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Evolution

Borrelia burgdorferi is clonal: Implications for taxonomy and vaccine development

(gene trees/lateral gene transfer/*ospA*/*p93*/flagellar gene)

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ABSTRACT **The chromosomal genes *fla* and *p93* and the *ospA* gene from a linear plasmid were sequenced from up to 15 isolates of *Borrelia burgdorferi*, which causes Lyme borreliosis in man. Comparison of the gene trees provides no evidence for genetic exchange between chromosomal genes, suggesting *B. burgdorferi* is strictly clonal. Comparison of the chromosomal gene trees with that of the plasmid-encoded *ospA* reveals that plasmid transfer between clones is rare. Evidence for intragenic recombination was found in only a single *ospA* allele. The analysis reveals three common clones and a number of rare clones that are so highly divergent that vaccines developed against one are unlikely to provide immunity to organisms from others. Consequently, an understanding of the geographic and genetic variability of *B. burgdorferi* will prove essential for the development of effective vaccines and programs for control. While the major clones might be regarded as different species, the clonal population structure, the geographic localization, and the widespread incidence of Lyme disease suggest that *B. burgdorferi* should remain the name for the entire array of organisms.**

Lyme borreliosis is the most common tick-borne infectious disease in North America, Europe, and northern Asia. The causative bacterial agent of this disease, *Borrelia burgdorferi*, was first isolated and cultivated in 1982 (1, 2). With that discovery, a wide array of clinical syndromes, described in both the European and American literature since the early 20th century, could be attributed to infection by *B. burgdorferi* (3–6).

The immune response to *B. burgdorferi* is characterized by an early, prominent, and persistent humoral response to the endoflagellar protein (fla) and to a protein constituent of the protoplasmic cylinder, p93. Both proteins are physically cryptic antigens, sheathed from the immune system by an outer membrane whose major protein constituents are OspA and OspB. Surprisingly, the immune response to these outer surface proteins tends to occur late in the disease (7, 8), although mice immunized with recombinant OspA produce a protective immune response (9). Consequently, OspA has become the dominant candidate for a genetically engineered vaccine.

Unfortunately, immunization with OspA from one strain does not necessarily confer resistance to a heterologous strain (10). Thus, a rational development of effective vaccines requires that the level of genetic variation in *B. burgdorferi* be determined, particularly in the antigenic epitopes at OspA and other outer surface proteins. Moreover, an understanding of the contributions made by mutation and recombination to variation is equally important, since these processes can potentially generate novel epitopes.

Fortunately, molecular evolutionists have developed methods for quantifying variation and deducing the effects of mutation, migration, selection, and recombination on the genetic structures of species. Using these techniques, we analyze the DNA sequences for three genes from a set of *B. burgdorferi* strains.‖ The genes encoding fla and p93 are located on the chromosome, whereas *ospA* is located on a 49-kilobase linear plasmid (11). Comparisons of the order of branching among the gene trees constructed for each gene allow us to determine whether recombination between genes and whether plasmid transfer between strains has occurred (12). An understanding of such processes is a prerequisite for the successful development of effective strategies for both control and prevention of Lyme disease (13).

MATERIALS AND METHODS

Strains. The strains (Table 1) PGau, K48, and DK29 were obtained from Russell Johnson, University of Minnesota, Minneapolis, Minnesota, and the antigenically characterized strains (18) PKa1, PHei, PTrob, PBo, and PKo were obtained from V.P.-M. Total genomic *B. burgdorferi* DNA was extracted as described from cultures of *B. burgdorferi* maintained in BSKII media (25).

Cloning and Sequencing of *ospA*. The *ospA* gene was amplified from genomic DNA by the polymerase chain reaction (PCR) in a Perkin–Elmer/Cetus thermal cycler. Briefly, 100-μl reaction mixtures contained 50 mM KCl, 10 mM TRIS·HCl (pH 8.3), 1.5 mM $MgCl_2$, 200 μM each NTP, 2.5 units of *Taq* I DNA polymerase (Amplitaq, Perkin–Elmer/Cetus), and 100 pmol each of the 5′ and 3′ primers. The 5′ and 3′ primers for cloning, 5′-GGAGAATATATTATGAAA-3′ (−12 to +6) and 5′-CTCCTTATTTTAAAGCG-3′ (+826 to +809), respectively, were determined from the sequence of *ospA* from B31 (25). The 5′ and 3′ ends of the gene are highly conservative (10, 16, 26). Amplification was performed as described (25), and the amplicon was visualized on an agarose gel by ethidium bromide staining.

Twenty nanograms of the chloroform-extracted PCR product were cloned directly into the PCR vector (Invitrogen) by

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‖The sequences reported for *B. burgdorferi* strains in this paper have been deposited in the GenBank data base (accession nos. for *ospA* are X62624 for strain K48, X63412 for DK29, X65600 for PHei, X62387 for PGau, X65598 for PTrob, X65599 for PKo, X65605 for PBo, X69606 for PKa1, and X70365 for IP3; accession numbers for *fla* are X69607 for strain PBo, X69608 for DK29, X69609 for PHei, X69610 for K48, X69611 for PKa1, X69612 for PGau, X69613 for PKo, and X69614 for PTrob; and accession numbers for *p93* are X69601 for strain PBo, X69602 for K48, X69803 for PKo, X69604 for PTrob, and X70365 for 25015).

Table 1. Strains used in phylogenetic analysis

Strain no.*	Strain name	Ref. for strain	Location and source	Refs. for protein sequence†		
				p93	OspA	Fla
1	B31	14	Shelter Island, NY. *Ixodes scapularis*	15	16	17
2	PKa1	18	Germany, human, CSF	ND	This study	This study
3	ZS7	19	Freiburg, Germany *I. ricinus*	ND	20	ND
4	N40	9	Westchester Co., NY	ND	21	ND
5	25015	22	Millbrook, NY *I. scapularis*	This study	10	ND
6	K48	None	Czechoslovakia *I. ricinus*	This study	This study	This study
7	DK29	18	Denmark, human, EM	This study	This study	This study
8	PHei	18	Germany, human, CSF	This study	This study	This study
9	Ip90	23	Khabarovsk, Russia *I. persulcatus*	ND	21	ND
10	PTrob	18	Germany, human, skin	This study	This study	This study
11	ACAI	24	Sweden, human, ACA	ND	21	ND
12	PGau	18	Germany, human, ACA	This study	This study	This study
13	Ip3	23	Khabarovsk, Russia *I. persulcatus*	ND	This study	ND
14	PBo	18	Germany, human, CSF	ND	This study	This study
15	PKo	18	Germany, human, EM	ND	This study	This study

ND, sequence not determined; ACA, isolated from skin of a patient with acrodermatitis chronica atrophicans; EM, isolated from skin of a patient with erythema migrans; CSF, isolated from cerebrospinal fluid of a patient with Lyme disease.
*The strain number is used to designate strains in Fig. 1.
†These give the references for sequences of the various proteins.

following the manufacturer's instructions. Two recombinant colonies containing the amplified fragment were selected, the plasmids were prepared, and the nucleotide sequence of *ospA* was determined by the dideoxy chain-termination technique using the Sequenase kit (United States Biochemical). Directed sequencing was performed with M13 primers followed by *ospA*-specific primers derived from sequences, previously obtained with M13 primers.

Cloning and Sequencing the *fla* Gene. A similar approach was used to clone *fla* and *p93*. The 5′ and 3′ primers for cloning are 5′-ATGATTATCAATCATAAT-3′ (+1 to +18) and 5′-TCTGAACAATGACAAAAC-3′ (+1008 to +991), giving 972 bases sampled for variation. The PCR products were purified by using *GeneClean* (Bio 101). PHei, PGau, K48, and PTrob were cloned into pVZ1 (27).

Cloning and Sequencing the 5′ End of *p93*. The first 1091 bases of *p93* were cloned and sequenced. The 5′ and 3′ primers for cloning are 5′-GGTGAATTTAGTTGGTAA-GG-3′ (−54 to −35) and 5′-CACCAGTTTCTTTAAGCT-GCTCCTGC-3′ (+1117 to +1092).

Phylogenetic Analysis. General discussions of methods used here for phylogenetic reconstruction and bootstrap analysis can be found in refs. 28 and 29. Maximum parsimony trees were constructed, and bootstrap analysis was done by using the phylogenetic analysis program PAUP (30). The bootstrap analysis records the percentage of random resampling where clusters remain together. A range of values that correspond to true phylogenetic clustering has not been agreed upon, but experience suggests that a range of 70–100% for trees built with maximum parsimony implies a monophyletic group.

RESULTS AND ANALYSIS

DNA Sequences. Alleles of the chromosomal gene *fla* and the first 1117 bases of the end of 5′ chromosomal gene *p93* were aligned without requiring any postulated insertions or deletions (Table 1). However, the length of plasmid-borne *ospA* alleles varied between 819 and 825 base pairs (bp), coding for proteins of 335, 336, or 337 amino acids. In four strains, K48, DK29, PHei, and IP90, *ospA* contained a three-nucleotide insert at positions 522–4 encoding a glycine residue. PHei contains a 2-bp deletion at 622 and 623 and a single base-pair deletion at 630 that reestablishes the reading frame. Strain 19857 has been postulated to have a 3-bp deletion at 318–320, a 6-bp deletion at 613–618, and two 3-bp insertions at 450 and at 492 (31).

***B. burgdorferi* Is Clonal.** All chromosomal genes within a strain will share a common evolutionary history in the absence of recombination. Consequently, alleles of different genes are expected to reproduce the same phylogenetic branching order. Recombination between genes brings alleles with different evolutionary histories together within a single strain, with the consequence that any similarity among the gene trees is disrupted. The topologies of the two chromosomal genes (*fla* and *p93*, Fig. 1) are identical, demonstrating that recombination is rare or nonexistent on an evolutionary time scale. Thus, *B. burgdorferi* is clonal.

Lateral Transfer of the Linear Plasmid. The tree for the gene on the linear plasmid, *ospA*, is very similar to the trees for the chromosomal genes. The exception is the position of strain 8. For *p93* and *fla*, strains 7 and 8 are very similar, and the common ancestor of these strains shares a common ancestor with strain 6. However, with ospA, strains 6 and 7 share a common ancestor before they share one with strain 8. Stability analysis using bootstrap resampling shows that these clusters are well defined, with *p93* of strains 7 and 8 stable 78% of the time and with *ospA* strains 6 and 7 stable 100% of the time. This suggests that the linear plasmid was transferred into strain 8 after the divergence of strains 7 and 8. Thus, while there is no evidence for chromosomal gene exchange, there is evidence of plasmid transfer.

Intragenic Recombination. The phenomenon of intragenic recombination is of great concern for vaccine development, because, if it occurs, *B. burgdorferi* might readily generate strains with novel epitopes. Recombination of short stretches of DNA into an allele need not affect phylogenetic position, which may still be determined by the bulk of the remaining sequence. However, transfers of short pieces of DNA create inconsistencies in parsimony trees because they represent a phylogenetic history that differs from the rest of the sequence.

To determine significantly long gaps and regions of significantly clustered sites, we used the test of Stephens (34). This test associates each polymorphic site with a particular partition or grouping of strains. All sites assigned to a particular partition are grouped together. The test detects gaps and regions of clustered inconsistencies that are significantly longer than expected based on the assumption that sites for a particular partition are randomly distributed along the

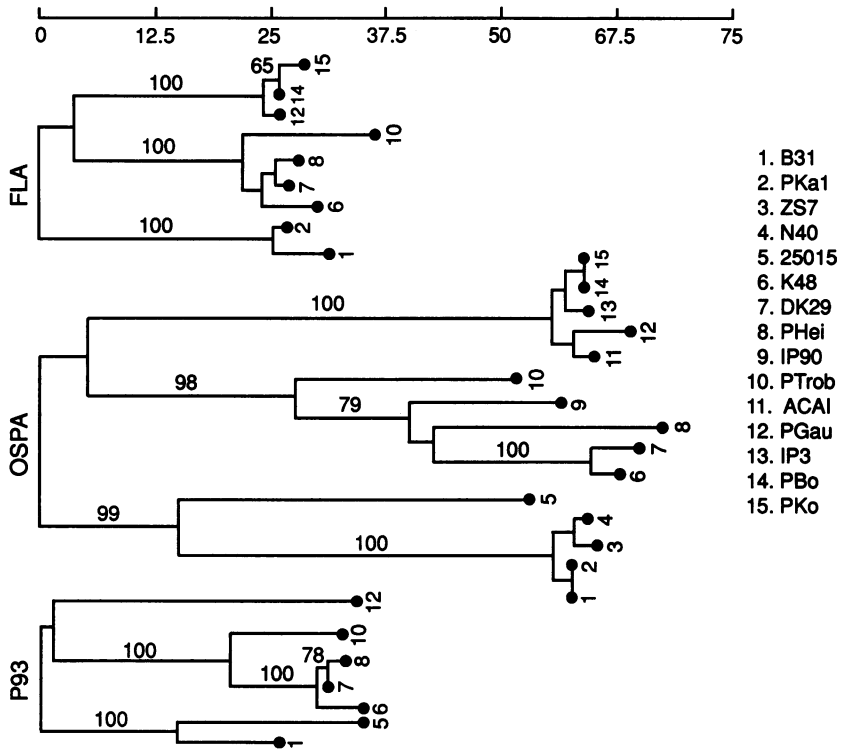

FIG. 1. Phylogenetic trees from the DNA sequences of *p93*, *ospA*, and *fla*. The scale is in changes per kilobase of DNA. The trees were generated by using the neighbor-joining (32) option on NTSYS-pc (33). The input distance matrix for each tree was the number of mismatches in the DNA sequence for each pair. The trees were rooted by midpoint rooting. The branch lengths were normalized to changes per kilobase of DNA for the figure. Thus, the trees are comparable as to rate. Strains represented as the ends of a straight line have the identical sequence. The numbers on the branches are the percent of trees retaining the cluster when the data is bootstrapped. The neighbor-joining trees were compared to trees generated by maximum parsimony using the program PAUP (30) and were found to be congruent. The neighbor-joining trees were better defined—i.e., undefined tricotomies by maximum parsimony were defined by neighbor joining. The bootstrap analysis was done by using maximum parsimony, giving a conservative estimation of the stability of nodes.

sequence. Such regions indicate the positions of possible recombination events.

The test is illustrated in cartoon form in Fig. 2 for a hypothetical tree of four sequences in which a short DNA segment was transferred between alleles 1 and 4. Fig. 2*A* illustrates a consistent partition, where bases unique to allele 1 are represented by Xs. In the center of the sequence is a gap. There are no sites for this partition in this region. Because of the transfer, this region is common to alleles 1 and 4. Fig. 2*B* shows an inconsistent partition in which alleles 1 and 4 share identical bases that are not shared by alleles 2 and 3. This configuration of gaps and clustering provides strong evidence that a piece of DNA was transferred between ancestors of allele 1 and allele 4 without specifying direction. Gaps without the complementary clustered sites can be caused by nonrandom mutations and alone are not sufficient evidence for transfer, while a cluster of inconsistent sites is good evidence. Note that clusters of inconsistent sites may be restricted to a single allele if the recombined DNA arises from an allele not otherwise represented in the phylogenetic tree.

When applying the Stephens test on DNA sequence data, all polymorphic sites must be tested for clustering to determine if there are regions of low variation and high variation along the sequence, so that regions of low variability do not appear as significantly long gaps. The test indicates no overall clustering of polymorphic sites for *p93* and *fla*. However, the first 75 bases of *ospA*, the region encompassing the amino-terminal signal sequence show significantly lower variability

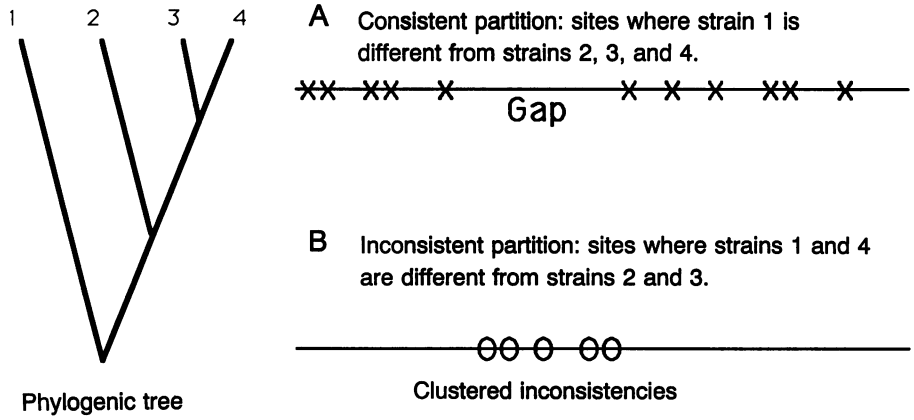

FIG. 2. Cartoon illustrating the test for intragenic recombination. An explanation is given in the text.

than the rest of the gene and these were eliminated from further analysis.

Gaps. There are no significant gaps or clustered inconsistencies for *p93*. There is one significant gap and no significant clustered inconsistencies for *fla*. This one significant gap ($P = 0.003$) is in the partition of strain 1 (B31) from all other strains. There are two significant gaps for *ospA*: partitioning strains 11–15 from the others reveals a significant gap between bases 555 and 701, and partitioning strains 1–4 from the others reveals a significant gap between bases 165 and 325. No complementary clustered inconsistencies were found that span any of these gaps. Consequently, they cannot be used as proof of recombination and are likely to be caused by clustered mutations.

Significant gaps will be found if contiguous nucleotides change during a single mutational event, since the null hypothesis assumes that mutations happen randomly along the sequence and each mutation changes a single nucleotide. This can be seen easily for the *fla* gap. Four of the five sites are contiguous (sites 499 and 500 and sites 932 and 933), suggesting five nucleotide changes from three mutational events.

Clustered Inconsistencies. Partitioning strain 8 from strains 6, 7, 9, and 10 reveals a cluster of inconsistent sites between bases 549 and 744, a region spanning the antigenic domain responsible for conferring resistance in OspA (35). Recombination appears to have introduced this region of *ospA* into strain 8 from another strain and this seems to be the only intragenic recombination in the *ospA* alleles.

This conclusion was confirmed by dividing the *ospA* genes in half and generating trees for each half. When there is intragenic recombination, the two halves are likely to be positioned differently on the trees. When the test is performed, the trees are identical to the tree generated when using the entire molecule except for the position of strain 8. For the 5′ end, strain 8 forms a group with strain 9, whereas for the 3′ end, strain 8 branches off near the base of the tree. A tree of *ospA* without the region from bases 549 to 744 clusters strains 8 and 9 with a stability of 76%, clusters strains 6 and 7 with a stability of 100%, and clusters strains 6, 7, 8, and 9 with a stability of 97%. A tree of bases 549–744 of *ospA* separated strain 8 from the cluster of strains 6, 7, 9, and 10 with a stability of 93%. Evidently, the sequence in the region between bases 549 and 744 diverged from the other *ospA* sequences of *B. burgdorferi* at about the same time as the major clusters diverged. This piece must have been introduced recently into the *ospA* carried by strain 8 because the analysis of the rest of this *ospA* shows that it is similar to the *ospA* in strain 9. While confirming that an intragenic recombination has occurred, this analysis also reinforces the contention that the plasmid was recently transferred because strain 8 groups with strain 9 even when the piece introduced by recombination is removed rather than strain 7 as expected from the data on chromosomal genes.

Groups. The phylogenetic trees (Fig. 1) show *B. burgdorferi* falls naturally into three major groups, with strains 1–5 in the first group, 6–10 in the second, and 11–15 in the third. Except for strain 5 in group one and strains 9 and 10 in group two, there is little variation within a group compared with the divergence between groups. Stability analysis using bootstrap resampling showed that these groups are stable and well defined. These three groups correspond to the "genospecies" of Baranton *et al.* (36). The *ospA* sequence of strain 19857 (31), which was isolated from rabbit kidney in the United States, is very different from all the others. If it were to be put on Fig. 1, it would diverge some 30 units before the divergence of the three major groups and would represent another "genospecies." Previous studies have shown three to seven distinct types of *B. burgdorferi* (31, 36–38).

DISCUSSION

Sequence studies of isolates of *Borrelia burgdorferi* from North America and Europe provide no evidence for lateral gene transfer of chromosomal genes on an evolutionary time scale. Thus, *B. burgdorferi* is strictly clonal. Although evidence for transfer of and recombination on a linear plasmid is presented, the general consistency of the *ospA* tree with the trees for the chromosomal genes suggests that transfers and recombinations are very rare events.

Variability and Vaccination. There is considerable variation displayed in OspA, including the regions conferring antigenicity. Consequently, the degree and frequency of protection from vaccination with OspA from a single strain will depend upon the response of the immune system to this variation and to the frequency of the diverse clones in the species. Indeed, mice immunized with recombinant OspA protein from N40 (strain 4) or from 25015 (strain 5) produced an immune response and were protected from infection by the same strain but not from a heterologous strain (9, 10). Recently, we have shown that strains K48 and DK29 are alike in their response to a panel of five monoclonal antibodies, whereas PTrob is very different and IP90 is intermediate (ref. 35; unpublished data). This suggests that *ospA* of PTrob will not provide cross-immunity for infections of cells similar to K48. Thus, the successful development of a vaccine against Lyme disease inevitably depends on an understanding of genetic variation in *B. burgdorferi* and how this variation influences cross-immunity.

If vaccines are developed, they should be effective for a considerable time. The lack of significant intragenic recombination, a process which might rapidly generate novel epitopes with changed antigenic properties, ensures that *B. burgdorferi* can only change antigenic type by accumulating mutational changes. But mutation is slow compared with recombination in generating very different types. Thus, new antigenic types will not rapidly arise and vaccines are expected to be effective for a long time.

Clonal Taxonomy. The results of this study agree with previous studies on the genetic diversity within *B. burgdorferi*. If strains 5 (Fig. 1) and 19857 (31) are excepted, the three major groups resemble the three "genospecies" reported earlier (37, 38). Strains 6–10 belong to "genospecies" II, which was given the species name *B. garinii* (37). Strains 1–4 belong to "genospecies" I, which would retain the species name, *B. burgdorferi*. Strains 11–16 belong to "genospecies" III, which has the common name, group VS461. The use of DNA hybridization as described (37) to give the "genospecies" species names is reasonable. There is 46–74% hybridization between these groups, which is in the range usually accepted for defining bacterial species.

However, taxonomic problems arise when naming species that are asexual because the criterion of a shared gene pool can not be used (12). Another important criterion that should be considered is ecological: lineages of asexual organisms that are ecologically equivalent should be considered a single species. There is no evidence that the various "genospecies" of *B. burgdorferi* are ecologically differentiated: all "genospecies" are associated with Lyme disease, and the suggested differences in the clinical manifestations of Lyme disease in North America and Europe could be due to differences in host and environmental factors rather than due to different frequencies of the "genospecies." All "genospecies" can be found in the same species of ticks. The only association between tick and clonal type noted at present is geographical.

Large differences between "genospecies" that are ecologically equivalent are expected when population size is large and migration low (39). *B. burgdorferi* seems to have a low migration rate. For example, while many isolates of *B.*

burgdorferi from the United States belong to the cluster represented by strains 1–4, strains genetically very similar to strain 5 were the only type isolated from a population of ticks near Millbrook, NY (40). This suggests that there is considerable geographic variation in frequencies of various clones. Presently, we suggest that *B. burgdorferi* remain the species name for all the spirochetal agents of Lyme disease until the "genospecies" can be differentiated ecologically and medically.

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