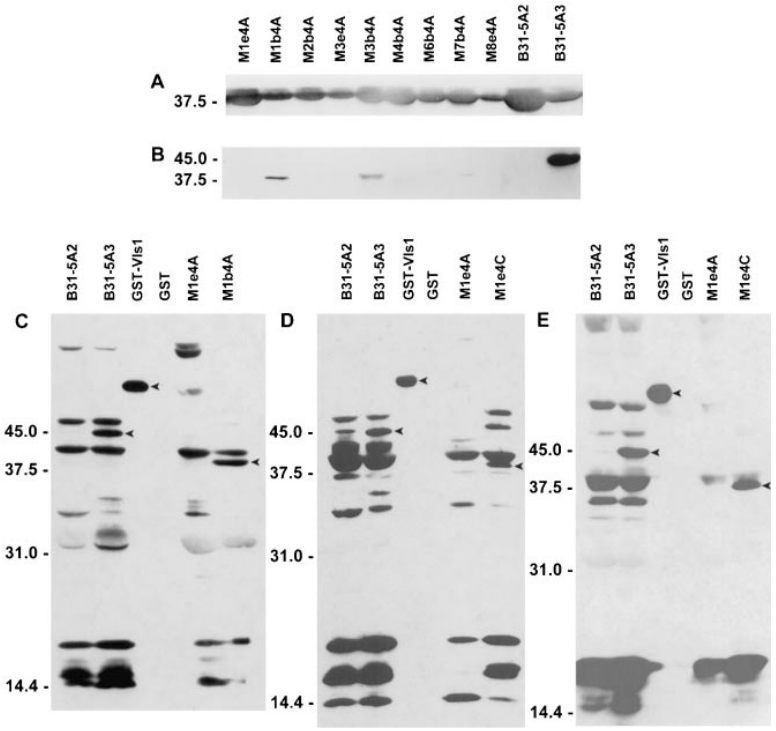

Figure 6. Altered VlsE Antigenicity of B. burgdorferi Clones (m1e4A through m8e4A) Isolated from C3H/HeN Mice 4 Weeks Postinfection

In (A) and (B), the antigenic reactivities of 9 clones isolated from mice (lanes 1–9) were compared with those of the parental clone B31–5A3 used for mouse inoculation (lane 11) and the low infectivity clone B31–5A2 (lane 10), which lacks the plasmid encoding VlsE. Two identical SDS–PAGE Western blots were reacted with (A) monoclonal antibody H9724 directed against the B. burgdorferi flagellin protein (Fla) as a positive control and (B) antiserum against the GST-Vls1 fusion protein. Prolonged exposures of the immunoblot shown in (B) indicated the presence of weakly reactive bands in all 9 mouse isolates (data not shown). B. burgdorferi proteins and the GST-Vls1 fusion protein were reacted with (C) serum from mouse 1 obtained 28 days after infection, (D) serum from a Peromyscus mouse infected with B31 via tick-bite, and (E) serum from a Lyme disease patient. The protein bands corresponding to VlsE and the GST-Vls1 fusion protein (as determined by reactivity with anti-GST-Vls1 antiserum; data not shown) are indicated by arrowheads. The relative locations of protein standards are shown in kilodaltons.

fully recognized by the immune response against the parental VlsE.

Discussion

We have identified an infectivity-associated 28 kb linear plasmid, lp28–1, in B. burgdorferi B31 by subtractive hybridization. DNA sequence analysis of cloned fragments from this plasmid revealed the *vls* locus consisting of an expressed *vlsE* gene and 15 silent *vls* cassettes. Subsequent experiments demonstrated that promiscuous recombination occurs in the *vlsE* cassette region in C3H/HeN mice, and that the sequence variation in the *vlsE* cassette region alters antigenicity of the VlsE variants, resulting in antigenic variation. Identification of the VlsE antigenic variation provides a possible explanation for persistence of B. burgdorferi infection in both human and laboratory animals. Although the *vls* locus has been characterized thoroughly only in one clonal population of B. burgdorferi B31, preliminary Southern hybridization results indicate that this locus is present in infectious strains of three well-defined Lyme disease borrelia genospecies (B. burgdorferi, B. afzelii, and B. garinii), despite the overall genetic heterogeneity among these organisms (Casjens et al., 1995; Xu et al., 1996).

The *vls* locus resembles the *vmp* system of B. hermsii in both sequence (Figure 3A) and genetic organization. Both the *vls* and *vmp* systems have a single expression site encoding a surface-localized lipoprotein, as well as multiple unexpressed sequences (Plasterk et al., 1985; Barbour et al., 1991a). Moreover, the expression sites for both systems are located near one of the telomeres of their respective linear plasmids (Kitten and Barbour, 1990; Barbour et al., 1991b). These observations suggest that the *vls* locus may provide the Lyme disease borreliae with the capability of antigenic variation analogous to the *vmp* system of B. hermsii (Barbour, 1993). The above similarities also indicate that the *vls* and *vmp* systems evolved from a common ancestral gene.

There are also several obvious differences between the *vls* and *vmp* systems. First, B. hermsii possesses at least two *vmp*-containing linear plasmids (Barbour, 1993), whereas only one *vls*-containing linear plasmid was detected in Lyme disease borreliae under our hybridization conditions (Figure 1B). Second, the silent *vmp* genes are separated by intergenic noncoding regions and arranged in either orientation (Barbour et al., 1991a), but the silent *vls* cassettes are organized head-to-tail as a single open reading frame throughout almost the entire region (Figure 2A). Third, the silent *vmp* genes lack promoter sequences, but most encode complete or nearly complete open reading frames with their own ribosome-binding sites (Barbour et al., 1991a). In contrast, the *vls* cassettes represent only the central third of the expression site. Finally, each phase of B. hermsii infection is caused predominantly by organisms expressing a single *vmp* allele (Barbour, 1993), whereas a high degree of *vlsE* allelic variation occurs among organisms isolated even from a small ear biopsy specimen during B. burgdorferi infection (Figure 5C).

Genetic variation involved in multi-gene families has been described in several other pathogenic microorganisms (Borst and Geaves, 1987; Borst et al., 1995). In the context of combinatorial recombination, the genetic variation at the *vlsE* site is similar to that of the pilin-encoding genes of Neisseria gonorrhoeae (Seifert and So, 1988). The gonococcal pilus is primarily composed of repeating subunits of an 18 kDa pilin protein and is required for adherence of the bacterium to a variety of human cells (Swanson and Koomey, 1989). While the

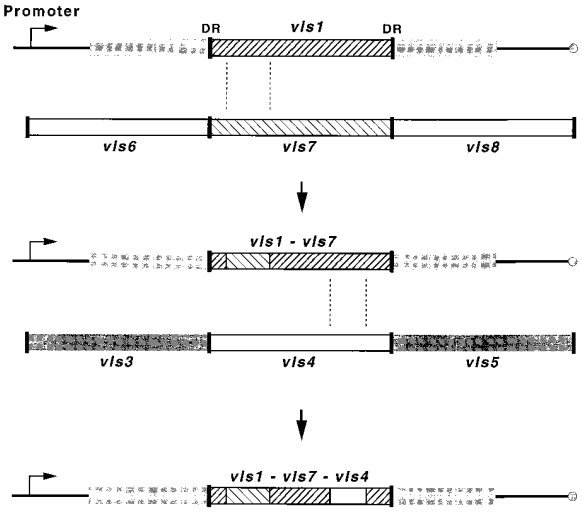

Figure 7. Proposed Model for Genetic and Antigenic Variation at the *vls* Locus

Recombination of segments of the silent *vls* cassettes *vls7* and *vls4* into the *vls1* cassette of B. burgdorferi B31–5A3 *vlsE* gene is shown. A series of similar recombination events would generate unique *vlsE* alleles consisting of a mosaic of segments from several different silent *vls* cassettes.

complete pilin genes are expressed only at two expression sites (*pilE1* and *pilE2*), multiple silent copies (*pilS*) containing portions of the pilin genes are found over a wide range on the gonococcal chromosome (Haas and Meyer, 1986). Through multiple combinatorial recombination events, a single gonococcal clone expressing one pilin serotype can give rise to a large number of progeny that express antigenically distinctive pilin variants (Meyer et al., 1982; Hagblom et al., 1985; Segal et al., 1986).

The coding sequences of the Neisseria pilin variants are divided into constant, semivariable, and hypervariable regions (Haas and Meyer, 1986), which are analogous to the conserved and variable regions of the *vls* cassettes (Figures 3B, 5B, and 5C). The constant regions and a conserved DNA sequence (Sma/Cla repeat) located at the 3′ end of all pilin loci are thought to pair the regions involved in recombination events (Wainwright et al., 1994). In this context, the 17 bp direct repeats (Figure 2C) and the conserved regions (Figure 3B) of the *vls* cassettes may play a similar role in recombination events. The silent loci of gonococcal pilin genes contain different regions of the complete pilin genes (Haas and Meyer, 1986), whereas the silent *vls* cassettes of B. burgdorferi represent only the central cassette region of the *vlsE* gene (Figure 3B). The recombination between the expression and silent loci occurs predominantly through a nonreciprocal gene-conversion mechanism (Haas and Meyer, 1986; Koomey et al., 1987).

Based on the available information, we postulate the following mechanism of genetic variation (Figure 7): (i) a *vls*-specific recombination mechanism is induced in the mammalian host; (ii) the conserved sequences facilitate recombination between the expressed and silent *vls* sequences, probably by a nonreciprocal gene conversion mechanism; (iii) the conserved 17 bp direct repeat sequences may be involved in alignment of the *vls* sequences during recombination or in binding of a proposed site-specific recombinase(s); and (iv) through multiple recombination events, portions of the expression site are replaced by segments from several silent *vls* cassettes, resulting in a vast array of potential *vlsE* alleles. The exact mechanism of *vls* recombination remains to be determined.

We have strong evidence that genetic variation at the *vls* locus generates antigenic variation. The structural and sequence similarities between the *vls* and *vmp* systems provided the initial indication that the *vls*-encoded protein was involved in antigenic variation. The apparent surface localization of VlsE and prolific recombination at the *vlsE* site in C3H/HeN mice supported the possibility of antigenic variation in Lyme disease borreliae. Decreased reactivity to antibody against the parental Vls1 cassette region among the clonal populations of mouse isolates demonstrated that genetic variation at the *vlsE* site resulted in changes in antigenicity of the VlsE variants (Figure 6B). The results obtained with sera from infected animals and humans (Figures 6C–6E) provided additional evidence to support the idea of antigenic variation. Further studies are necessary to determine the significance of the *vls* genetic and antigenic variation in the mammalian host.

Variation of B. burgdorferi surface proteins such as VlsE may also affect the organism's virulence and its ability to adapt to different microenvironments during infection of the mammalian host. Recent studies of a Borrelia turicatae mouse infection model that resembles Lyme disease showed that one serotype expressing VmpB exhibited more severe arthritic manifestations, whereas another expressing VmpA had more extensive central nervous system involvement (Cadavid et al., 1994). The numbers of borreliae present in the joints and blood of serotype B-infected mice were much higher than those of mice infected with serotype A, consistent with a relationship between Vmp serotype and disease severity (Pennington et al., 1997). Antigenic variation of Neisseria pilin (Rudel et al., 1992; Nassif et al., 1993; Jonsson et al., 1994) and Opa proteins (Kupsch et al., 1993) is known to affect adherence of the organisms to human leukocytes and epithelial cells.

VlsE (or, potentially, other proteins encoded by lp28–1) appears to be required for infectivity of Lyme disease borreliae in the mammalian host (Figure 1) but not for in vitro growth, since B. burgdorferi Sh2–82 clones containing and lacking lp28–1 had virtually identical growth rates in in vitro cultures (Norris et al., 1995). However, low infectivity clones lacking lp28–1 do not propagate in severe combined immunodeficiency (SCID) mice, indicating that the required factor(s) provides an important function unrelated to evasion of the adaptive immune system (Norris et al., 1995). Also, in vivo selection against B. burgdorferi clones lacking lp28–1 appears to occur early in infection (within the first week), before the adaptive immune response would be expected to exert significant selection pressure. Therefore, it is likely that VlsE plays an important role in some aspect of infection (e.g., colonization, dissemination, adherence, extravasation, evasion of innate immune mechanisms, or nutrient acquisition), and that antigenic variation merely permits surface expression of this protein without leading to

elimination of the bacteria by the host's immune response. Retention of this activity would require that the variation in amino acid sequences would not interfere with the active site(s) of the protein; this requirement may explain the existence of highly conserved regions at the N- and C-termini and within the *vls* cassette. Sequence variation as a mechanism of maintaining surface protein function in the face of a hostile immune response may be a common strategy among pathogenic microorganisms.

Experimental Procedures

Bacterial Strains

B. burgdorferi strains B31, Sh-2–82, and N40 were originally isolated from Ixodes scapularis ticks as summarized previously (Norris et al., 1995; Xu et al., 1996). Nine B31 and 10 Sh-2–82 passage 5 clones had been characterized according to infectivity and described previously by Norris et al. (1995). Infectious B. afzelii ACA-1 and B. garinii IP-90 clones were obtained by subsurface plating of organisms following isolation from experimentally infected C3H/HeN mice (A. G. B., unpublished data). Spirochetes were cultured in BSK II medium as described (Norris et al., 1995). The E. coli strains XL1-blue MRF′ (Stratagene, La Jolla, CA) and BL-21(DE3) (Novagen, Madison, WI) were used for DNA cloning and fusion protein expression, respectively.

Subtractive Hybridization

Subtractive hybridization was performed according to the procedure of Seal et al. (1992). B. burgdorferi total DNA was isolated as described previously (Walker et al., 1995). Total DNA of the high passage B. burgdorferi B31 was subjected to ultrasonic disruption, and the resulting 0.5–1 kb fragments were utilized as driver DNA. The driver DNA (50 μg) was then mixed with 1 μg of total DNA from the low passage B31 digested to completion with Sau3AI (target DNA). The target–driver DNA mixture was denatured and reannealed under the conditions described previously (Seal et al., 1992). The resultant DNA mixture was ligated into BamHI-digested pBluescript II SK(−) (Stratagene). The ligation mixture was used to transform E. coli XL1-blue MRF′-competent cells (Stratagene), and the transformants were plated on Luria-Bertani (LB) agar containing 100 μg/ml ampicillin, 0.5 mM IPTG, and 20 μg/ml X-gal. LB broth cultures inoculated with white colonies were blotted to Hybond-N^+ nylon membranes (Amersham, Arlington Heights, IL) with a Bio-Slot apparatus (Bio–Rad, Hercules, CA) and probed with ^{32}P-labeled driver and target DNA. The clones that hybridized only to target probe but not to driver probe were partially sequenced using vector–sequence based T3 and T7 primers.

DNA Electrophoresis and Southern Hybridization

For plasmid analysis, B. burgdorferi total DNA was prepared in agarose inserts and separated in 1% Fastlane agarose gels (FMC, Rockland, ME) by pulsed-field electrophoresis as described previously (Norris et al., 1995). Restriction enzyme-digested DNA fragments were separated by standard agarose gel electrophoresis (Sambrook et al., 1989). DNA bands were visualized by ethidium bromide staining. For Southern hybridization, DNA was blotted to Hybond-N^+ nylon membranes by the alkaline transfer method (Sambrook et al., 1989). The blots were hybridized as described previously (Walker et al., 1995).

DNA Cloning and Sequence Analysis

The total plasmid DNA of B31–5A3 was prepared and treated with mung bean nuclease to open the covalently linked telomeres of the linear plasmids according to Hinnebusch et al. (1990). The resulting plasmid DNA was filled in with the Klenow fragment of DNA polymerase, and an EcoRI linker (5′-CCGGAATTCCGG-3′) was ligated onto the plasmid ends using T4 ligase. The preparation was then digested with EcoRI and ligated into EcoRI-treated λDASH II vector (Stratagene). The recombinant phages were propagated and screened by plaque hybridization with the pJRZ53 probe according to the vector manufacturer's instructions. Lambda phage DNA was purified by CsCl-gradient purification method (Sambrook et al., 1989).

For random cloning of the λDASH-Bb12 insert, the purified bacteriophage DNA was treated with DNaseI, cloned into EcoRV-digested pBluescript II SK(−), and screened with λDASH-Bb12 probe as described previously (Demolis et al., 1995). Positive clones were sequenced as described below using T3 and T7 primers. In some instances, unsequenced regions were filled in by primer walking. The sequenced fragments were assembled using the GELASSEMBLE program of GCG (Version 8, Genetics Computer Group, Madison, WI). High stringency settings were applied to discriminate identical sequences from highly homologous sequences.

All plasmid and PCR templates were purified by Wizard columns (Promega, Madison, WI). DNA sequences were determined with an ABI 377 automatic DNA sequencer (Perkin-Elmer/ABI, Foster City, CA) in the DNA Core Laboratory, Department of Microbiology and Molecular Genetics, University of Texas Medical School at Houston. The GAP and PILEUP programs of GCG were used to determine sequence homology (percent similarity and identity) and to perform multiple sequence alignments, respectively. Graphical output of alignments was prepared in part through the use of the BOXSHADE program (originally programmed by K. Hofmann at Bioinformatics Group, Isrec, Switzerland, and M. D. Baron at the Institute of Animal Health, Pirbright, U. K., and compiled in Pascal version for Sun Solaris/Pascal by P. A. Stockwell at University of Otago, Dunedin, New Zealand). Searches for sequence similarity were performed at the National Center for Biotechnology Information using the BLAST programs (Altschul et al., 1990).

PCR Techniques

All PCR amplifications were performed using the thermalase PCR kit (Amresco, Solon, OH) in a Minicycler from MJ Research (Watertown, MA). For primer pairs containing 5′-end nested sequences (F4120 and R4121), a two-step program was used as follows: 96°C for 3 min, 5 cycles of denaturation at 95°C for 40 s, annealing at 56°C for 40 s, and extension at 72°C for 2 min, followed by 30 cycles at a higher annealing temperature of 65°C. For primer pairs without nested sequences (F4064 and R4066), 35 amplification cycles of denaturation at 95°C for 40 s, annealing at 60°C for 40 s, and extension at 72°C for 2 min were used. The final cycles of both programs were followed by extension at 72°C for 10 min.

For RT–PCR, total RNA was extracted from late log-phase cultures of B. burgdorferi B31–5A3 with an RNA purification kit (Amresco). The resulting RNA preparation was used to produce cDNA with the R4066 primer (5′-CTTTGCGAACGCAGACTCAGCA-3′) (Figure 2C) and murine leukemia virus reverse transcriptase (Promega) according to the supplier's instructions. Primer F4064 (5′-ATCCGATTG CTGCTGCTATTGGG-3′) (Figure 2C), primer R4066, and the RT reaction were used for PCR reaction as described above. The PCR product was then cloned into the pCR-II vector (Invitrogen, San Diego, CA) according to the supplier's manual, and the resulting clones were sequenced.

GST Fusion Protein Expression

A 614 bp fragment containing the *vls1* cassette was amplified by PCR using (+) strand primer F4120 (5′-GCGGATCCAGTACGACGG GGAAACCAG-3′) and (−) strand primer R4121 (5′-GCGGATCCCCT TCTCTTTCTCACCATCC-3′) (Figure 2C). For cloning purposes, we added an 8 bp sequence (underlined) at the 5′-ends of both primers to create BamHI sites. The resultant PCR product containing the entire *vls1* cassette region was cloned into the BamHI site of the pGEX-2T expression vector (Pharmacia, Piscataway, NJ) to produce a GST fusion protein (designated GST-Vls1) in E. coli strain BL-21(DE3) according to the supplier's instructions. The insert sequence of the recombinant plasmid was verified prior to use for protein expression. The fusion protein was purified by glutathione-Sepharose 4B column (Pharmacia) according to the manufacturer's instructions.

Antibodies and Immunoblotting (Western Blotting)

Antisera against the GST-Vls1 fusion protein and GST as a control were prepared in rabbits by standard methods (Sambrook et al.,

1989). Nonspecific reactivity of the antiserum was removed by absorption with cell lysate of a low infectivity B31 clone 5A2 lacking lp28–1 plasmid as described previously (Carroll and Gherardini, 1996). Antiserum against recombinant OspD was prepared in a similar manner, and monoclonal antibody H9724 reactive with B. burgdorferi flagellum protein (Fla) was graciously provided as a hybridoma culture supernatant by D. D. Thomas (University of Texas Health Science Center at San Antonio). Serum samples from Peromyscus leucopus mice infected with B. burgdorferi B31 via tick bite and from Lyme disease patient sera were generously provided by T. Schwan of the Rocky Mountain Laboratories (Hamilton, MT) and P. Mitchell of Marshfield Laboratories (Marshfield, WI), respectively. The immunoblots of B. burgdorferi cultures were prepared according to Norris et al. (1992) and detected with secondary antibodies (for C3H/HeN mouse sera and human sera) or Protein A (for Peromyscus sera) using an ECL Western blot kit from Amersham according to the supplier's instructions.

Surface Proteolysis

Proteinase K digestion of B. burgdorferi B31–5A3 was performed as described previously (Norris et al., 1992). Proteins of the treated organisms were separated by SDS–PAGE, electrotransferred to PVDF, and reacted with antisera against GST-Vls1 or OspD, or with monoclonal antibody H9724. Reactions were visualized using the ECL Western blot kit.

Mouse Infections

The original stock of B. burgdorferi B31–5A3 (Norris et al., 1995) was cultured in BSK II broth for 7 days, and 10^5 organisms were used to inoculate each of eight 3-week-old female C3H/HeN mice by subcutaneous injection. Four weeks after infection, the organisms were isolated by inoculating 50 μl of blood or a full-thickness biopsy of the ear into 6 ml of BSK II broth. Clonal populations of B. burgdorferi isolates from C3H/HeN mice were obtained by subsurface plating (Norris et al., 1995). The first passages of these cultures were frozen in BSK II medium with 15% glycerol at −70°C as stocks for further study. The *vlsE* cassette region was amplified by PCR using primers F4120 and R4066 (Figure 2C) and sequenced using the same set of primers. Samples of the frozen stocks (~3 μl) were scraped from the surface, thawed, and added directly into PCR tubes as the DNA template source to minimize possible variation during in vitro cultivation. Serum samples were also collected from each mouse before infection and 4 weeks after initial infection, and stored at −70°C for immunoblot analysis.

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GenBank Accession Numbers

The sequences for the *vlsE* gene (allele *vlsE1*) and the 15 silent *vls* cassettes of B. burgdorferi B31 clone 5A3 are contained in the GenBank entries U76405 and U76406, respectively. The nucleotide sequences for 15 *vlsE* alleles of the mouse isolates are contained in the GenBank entries U84553 (*m1b4A*), U84554 (*m1e4A*), U84555 (*m1e4B*), U84556 (*m1e4C*), U84557 (*m1e4D*), U84558 (*m1e4E*), U84559 (*m2b4A*), U84560 (*m3b4A*), U84561 (*m3e4A*), U84562 (*m4b4A*), U84563 (*m4e4A*), U84564 (*m6b4A*), U84565 (*m7b4A*), U84566 (*m8e4A*), and U84567 (*m5e4A*).