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Population Structure of the Relapsing Fever Spirochete *Borrelia hermsii* as Indicated by Polymorphism of Two Multigene Families That Encode Immunogenic Outer Surface Lipoproteins

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The tick-borne relapsing fever spirochete *Borrelia hermsii* evades the mammalian immune system by periodically switching expression among members of two multigene families that encode immunogenic, antigenically distinct outer surface proteins. The type strain, *B. hermsii* HS1, has at least 40 complete genes and pseudogenes that participate in this multiphasic antigenic variation. Originally termed *vmp* (for variable major protein) genes, they have been reclassified as *vsp* (for variable small protein) and *vlp* (for variable large protein) genes, based on size and amino acid sequence similarities. To date, antigenic variation in *B. hermsii* has been studied only in the type strain, HS1. Nucleotide sequence comparisons of 23 *B. hermsii* HS1 genes revealed five distinct groups, the *vsp* gene family and four subfamilies of *vlp* genes. We used PCR with family- and subfamily-specific primers, followed by restriction fragment length polymorphism analysis, to compare the *vsp* and *vlp* repertoires of HS1 and seven other *B. hermsii* isolates from Washington, Idaho, and California. This analysis, together with pulsed-field gel electrophoresis genome profiles, revealed that the eight isolates formed three distinct groups, which likely represent clonal lineages. Members of the three groups coexisted in the same geographic area, but they could also be isolated across large geographical distances. This population structure may result from immune selection by the host, as has been proposed for other pathogens with polymorphic antigens.

Relapsing fever is the medical term for a disease characterized by a cyclic rise and fall in body temperature caused by arthropod-transmitted spirochetes of the genus *Borrelia*. Louseborne or epidemic relapsing fever is solely a human disease, whereas tick-borne relapsing fever, with one exception, is a zoonosis that can be transmitted to humans. Several tick-borne relapsing fever *Borrelia* species, each associated with a different tick vector and mammalian hosts, occur within discrete geographical areas throughout the world (6). *Borrelia hermsii*, for example, persists in natural cycles that involve the soft tick *Ornithodoros hermsi*, chipmunks, and tree squirrels in high-altitude forests of California, Arizona, Nevada, Colorado, Oregon, Idaho, Washington, and British Columbia.

After injection into a mammal by an infected, feeding tick, *B. hermsii* multiplies in the blood stream, achieving numbers that can exceed 10 million per ml of peripheral blood (48, 53). Fever occurs as these organisms are cleared by an immune response directed against a prominent lipoprotein that coats the outer surfaces of spirochetes. Spirochetes virtually disappear from the blood, and the fever subsides; however, spirochetemia and fever recur 4 to 7 days later. This second population consists of spirochetes that are coated with a new lipoprotein which is antigenically different from the one expressed by the original infecting bacteria. This antigenic variation is multiphasic; relapse and recovery can repeat for several cycles, and each relapse population of spirochetes expresses an antigenically distinct surface lipoprotein that is encoded by a separate gene (4). At least 40 distinct serotypes have previously been identified in the progeny of a single cell of *B. hermsii* HS1, and most of the corresponding genes have been cloned and sequenced (7, 41). These genes were originally named variable major protein genes (*vmp1*, *vmp2*, *vmp3*, etc.). A recent comparison of their amino acid sequences indicated that the *vmp* genes comprise two distinct multigene families; therefore, they have been redesignated *vsp* and *vlp* genes (for variable small and variable large proteins, respectively) (2, 15).

Genes of both families are found in two locations on linear plasmids in *B. hermsii* HS1. In one locus, the expression site, a single *vsp* or *vlp* gene is positioned immediately downstream of a promoter near one end (or telomere) of a 28- to 30-kb linear plasmid and only that gene is expressed (27). A second copy of the expressed gene and all other genes of the two gene families exist in nonexpressed or silent form at nontelomeric locations on the same linear plasmid or on different linear plasmids of about the same size. Antigenic variation results from inter- or intraplasmid DNA rearrangements that replace the gene at the telomeric expression site with a different *vsp* or *vlp* gene, augmented in some cases by postswitch mutations of this previously silent gene (5, 27, 36, 40–42). Any silent *vsp* or *vlp* gene can replace any expressed *vsp* or *vlp* gene, although with different probabilities (3, 8, 53).

Our understanding of the genetic mechanisms of antigenic variation of relapsing fever *Borrelia* spp. in the vertebrate host

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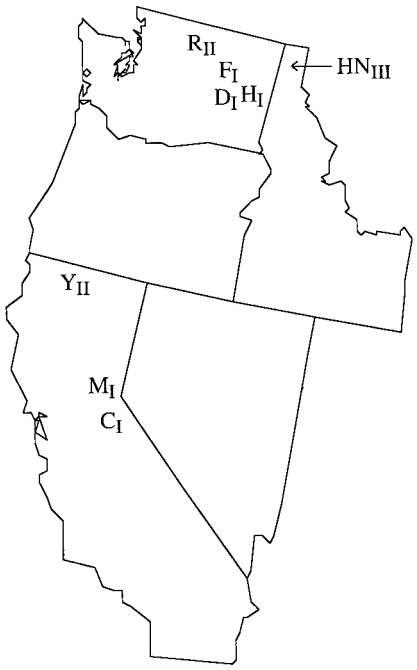

FIG. 1. Map of western United States showing the geographic origins of the eight *B. hermsii* isolates used in this study. A Roman numeral subscript indicates the group to which an isolate belongs, based on the similarity of the *vsp* and *vlp* gene repertoire. C, CON; D, DAH; F, FRO; H, HS1; HN, HAN; M, MAN; R, REN; Y, YOR.

has emerged from studies of a single isolate of *B. hermsii*, the type strain HS1. Here we begin an analysis at the population level of the genes involved in *B. hermsii* antigenic variation. We compared the *vsp* and *vlp* repertoire of the type strain HS1 to those of seven other isolates of *B. hermsii* from different enzootic foci in the western United States. Parasite genes that encode surface antigens often seem to be subject to a faster molecular evolutionary clock than do genes for nonimmunogenic proteins, probably because allelic (genetic) polymorphism arises through immune selection of escape variants in previously exposed hosts (12, 57). We reasoned, therefore, that comparisons of the *vsp* and *vlp* genes would reveal finer phylogenetic differences than would comparisons of conserved, presumably less variable markers, such as 16S DNA coding for rRNA or flagellin genes. The results showed extensive variation in the *vsp* and *vlp* genes of different isolates. The variation was not continuous, however. The eight isolates examined exhibited one of three basic *vsp* and *vlp* profiles, suggesting a population composed of clones or families of closely related clones.

A second motive for this study arose from our interest in the interaction of *B. hermsii* with its tick vector. Originally isolated in 1968 (56), present stocks of the type strain HS1 have decreased infectivity for ticks, probably as a result of laboratory passage in vitro and in mice (6). To investigate the role of variable surface lipoproteins in the invertebrate host, the tick *O. hermsi*, we utilized specific Vsp and Vlp antibodies and gene sequences derived from analysis of the HS1 strain. The present study identified a new isolate, *B. hermsii* DAH, that appears to be identical to *B. hermsii* HS1 in its *vsp* and *vlp* repertoire and that is infectious for both invertebrate and vertebrate hosts.

MATERIALS AND METHODS

***Borrelia* strains.** All *Borrelia* strains were from the culture collection of the Rocky Mountain Laboratories. The eight isolates of *B. hermsii* used in this study, their geographic origins, and dates of isolation are shown in Fig. 1 and Table 1. The histories, identifications, and characterizations of some of these isolates have been described previously (47). A clone of *B. hermsii* HAN was obtained by limiting dilution in BSK medium; *B. hermsii* HS1 has also been previously cloned (53). Other tick-borne relapsing fever species used were *B. turicatae* and *B. parkeri*, (from western North America), *B. crocidurae* (from Africa and the Middle East), and *B. anserina*, the agent of avian borreliosis (found worldwide). *B. coriaceae* is the putative cause of tick-borne epizootic bovine abortion (29), and *B. burgdorferi* B31 is the type strain of the Lyme disease agent (13).

***B. hermsii* HS1 *vsp* and *vlp* gene sequence comparisons.** The nucleotide sequences of 23 *vsp* and *vlp* genes previously cloned from *B. hermsii* HS1 (7, 14, 41, 43) were compared by using the multiple-sequence analysis programs PILEUP and PRETTY of UNIX (version 7; Genetics Computer Group, Inc., Madison, Wis.). The individual genes and their GenBank/EMBL accession numbers are as follows: *vsp1*, L33870; *vsp2*, L33897; *vsp3*, L04789; *vsp6*, L33898; *vsp8*, L33899; *vsp11*, L33900; *vsp13*, L33901; *vsp22*, L33902; *vsp24*, L04786; *vlp4*, U51926; *vlp5*, U52035; *vlp7*, X53926; *vlp9*, U52036; *vlp10*, U52037; *vlp12*, U52038; *vlp14*, U52148; *vlp15*, U52039; *vlp17*, L04788; *vlp18*, U52149; *vlp19*, U52040; *vlp21*, M57256; *vlp23*, U52041; and *vlp25*, L04787.

PCR analysis of the *vsp* and *vlp* genes. Genomic DNA was isolated by a standard method from *Borrelia* cultures in BSK II medium (36). PCR (30 cycles) was performed with 50 ng of *Borrelia* DNA by using a thermal cycler (Perkin-Elmer, Foster City, Calif.) and *vsp* family- and *vlp* subfamily-specific primer sets designed from *B. hermsii* HS1 sequences (Table 2). A 659-bp fragment of the flagellin gene was also amplified from *Borrelia* species with a generic primer set homologous to conserved regions of the gene (39). A portion of each PCR was electrophoresed on 4% polyacrylamide gels in TBE buffer (90 mM Tris [pH 8.0], 90 mM borate, 2 mM EDTA).

RFLP analysis of the *vsp* and *vlp* genes. PCR-amplified DNA fragments were ethanol precipitated, washed with 70% ethanol, and resuspended in TE (10 mM Tris [pH 8], 1 mM EDTA). DNAs from *vsp* family- and *vlp* subfamily-specific PCRs were digested in separate reactions with the following restriction endonucleases: *Dde*I and *Rsa*I (*vsp* family); *Hin*dIII and *Pst*I (*vlp*α); *Sau*3AI, *Sca*I, and *Ssp*I (*vlp*β); *Bgl*II, *Dde*I, and a *Pvu*II-*Hae*III double digest (*vlp*γ); and *Sau*3AI and *Ssp*I (*vlp*δ). DNAs from flagellin gene PCRs were digested with *Alu*I, *Pvu*II, *Rsa*I, and *Sau*3AI. Digests were electrophoresed on 3% SeaPlaque GTG agarose (FMC BioProducts, Rockland, Maine) or 12% polyacrylamide gels with TBE buffer. Restriction fragment length polymorphism (RFLP) patterns of DNA amplified from each *B. hermsii* isolate were individually compared with that of the HS1 strain to determine a similarity coefficient (*S*), the proportion of shared restriction fragments, as a measure of genetic distance. Each DNA band was treated as a separate character, and bands of the same electrophoretic mobility were considered to be shared, regardless of differences in staining intensity. We used the formula of Nei and Li (37), $S(x,y) = 2n_{xy}/(n_x + n_y)$, in which n_{xy} is the number of fragments shared by the two isolates and n_x and n_y are the total numbers of fragments produced from isolates x and y, respectively.

Pulsed-field gel electrophoresis (PFGE) and Southern blotting. *Borrelia* cells from BSK II cultures were centrifuged, washed twice with TN (50 mM Tris [pH 8], 150 mM NaCl), and resuspended in TN to a concentration of approximately 10^9 per ml. An equal volume of molten (37°C) 1% InCert low-melting-temperature agarose (FMC) was mixed with the cell suspension, which was aliquoted to a mold and allowed to gel in the form of agarose blocks. Cells were lysed in situ by incubating agarose blocks for 16 h at 45°C in 50 mM Tris (pH 8)–50 mM EDTA–1% sodium dodecyl sulfate (SDS) containing 1 mg of proteinase K per ml (19). Blocks were washed four times, 1 h each, with TE. Intact genomic DNA from blocks was separated on a 1% agarose gel by transverse alternating field electrophoresis by using the Geneline II system (Beckman Instruments, Palo Alto, Calif.) and a previously described protocol (33). After transverse alternating field electrophoresis, the DNA on the gel was stained with ethidium bromide, photographed, and then transferred from the gel to a Hybond-N membrane

TABLE 1. *B. hermsii* isolates used in this study

Isolate	Geographic origin	Source	Yr of isolation	Reference
HS1	Spokane, Wash.	*O. hermsi* tick	1968	56
DAH	Northwest of Cheney, Wash.	Human blood	1991	47
FRO	Eastern Washington	Human blood	1987	46
MAN	Sierra Nevada Mountains, Calif.	Human blood	1960s	46
CON	Sierra Nevada Mountains, Calif.	Human blood	1960s	46
HAN	Bonner's Ferry, Idaho	Human blood	1990	47
YOR	Siskiyou Mountains, Calif.	Human blood	1964	46
REN	Winthrop, Wash.	Human blood	1992	47

TABLE 2. PCR primers

Target	Sequence[a]	Annealing temp (°C)	References
vsp family	5′-AAGTCTGACGGAACAGTACT-3′ 5′-GTTATTTTGAGAAGGTTTTTC-3′	50	7, this study
vlp family			
α subfamily	5′-CTAATGATAGGWTGTGGACAAC-3′ 5′-CTCAAGAACATTCTTTACTGTC-3′	55	7, this study
β subfamily	5′-GTGATGCATTAGGATTTAATGC-3′ 5′-CCTAATACCTTATTTACWGCAC-3′	55	7, this study
γ subfamily	5′-CTAGTGACCCAATTGCTAATGT-3′ 5′-TCTAGTGCTTTAGTAATTGCAC-3′	55	7, this study
δ subfamily	5′-ATACTAAGAAAAGTGATATAGG-3′ 5′-CCATTGCTCGCAGTGCAATGCC-3′	50	7, this study
Flagellin gene	5′-ACATATTCAGATGCAGACAGAGGT-3′ 5′-GCAATCATTGCCATTGCAGATTGT-3′	60	39, this study

[a] W, A or T.

(Amersham, Arlington Heights, Ill.) by vacuum blotting (VacuGene; Pharmacia Biotech, Piscataway, N.J.).

For use as probes on Southern blots, *vsp* and *vlp*-specific PCRs were diluted 1:1,000 and reamplified. Reamplified DNA fragments were purified by using Sephacryl S-400 microspin columns (Pharmacia) and labelled with [α-^{32}P]dATP by means of a random primer labelling kit (Boeringher Mannheim, Indianapolis, Ind.). Southern hybridization was performed at 37°C for 16 h in 50% formamide–6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5× Denhardt's solution–0.5% SDS–0.1 mg of denatured salmon sperm DNA per ml. Final washes were done with 0.1× SSC–0.1% SDS–0.1 mM EDTA at 63°C.

RESULTS

Four subfamilies of the *vlp* gene family of *B. hermsii* HS1. The nucleotide sequences of 9 *vsp* genes (*vsp1*, *-2*, *-3*, *-6*, *-8*, *-11*, *-13*, *-22*, and *-24*) and 14 *vlp* genes (*vlp4*, *-5*, *-7*, *-9*, *-10*, *-12*, *-14*, *-15*, *-17*, *-18*, *-19*, *-21*, *-23*, and *-25*) of *B. hermsii* HS1 (2, 7, 43) were compared by using multiple-sequence analysis programs of the Genetics Computer Group. As previously described (43), the nine *vsp* genes comprised one family of genes that ranged from 632 to 654 bp, with 68 to 85% nucleotide sequence similarity. The 14 *vlp* genes examined ranged from 1,023 to 1,110 bp and formed four distinct groups, labelled subfamilies α to δ (Fig. 2). The branchpoints that separate the four *vlp* subfamilies occur much deeper in the dendrogram than do the branchpoints that separate individual gene sequences within each subfamily. This is a reflection of the 70 to 87% similarity between members of each particular *vlp* subfamily, compared to the 39 to 51% nucleotide sequence similarities of *vlp* genes from different subfamilies.

Polymorphism of *vsp* and *vlp* genes among *B. hermsii* isolates. A schematic comparison of *vsp* and *vlp* genes is shown in Fig. 3. Probably because of common transport, lipidation, and membrane-anchoring mechanisms of these surface lipoproteins (14), the 5′ ends of all of the *vsp* and *vlp* genes examined are conserved. All 23 genes are identical for the first 26 bp, and all, except for *vlp* subfamily α genes, are identical for the first 77 bp. Downstream from this constant leader sequence, the 5′ and 3′ portions of the genes of the *vsp* family or a particular *vlp* subfamily are additionally conserved (7, 43). Nucleotide sequence alignments confirmed that most of the variation between individual *vsp* family and *vlp* subfamily members occurs in a central region of the genes. From the conserved 5′ and 3′ regions, sequences were identified for use as *vsp* family- and *vlp* subfamily-specific PCR primers. Each of the five primer sets was predicted to amplify the central variable segment of every member of the *vsp* family or a particular *vlp* subfamily of the HS1 strain (except for *vsp* family primers, which amplify all *vsp* genes except for *vsp11*) but not those of genes outside of the subfamily. Because the primer sequences occurred within genes, both silent and expressed forms were amplified.

To detect genetic variability of the *vsp* and *vlp* repertoire among isolates of *B. hermsii*, we performed PCR with each of the five primer sets and genomic DNAs isolated from *B. hermsii* HS1 and seven other *B. hermsii* isolates (Table 1). Because the collection of *vsp* or *vlp* products generated in each PCR differed in size by 5% or less, they usually migrated as a single band on agarose gels (data not shown). Greater resolution was achieved on polyacrylamide gels, on which amplified *vsp* and *vlp* fragments yielded family or subfamily profiles (Fig. 4). This analysis revealed polymorphism in the *vsp* and *vlp* genes among isolates of this species. *B. hermsii* DAH and FRO displayed profiles similar to that of the type strain HS1, but the five other *B. hermsii* isolates generated different patterns. The profiles of *B. hermsii* YOR and REN appeared to be identical; the remaining three isolates yielded unique *vsp* and *vlp* profiles. Some of the polymorphism evident in Fig. 4 could be due to heteroduplex formation or to amplification of intergenic sequences if adjacent silent genes were arrayed in opposite orientations. Nevertheless, identical profiles resulted from each of three independent PCR experiments.

Two other relapsing fever *Borrelia* species from the western United States were tested (Fig. 4). *B. parkeri* yielded a product only with the *vlp*α subfamily primers, and *B. turicatae* yielded a product only with the *vlp*γ subfamily primers. *B. crocidurae*, a relapsing fever agent from the Mediterranean region, and *B. coriaceae*, the putative agent of epizootic bovine abortion isolated from California, generated products from both the *vlp*β and *vlp*γ subfamily primer sets. *B. burgdorferi*, the agent of Lyme borreliosis, and *B. anserina*, the agent of avian borreliosis, were negative for all five PCRs.

The PCR analysis discussed above utilized conserved 5′ and 3′ sequences to amplify the central variable regions of all the *vsp* and *vlp* genes. Many of the individual products were pre-

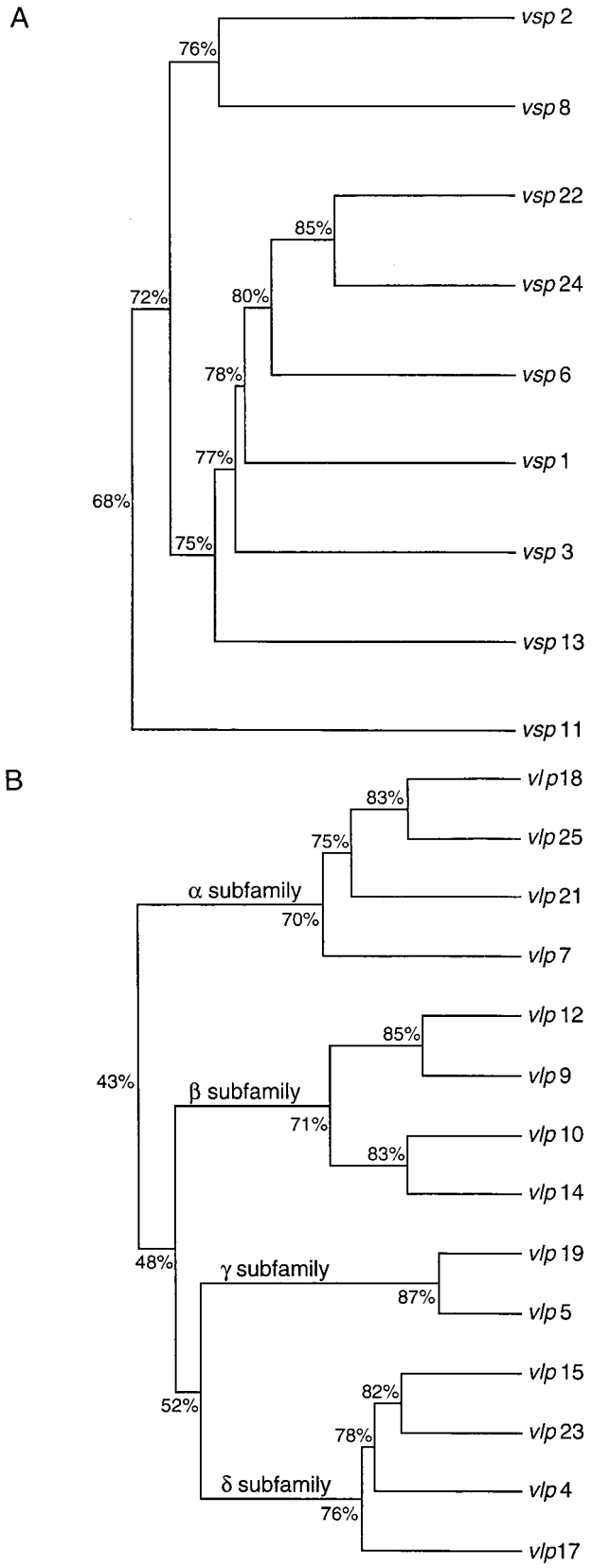

FIG. 2. Dendrograms comparing the genetic relatedness of 9 *vsp* (A) and 14 *vlp* (B) gene sequences of *B. hermsii* HS1. The *vlp* genes are subdivided into four subfamilies, labelled α to δ. The percentage of nucleotide sequence similarity between genes or gene clusters is indicated at each branchpoint.

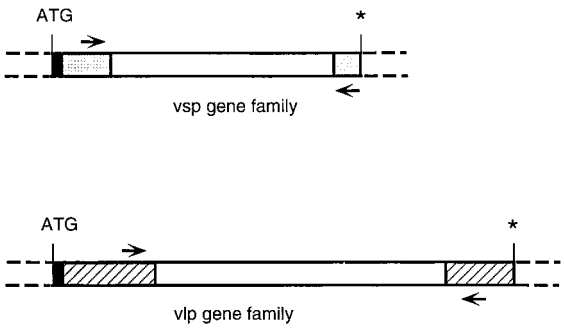

FIG. 3. Nucleotide sequence features of *vsp* and *vlp* genes of *B. hermsii* HS1. The central, most variable segment of these genes is flanked by conserved regions (shaded areas for *vsp* and hatched areas for *vlp*) that contain the *vsp* family- or *vlp* subfamily-specific PCR primer binding sites (arrows). The short 5′ leader sequence shared by genes of both families is indicated by a black box, and asterisks denote stop codons.

dicted to be nearly the same size and not resolvable, even by polyacrylamide gel electrophoresis. Thus, the observed number of PCR fragments generated from the HS1 strain (Fig. 4) was less than the number of known genes (Fig. 2). To further compare the *vsp* and *vlp* repertoires of isolates, portions of the PCRs of Fig. 4 were digested with restriction endonucleases prior to gel electrophoresis. Because the most variable regions of these genes were amplified, we expected this subsequent RFLP analysis to increase observable differences among isolates. Restriction enzymes with known sites in HS1 sequences were chosen. The RFLP profiles for the *vsp* family are shown in Fig. 5; the results for the *vlp* subfamilies are not shown. The RFLP patterns generated from the type strain HS1 included all of the fragments predicted from the known sequences of the 23 genes analyzed and a few fragments presumably derived from other *vsp* and *vlp* genes. The RFLP patterns of the seven previously uncharacterized *B. hermsii* isolates were compared to that of HS1 by calculating an estimate of genetic distance, *S*, the proportion of restriction fragments shared by the pair of isolates (37). *S* values range from 1.0 (complete identity) to 0 (total dissimilarity). For comparison, RFLP analysis was also conducted with a 659-bp fragment amplified from the flagellin gene of each of the eight *B. hermsii* isolates. RFLP analyses of two separate sets of PCRs were identical.

Several points can be made from the results (Fig. 5; Table 3). *B. hermsii* DAH was identical and *B. hermsii* FRO was nearly identical to the type strain HS1 across the entire *vsp* and *vlp* repertoire. *B. hermsii* MAN and CON were related to HS1 (S = 0.83 and 0.77, respectively, for the entire repertoire). These related isolates originated from eastern Washington state, except for the central California isolates MAN and CON (Fig. 1). *B. hermsii* YOR (from northern California) and REN (from northcentral Washington) were identical to each other but were not closely related to HS1. *B. hermsii* HAN (from northern Idaho) was not similar to any of the other isolates and yielded fewer total restriction digest products. The greatest heterogeneity among all these isolates was seen in genes of the smallest subfamily, *vlp*γ. Genetic variability among *vsp* and *vlp* genes appeared to be much greater than that among the flagellin genes of the eight isolates. *B. hermsii* HS1, DAH, FRO, MAN and CON were identical in their flagellin RFLP profiles for all four restriction enzymes used. *B. hermsii* HAN, YOR, and REN were identical and differed from the others by a single *Rsa*I site. The nucleotide sequence of a 220-bp portion of the flagellin gene of *B. hermsii* YOR has previously been

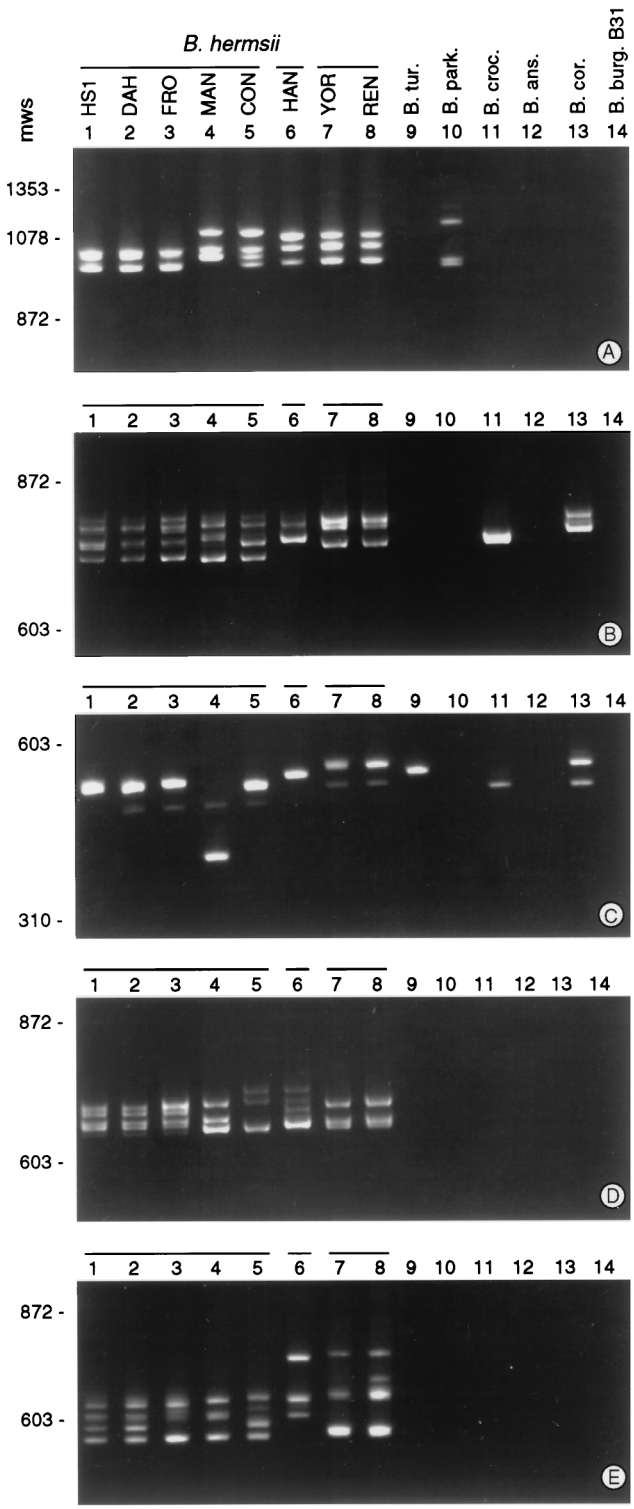

FIG. 4. PCR profiles of *vsp* and *vlp* genes of relapsing fever *Borrelia* species. PCRs with primer sets specific for *B. hermsii* HS1 *vlp* subfamilies α through δ (A through D, respectively) and *vsp* family (E) were performed with DNAs isolated from the indicated *Borrelia* species. PCRs were analyzed on 4% polyacrylamide gels. Lanes 1 through 8, the indicated isolates of *B. hermsii*; lanes 9 through 11, relapsing fever agents *B. turicatae* (B. tur.), *B. parkeri* (B. park.), and *B. crocidurae* (B. croc.), respectively; lanes 12, *B. anserina* (B. ans.); lane 13, *B. coriaceae* (B. cor.); lane 14, from *B. burgdorferi* (B. burg.) B31. Horizontal bars designate the three groups of related *B. hermsii* isolates. The sizes (in base pairs) of selected *Hae*III-digested ϕX170 DNA molecular weight standards (mws) are shown on the left.

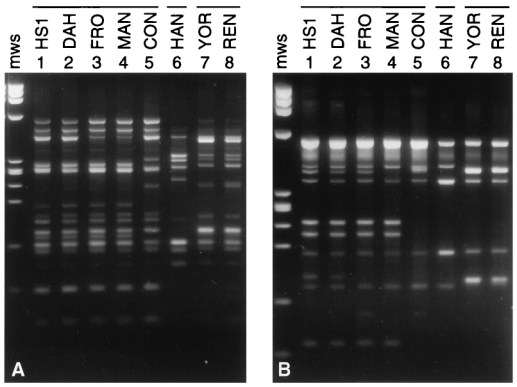

FIG. 5. *vsp* gene family RFLP profiles of eight *B. hermsii* isolates. Gene fragments amplified by PCR with *vsp* family-specific primers were digested with *Dde*I (A) or *Rsa*I (B) and analyzed on ethidium bromide-stained 3% agarose gels. Lanes 1 through 8, the indicated *B. hermsii* isolates (horizontal bars designate the three groups of related isolate); lanes mws, *Hae*III-digested ϕX170 DNA molecular weight standards.

determined; it differs from the HS1 flagellin gene sequence at five nucleotide positions (2.3% base substitution) (39).

Linear plasmid location of the *vsp* and *vlp* gene families. Silent and expressed *vsp* and *vlp* genes of *B. hermsii* HS1 have previously been mapped to linear plasmids (27, 40). To see whether this is true for the other isolates, PFGE was performed with genomic DNA from each of the eight isolates, followed by Southern hybridization with HS1 *vsp* and *vlp* gene probes. The ethidium bromide-stained gel showed that the linear chromosome and linear plasmid profiles of the eight isolates correlated with the classification described above for the *vsp* and *vlp* gene family repertoire. Isolates HS1, DAH, FRO, MAN and CON formed one group, YOR and REN formed a second group, and the unique HAN isolate formed a third group (Fig. 6A). Among the HS1 group, the largest linear plasmid (180 to 200 kb) of the CON isolate was noticeably smaller than those of the other four isolates. Strain HS1 had a linear plasmid of about 40 kb that was not present in the others (Fig. 6). This difference, however, was due to a size increase of the HS1 expression plasmid from 30 to 40 kb, which occurred during in vitro passage, probably as a result of intragenic plasmid recombination (2).

PCR-amplified gene fragments of the HS1 *vsp* family and each of four *vlp* subfamilies hybridized to two to four linear plasmids of approximately 30 to 50 kb in the five HS1-related isolates and more weakly to linear plasmids of 30 to 90 kb in the three isolates more distantly related to HS1 (23). Thus, genes of the *vsp* family and of each *vlp* subfamily are apportioned to different linear plasmids; in other words, individual linear plasmids typically contain a mixture of *vsp* and *vlp* genes. Although not all of these plasmids contained representatives of every group, even the smallest *vlp* subfamily, *vlp*γ (Fig. 2B), mapped to at least two linear plasmids. A composite in which the hybridization probe was a mixture of the products of all five *vsp* and *vlp* PCRs from HS1 is shown in Fig. 6B. The hybridization patterns and linear-plasmid profiles are consistent with and thus reinforce the grouping of isolates based on *vsp* and *vlp* polymorphisms. Individual *vlp* subfamily probes (but not the *vsp* family probe) also hybridized weakly to the chromosomes of the five related strains or to a DNA species that comigrated with the chromosome on PFGE gels. Whether this was due to the occurrence of *vlp*-related sequences on the chromosome or minor amplification of an unrelated chromosomal segment during PCR generation of the probes is not known.

TABLE 3. RFLP *S* values of seven *B. hermsii* isolates compared to the type strain HS1

Genetic locus	*S* value for[a]:						
	DAH	FRO	MAN	CON	HAN	YOR	REN
vsp family	1.0 (27)	0.96 (27)	0.96 (25)	0.67 (24)	0.56 (16)	0.55 (24)	0.55 (24)
vlp family							
α subfamily	1.0 (15)	1.0 (15)	0.73 (15)	0.71 (13)	0.40 (8)	0.71 (16)	0.71 (16)
β subfamily	1.0 (36)	0.94 (32)	0.83 (34)	0.89 (38)	0.57 (27)	0.54 (26)	0.54 (26)
γ subfamily	1.0 (11)	0.91 (12)	0.57 (10)	0.91 (12)	0.30 (9)	0.07 (17)	0.07 (17)
δ subfamily	1.0 (23)	1.0 (23)	0.86 (21)	0.65 (11)	0.61 (13)	0.62 (16)	0.62 (16)
Total	1.0 (112)	0.96 (109)	0.83 (105)	0.77 (98)	0.54 (73)	0.53 (99)	0.53 (99)
Flagellin gene	1.0 (23)	1.0 (23)	1.0 (23)	1.0 (23)	0.93 (22)	0.93 (22)	0.93 (22)

[a] Data are the proportions of restriction fragments shared by the indicated isolate and HS1. Values range from 1.0 (identical to HS1) to 0 (totally dissimilar to HS1). See Materials and Methods for details. Parenthetical data are the total numbers of different restriction fragments from each isolate. The numbers of fragments for HS1 were the same as those for the DAH isolate.

DISCUSSION

The antigenic variation of *B. hermsii*, revealed by many years of studying the type strain HS1, is due to frequent gene conversions in which an expressed gene at a recombinogenic telomeric expression locus is replaced by a different, previously silent gene (27, 40). The 23 *vsp* and *vlp* genes of *B. hermsii* HS1 that we compared compose five distinct groups, based on nucleotide sequence similarity (Fig. 2). In an earlier study, the order of *vsp* and *vlp* genes expressed during relapsing fever did not follow a preordained program nor was it completely random (3, 8, 53). The three most common first-relapse serotypes, however, resulted from switches to *vlp7* (α subfamily), *vsp2*, or *vlp17* (δ subfamily). Since Vsp proteins or Vlp proteins of the same subfamily can share epitopes (9), switching to an antigenically unrelated gene of another family or subfamily may be important in prolonging infection.

All previous work with the *vsp* and *vlp* genes of *B. hermsii* has been done with HS1, the type strain. This study is the first investigation at the population level of these two *B. hermsii* gene families that underlie the outer surface protein antigenic variation which occurs during relapsing fever. Allelic polymorphism of immunogenic outer surface protein genes at the population level has previously been observed in viruses (1, 44, 45), protozoa (16), and bacteria (12), including the circular plasmid-borne gene encoding outer surface lipoprotein OspC of the Lyme disease spirochete *B. burgdorferi*. Like the Vsp and Vlp lipoproteins of *B. hermsii*, OspC appears to be the predominant outer surface lipoprotein during initial infection of a mammal (20, 38, 49), and different isolates of *B. burgdorferi* exhibit extensive allelic polymorphism of this gene (24, 31, 52, 54, 55, 58). To extend the comparison further, *ospC* of *B. burgdorferi* has previously been shown to be similar in sequence to *vsp33* of *B. hermsii* HS1 (17, 34).

We compared the *vsp* and *vlp* repertoires of seven isolates of *B. hermsii* from California, Idaho, and Washington (47) to that of the HS1 type strain, originally isolated in 1968 near Spokane, Wash. (56) (Fig. 1). This comparison revealed another type of genetic variation of *vsp* and *vlp* genes, polymorphism at the population level. The polymorphism was not continuous but discrete; the eight isolates appeared to represent three groups. The HS1-related group contained DAH, FRO, MAN, CON, and the type strain. These five had not only 77% or greater RFLP similarities in *vsp* and *vlp* profiles but also identical flagellin gene RFLP profiles (Fig. 5; Table 3) and similar genome profiles, with *vsp* and *vlp* genes located on linear plasmids of similar sizes (Fig. 6). Of these five isolates, *B. hermsii* DAH was identical to HS1 in its *vsp* and *vlp* profile. For unknown reasons which do not involve apparent changes in *vsp* or *vlp* gene repertoire, laboratory passage of *B. hermsii* HS1 (as well as Lyme disease *Borrelia* spp.) can result in loss of infectivity for mammals and serial passage in the vertebrate host can result in loss of infectivity for ticks (6, 46). Thus, low-passage, fully infectious *B. hermsii* DAH should be useful in studying relapsing fever pathogenesis and transmission, because the *vsp*- and *vlp*-specific antisera and sequence data accrued from many years of work with the type strain can potentially be utilized. The type strain-related group also included *B. hermsii* FRO, which was 96% identical to HS1 in its *vsp* and *vlp* profile, and the MAN and CON isolates, which were 83 and 77% identical to HS1, respectively. A second group was composed

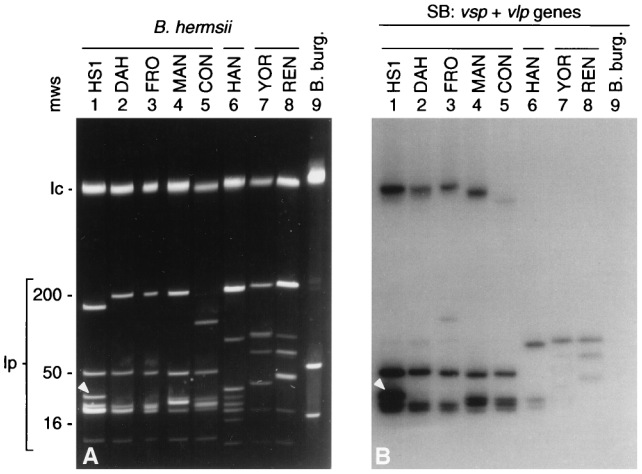

FIG. 6. (A) PFGE gel showing linear-chromosome (lc) and linear-plasmid (lp) profiles of eight *B. hermsii* isolates (lanes 1 through 8) and high-passage *B. burgdorferi* (B. burg.) B31 (lane 9). Arrowheads indicate the HS1 expression plasmid. During in vitro passage after isolation, the size of this plasmid increased by about 10 kb. (B) Southern blot (SB) probed with the total collection of PCR-amplified *B. hermsii* HS1 *vsp* and *vlp* gene fragments (*vsp* + *vlp* genes). The sizes (in kilobases) of selected lambda DNA concatemers and the 16-kb linear plasmid of *B. burgdorferi* B31, used as molecular weight standards (mws), are shown on the left. Horizontal bars designate the three groups of related *B. hermsii* isolates.

of *B. hermsii* YOR and REN. These two isolates had identical *vsp* and *vlp* profiles, which were only 53% similar to the HS1 profile, and identical genome profiles. The third group was represented by a single isolate, *B. hermsii* HAN, which had a *vsp* and *vlp* profile that was only 54% similar to that of HS1 and had a unique genome profile.

There are interesting parallels between the antigenic variation of this spirochete at the clonal level and that at the population level. A single *B. hermsii* cell contains distinct groups of outer surface antigen genes (Fig. 2), and the total population appears to be made up of distinct groups of isolates with different allelic repertoires of these genes. Just as the ability of a single spirochete to switch expression among antigenically distinct *vsp* and *vlp* genes allows escape from an individual host's immune response, allelic polymorphism or genetic variability of *vsp* and *vlp* genes within the total spirochete population may help to evade herd immunity (21, 22). This may be particularly important for a parasite, such as *B. hermsii*, that is transmitted within discrete enzootic foci by a nest-dwelling tick.

A population composed of independent strains with nonoverlapping repertoires of polymorphic antigenic determinants is characteristic of several pathogens. African trypanosomes, which exhibit multiphasic antigenic variation of variable surface glycoproteins (Vsg proteins) during infection in a manner strikingly similar to that of *B. hermsii* (11, 27), provide one example. Populations of *Trypanosoma* species are made up of different serodemes, or strains with distinct repertoires of *vsg* alleles (35), which are analogous to the three groups of *B. hermsii* delineated in this study. A stable collection of strains with allelic differences in immunogenic surface antigen genes also typifies populations of *Plasmodium falciparum* (variable antigen types) and the bacterium *Neisseria meningitidis* (polymorphic epitopes of the outer membrane protein PorA) (21). Immune selection by the host has previously been proposed as the driving force that organizes a parasite population into independently transmitted strains that do not share alleles. According to this model, exposure to one strain leads to complete or partial cross-protection against all members of the same strain but the host remains susceptible to other strains circulating in the herd (21). A second model is suggested by the fact that the three species of North American relapsing fever borreliae have complete specificity for three species of *Ornithodoros* ticks (6). Likewise, the groups of *B. hermsii* detected here could have resulted from coevolution with distinct, reproductively isolated strains of the *O. hermsi* vector. Although little is known about the population genetics of *O. hermsi*, the coexistence of all three *B. hermsii* groups within the same enzootic focus (Fig. 1) appears to be inconsistent with this hypothesis.

Because the population structure described here has previously been observed for both sexual (frequently recombining) and clonal (rarely recombining) pathogens, the *B. hermsii* groups we detected cannot be assumed to consist of independent clonal lineages. Nevertheless, our results suggest that *B. hermsii*, like *B. burgdorferi* (10, 18, 30), has a clonal population structure. Clonality of bacteria at the population level refers to infrequent genetic exchange and recombination between different cell lines (50, 51). One criterion of a clonal population is linkage disequilibrium, the nonrandom association of genetic markers. Although only eight isolates were examined and their individual *vsp* and *vlp* gene sequences were not compared, evidence of linkage disequilibrium was detected. Three *B. hermsii* groups were apparent by RFLP analysis of the *vsp* and *vlp* genes, and this conformed with the classification based on other markers, including RFLP patterns of the flagellin gene, PFGE genome profiles, and hybridization patterns of the two multigene families (Fig. 6). In addition, previous characterizations of plasmid and total protein profiles, Western and Southern blot reactivities, genomic DNA RFLP profiles, and PCRs of *vlp7* and *vlp21* genes of the eight isolates were consistent with the classification based on *vsp* and *vlp* polymorphisms (46, 47). Further evidence for or against linkage disequilibrium and a clonal population structure will come from comparing phylogenetic trees of the *vsp* and *vlp* gene sequences with those of other genetic markers, such as 16S DNA coding for rRNA and flagellin genes, as well as from other population genetics analyses, such as multilocus enzyme electrophoresis. A second characteristic of clonal populations is the ability to recover isolates of identical genotypes over large geographic areas and long periods (50). Perhaps most telling are the allopatric northern Californian YOR and Washington state REN isolates, which appeared to be identical in *vsp* and *vlp* genes even though they were separated by over 400 miles (Fig. 1). *B. hermsii* is not spread rapidly by birds or other migratory hosts, suggesting that this dispersal required many years and generations. The apparent genetic identity of the sympatric *B. hermsii* HS1 and DAH isolates, which were isolated from the Spokane, Wash., area 24 years apart, is also evidence of infrequent gene flow.

Although the eight isolates, except for HS1 and HAN, have not been cloned by limiting dilution, it is unlikely that the *vsp* and *vlp* polymorphism seen is due to the isolates being composed of a mixture of clones. Rather, the results indicate that the human blood samples from which the new isolates were cultured contained clonal populations, as evidenced by the identity of independent, geographically and temporally distant isolates. The isolation procedure itself likely entails cloning. Greater than 99% of the population in a spirochetemia express the same lipoprotein gene (41), indicating they are the progeny of a single cell. During isolation, spirochetemic human blood was first used to infect mice and spirochetemic mouse blood was then inoculated into BSK II medium (47). As evidence of this, clones of *B. hermsii* HAN were identical to the parent isolate in our analyses.

Simple genetic drift might be expected to affect all the *vsp* and *vlp* genes equally, with the consequence that the estimated genetic distance between isolates would be equivalent for each multigene family and subfamily. In fact, marked differences were seen. For example, within the HS1-related group, the *vsp* genes of the MAN isolate had an *S* value of 0.96 by RFLP to the HS1 *vsp* genes; however, the *S* value for their *vlp*γ genes was only 0.57 (Table 3). Conversely, the *vsp* genes of the CON isolate had an *S* value of only 0.67 to the HS1 *vsp* genes, whereas the *S* value for their *vlp*γ genes was 0.91. A greater disparity was evident in the *vlp*α and *vlp*γ genes of the group composed of the identical YOR and CON isolates, compared to those of HS1. Their *vlp*α genes had an *S* value of 0.71, but the *S* value for their *vlp*γ genes was only 0.07. The reason for the apparent inconsistency in genetic relatedness across the *vsp* family and *vlp* subfamilies is unclear at this level of analysis. One possibility is that genetic exchange can occur between isolates. Evidence for possible horizontal transfer of plasmid-borne outer surface protein gene sequences of *B. burgdorferi* has previously been reported (18, 25, 31, 32). Alternatively, intra- and interplasmid recombination events (28, 41, 42) that differentially affect the multigene families in clones may result in different mutation rates.

The following relapsing fever *Borrelia* species contained sequences that were PCR amplified with the *B. hermsii* HS1 *vlp* subfamily-specific primer sets indicated (Fig. 4): *B. turicatae* (γ subfamily) and *B. parkeri* (α subfamily), which (like *B. hermsii*)

are from the western United States; and *B. crocidurae* (β and γ subfamilies), which is from the Mediterranean region. The suspected agent of epizootic bovine abortion in the western United States, *B. coriaceae*, also yielded a product with the *vlp*β and *vlp*γ subfamily PCR primers. In a previous study, HS1 *vlp7* and *vlp21* gene probes (α subfamily) hybridized weakly to *B. turicatae* and *B. coriaceae* DNAs (47). Our results support the conclusion (47) that *B. coriaceae* is more closely related to *B. hermsii* than is *B. anserina*, the cause of avian borreliosis, or *B. burgdorferi*, neither of which were positive in any of the PCRs. RFLP analysis of the PCR products derived from the other *Borrelia* species examined revealed that they were not highly similar to the analogous *B. hermsii* products (23).

Finally, an ongoing controversy in molecular evolution is the degree to which genetic variation, such as *vsp* and *vlp* polymorphism, is selected, in this case by the host's immune system (positive or Darwinian selection), or results from random fixation via genetic drift (neutral theory of evolution) (26). Evidence has previously been found for positive selection of *B. burgdorferi ospC* variants (54). An ideal test case of the positive-selection hypothesis may be provided by the *vsp* and *vlp* genes, because relapsing fever spirochetes rely on them to thwart the mammalian immune response and thus to prolong infection and increase the likelihood of transmission. Answers to these and other questions raised by this study await nucleotide sequence analysis of these two multigene families in multiple *B. hermsii* isolates.

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