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The *vls* antigenic variation systems of Lyme disease *Borrelia*: eluding host immunity through both random, segmental gene conversion and framework heterogeneity

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Summary

Spirochetes that cause Lyme borreliosis (also called Lyme disease) possess the *vls* locus, encoding an elaborate antigenic variation system. This locus contains the expression site *vlsE* as well as a contiguous array of *vls* silent cassettes, which contain variations of the central cassette region of *vlsE*. The locus is present on one of the many linear plasmids in the organism, e.g. plasmid lp28-1 in the strain *B. burgdorferi* B31. Changes in the sequence of *vlsE* occur continuously during mammalian infection and consist of random, segmental, unidirectional recombination events between the silent cassettes and the cassette region of *vlsE*. These gene conversion events do not occur during in vitro culture or the tick portion of the infection cycle of *Borrelia burgdorferi* or the other related *Borrelia* species that cause Lyme disease. The mechanism of recombination is largely unknown, but requires the RuvAB Holliday junction branch migrase. Other features of the *vls* locus also appear to be required, including *cis* locations of *vlsE* and the silent cassettes and high G+C content and GC skew. The *vls* system is required for long-term survival of Lyme *Borrelia* in infected mammals and represents an important mechanism of immune evasion. In addition to sequence variation, immune selection also results in significant heterogeneity in the sequence of the surface lipoprotein VlsE. Despite antigenic variation, VlsE generates a robust antibody response, and both full length VlsE and the C6 peptide (corresponding to invariant region 6) are widely used in immunodiagnostic tests for Lyme disease.

Antigenic variation is defined as a hereditable, reversible variation in an antigenic structure that occurs during the course of infection at a rate higher than would be expected for standard recombination or mutation mechanisms. Many bacterial and protozoal pathogens have developed antigenic variation systems in which surface antigens can be continually altered as a means of evading the constant onslaught of adaptive antibody and T cell responses (1). In 1997, an elaborate antigenic variation system was identified in *B. burgdorferi* B31 (2). Because of sequence similarity between this system and the previously characterized Variable Major Protein (VMP) system of relapsing fever bacteria, it was termed the VMP-like sequence (*vls*) locus. Its expression site, called *vls* Expressed (*vlsE*), undergoes remarkable sequence variation involving segmental gene conversion events from *vls* silent cassettes. This chapter will describe what is currently known about the structure,

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properties, role in host-pathogen interactions, recombination process, and evolution of the *vls* system.

Lyme borreliosis

Lyme borreliosis (LB; also called Lyme disease) is a multistage, tick-transmitted infection caused by spirochetes in the genus *Borrelia*. *Borrelia burgdorferi* is the principal human pathogen in North America, whereas *B. garinii*, *B. afzelii*, and *B. burgdorferi* all give rise to Lyme borreliosis in Euroasia (3-5). These organisms are transmitted by hard-bodied ticks of the genus *Ixodes*; *I. scapularis* and *I. pacificus* are the transmitting ticks in North America, whereas *I. ricinus* and *I. persulcatus* are most active in Europe and Asia, respectively. *B. spielmanii, B. bissettii* and *B. valaisiana* have also been associated with rare cases of human infections (6). There are many additional Lyme *Borrelia* species that are not known to cause human disease. All of the Lyme *Borrelia* species are referred to collectively as *Borrelia burgdorferi* sensu lato (in a broad sense), whereas *B. burgdorferi* sensu stricto (in a strict sense) refers only to the type species of the group. Relapsing fever *Borrelia* (including *B. hermsii*, *B. crocidurae*, and *B. recurrentis*), although related to Lyme *Borrelia*, cause an entirely different disease transmitted by soft-bodied, fast-feeding *Ornithodoros* ticks.

B. burgdorferi and other Lyme *Borrelia* survive by contiguous transmission between *Ixodes* ticks and susceptible mammalian hosts. Infection of humans occurs through the bite of an infected tick (usually at the nymphal stage), causing a localized infection and a resulting expanding red rash called erythema migrans (Table 1). The spirochetes multiply locally, but even at these early stages of infection are able to penetrate blood vessels and lymphatics and thereby disseminate to other tissues. The erythema migrans lesion will eventually clear. However, most patients will go on to develop disseminated symptoms, including a variety of musculoskeletal, neurologic, and cardiovascular manifestations. Months to years later, persistent infection causes Lyme arthritis, which is the most prominent late symptom in North American patients infected with *B. burgdorferi. B. garinii* infection tends to cause neurologic signs, whereas most cases of the skin lesion acrodermatitis chronica atrophicans (ACA) are caused by *B. afzelii*. The manifestations shown in Table 1 really form a continuum, and the disease properties differ greatly from patient to patient. Lyme *Borrelia* are present at high concentrations only in erythema migrans skin lesions, and otherwise are typically present in small numbers and can be distributed to almost any tissue. The organisms produce no known toxins; rather, pathogenesis appears to be primarily due to the induction of inflammatory reactions in the infected mammalian host (7). During the transitions between the tick and mammalian hosts, Lyme *Borrelia* undergo massive changes in gene expression (8), resulting in concomitant shifts in the proteins required for survival and growth in the arthropod or warm-blooded animal environments.

While it is not known how long humans can be infected with Lyme *Borrelia*, viable spirochetes can be cultured from almost any tissue of experimentally infected mice throughout their lifetimes; thus it is likely that, without treatment, humans can carry viable organisms for years. Lyme *Borrelia* thus fall in a category of persistent, nontoxigenic pathogens that also includes the syphilis spirochete *Treponema pallidum* subsp. *pallidum* (9).

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Persistence requires mechanisms for evading host immune responses, particularly the adaptive immune response. Immune evasion mechanisms that have been described in Lyme *Borrelia* include Complement Regulator-Acquiring Surface Proteins (CRASPs), which bind Factor H and Factor H-like protein 1 (FHL-1) and thus inhibit the activation of the complement cascade (10,11). Another mechanism involves the down-regulation of the antigenic tick phase-associated outer surface lipoproteins OspA and OspB, as well as OspC, which is required for survival of *B. burgdorferi* during the early phase of mammalian infection (12,13).

vls system of *B. burgdorferi* B31

The B31 strain of *B. burgdorferi* was isolated from *I. scapularis* (then called *I. dammini*) ticks collected on Shelter Island, New York, and was the first strain of Lyme *Borrelia* described(14). In early studies of this organism, it was determined that its genome consisted of a linear chromosome and multiple linear and circular plasmids ranging in size from 56 kbp to 5 kbp. Many of these plasmids were easily lost during in vitro culture, and absence of some of these plasmids correlated with loss of infectivity in animal models (15-23). The availability of the genome sequence of *B. burgdorferi* B31 showed that the organism contained 12 linear and 9 circular plasmids (with a total of over 600 kbp) as well as a 972 kbp linear chromosome (24,25). The linear replicons of *Borrelia* were found to have covalently closed, hairpin telomeres in which a 5'-3' bond is present between the positive and negative strands (25-27). Replication of these linear molecules occurs through the formation of a circular intermediate that undergoes asymmetric single-stranded cleavage by the plasmid-encoded telomere resolvase ResT at specific sites near the telomere, followed by separation of the two plasmid copies, snap-back hybridization, and ligation (28).

The *vls* system was first discovered prior to the availability of the genomic sequence as the result of a subtractive hybridization study aimed at identifying sequences that were present in low passage, infectious clones of *B. burgdorferi* B31 but absent in a high passage, noninfectious B31 clone (2). A single recombinant plasmid called pJRZ53 was found to encode an amino acid sequence with a low but significant sequence identity with the *B. hermsii* antigenic variation protein Vlp17. The pJRZ53 insert hybridized with several restriction fragments of lp28-1, a 28 kbp linear plasmid. Cloning of the 10 kbp region containing the *vls* sequences into a lambda phage vector followed by sequencing revealed the presence of a single telomeric copy of the expression site *vlsE* as well as a contiguous array of 15 sequences that shared 90.0% to 96.1% nucleotide sequence identity and 76.9% to 91.4% encoded amino acid sequence identity with the central "cassette" region of the *vlsE* gene (Fig. 1A). The 15 unexpressed (silent) cassette regions are 474 to 594 bp in length and are demarcated by 17 bp direct repeats found also at either end of the cassette region of *vlsE*. They are present in a head to tail arrangement, forming a long contiguous open reading frame interrupted only by one stop codon (in cassette *vls11*) and two frame shifts (in cassettes *vls14* and *vls16*). Alignment of the nucleotide or encoded amino acid sequences of the *vls* cassettes reveals the presence of 6 variable regions (VRs) interspersed among 7 relatively invariant regions (IRs) (Fig. 1B). In the variable regions, up to 6 different amino acids can be encoded in some positions of the aligned sequence. Nearly all indels are in multiples of three base pairs, indicating the importance of maintaining an intact open

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reading frame. Some of the silent cassettes are truncated or have long internal deletions; these regions were excluded from the analysis shown in Fig. 1B, because they do not appear to participate in *vlsE* recombination events.

The *vlsE* gene itself encodes a 36 kDa protein with a lipoprotein leader sequence; further studies verified that the VlsE product is lipidated and localized on the outer surface of the outer membrane (2). The gene has a consensus sigma 70 promoter region, and primer extension analysis showed that transcription is initiated with nearly equivalent efficiency at two adjacent thymidine residues at 13 and 14 bp downstream from the beginning of the −10 sequence(29). Interestingly, inverted repeats that include portions of the *vlsE* promoter region and the 5’ end of the first silent cassette are predicted to form a 51-bp stem loop structure; this feature is likely involved in *vlsE* transcription and/or recombination.

To date, no *vlsE* recombination events have been reported in in vitro cultures of Lyme *Borrelia* or in infected ticks (2,30-32). In contrast, *vlsE* sequence changes have been detected as early as 4 days post inoculation of C3H/HeN mice with *B. burgdorferi*; by 28 days of infection, few (if any) organisms expressing the ‘parental’ *vlsE* sequence are present (30,33). Similar results have been obtained with infected rabbits, with multiple *vlsE* sequence changes observed at 2 weeks, the earliest post-inoculation time point in these experiments (34). In contrast to relapsing fever *Borrelia,* in which the typical pattern is the sequential outgrowth of clones expressing a single VMP type, mammalian infection with Lyme *Borrelia* results in the outgrowth of a myriad of clones each expressing a different VlsE variant. Indeed, the degree of sequence variation is such that it is difficult to find two clones with the same *vlsE* sequence at time points beyond 4 weeks post inoculation, even in the same tissue biopsy (2,30,33).

Given the presence of 15 silent cassettes that represent variants of the *vlsE* cassette region sequence, the initial hypothesis was that each of these silent cassettes could replace the *vlsE* cassette, resulting in 15 different variants. Instead, the sequencing of *vlsE* cassette regions from over 1,400 *B. burgdorferi* clones has revealed that sequence variation occurs through the replacement of segments of DNA in the expression site with segments of the corresponding regions of the silent cassettes (Fig. 1C) (2,30,33). These segments can range in size from a few base pairs to nearly the entire length of the *vlsE* cassette region (33). The recombinations represent gene conversion events, in that the sequences of the silent cassettes are not altered (35), as will be discussed further in the context of recombination mechanisms. *vlsE* gene conversion events appear to occur continuously during the course of mammalian infection, resulting in a mosaic of overlapping recombinations that are difficult to decipher. An Excel spreadsheet-based Visual Basic program has been developed to help determine which silent cassettes could have been the source of sequence replacements (33). Because the silent cassettes have many regions of sequence identity, it is often not possible to attribute a given recombination event to a single silent cassette; multiple cassettes could have served as potential ‘donors’. For the same reason, the boundaries of the recombination usually cannot be determined with confidence, so recombination events are defined as minimal recombination regions (including the nucleotides that differ from the parental sequence) and maximal recombination regions (encompassing the minimal region plus the surrounding nucleotides that are identical between the parental sequence and the silent

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cassette donor) (33). The sequence variation is restricted to the *vlsE* cassette region, in that 5' and 3' ends of the gene do not undergo sequence changes (35).

vls systems of other Lyme *Borrelia*

vls sequences have been identified in every Lyme *Borrelia* organism for which a complete or draft genomic sequence is available, indicating that this antigenic variation system is ubiquitous among all Lyme disease *Borrelia*. After the initial description of the *B. burgdorferi* B31 *vls* system, the identification of *vls* sequences in recombinant DNA libraries derived from *B. burgdorferi* 297 (36), *B. garinii* IP90 (37), and *B. garinii* A87SA (38) were reported. Comprehensive analysis of *vls* sequences of *B. garinii* IP90 and *B. afzelii* ACA-1 revealed the near-complete sequence of the silent cassettes of these two strains (39). The intact *vlsE* genes of these two strains were not isolated, but partial sequences were obtained by the cloning of cDNA products and by PCR analysis (39).

All of the 26 Lyme *Borrelia* strains from which extensive plasmid DNA sequences have been obtained (24,40-45) contain *vls* sequences, greatly increasing the available information regarding the characteristics of *vls* systems. Shotgun sequencing of genomes has yielded nearly complete sequences of *vls* silent cassette arrays. However, *vlsE* sequences are typically missing from genomic sequences, probably because of their location near a telomere or predicted stem-loop structures and resulting poor cloning and sequencing efficiency.

Currently, complete or nearly complete *vlsE* sequences are available in GenBank for 16 Lyme *Borrelia* strains. In addition, the *B. burgdorferi* 29805 silent cassette *vlsS1* is contiguous with a region homologous to the 5' constant (non-cassette) region of *vlsE*, so it is also included in this comparison. The phylogenetic tree of the predicted VlsE amino acid sequences with the *B. hermsii* Vlp sequences as outliers (Fig. 2) reveals the conservation and diversity among the VlsE sequences. VlsE proteins of the German *B. burgdorferi* isolates PKa2, PAbe, PBoe, and PBre all exhibit a high sequence identity/similarity with the United States isolate B31 (83-93% and 90-95%, respectively), with nearly all the sequence differences existing in the variable regions. This result indicates that a closely related clade of *B. burgdorferi* clones exists in the North American and European continents, such that the VlsE sequence differences are essentially the same as are observed among B31 antigenic variants (33). Indeed, the availability of several genomic sequences from this clade indicate a pan-genomic clonality, consistent with the recent geographic spread of this group of Lyme *Borrelia* (43). In contrast, the VlsE sequences of North American *B. burgdorferi* isolates B31 and 297 exhibit a high degree of divergence, with only 46% identity and 53% similarity. A similar level of divergence is observed between the Lyme *Borrelia* species (Fig. 2). Again using *B. burgdorferi* B31 for comparison, the *B. garinii*, *B. afzelii*, and *B. spielmanii* strains have only 35-49% identity and 41-59% similarity, indicative of a high level of diversification in "framework" regions outside of the variable regions. *B. valaisiana* VlsE was the most divergent from the B31 sequence (33% identity, 41% similarity). A very similar pattern of diversification between strains (not shown here) is evident in the much larger group of available *vls* silent cassette sequences, and also when nucleotide sequences are used for comparison.

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The degree of diversity among *vlsE* sequences is higher than that of any other orthologous gene group in the Lyme *Borrelia* strains examined. *ospC* is the next most divergent, with the lowest OspC identity and similarity among available full-length sequences being 68% and 79% respectively (between *B. garinii* TCLSK and *B. valaisiana* VS116). The high diversity in *vlsE* (and *ospC*) is indicative of a strong selective pressure, most likely driven by mammalian host adaptive immune responses as discussed later in this chapter.

The sequences of only three intact *vls* systems (including both *vlsE* and the *vls* silent cassettes) are currently available (Fig. 3). In each of these, the arrangement is similar to the B31 system, with *vlsE* and the silent cassettes facing in opposite directions and separated by a short DNA segment. This region includes inverted repeats that could form a stem loop structure; however, these repeats are comprised of different sequences in each case. For example, the possible stem loop structure in strain B31 involves portions of the *vlsE* promoter on one side and a segment upstream of the first *vls* silent cassette region on the other. All three strains lack an intact promoter for the silent cassette region. However, both *B. burgdorferi* JD1 and *B. garinii* Far04 have sequences identical to the 5'-end of the *vlsE* open reading frame that are contiguous with the first silent cassette. In JD1, the region complementary with *vlsE* lacks a start codon but includes a 476 nt region of identity (with 2 nt differences) with the 5' end *vlsE* and a portion of the first *vls* cassette. The Far04 silent cassette region likewise has a 476 nt region of identity to the 5' end of *vlsE* and part of the cassette region, but also encompasses the ribosome binding site and −10 sequence. The B31 strain silent cassette region also has a shorter segment of sequence identical to the 5' end of *vlsE*. The 'loop' region between the complementary 'stem' sequences is 300 nt and 299 nt in JD1 and Far04, respectively, but is only 6 nt long in the B31 sequence. The significance and potential functionality of the duplication of the 5' end of *vlsE* is not known, but the fact that it is conserved to a varying extent in each organism indicates that its preservation is favored and that 'purifying' recombinations may maintain the structure. Of course, there are many additional regions of sequence identity between *vlsE* and the silent cassettes that likely participate in the *vlsE* gene conversion events.

Many of the silent cassettes in both *B. garinii* Far04 and *B. burgdorferi* JD1 are separated by frame shifts (Fig. 3), and this pattern is found in the *vls* loci of other Lyme *Borrelia* organisms as well. Thus the presence of a nearly contiguous open reading frame in the *vls* cassette region as seen in *B. burgdorferi* B31 is not required for *vlsE* recombination and locus functionality. The *B. garinii* Far04 *vls* locus is found close to a telomere, as is the B31 locus; the *vlsE* sequence in JD1 is incomplete, so the nature of the downstream sequence is not known. *vls* silent cassette regions have been identified in every other complete or near complete genomic sequence from Lyme *Borrelia* organisms, currently including 2 *B. afzelii* , 13 *B. burgdorferi*, 4 *B. garinii*, 2 *B. spielmanii*, and 1 *B. valaisiana* strains (data not shown). In all of these cases, the silent cassette arrangements are similar to those shown in Fig. 3. As mentioned previously, the lack of a contiguous sequence containing *vlsE* appears to be due to either its telomeric location or the inverted repeats, which interferes with sequencing reactions. Overall, the general properties of *vls* loci in Lyme *Borrelia* are well conserved, although substantial sequence diversity is present.

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VlsE structure and the localization of variable regions

Determination of the three dimensional structure of *B. burgdorferi* B31 VlsE (46) revealed one of the most remarkable examples of immune evasion through the optimized localization of variable epitopes. As mentioned previously, VlsE is a surface localized lipoprotein, and is thereby anchored to the outer membrane surface by lipid moieties covalently linked to the N-terminal cysteine residue of the processed protein. Although the unit cell of the crystal structure consists of four monomers with two side-by-side pairs (46), VlsE appears to exist as a monomer rather than a dimer in its native state. The N- and C-terminal sequences and the relatively invariant regions within the cassette form α-helices that constitute the structural framework of VlsE (Fig. 4A). Within this framework, the six VRs constitute random coil regions that form a 'dome' on the membrane distal surface of the protein. In the space-filling model (Fig. 4B), these variable regions essentially cover the top of the protein. Therefore, the region of the polypeptide that is most likely to interact with antibodies is precisely the area that undergoes rapid sequence variation during the course of mammalian infection.

It is not known to what degree the relatively invariant lateral surfaces of this elongated protein are exposed to the external fluid phase in the intact organism. The surface lipoproteins may form a contiguous layer during infection that would prevent antibodies from accessing the lateral surfaces. Such a 'forest' of outer surface proteins has been documented by cryo electron tomography (cryo ET) in in vitro cultured *B. burgdorferi* (47), but under these conditions the lipoproteins OspA, OspB, and OspC are highly expressed. During mammalian infection, OspA and OspB are dramatically down-regulated, OspC appears to be transiently expressed, and VlsE expression is thought to increase (29,48,49); however, to date the surface topology of mammalian host-adapted *Borrelia* has not been examined in detail by cryo ET or other means. It has been shown that antibodies against the invariant regions, particularly the highly immunogenic IR6 region, do not react with VlsE in intact organisms, although they will bind to recombinant VlsE in solution (37)(50). IR6 forms a compact α-helix that is 'buried' underneath the variable region random coil structures (46), and thus may lack the surface exposure necessary to permit antibody binding. Recombinant VlsE is difficult to crystallize; it is possible that a significant amount is misfolded and thus may have exposed IR6 available for antibody reactions.

Role in pathogenicity

The earliest indication that the *vls* system was required for full infectivity was that loss of the encoding plasmid (a 28 kb linear plasmid, lp28-1, in most Lyme *Borrelia*) resulted in an intermediate infectivity phenotype in experimentally infected mice (15,18,20-23). Needle inoculation of immunocompetent C3H/HeN mice with lp28-1^{-} clones of *B. burgdorferi* strains B31 or 297 results in a transient infection in which organisms can be cultured from joints and, occasionally, other tissues for up to two weeks post inoculation (but not thereafter). Anti-*B. burgdorferi* antibody responses occur in the animals infected with the lp28-1^{-} clones, consistent with the establishment of sufficient numbers of spirochetes to induce B cell responses. Surprisingly, lp28-1^{-} clones cause long-term infections in severe combined immunodeficiency (SCID) mouse strains (21,30,51). *Trans*-complementation with

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vlsE alone on a shuttle vector does not alter the infectivity phenotype of a lp28-1⁻ clone, indicating that VlsE expression is not sufficient to restore full infectivity (51). Loss of lp28-1 does not affect the colonization of *I. scapularis* ticks (52). Taken together, these results show that lp28-1 carries virulence determinant(s) that protect Lyme *Borrelia* against adaptive immune responses in mammalian hosts.

More definitive evidence of the role of the *vls* system in mammalian infection was provided by elegant studies reported by Bankhead and Chaconas in 2007 (53). Using a telomere resolvase-mediated targeted deletion approach, they were able to delete the entire *vls* locus from the right end of lp28-1 in *B. burgdorferi* B31. As a control, they also generated mutants lacking genes (*BBF01-BBF19*) from the left end of lp28-1, upstream from the region required for plasmid replication. The two *vls* mutant clones tested exhibited the same intermediate infectivity phenotype in C3H/HeN mice as the control lp28-1⁻ strain, whereas the mutants lacking the left end of lp28-1 were fully infectious. Expression of VlsE by itself in the *vls* background was not sufficient to restore full infectivity. These results clearly indicate that an intact *vls* system is required for persistent infection.

Antibody responses against VlsE

Immune evasion through antigenic variation systems is thought to act through the alteration of surface-exposed epitopes so that antibodies induced against one form of the antigen will not react with subsequent variants. An unexpected finding was that VlsE induces a robust antibody response in human Lyme borreliosis patients and naturally and experimentally infected animals (2,37,54-60). The antibody responses are predominantly against the conserved, non-variable regions of VlsE, particularly the IR6 region (also called C6) within the *vls* cassette (61,62); serologic reactivity has also been demonstrated against the N-terminal and C-terminal regions outside the central cassette (63). However, McDowell et al. (64) established by VlsE variant cross-absorbtion studies that the variable regions of VlsE also induce antibody responses. Antibodies against conserved regions are typically cross-reactive with other VlsE sequences, and either full-length VlsE or the C6 peptide have been incorporated into many serological diagnostic tests for Lyme borreliosis in humans or dogs (55,56,58,59).

In studies conducted to date, immunization with recombinant forms of VlsE or the C6 peptide has not has not resulted in protection of mice against infection with *B. burgdorferi* expressing the homologous VlsE variant (65) (S.J.N., unpublished data). Also, *B. burgdorferi* strains with a functional *vls* system that are experimentally modified to constitutively express an invariant form of VlsE cause persistent infection (51,53). It could be expected that *Borrelia* expressing invariant VlsE would be eliminated by antibodies against that allele, but that is not the case. Liang et al. (37) have hypothesized that suggested that IR6 may serve as a “decoy” epitope that diverts the immune response away from protective responses to other epitopes on VlsE or other antigens. Although the recombinant B31 VlsE or a single C6 peptide is effective in detecting antibodies induced in humans and dogs infected with a variety of Lyme *Borrelia* species and strains, Baum et al. (66) found that the white-footed mouse *Peromyscus leucopus*, a natural host of *B. burgdorferi* in North America, often produces a weak response to infection with several different *B. burgdorferi*

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strains. They suggest that coevolution of the pathogen and host has resulted in the elimination of sequences that induce protective anti-VlsE responses. Overall, much remains to be understood about the nature of the immune response against VlsE and host-pathogen interactions.

VlsE expression

As is the case for many Lyme *Borrelia* proteins, the expression of VlsE appears to be highly regulated during the organism's infectious cycle (31,48,67). When *B. burgdorferi* is acquired by tick larvae, it continues to express VlsE for approximately 96 hours, after which the percentage of VlsE-positive decreases (48). VlsE is expressed at very low levels by the spirochete in the tick in between feedings. Disparate results were obtained regarding VlsE level changes occurring when an infected tick acquires a blood meal from mice. In two studies, the expression increased significantly within 24 hours, in a manner similar to the increase in OspC expression (31,67); in a third report, the percent of VlsE-positive organisms remained <10% during feeding, but increased to ~90% in the skin of the mice at 72 hours after feeding was initiated (48). Piesman et al. (68) found that *vlsE* transcript levels were higher in *B. burgdorferi* in the salivary glands as compared to the levels in the midgut of feeding ticks, suggesting that the salivary gland environment is stimulatory toward *vlsE* expression. Similarly, Ko i et al. (67) observed *vlsE* transcript increases during tick feeding in *I. ricinus* ticks infected with *B. afzelii*. In vitro studies are contradictory, with opposite changes in VlsE expression occurring when *B. burgdorferi* were incubated at pH 7 or pH 8 at 34° C (31,48). However, the reports were in agreement that VlsE expression was high at pH 7.5-8 at 23° C, conditions thought to mimic the tick midgut.

Hudson et al. (29) determined that *vlsE* is transcribed at higher levels in *B. burgdorferi* B31 in the presence of endothelial cells than in cell-free liquid culture, suggesting that the presence of these cells or their products triggers an up-regulation of *vlsE* expression. No *vlsE* recombination was detected in these studies.

The *vlsE* promoter region has predicted −35 and −10 sequences typical of RpoD recognition sites (Fig. 5A). The transcriptional initiation site was mapped by Bykowski et al. (48) by primer extension and was found to be located at two adjacent T residues 6 nt downstream of the −10 sequence. One can speculate that the predicted stem loop structure in the B31 has regulatory effects on *vlsE* expression. However, as mentioned previously, the location and nature of inverted repeats is variable in the few organisms in which the intact sequence of this region is available. Factors that bind to the promoter region have not as yet been reported. Jutras et al. (69) used electrophoretic mobility shift assays (EMSAs) to demonstrate that SpoVG binds to a dsDNA region of the *B. burgdorferi* B31 *vlsE* open reading frame; it is thus possible that this factor may affect transcription after initiation (see further discussion of SpoVG below).

Crother et al. (49,70) performed novel studies on the expression of *B. burgdorferi* lipoproteins during mouse or rabbit infection, in which the tissue specimens were extracted by Triton X114 phase partitioning, which preferentially extracts lipoproteins. In the first study (70), SCID mice were inoculated to increase the yield of organisms, and infected joint,

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heart, ear, and skin tissues were utilized. The extracted proteins were detected by immunoblot analysis with polypeptide-specific or anti-*B. burgdorferi* polyspecific antisera. In these experiments, VlsE was the most abundant borrelial protein recovered from all tissues except the heart. In the heart, the levels were low throughout the course of infection. In the second study, rabbits were infected by intradermal injection, and rabbits were sacrificed and the skin sites analyzed on days 5, 7, 9, 11, 14, and 21 post inoculation (PI). By this methodology, VlsE protein levels increased dramatically on day 7 PI, and remained high throughout the experiment. Conversely, OspC levels peaked on day 7 PI and decreased rapidly thereafter. When VlsE was analyzed using VlsE-specific antiserum and two dimensional gel electrophoresis, it appeared as a series of spots over a broad pH range; this may reflect the presence of multiple variant VlsE proteins with different isoelectric points (pI's). In addition, low molecular weight isoforms of VlsE were also present, but it was unclear whether these were degradation products or truncated forms. The results of this study provided evidence that OspC levels are downregulated and VlsE levels are upregulated during the course of experimental rabbit infection.

Further studies by Liang et al. (71,72) examined transcript levels of *vlsE*, *ospC* and other lipoprotein genes during the course of *B. burgdorferi* infection in normal mice, B-cell deficient mice, SCID mice, and SCID mice treated with sera from infected animals. In SCID mice, transcript levels of most lipoprotein genes, including *vlsE* and *ospC*, remain at high levels; the same results were obtained with *vlsE* and *ospC* in B cell-deficient mice (72). In contrast, *ospC* transcripts were decreased below detectable levels and *vlsE* transcript levels are reduced in immunocompetent mice at day 33-40 PI. Treatment of SCID mice with sera from *B. burgdorferi* infected mice resulted in dramatic decreases (up to 76-fold) in *ospC* mRNA levels and increases in *vlsE* transcripts (up to 44-fold) relative to SCID mice treated with prebleed mouse sera (72). Similar results were obtained with a monoclonal antibody against OspC, indicating that anti-OspC antibodies were principally responsible for this effect. Interestingly, the effects varied according to the tissues sampled; for example, neither of the antisera had a significant effect on *vlsE* transcript levels in joint tissue.

Overall, studies to date indicate that *vlsE* transcription is dramatically affected by antibody responses, most likely through the direct or indirect effects of OspC transcript or protein expression levels. It is not known currently if these effects are due to the selection of OspC-underexpressing cells, or a generalized down-regulation of OspC expression in the infecting population. Studies by Anguita et al. (73) suggest that the inflammatory process (in particular, interferon-Y expression) may also affect spirochete tissue adaptation and *vlsE* recombination. Clearly, additional studies are needed to gain a better understanding of this interesting and novel phenomenon.

The *vlsE* recombination process

Gene conversion clearly represents the major mechanism of *vlsE* sequence variation, although some have suggested that point mutations may also play a role (74). In terms of the recombination results, *vlsE* recombination most closely resembles that in the *Neisseria gonorrhoeae pilE* system and the *var* system of *Plasmodium falciparum*, in that the region of DNA replacement is variable in both length and region; in each cases, the sequence

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changes appear to be restricted to a given area (e.g. the cassette region in *vlsE*). The *vls* cassette regions uniformly exhibit high GC content (~50%) and marked GC skew relative to the rest of the *B. burgdorferi* sensu lato genomes, and the existence of G quadraplex (G4) structure similar to that in neisserial *pilE* loci has been suggested (75). Beyond these similarities, little is known about the *vlsE* recombination process. It does not require the extensive array of DNA recombination and repair proteins that are involved in *pilE* sequence variation (76,77), nor has the involvement of a small RNA (as recently described in *N. gonorrhoeae*(78)) been implicated. Unlike *Neisseria* species and most other antigenic variation systems described, *vlsE* recombination is not detectable during in vitro culture (or during tick infection), indicating that the process activated in some manner by conditions in the mammalian host. However, to date the factor(s) contributing to this activation have not been identified, limiting the study of *vlsE* recombination to animal infection experiments. Analysis of the recombination process is further hampered by the recalcitrance of the *vls* locus to genetic manipulation, other than its elimination through upstream insertion of telomere sequences (53).

As a result, the present understanding of *vlsE* recombination requirements is limited to the aspects listed in Table 2. These points are addressed in greater detail below.

Cis arrangement of *vlsE* and *vls* silent cassettes

Complete (or nearly complete) *vls* loci sequences have only been determined for three *Borrelia* strains: *B. burgdorferi* B31, *B. burgdorferi* JD1, and *B. garinii* Far04. In each of these cases, the reading frames of *vlsE* and the silent cassettes are arranged in opposite directions, pointing away from one another (Fig. 3). The silent cassette region also begins with a region highly homologous to the 5’ end of the *vlsE* open reading frame; the promoter, however, is not retained. This region may be a remnant of the initial duplication event that gave rise to the first silent cassette. As described previously, the region at the junction of *vlsE* and the *vlsE* cassettes in each strain contains an inverted repeat of 51, 476 and 476 nt respectively, although these differ in location and composition. In the B31 strain, the inverted repeat is in the region between *vlsE* and the *vls* silent cassettes, with inclusion of the −35 sequence of the *vlsE* promoter (Fig. 5A; (29,48); J. K. Howell and S. J. Norris, unpublished data). In the JD1 and Far04 strains, the inverted repeats encompass the 5’ ends of the open reading frames of *vlsE* and the first silent cassette, ending at the sequence differences within the cassette regions. As mentioned previously, the genes at the far end of lp28-1 in *B. burgdorferi* B31 are not required for *vlsE* sequence changes, in that removal of *BBF01-BBF19* from lp28-1 did not interfere with *vlsE* recombination or infectivity of the clone in immunocompetent mice (53).

Based on the limited sampling of intact *vls* loci, the *cis* ‘head to head’ arrangement of *vlsE* and the silent cassettes may be required for *vlsE* recombination. No sequence changes were detected when mice were infected with *B. burgdorferi* B31 harboring an intact *vlsE* gene in a shuttle vector (51), suggesting the importance of the *cis* arrangement. A possible model is that *vlsE* “doubles back” onto the silent cassette, promoting close proximity of the donor and recipient regions and thereby strand invasion as part of the gene conversion process. It is conceivable that the inverted repeats could form hairpin loops and in some way facilitate the

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doubling back. However, one would expect the nearest silent cassettes to recombine more frequently with *vlsE* in this scenario, but that is not the case (33). If site-directed mutagenesis of the *vls* locus becomes feasible, then the potential roles of the head to head arrangement and inverted repeat regions in *vlsE* recombination could be examined more thoroughly.

High G+C content, GC skew, and potential G4 structures

The *vlsE* cassette region and the *vls* silent cassettes have a remarkably high G+C content and GC skew compared to the remainder of the *B. burgdorferi* B31 genome (2,39,75) (Fig. 5B). At 49.7% compared with the 28.2% value for the overall genome, the average percent G+C is 20.5% higher in the silent cassette regions. Although the 3' end of the *vlsE* also has a high G+C content, the coding strand GC skew (G-C/G+C) is preserved only in the cassette region. The cassette region GC skew of 0.55 is much higher than that in the leading strand of DNA replication (e.g. 0.18 in the *B. burgdorferi* B31 chromosome). This pattern is conserved in all of the *vls* sequences that have been characterized to date, despite significant sequence divergence (as low as 40/49 percent identity/similarity). The preservation of this pattern is therefore likely to be important in the promotion of *vlsE* recombination.

In *N. gonorrhoeae*, the segmental recombination of *pilS* cassette sequences into the *pilE* pilus protein expression site requires the formation of a G-quadruplex (G4) structure in a region just upstream of the *pilE* open reading frame (79,80). This conformational change involves the formation of intrachain G-G hydrogen bonds in neighboring G-rich regions to create a complex of four antiparallel segments. It is promoted by the transcription of a small RNA that overlaps with the G4 region (78).

The high GC content and skew in the *vls* cassette region suggests that similar secondary structure changes may be involved in *vlsE* recombination. In 2013, Walia and Chaconas (75) explored the possible occurrence of G4 structure in *vls* sequences. First, they noted that PCR amplification of *B. burgdorferi* B31 *vlsE* resulted in an unexpected lower molecular weight band in which the variable region and one of the 17 bp direct repeats were missing. This phenomenon is apparently due to aberrant base pairing between the two identical direct repeats during the amplification process. Thus it is not thought to occur during *vlsE* recombinations; rather, it indicates unusual base-pairing properties of the 17 bp repeat, which includes a stretch of five G residues. In solution, the 17 bp region of *B. burgdorferi* B31 (or a shorter 14 bp oligonucleotide that also encompasses the G_5 stretch) forms a stable, higher order complex in the presence of high concentrations of KCl, conditions that favor G4 formation (75). The occurrence of hydrogen bonding between the G-rich regions was indicated by protection against methylation by methylsulfoxide. It should be noted, however, that direct repeats at the ends of *vlsE* cassette regions are variable both between and within strains; their lack of conservation argues against a sequence-specific role of these regions (e.g. recognition by a protein involved in the recombination process). Walia and Chaconas (75) further reported that stretches of 3 or more G's are found at very high frequencies in the coding strand of the *vlsE* cassette regions of *B. burgdorferi* strains B31, N40, and JD1; indeed, this is a common property of all known *vls* sequences. Whereas all of these findings support the potential formation of G4 or other intrastrand secondary structures

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in *vls* sequences, to date no G4 structures have been demonstrated either conceptually (based on sequence) or biophysically using native *vls* regions. This represents a promising area of future research.

DNA repair and recombination proteins

Dresser et al. (76) and Lin et al. (77) examined a comprehensive list of *B. burgdorferi* B31 genes encoding predicted DNA repair and recombination proteins for their potential role in *vlsE* recombination. Mutations in these genes were introduced by either site-directed mutagenesis (76) or random transposon mutagenesis (77). Surprisingly, only a few genes were found to have a marked effect on *vlsE* sequence changes during mouse infection (and hence also on survival of the *B. burgdorferi* strains in immunocompetent mice). Both studies identified *ruvA* and *ruvB* as important genes in the recombination process, in that the rate of accumulation and diversity of *vlsE* sequence changes were dramatically reduced in clones in which these genes were mutated (76,77). RuvA and RuvB form a Holliday junction branch migrase. Holliday junctions are mobile junctions between two homologous DNA duplexes, in which strand exchange results in base pairing between homologous regions and formation of a structure with four double-stranded branches. RuvAB branch migrases promote the release and reformation of the base pairs, resulting in migration of the branch point in a zipper-like manner. This branch migration process may be important in the random 'selection' of regions of the silent cassette sequences for exchange with the parental *vlsE* sequences. In most bacterial systems, the endonuclease RuvC is required to resolve the Holliday junction and complete homologous recombination. *Borrelia* lack an obvious *ruvC* homologue; it possible that putative prophage nucleases on the multiple cp32 plasmids could fulfill this function (76). In the Lin et al. study (77), mutation of *mutS* also appeared to have a relatively small effect on the rate of *vlsE* sequence variation during infection. The other genes examined had no obvious effect on *vlsE* recombination; this list includes genes encoding the recombination proteins RecA, RecG and RecJ, repair proteins MutL and MutS2, general DNA replication/processing proteins NucA, Mag, Mfd, Nth, SbcC, SbcD, and PriA, plasmid-encoded putative recombinases BBD20 and BBG32, predicted DNA methyltransferase BBE29, and Rep helicase. The finding that the single strand DNA-binding protein RecA is not necessary for *vlsE* sequence changes was also determined previously by Liveris et al. (81). The apparent lack of involvement of this long list of genes differs from the *N. gonorrhoeae pilE* system, in which many proteins of the common recombination pathways (including RecA) are required (see (75,80)). This pattern, coupled with the induction of *vlsE* recombination during mammalian infection, suggests that this process may utilize as yet undiscovered recombinase(s) or other proteins (or RNAs) that become active in the mammalian environment and are specific for the *vls* sequences.

Potential role of SpoVG

It is likely that proteins or small RNAs that specifically recognize *vls* sequences are involved in the recombination process. SpoVG was initially described as a cytoplasmic protein that is required for normal sporulation, vegetative cell structure, and cell division in *Bacillus subtilis*. However, homologs of this protein are found in most eubacteria. In a search for proteins that bind to *vlsE* DNA sequences, Jutras et al. (69) identified a *B. burgdorferi* homologue of SpoVG that binds to an 18-bp sequence within the 5' region of the B31 *vlsE*

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gene, just upstream of the cassette region. The predicted SpoVG sequence is highly conserved in all *Borrelia* species, and also has high similarity with SpoVG sequences in other bacteria. However, the B31 *vlsE* sequence to which SpoVG binds is not well conserved in other Lyme disease *Borrelia* outside the B31 'clade'. Further study is thus necessary to determine if the observed binding activity is related to a conserved mechanism among *B. burgdorferi* sensu lato for regulation of *vlsE* expression or recombination.

Induction of *vlsE* expression and recombination

As mentioned previously, *vlsE* recombination has not been detected consistently in in vitro cultures or in infected ticks, yet can be detected as early as three days after the initiation of mammalian infection (30,33,34). The fact that the recombination can be detected as soon as organisms can be cultured, combined with its occurrence in SCID animals, argues against these results being due to immune selection in the infected animals, although it is clear that the immune response accelerates the clearance of the parental clone used for inoculation (33). Thus recombination appears to be induced in the mammalian environment by unknown mechanisms that appear to be unrelated to temperature, CO_2 concentration, presence of serum components, or other simple explanations. This situation differs from that of relapsing fever organisms and *N. gonorrhoeae*, in which recombination of variable major protein genes and *pilE* occur readily during in vitro culture. *vlsE* sequence changes have been detected by incubation of *B. burgdorferi* with mouse or rabbit tissue explants using sensitive PCR procedures, but it is as yet unclear whether these exceedingly rare events are occurring at rates higher than in standard in vitro cultures. As described previously, *vlsE* transcription and VlsE protein expression is increased during mammalian infection, and it is possible that gene expression facilitates the recombination process. Attempts to mutate *vlsE* in its native lp28-1 location have thus far been unsuccessful, but would be useful in determining whether transcription and/or translation contribute to the occurrence of cassette region sequence changes, as well as in identifying important *cis* elements.

Evolution of the *vls* system

Lyme borreliosis (LB) and relapsing fever (RF) *Borrelia* are closely related, with nearly complete synteny across the chromosomes and a high degree of homology among the protein products. Plasmid structure and content in these groups differ considerably and likely account in part for the biological differences observed, including the colonization and transmission by *Ixodes* vs. *Ornithodoros* ticks and distinct patterns of clinical manifestations. However, recent characterizations of *B. miyamotoi* and strain LB-2001 genome sequences indicate a blurring of these phenotypic and genotypic lines, in that both organisms are firmly positioned within the RF group genetically but are transmitted by *Ixodes* ticks (82). Overall, the phylogeny of *Borrelia* species clearly indicate that they are monophyletic, i.e. arose from a common primordial ancestor distinct from those that gave rise to other major groups of spirochetes, including the *Spirochaeta/Treponema/Sphaerochaeta* group, the *Leptospira/Leptonema/Turneriella* group, and *Brachyspira*.

All Lyme disease organisms characterized to date have *vls* antigenic variation systems, and all relapsing fever organisms have variable major protein (VMP) antigenic variation systems, which feature a single expression site in which either variable large protein (*vlp*) or

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variable small protein (*vsp*) open reading frames can be inserted. The two systems differ fundamentally, in that *vlsE* sequence changes arise from replacement with random segments from silent cassettes, whereas most recombinations in the VMP system involve replacement of the expression site gene with a nearly complete open reading frame from one of ~ 40 *vlp* and *vsp* promoterless gene copies (83). These recombination events predominantly utilize two sites termed Upstream Homology Sequences (UHS) and Downstream Homology Sequences (DHS) that are shared among nearly all of the 'donor' *vlp* and *vsp* copies. This feature is remarkable, in that the *vlp* and *vsp* sequences are otherwise essentially unrelated to each other.

Despite this diversity in the LB and RF recombination mechanisms, the *vlp* and *vsp* RF lipoprotein gene families each have relatives in LB organisms. *vlsE* has a moderate level of sequence homology with the *vlp* genes; for example, comparison of the originally described variant *vlsE1* with *B. hermsii vlp17* reveals nucleotide sequence identity of 58.8% and deduced amino acid sequence identity and similarity of 37.4% and 58.8%, respectively (2). In addition to the multiple *vsp* copies, RF organisms also contain a *vsp*-related 'variable tick protein' (*vtp*) gene that does not participate in *vlp*/*vsp* recombination but is preferentially expressed in the tick environment. Interestingly, LB organisms have a prominent *vsp* homolog encoding outer surface protein C (*ospC*), which is predominantly expressed during the transmission of *Borrelia* from the tick to the mammal and in the mammal during the early stages of infection. Therefore the *vlp* and *vsp* gene families each must have evolved from single primordial *vlp* and *vsp* genes in the common *Borrelia* ancestor and then undergone duplication and remarkable sequence and functional diversification during the development of the separate LB and RF *Borrelia* lineages. This scenario is shown diagrammatically in Fig. 6.

Is there any evidence of a common *vlp* precursor? Surprisingly, there is a paralogous gene in LB *Borrelia* that may be a remnant of this ancient ancestral gene. In *B. burgdorferi* B31, it is a fragmented pseudogene. However, in several strains, including *B. burgdorferi* JD1, the reading frame is intact; some strains, including *B. burgdorferi* WI91-23 and *B. garinii* PBr, have two intact copies. These genes, located on 28 kb, 38 kb, or 36 kb plasmids, often have been mistakenly annotated as *vlsE* in genomic sequences; it is suggested that they be referred to as *vls* homologues or *vlsH*. In JD1, the lp28-6 encoded gene BbuJD1_Z01 encodes a 40.6 kDa lipoprotein (e.g. BbuJD1_Z01) that is 28% identical and 43% similar to B31 VlsE1. One conserved region is contained in Invariant Region 6 (IR6, also called C6), a 25 aa region that forms an alpha helix embedded in the membrane distal region of VlsE (46). This region of VlsE is also highly immunogenic and is used as a diagnostic antigen in immunologic tests for Lyme disease. Several strains, including *B. burgdorferi* B31, N40, 29805, and 64b, have an identical frameshift following the 56th codon; this occurrence may indicate ancestral inheritance of this damaged gene from a common progenitor. The *vlsH* genes and their products have not been studied; it would be of interest to examine their expression and other properties.

Both OspC and VlsE are surface-localized lipoproteins that are thought to be expressed sequentially during mammalian infection, with OspC being the predominant surface protein in the first days or weeks of infection followed by replacement by VlsE (84). Likely due to

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the constant assault of the host antibody response, these proteins are the most heterogeneous of all LB *Borrelia* polypeptides. The natural hosts of LB *Borrelia* (such as the white-footed mouse *Peromyscus leucopus*) are continually exposed to different strains of *Borrelia* delivered through multiple tick bites. Thus antibodies against OspC and the 'framework' portions of VlsE from prior infections would select for organisms that expressed different epitopes, promoting the fixation of mutations that change the antigenic structure without destroying the proteins' structural integrity. This constant immune pressure thus resulted in strains expressing increasingly heterogeneous versions of this surface proteins. In the case of VlsE, this selection not only drove the development of the *vls* antigenic variation system, but also accelerated the evolution of diverse framework regions of this protein.

Conclusions

The *vls* system represents one of the most elaborate and elegant antigenic variation systems in bacterial pathogens. Its consistent presence in LB organisms indicates its importance in the tenuous survival of these spirochetes during their tightrope act of continual transmission between mammalian and arthropod hosts. The intense immune selection not only drove the parallel but divergent evolution of two different antigenic variation systems in RF and LB *Borrelia,* but also has promoted VlsE framework divergence within different LB species and strains. In addition, the patterns of expression of OspC in LB spirochetes and Vtp in RF organisms could be considered forms of phase variation to evade the immune response; OspC heterogeneity provides yet another example of the power of antibody reactions in the selection of antigenic variants. Together, these elaborate adaptations promote survival of Lyme borreliosis organisms for months to years in mammalian hosts, thus assuring the passage of future generations to arthropods and back again to sylvan mammals and, as accidental hosts, humans.

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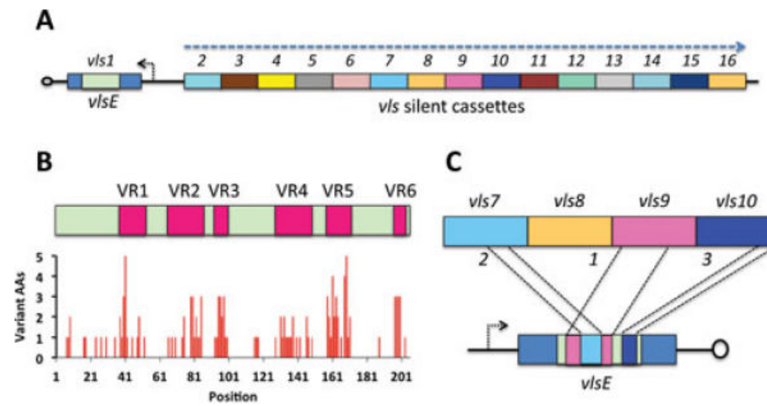

Fig. 1.

Characteristics of the *vls* locus of *B. burgdorferi* B31. A. Arrangement of the *vlsE* expression site and the 15 silent cassettes near the telomere of the linear plasmid lp28-1. The promoter for *vlsE* is indicated by the short arrow and the orientation of the silent cassettes is shown by the large arrow. B. The cassette regions contain six variable regions (VR1 through VR6) separated by relatively invariant regions (IRs). The graph indicates the number of different amino acids encoded by the silent cassettes at each codon in the aligned sequences. C. Unidirectional, random, segmental recombination occurs sequentially during mammalian infection, as indicated by this hypothetical example of sequential recombination between *vlsE* and silent cassettes 9, 7 and 10.

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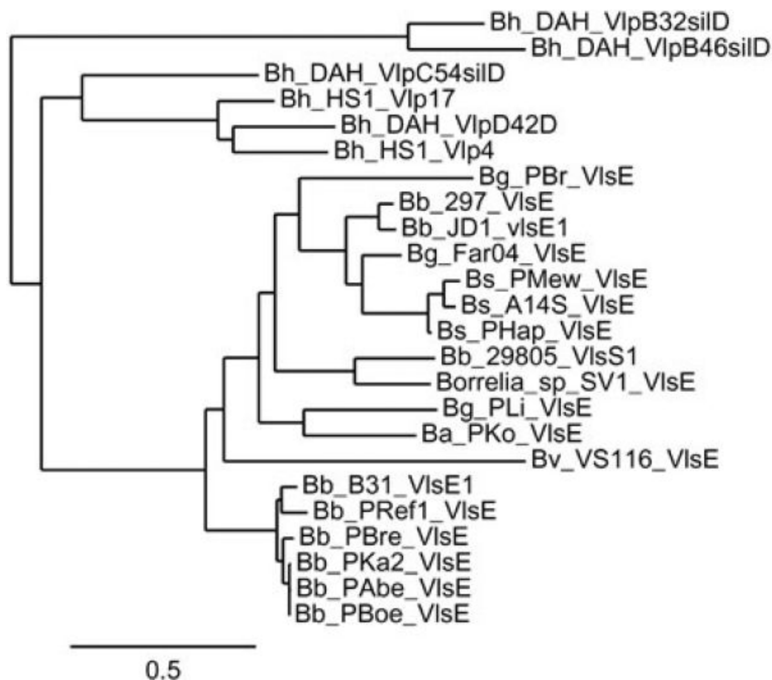

Fig. 2.

Dendrogram depicting the conservation and diversity of VlsE amino acid sequences and their relatedness to Vlp proteins from relapsing fever organisms. Representative Vlp proteins from *B. hermsii* (Bh) strains DAH and HS1 are clustered into two groups at the top of the dendrogram. The B31 strain of *B. burgdorferi* (Bb) and closely related European strains form a distinct grouping at the bottom, whereas the remaining Lyme disease *Borrelia* VlsE sequences exhibit considerable diversity. Additional species abbreviations: Bg = *B. garinii*, Bs = *B. spielmanii*, Ba = *B. afzelii*, and Bv = *B. valaisiana*.

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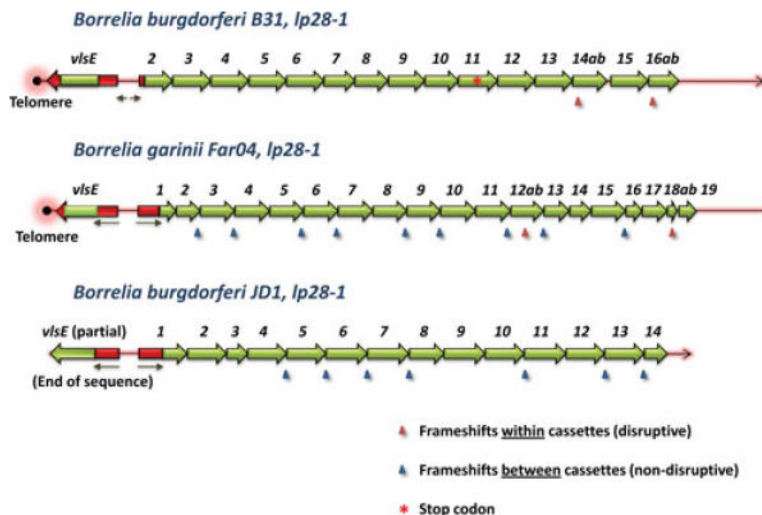

Fig. 3.

Arrangement of *vlsE* and the silent cassettes in *B. burgdorferi* B31, *B. garinii* Far04, and *B. burgdorferi* JD1. Cassette region sequences are shown in green, whereas flanking 5’ and 3’ *vlsE* sequences and homologous 5’ sequences at the beginning of the silent cassette arrays are shown in red. Arrows indicate the locations of inverted repeats. Arrowheads correspond with frameshifts, with the blue arrowheads indicating frameshifts between silent cassettes and red arrowheads showing those located within silent cassettes (as also indicated by the *a* and *b* designations of the ORFs before and after the frameshift). The B31 silent cassette 11 contains a stop codon (red asterisk).

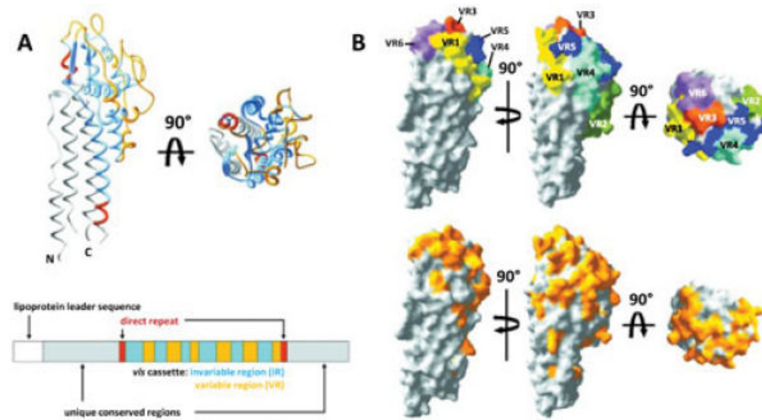

Fig. 4.

Localization of the variable regions (VRs) in the three-dimensional structure of the *B. burgdorferi* B31 allele VlsE1. A. Ribbon diagram showing the abundance of alpha helices and the location of the cassette variable regions (VR) (yellow) and invariant regions (blue). Amino acids encoded by the direct repeats at either end of the cassette region are shown in red. The protein is anchored to the outer membrane by lipid moieties associated with the N-terminal cysteine. A schematic of the cassette region and flanking sequences is shown below the 3D structure. B. Space-filling models indicating the locations of variable regions (VRs) 1 through 6. The VRs cover most of the membrane distal surface of the protein. The bottom panel shows the locations of variable amino acid residues in yellow. Modified from Eicken et al. (46) with permission.

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Strain	Direct Repeat
B. burgdorferi B31	TGAGGGGGCTATTAAGG
B. burgdorferi 297	TGGGAAGGTTGTTAAGG
B. burgdorferi N40	GATGCAAATGTGAAG
B. garinii Ip90	AAGGGGACTGTTAAGAA
B. garinii A87S	GTTGTGGCTGCTGTTAAG
B. afzelii ACAI	GAGAGTGCTGTTGATGA

Fig. 5.

Properties of the *vls* locus potentially involved in recombination and regulation of transcription. A. Inverted repeat between *vlsE* and the *vls* silent cassettes of *B. burgdorferi* B31 (48);(S.J. Norris and J.K. Howell, unpublished data). The predicted stem loop structure includes the −35 region of the *vlsE* promoter. B. G+C content and GC skew of *vlsE* and the *vls* silent cassettes in *B. burgdorferi* B31. C. Lack of conservation of the *vls* direct repeat sequences in Lyme disease *Borrelia* suggests a limited role of these regions in *vlsE* recombination (38). Representative sequences are shown.

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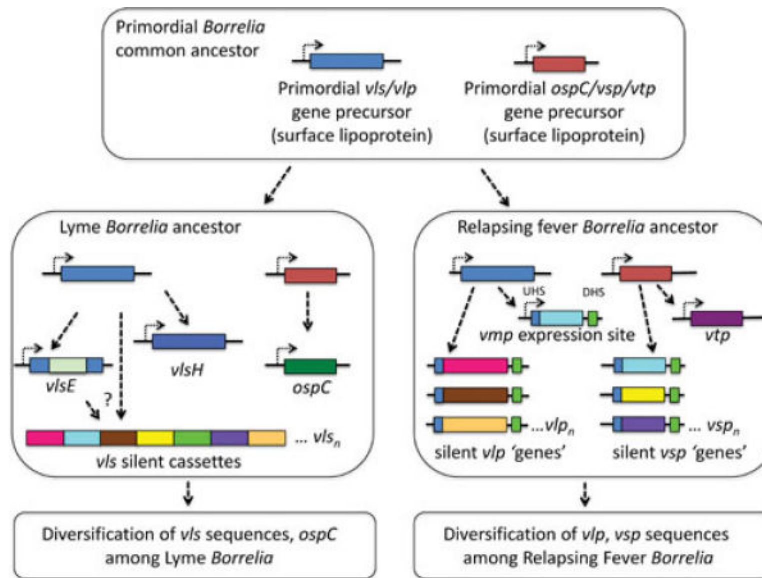

Fig. 6.

Model for the evolution of *vlp*- and *vsp*-related genes in *Borrelia*. It is hypothesized that a common ancestor of LB and RF organisms contained single copies of primordial *vlp* and *vsp* homologues. Following the divergence of LB and RF groups, these precursor genes duplicated and developed into the *vls* system and *ospC* in a primordial LB organism, and into the VMP system and *vtp* in a primordial RF ancestor. Each of these antigenic variation systems and related surface proteins continued to evolve and diverge under the pressure of immune selection.

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Table 1

Stages of Lyme borreliosis

Localized (days to weeks post infection)
Erythema migrans skin lesion
Headache, malaise, fatigue, muscle and joint pain
Disseminated (weeks to months post infection)
Secondary annular skin lesions
Neuroborreliosis – meningitis, facial palsy, radiculoneuritis
Migratory musculoskeletal pain
Atrioventricular nodal heart block
Lymphocytoma[a]
Eye manifestations
Persistent (months to years post infection)
Migratory arthritis of large joints
Neuroborreliosis – meningitis, encephalitis, facial palsy, radiculoneuropathy, polyneuritis
Atrioventricular nodal heart block
Acrodermatitis chronica atrophicans skin lesions[a]

Information from Steere et al. (3) and Müllegger et al. (5)

[a]Found in Eurasia

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Table 2

Observations regarding the mechanisms of *vlsE* recombination

1) The *cis* arrangement of *vlsE* and the *vls* silent cassettes appears to be required for recombination to occur.
2) Inverted repeats with the potential of forming stem loop structures are present between *vlsE* and the *vls* cassette arrays in the three strains in which intact *vls* region sequences have been obtained, but these inverted repeats vary in sequence, location and length.
3) The uniform existence of high GC content and GC skew in *vls* cassette sequences indicates the involvement of these features in recombination.
4) Among an extensive list of DNA recombination and repair gene products examined to date, only RuvAB (a Holliday junction resolvase) and potentially MutS (77) have a substantial effect on the *vlsE* recombination process (76,77).
5) The regulatory protein SpoVG binds to a sequence in the *vlsE* reading frame upstream of the cassette region of *B. burgdorferi* B31 (69); however, this sequence is not uniformly present in other Lyme disease *Borrelia vlsE* genes, and the potential effects of SpoVG on *vlsE* expression and recombination are currently unknown.
6) *vlsE* expression and recombination are markedly upregulated during infection of mammalian hosts, but the mechanism(s) of these effects are not known.