

## Genetic Variation at the *vlsE* Locus of *Borrelia burgdorferi* within Ticks and Mice over the Course of a Single Transmission Cycle

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The Lyme disease spirochete, *Borrelia burgdorferi*, causes a persistent infection in the vertebrate host even though infected animals mount an active immune response against the spirochete. One strategy used by the spirochete to evade vertebrate host immunity is to vary the structure and expression of outer membrane antigens. The *vlsE* locus represents the best-studied example of antigenic variation in *B. burgdorferi*. During vertebrate host infection, recombination between the active *vlsE* locus and silent, partial *vlsE* copies leads to gene conversion events and the generation of novel alleles at the expression site. In the present study, we followed a population of *B. burgdorferi* organisms moving through vertebrate host and tick stages to complete one transmission cycle. The major goal of the study was to determine if the *vlsE* locus was subject to different selective pressure and/or recombination frequency at different stages of the spirochete's life cycle. We report here that the *vlsE* genetic diversity generated within the rodent host was maintained through the larval and nymphal tick stages. Therefore, naturally infected ticks are likely to transmit spirochete populations with multiple *vlsE* alleles into naive vertebrate hosts. Although *vlsE* genetic diversity in mice was maintained through tick stages, the dominant *vlsE* alleles were different between tick stages as well as between individual ticks. We propose that population-level bottlenecks experienced by spirochetes, especially during the larval-to-nymphal molt, are responsible for individual infected ticks harboring different dominant *vlsE* alleles. Although *vlsE* genetic diversity is maintained through tick stages, the VlsE protein is unlikely to be of functional importance in the vector, because the protein was expressed by very few (<1%) bacteria in the vector.

Pathogens alter the structure of surface antigens to adapt to different niches within the vertebrate host and to evade destruction by the host's immune system (3, 38). The Lyme disease spirochete, *Borrelia burgdorferi*, causes a persistent infection in the vertebrate host, even in the face of a robust immune response (4). The *B. burgdorferi* genome codes for a large number of membrane proteins, many of which are surface exposed (12). The *vlsE* locus codes for a surface lipoprotein and represents the best-studied system of antigenic variation in *B. burgdorferi* (40–42).

*B. burgdorferi vlsE* is homologous to the variable membrane protein (*vmp*) genes of *Borrelia hermsii* and other relapsing fever spirochetes (40). The *vls* and *vmp* systems are similar in that they are both gene families present on linear plasmids (27, 40). However, the *B. burgdorferi* system in strain B31 consists of a functional expression locus flanked by 15 silent partial gene copies, whereas *B. hermsii* has a functional expression site and at least 40 silent copies scattered in different parts of the genome (3, 27). DNA recombination events between the silent genes and expression site lead to partial or complete gene conversion events and the production of novel alleles at the expression site (17, 27, 40–42). In the *B. burgdorferi* B31 strain, the *vlsE* locus is present near the telomere of plasmid lp28-1

(40). The expression site consists of a promoter and an open reading frame that has conserved 5' and 3' domains and a central variable cassette that is flanked by 17-bp direct repeat sequences (40). Directly upstream of the expression site are 15 promoterless *vlsE* cassettes, most of which also have the 17-bp direct repeats at the 5' and 3' ends (40). Recombination between the silent gene and expressed site leads to partial gene conversion events and the generation of alleles at the expression site that are a mosaic of the silent copies (41, 42). In theory, this mechanism could lead to the generation of over 10<sup>30</sup> different alleles at the expression site, although in practice, this diversity appears to be constrained for reasons that are not understood (36).

The primary role of recombination at the *vlsE* locus is, most likely, immune evasion, because the variable regions in the central domain are antigenic and form epitopes on the exposed surface of the protein (11, 19, 21, 22). The *vlsE* locus undergoes extensive recombination over the course of an infection in the vertebrate host, which results in the generation of a bacterial population with multiple, novel *vlsE* alleles (40–42). In contrast, novel recombinants have not been observed among spirochetes grown in culture, indicating that the recombination mechanism may be inactive or that the rate of recombination may be too low in culture to be detected.

A few studies have focused on *vlsE* alleles within infected nymphal ticks (15, 24). Individual wild ticks carry spirochete populations with multiple *vlsE* alleles, which may be indicative of recombination within the tick or the tick acquiring a heter-

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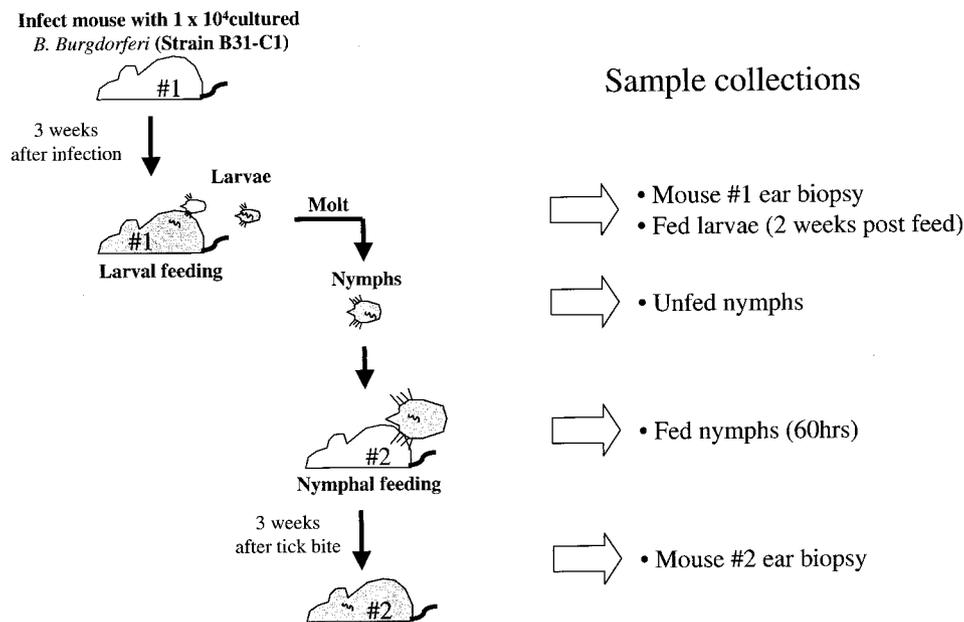


FIG. 1. Experimental design. Three weeks after infection with *B. burgdorferi* strain B31-C1, 100 larval ticks were placed on mouse 1. Most of the larvae fed to repletion and successfully molted to the nymphal stage. Mouse 2 was challenged with 20 infected nymphal ticks.

ogeneous population from the vertebrate host (16). In studies with naturally infected nymphs, we observed a few *plsE* variants in unfed nymphs and many variants in partially fed nymphs (24). This result suggested that *plsE* recombination may be stimulated during nymphal feeding or that the *plsE* locus may be subjected to different selective forces within unfed and partially fed nymphs. In a related study, Indest et al. (15) used capillary feeding to introduce a clonal population of spirochetes with a single *plsE* allele into ticks (15). When capillary-infected nymphs were tested, only the original allele was detected, indicating that *plsE* recombination did not occur within capillary-infected nymphs (15). In the present study, we have introduced a clonal population with a single *plsE* allele into a mouse and followed changes in the expression and genetic structure of alleles at a population level as the spirochetes (i) moved from the rodent host into larval ticks, (ii) were transstadially maintained through the molt from larvae to nymphs, and (iii) moved from nymphal ticks back into a naive mouse to complete a single transmission cycle. The major goal of the study was to determine if the *plsE* locus was subject to different selective pressures and/or recombination frequencies at different stages of the spirochete's life cycle.

#### MATERIALS AND METHODS

***B. burgdorferi*.** A low-passage B31 strain of *B. burgdorferi* (Centers for Disease Control and Prevention, Fort Collins, Colo.) was grown on solid Barbour-Stoenner-Kelly II (BSK II) medium (2, 18), and a single clone designated B31-C1 was isolated and used in the present study. *B. burgdorferi* B31-C1 was inoculated into liquid BSK II medium and grown to midexponential phase ( $1.45 \times 10^7$  cells per ml). This culture was frozen and used as a stock for subsequent studies.

***Ixodes scapularis* ticks.** The ticks used in this study originated from females collected in Bridgeport, Conn. The larvae used were the F<sub>1</sub> generation of the wild ticks.

**Study design.** A single transmission cycle was completed in the laboratory, and samples were collected at different stages as outlined in Fig. 1. A clonal population of strain B31 spirochetes (B31-C1) was used to initiate the cycle. A

C3H/HENj mouse was infected by subcutaneous injection of  $10^4$  spirochetes. Three weeks after infection, 100 larval ticks derived from a single female tick were allowed to feed on the mouse. The larvae acquired spirochetes from the mouse during the blood meal. Engorged larvae were collected and kept in a humid chamber at 21°C until they molted to the nymphal stage. Two weeks after emergence, 20 infected nymphs were placed on a naive mouse and allowed to feed to repletion. During the blood meal, the nymphs transmitted the infection to the naive mouse. Ear biopsies were collected from the first mouse 2 days before larval placement and from the second mouse 3 weeks after the nymphal blood meal. Infected ticks were collected for analysis 2 weeks after the larval blood meal (larvae), prior to the nymphal blood meal (unfed nymphs), and 60 h into the nymphal blood meal (partially fed nymphs) (Fig. 1). Three ticks were collected from each stage. To culture spirochetes, the biopsies and individual ticks were inoculated into BSK II medium containing antibiotics (fosfomycin and rifampin) and antifungal (amphotericin B) agents to reduce contamination (Sigma Chemical Co., St. Louis, Mo.). Positive cultures were plated on solid BSK II medium to obtain single colonies of spirochetes. B31-C1 used to start the cycle was also plated on BSK II plates to obtain single colonies.

**RFLP analysis of *Borrelia* clones.** Each BSK II plate with *Borrelia* colonies was derived from a single mouse or tick. The *plsE* locus was amplified from individual colonies that were randomly picked from each plate. The numbers of colonies picked from each plate were as follows: mouse 1—24 colonies; larvae 1, 2, and 3—9, 9, and 10 colonies, respectively; unfed nymphs 1, 2, and 3—10, 14, and 20 colonies, respectively; partially fed nymphs 1, 2, and 3—19, 20, and 29 colonies, respectively; and mouse 2—19 colonies. Furthermore, 52 colonies were picked from the plate with cultured B31-C1 colonies. The colonies were picked by touching isolated colonies with a sterile toothpick. The tip of the toothpick was dipped into a PCR mixture to amplify the *plsE* gene with primers *plsE*-F and *plsE*-R (24), which annealed to conserved regions just outside the variable central cassette domain. The amplified PCR fragments were digested with restriction enzymes *AluI* and *MboI*. The clones with identical restriction fragment length polymorphism (RFLP) patterns were grouped together, and each group was given a numerical identity.

**Calculation of *Borrelia plsE* allele diversity in mouse and tick stages.** The RFLP data were used to calculate the *plsE* allele heterogeneity within individual mice and ticks as well as at particular life stages (mouse, larvae, unfed nymph, and fed nymph). The *plsE* allele diversity was calculated by using Simpson's index of diversity (SID) (34). The index is calculated from the following equation:  $1 - D = 1 - \sum n_i(n_i - 1)/N(N - 1)$ , where  $D$  is the diversity index,  $n_i$  is the number of the  $i$ th type, and  $N$  is the total number of individuals in the population. SID measures the probability of drawing a pair of individuals of different species from

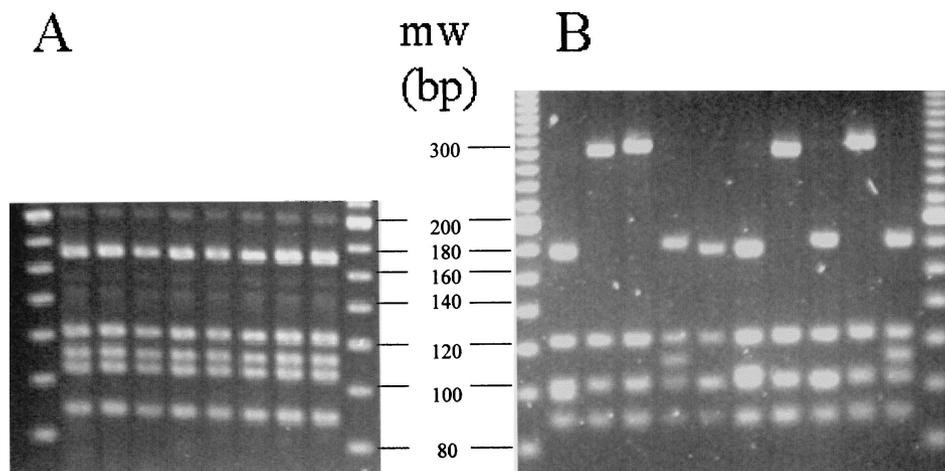


FIG. 2. RFLP typing of representative *vlsE* alleles. *Borrelia* colonies were individually analyzed by PCR-RFLP typing with *AluI* and *MboI* restriction enzymes. The original parent clonal B31-C1 culture was plated, and eight individual colonies were analyzed (A). Spirochetes were also cultured from a mouse 3 weeks after infection with B31-C1, and 10 individual colonies were analyzed (B). Note that all original parent B31-C1 colonies shared the same RFLP pattern, whereas the clones from the mouse have different RFLP patterns, indicating recombination at the *vlsE* locus. The extreme left and right lanes in each panel display a 20-bp molecular size ladder (MW).

a sample. The statistical significance of the differences in the SID between different animals or life stages was compared by using SAS software and the Bootstrap method (JMP, version 4.0, SAS Institute, Cary, N.C.).

**DNA sequencing of selected *vlsE* clones.** We sequenced the B31-C1 parent clone as well as 11 other clones with known RFLP patterns from unfed and partially fed nymphs to verify that the RFLP assay distinguished between clones with similar and different sequences. The *vlsE* variable central cassette was PCR amplified from each clone, and the PCR products were cloned into a TOPO cloning vector, pCR2.1. (Qiagen, Valencia, Calif.) for sequencing. Cloned fragments were sequenced with the *vlsE*-F and *vlsE*-R primers described above at the University of North Carolina Automated DNA Sequencing Facility. The sequences of the selected clones were aligned by using the multiple-alignment software ClustalW, version 1.4 (37). The parental B31-C1 clone used in our study had a sequence that was identical to a B31 *vlsE* sequence retrieved from GenBank (U76405) (40).

**Artificial capillary feeding of nymphal ticks.** *Borrelia* clone B31-C1 was introduced into *Borrelia*-free nymphs by a previously described artificial capillary feeding method (6). Briefly, a suspension of spirochetes at a density of  $5.0 \times 10^7$  bacteria per ml was introduced into a glass capillary tube. The tube was placed over the hypostome of nymphal ticks for 2 h at 37°C. Some of the artificially infected ticks were placed on a naive mouse and allowed to feed to repletion. Artificially infected nymphs were collected before and immediately after the blood meal for RFLP analysis. One group of infected nymphs was starved for 7 days before analysis. The ticks were cultured for *Borrelia*, and the *vlsE* alleles were analyzed as described above.

**Detection of VlsE protein on spirochetes by IFA.** Cultured *B. burgdorferi* organisms as well as gut and salivary glands from unfed and partially fed infected nymphs were prepared for indirect immunofluorescence assay (IFA) as previously described (24). In brief, the slides were blocked with 5% fetal calf serum-phosphate-buffered saline (PBS) prior to incubation with a rabbit serum raised against the IR6 domain of the VlsE protein (20) (kindly provided by Mario Phillip, Primate Center, Tulane University). After four washes with PBS, the slides were incubated with goat anti-rabbit immunoglobulin G conjugated with Alexa 594 (MolecularProbes, Eugene, Oreg.) and fluorescein isothiocyanate (FITC)-conjugated anti-*Borrelia* antibody (KPL, Gaithersburg, Md.) before being viewed with an epifluorescence microscope (ECLIPSE 600, Nikon, Tokyo, Japan).

**Nucleotide sequence accession number.** The *vlsE* nucleotide sequences of the 11 *B. burgdorferi* clones derived from unfed and partially fed nymphs have been deposited in the GenBank database under accession no. AY179870 (U2001), AY179871 (U2002), AY179872 (U2003), AY179873 (U2004), AY179874 (U2005), AY179875 (U2010), AY179876 (F2002), AY179877 (F2006), AY179878 (F2008), AY179879 (F3001), and AY179880 (F3007).

## RESULTS

**RFLP assay for genotyping *vlsE* alleles.** To date, investigators studying antigenic variation at the *vlsE* locus have used DNA sequencing to characterize different alleles. Given the large number of samples we had to analyze for the present study, we decided to use an RFLP assay to characterize *vlsE* alleles. The *vlsE* RFLP type was determined by PCR amplification of the variable central cassette region and then digesting the PCR product with the restriction enzymes *AluI* and *MboI*. All colonies (52 individual clones) selected from culture-grown B31-C1 had the same RFLP pattern, whereas the colonies derived from the infected mouse had highly variable RFLP patterns, indicating that the RFLP assay was sensitive enough to detect recombination occurring in the vertebrate host (Fig. 2). We also sequenced a subset of clones from unfed and partially fed nymphs to further validate the RFLP assay. The *vlsE* locus from 11 clones that had been grouped into RFLP types was sequenced. Clones with identical RFLP patterns shared >97% identity (Fig. 3). For example, six clones belonging to RFLP type 15 were identical, except for one clone (clone U2002), which differed by a two-nucleotide nonsynonymous change from the other RFLP type 15 clones at positions 226 to 227 (Fig. 3). Clones with different RFLP types were less closely related. For example clones belonging to RFLP types 26 (clone F2002) and 18 (clone F2008) shared only 91.67% nucleotide identity (Fig. 3) and 83% amino acid identity, indicating that most changes between the clones were nonsynonymous (data not shown). These results confirm that the RFLP assay is a rapid and inexpensive method for separating *vlsE* alleles that are closely related if not identical from those that are more distantly related.

**Changes in the type and diversity of *vlsE* alleles at different stages of the *B. burgdorferi* transmission cycle.** To detect changes in *vlsE* alleles generated over the course of the transmission cycle, spirochetes were cultured from the original do-

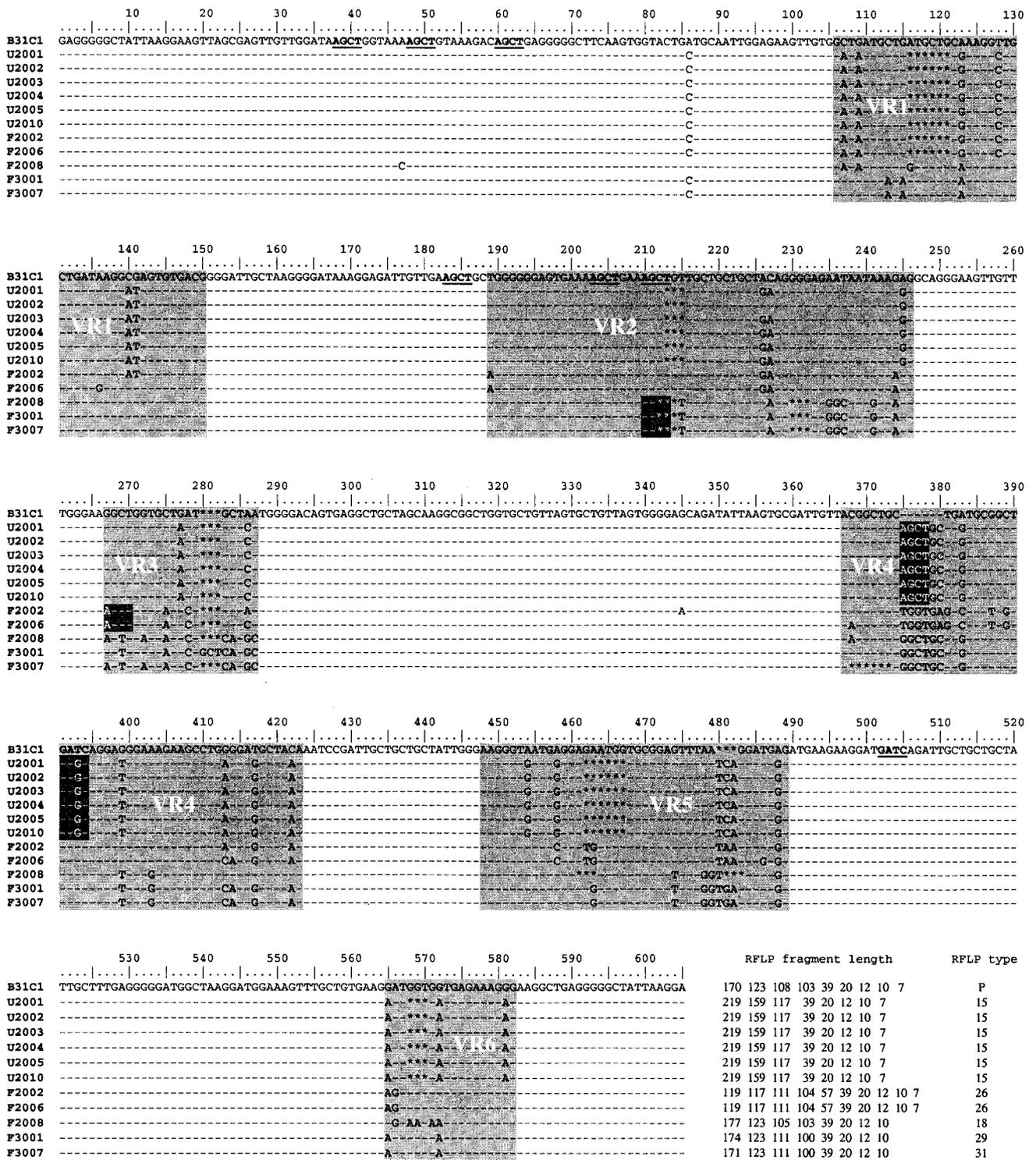


FIG. 3. Validation of *vsE* RFLP assay by DNA sequencing. Twelve clones with known RFLP patterns were sequenced. The clones were derived from cultured B31-C1 (1 clone), unfed nymphs (6 clones: U2001, U2002, U2003, U2004, U2005, and U2010), and partially fed nymphs (5 clones: F2002, F2006, F2008, F3001, and F3007). The sequences were aligned by using a multiple-alignment software, ClustalW, version 1.4. Nucleotides that are identical to the parental B31C1 strain are marked as dashes, and changes are indicated with the letter of the new nucleotide. Gaps are indicated by asterisks. *AluI* (AGCT) and *MboI* (GATC) sites on the B31C1 sequence are underlined. Nucleotide changes in the tick clones that lead to the creation or destruction of an *AluI* or *MboI* site are indicated in white text. The variable domain consists of six hypervariable regions, which are shaded and labeled VR1 to VR6. The expected fragment sizes for each clone following *AluI* and *MboI* digestion and the designated RFLP type are indicated at the end of the sequence.

TABLE 1. *vlsE* RFLP types observed in individual ticks and mice over the course of a single transmission cycle<sup>a</sup>

RFLP type	No. of clones with same RFLP type										
	1st Mouse	Larvae			Nymphs						2nd Mouse
		L1	L2	L3	Unfed			Fed			
				U1	U2	U3	F1	F2	F3		
P	1										
1	11										
2	3										
3	2										
4	1						7				
5	1										
6	2	1				1					
7	2										
8	1										
9		3	2	3		1	9		11	3	8
10		1		2							
11		3	4	2							
12		1	1	1							
13			1	1							
14		1	1	1							
15					10						
16						12					
17							6				
18							2		4		
19							2				
20							1				
21								1	1		
22								1			
23								5			
24								4			
25								1			
26									2		
27									1		
28									1		
29										7	
30										7	
31										8	5
32										2	
33										2	
34											3
35											2
36											1
Total	24	9	9	10	10	14	20	19	20	29	19

<sup>a</sup> A total of 183 clones derived from mouse and tick stages were analyzed, and 37 distinct RFLP patterns, including the parental type (P type), were identified.

nor mouse, larvae, and unfed and partially fed nymphs and from the second mouse, which acquired the infection from the nymphs (Fig. 1). Thus, all of the spirochetes analyzed were derived from a clonal population initially injected into the single donor mouse. Furthermore, all the ticks used were derived from an egg mass from one tick. A total of 183 *Borrelia* clones isolated from individual ticks and mice were examined by PCR-RFLP typing, and 37 (36 new types plus the parental B31-C1 type) different RFLP patterns were identified (Table 1). Twenty-four independent clones from the first, donor, mouse belonged to nine RFLP types, with the majority belonging to type 1. The larvae that fed on the mouse had *vlsE* RFLP types that were mostly distinct from the types in the mouse. Of the 28 clones analyzed from three larval ticks, only one clone (L1, type 6) had an RFLP type that was observed in the donor mouse (Table 1). RFLP types were shared between individual

TABLE 2. Genetic diversity at the *vlsE* locus of spirochetes isolated from culture, tick stages, and mice<sup>a</sup>

Stage(s)	No. of clones tested	No. of RFLP types <sup>b</sup>	Diversity index <sup>c</sup>	Mean (SD)
B31C-1	52	1	0	
1st Mouse	24	9	0.78	
Larvae				
1	9	6	0.83	0.84 (0.04)
2	9	5	0.81	
3	10	6	0.89	
Unfed				
1	10	1	0	0.41 (0.36)
2	14	3	0.27	
3	20	5	0.72	
Fed				
1	19	6	0.78	0.76 (0.07)
2	20	6	0.67	
3	29	6	0.82	
2nd Mouse	19	5	0.76	

<sup>a</sup> There is no significant difference in diversity between tick stages and between mouse and tick stages (ANOVA followed by Tukey-Kramer HSD; *P* < 0.05).

<sup>b</sup> Number of distinct RFLP types observed in the clones tested.

<sup>c</sup> SID was calculated as described in the text.

larval ticks (Table 1). Of the seven different RFLP types observed in the larvae, six were present in more than one tick.

When the larvae molted to the nymphal stage, only two of the eight RFLP types observed in the nymphs (donor mouse and larvae) (Table 1). Unlike the larvae, little overlap was observed in the RFLP types between individual nymphal ticks. When the nymphs fed, 16 RFLP types were observed, and only 3 of these types overlapped with preceding stages. As in the case of the unfed nymphs, little overlap was observed between individual feeding nymphs. At the completion of the cycle with the infection of the second mouse, five RFLP types were observed among 19 clones tested, and three of these types were not observed in the preceding stage. Of the 37 RFLP types, only 5 types (types 4, 6, 9, 18, and 31) were observed in more than one life stage.

We used SID (34) to calculate the genetic diversity at the *vlsE* locus in spirochete populations isolated from different stages (Table 2). All of the clones (52 clones) from the cultured B31-C1 parent had the same RFLP type and therefore an SID of 0 (Table 2). When B31-C-1 was injected into a mouse and spirochetes were cultured 3 weeks after infection, nine different RFLP types were detected among 24 clones tested (SID = 0.78). Thus, as expected, cultured spirochetes became genetically more diverse at the *vlsE* locus in the rodent host. The diversity generated in the mouse was maintained in larval ticks, since individual larvae had SIDs that ranged from 0.81 to 0.89. Following the larval molt to the nymphal stage, the SID was variable in individual nymphs. The three nymphs tested had SIDs of 0.00, 0.27, and 0.72, respectively. Following the blood meal, the diversity among the partially fed nymphs was high and ranged from 0.67 to 0.82. When the nymphs transmitted the spirochetes to the second mouse, the SID was 0.75, which

was similar to the SID of the first, donor mouse. Previously we reported an increase in the number of *vlsE* alleles within ticks following the nymphal blood meal (24). In the present study, we observed average SIDs of 0.41 in unfed ticks and 0.76 in partially fed nymphs, indicating a possible increase in diversity following the blood meal. However, this difference was not statistically significant, possibly because of the small sample size (three ticks per group) as well as the large tick-to-tick variation among unfed nymphs. In fact, statistical analysis of the indices of diversity demonstrated no significant differences between any of the stages in ticks and mice (analysis of variance [ANOVA] followed by Tukey-Kramer highly significant differences [HSD]test;  $P < 0.05$ ) (JMP, version 4.0, SAS Institute). Because the SID of the unfed nymphs was highly variable, we tested another four unfed nymphs. These four unfed nymphs had SIDs of 0.56, 0.65, 0.61, and 0.37, respectively, confirming that the SID was, indeed, highly variable between individual unfed nymphs.

**Overlapping *vlsE* alleles at different stages of the transmission cycle.** Several *vlsE* RFLP types (types 4, 6, 9, 18, and 31) were observed in more than one stage of the transmission cycle (Table 1). The most striking example of this was type 9, which was present in the larval, unfed and fed nymphal, and 2nd mouse stages (Table 1). However, when representative clones of RFLP type 9 were sequenced from different stages, the sequences were not closely related, indicating that type 9 represents a rare case in which clones with different sequences generate restriction fragments of similar sizes (data not shown). Similarly, RFLP types 4, 6, and 18 also represent cases in which restriction fragments of similar sizes were generated by divergent sequences. In contrast, RFLP type 31 represents a case in which a *vlsE* allele detected in a feeding tick was subsequently detected in the mouse 3 weeks after transmission. When clones of RFLP type 31 from fed nymphs (F2) and mouse 2 were sequenced, the variable domain was >99% identical at the nucleotide level (data not shown).

***vlsE* alleles in artificially infected nymphs.** To directly test if nymphal feeding stimulated *vlsE* recombination and an increase in *vlsE* allele diversity, we used a capillary feeding method to introduce clonal *Borrelia* with a single *vlsE* allele into unfed nymphs. Some of the artificially infected nymphs were allowed to feed on a naive mouse. All *Borrelia* clones from these artificially infected ticks that had not fed (44 clones from two ticks) or had partially fed (53 clones from three ticks) had only the single parental B31-C1 RFLP type, indicating that recombination or selection did not occur in capillary-fed nymphal ticks or occurred at a frequency that was too low to be detected with the sample sizes used here.

***vlsE* expression in the tick vector.** The VlsE protein is expressed and under immune selection in the vertebrate host and, therefore, is likely to be of functional importance in the vertebrate. Experiments were done to determine if the protein was produced by spirochetes within ticks too. Spirochete homogenates were prepared from unfed and partially fed nymphal ticks as well as from culture-grown bacteria. The samples were fixed and stained by double-labeling IFA using an antibody directed against a conserved region of VlsE and as well as a polyclonal antiserum raised against whole spirochetes. The majority of cultured spirochetes (>90%) produced VlsE (Fig. 4). In contrast, very few bacteria (< 1%) in the unfed or

partially fed nymphs produced the protein (Fig. 4). The analysis was extended in partially fed nymphs by staining guts and salivary glands separately, and <1% of the spirochetes in each organ produced the protein. Even the few positive spirochetes had very faint fluorescent signals indicating low levels of protein compared to those in cultured bacteria.

## DISCUSSION

The goal of the present study was to characterize population-level changes in the genetic structure and expression of the *B. burgdorferi vlsE* locus during a single transmission cycle involving the vertebrate host and invertebrate vector. A complete cycle consists of the spirochetes moving from an infected mouse into larvae, the infection being maintained transstadially through the larval-to-nymphal molt, and the spirochetes being transmitted to a new mouse during the nymphal blood meal. To minimize variation due to the genetic background of the pathogen and vector, we used a cloned population of spirochetes to initiate the cycle and used a tick population derived from a single egg mass for the experiment.

***vlsE* alleles in mice and larval ticks.** Fifty-two clones derived from cultured B31-C1 had the same RFLP type, indicating that recombination did not occur in vitro or occurred at a frequency that was too low to be detected in a sample of 52 clones. A similar observation has been made by others who have examined the *vlsE* locus of spirochetes grown in culture (15, 40–42). When these spirochetes were injected into a mouse, multiple *vlsE* alleles were observed, indicating that recombination and selection in the rodent host lead to the emergence of a genetically heterogeneous population of spirochetes with multiple *vlsE* alleles (24, 40–42). We determined if the major *vlsE* alleles present within a mouse were also acquired by feeding larvae. Very little overlap was observed between *vlsE* alleles from the mouse and larvae that fed on the mouse. Among the seven RFLP types observed within larvae, only one type overlapped with the RFLP types present in the donor, mouse 1. After 3 weeks of infection, the mouse has a population of spirochetes with a large repertoire of *vlsE* alleles. It is unclear how much spatial heterogeneity exists in the animal with respect to the distribution of spirochetes with different *vlsE* genotypes. The mouse may be infected with distinct genotypes of *Borrelia* at different sites in the body, and extensive spatial heterogeneity may explain the lack of overlap in *vlsE* alleles in spirochetes cultured from larvae and mouse ear biopsies. It is also plausible that novel *vlsE* alleles that appear in the mouse may rapidly distribute throughout the animal so that the same dominant alleles are present in different regions of the animal. Under this scenario, a selection event occurring within the mouse or larval tick may be responsible for the differences in the *vlsE* genotype of spirochetes in the donor mouse and the larvae.

We also compared the *vlsE* alleles dominant in each larval tick. When larvae were tested 2 weeks after infection, we observed extensive overlap of RFLP types between larvae (Table 1). Of the seven RFLP types observed, six were present in more than one larval tick. The extensive overlap in *vlsE* alleles between individual larvae points to cofeeding larvae sampling the same spirochete populations from a mouse or a selection

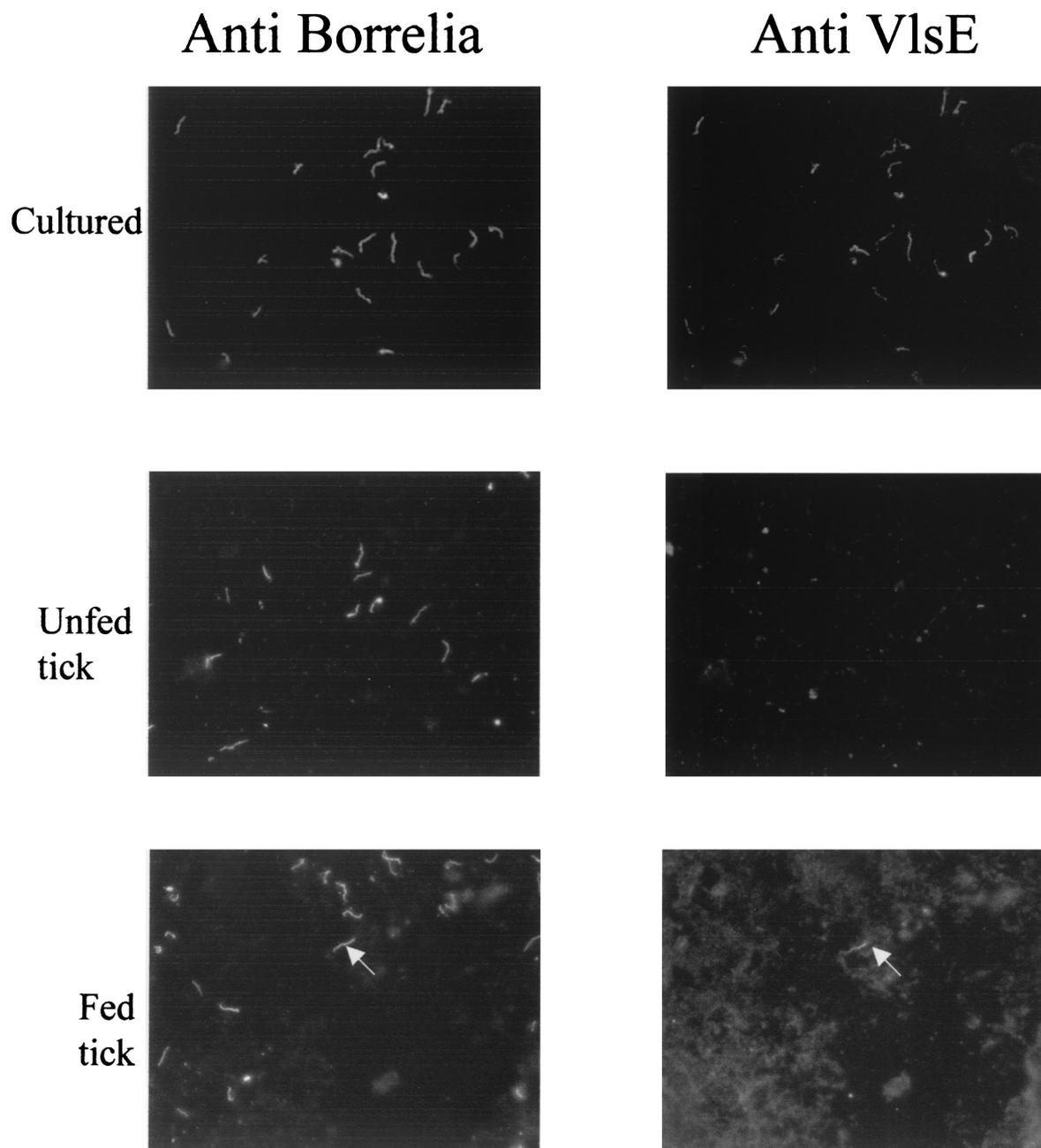


FIG. 4. Detection of VlsE protein on spirochetes within ticks and spirochetes grown in culture. Spirochetes in culture or within unfed or partially fed (48 h) nymphs were stained by double-labeling IFA with antibodies directed against a conserved region on the VlsE proteins and a polyclonal serum against whole *Borrelia*. Most spirochetes grown in culture stained with the VlsE antibody, whereas very few bacteria (an arrowhead points to a VlsE-positive bacterium) within ticks stained with the antibody.

event in the mouse or larvae favoring the survival of spirochetes expressing a specific subset of *vlsE* alleles.

***vlsE* alleles in nymphal ticks.** In the laboratory, larvae molt to the nymphal stage 3 to 4 weeks after the larval blood meal. We observed little overlap between RFLP types in larvae and nymphs as well as between individual nymphs. Currently, we do not understand why RFLP types were observed in common between individual larvae but not between individual nymphs (Table 1). The larval molt subjects spirochetes to unknown

selective forces, because the bacterial population size drops by approximately 10-fold (from >2,500 spirochetes to <300 spirochetes) during the molt (26), and individual nymphs end up with low numbers of bacteria that widely vary from tick to tick. As the spirochete numbers drop, many *vlsE* alleles may become extinct, and such a population-level bottleneck may explain why the *vlsE* diversity index was highly variable, with some unfed nymphs having very low diversity indices and others having high diversity indices.

Previously, we reported that unfed nymphs had few *vlsE* alleles in comparison to partially fed nymphs that had many alleles (24). In the present study, too, we observed an increase in diversity (average SID of 0.41 in unfed ticks and 0.76 in partially fed nymphs) during nymphal feeding. However, this difference was not statistically significant. Thus, we conclude that the *vlsE* allele diversity does not decrease in individual unfed nymphs, but rather it becomes more variable, with some nymphs having populations with a low SID and others continuing to maintain a high SID. When the diversity indices for the *vlsE* locus were compared between all stages (mice, larvae, and unfed and fed nymphs), none of the stages was significantly different from any other stage. The most conservative interpretation of these results is that, at a population level, the high-level *vlsE* variability generated within an infected mouse is maintained through all tick stages and the tick transmits a population of spirochetes into the second mouse that is as variable as the population in the first, donor, mouse.

A *vlsE* allele present in fed ticks (RFLP type 31) was observed in mouse 2 3 weeks after transmission (Table 1). RFLP type 31 clones from the feeding nymph and mouse were >99% identical at the nucleotide level (data not shown). These results indicate that certain *vlsE* alleles can persist in the vertebrate host for as long as 3 weeks and not be subject to immune clearance. In studies with syringe-infected mice, the alleles in the inoculum have rarely been observed several weeks after infection. Further studies are needed to understand why certain *vlsE* alleles may persist following tick but not syringe infection.

**Does the *vlsE* locus recombine in the tick vector?** Artificial capillary feeding experiments support the conclusion that novel *vlsE* alleles are not generated de novo within the tick vector. Indest and colleagues recently used an artificial capillary feeding method to infect nymphal ticks with a clonal population of spirochetes with a single *vlsE* allele (15). When these capillary-infected ticks were allowed to engorge to repletion and tested 10 days later, only the parental *vlsE* allele was recovered, indicating that recombination did not occur in the tick or occurred at a frequency that was too low to be detected by these assays (15). We also used the capillary method to infect nymphs and to test for *vlsE* recombination. Unlike Indest and colleagues, who tested the partially fed nymphs 10 days after the blood meal, we tested the nymphs in the process of active feeding because the recombinants may be selectively transmitted to the vertebrate host and may not be retained in the tick after the blood meal. However, we still failed to detect any evidence for *vlsE* recombination, indicating that novel alleles are not likely to be generated within capillary-infected nymphs. However, it is premature to conclude that recombination does not occur in the tick during natural infection, because spirochetes introduced by natural infection and capillary feeding are not the same. Naturally infected ticks acquire spirochetes as larvae, and the bacteria represent a population that has infected the vector for several months as well as a population that has survived through a tick molt. Capillary-delivered spirochetes enter the nymphal stage and have infected the vector for a relatively short time before the nymphal blood meal. We recently found that spirochetes introduced by the capillary method are less efficiently transmitted than naturally infected nymphs (6). Additional studies are needed to

determine if all the *vlsE* variants observed in the vector were generated in the vertebrate host or if *vlsE* recombination occurs in naturally infected nymphs.

The VlsE protein expression data support the conclusion that the locus is unlikely to be of functional importance or directly under selective pressure in ticks. Indest and colleagues reported that the VlsE protein is produced by spirochetes within nymphs, but at low levels (15). Using double-labeled IFA microscopy, we report here that only a small fraction (<1%) of spirochetes produce VlsE, and even the few bacteria that were positive produced a faint signal indicating low-level expression. We propose that the *vlsE* locus falls into the growing category of *Borrelia* genes that are preferentially expressed in the host and not the vector (10, 13). Several studies have identified temperature and pH as signals that regulate the expression of *Borrelia* genes (7, 8, 28, 35, 39). These signals are likely to play a role during natural transmission, because spirochetes experience an increase in temperature and decrease in pH as they move from the vector to the vertebrate. To identify the entire set of *B. burgdorferi* genes differentially regulated by temperature and pH, two groups have recently probed *Borrelia* microarrays with cDNA prepared from spirochetes grown in culture (25, 29). These studies have led to the identification of a large number of differentially expressed genes, including those such as *ospC*, known to be differentially produced during tick feeding. However, the *vlsE* locus did not stand out as a locus that was differentially produced in response to temperature or pH (25, 29). When cultured spirochetes were compared to spirochetes harvested from chambers implanted in a mouse, the expression of the *vlsE* locus was increased approximately threefold in the mammal, indicating that the *vlsE* locus may be activated after the bacteria leave the vector and enter the rodent host (29). The only signal that has increased the expression of *vlsE* in vitro is derived from endothelial cell membranes, further lending support to the idea that transcription is activated in the vertebrate host and not the tick vector (14).

Antigenic variation systems similar to the *vlsE* system are found in other vector-borne bacteria (3). The *mvp2* system in *Anaplasma marginale* is based on partial gene conversion events leading to the generation of a large number of *mvp2* variants in the vertebrate host (5, 23). Although some studies have reported evidence for tick-specific *mvp2* variants (9, 30), other studies indicate that the dominant *mvp2* alleles in the vertebrate host are passively maintained in the tick (1, 31). It would not be surprising to discover that tick-specific *mvp2* alleles are the norm rather than the exception, because, unlike the *vlsE* locus, the *mvp2* locus is expressed by *A. marginale* colonizing the vector (31). The variable membrane protein (*vmp*) antigenic variation system of the relapsing fever spirochete, *B. hermsii*, consists of a tick-specific *vmp33* expression site that is genetically stable and not subject to high-frequency recombination and a separate vertebrate *vmp* expression locus that recombines with over 40 different silent genes to generate novel alleles (3, 33). The major *vmp* alleles expressed in the vertebrate host at the time of tick feeding are acquired by the tick, but within the tick, transcription switches from the mammalian expression locus to the tick-specific *vmp33* locus (32, 33). The mammalian *vmp* alleles are passively maintained through the tick, and transcription is activated again after

transmission to the next vertebrate host (32, 33). Thus, the *vlsE* and *vmp* antigenic variation systems of *Borrelia* are active and under selection in the vertebrate host but only passively maintained through vector stages of the life cycle.

In summary, we have demonstrated here that the VlsE protein is unlikely to be of functional importance in the vector because the protein was expressed by very few bacteria in ticks. Furthermore, *Borrelia* strains missing the linear plasmid (lp28-1) coding for *vlsE* show a decreased infectivity to mice but not ticks, indicating a role for this plasmid in the vertebrate host and not the vector (15). DNA recombination and selection lead to the generation of a large number of *vlsE* alleles within the rodent host. We also demonstrate here that *vlsE* genetic diversity generated within the vertebrate host was maintained through larval and nymphal stages so that feeding nymphs are likely to inject a population of spirochetes with many different alleles into the vertebrate. Although *vlsE* genetic diversity in mice was maintained through tick stages, the dominant *vlsE* alleles were different between tick stages and individual ticks. We propose that large decreases in the spirochete population size, especially during the larva-to-nymph molt, lead to the random survival of some *vlsE* genotypes and not others and the observed differences in the dominant *vlsE* alleles in individual ticks.

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