Genetics of *Borrelia burgdorferi*

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Abstract

The spirochetes in the *Borrelia burgdorferi* sensu lato genospecies group cycle in nature between tick vectors and vertebrate hosts. The current assemblage of *B. burgdorferi* sensu lato, of which three species cause Lyme disease in humans, originated from a rapid species radiation that occurred near the origin of the clade. All of these species share a unique genome structure that is highly segmented and predominantly composed of linear replicons. One of the circular plasmids is a prophage that exists as several isoforms in each cell and can be transduced to other cells, likely contributing to an otherwise relatively anemic level of horizontal gene transfer, which nevertheless appears to be adequate to permit strong natural selection and adaptation in populations of *B. burgdorferi*. Although the molecular genetic toolbox is meager, several antibiotic-resistant mutants have been isolated, and the resistance alleles, as well as some exogenous genes, have been fashioned into markers to dissect gene function. Genetic studies have probed the role of the outer membrane lipoprotein OspC, which is maintained in nature by multiple niche polymorphisms and negative frequency-dependent selection. One of the most intriguing genetic systems in *B. burgdorferi* is *vls* recombination, which generates antigenic variation during infection of mammalian hosts.

INTRODUCTION

Borrelia burgdorferi, the causative agent of Lyme disease (Lyme borreliosis), belongs to an ancient phylum of bacteria called spirochetes. The long, thin serpentine morphology is the signature feature shared among spirochetes (**Figure 1**), whereas its many other characteristics, such as genome organization, lifestyle, and disease pathogenesis (if present), are quite diverse. *Borrelia* species are the only members of this phylum that must be transmitted among vertebrate hosts, including humans, by an arthropod vector.

Several *B. burgdorferi* sensu lato genospecies have been described (**Figure 2**) (8, 29, 58, 79, 80, 96, 100, 104, 123, 134, 168), all of which are transmitted among vertebrate wildlife species by ixodid ticks. However, only three of these species, *B. burgdorferi* sensu stricto, *Borrelia garinii*, and *Borrelia afzelii*, are regularly associated with human infections (27, 165, 172). The remaining genospecies also infect multiple vertebrate species but are rarely found in human patients (43). Several *Borrelia* isolates may be novel genospecies, including genomospecies 1 and genomospecies 2 from California (122) and a European group (102, 127).

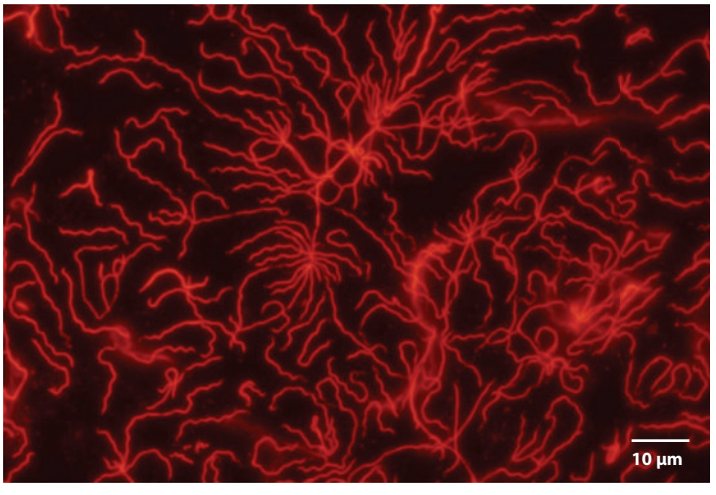

Figure 1

Live *Borrelia burgdorferi* sensu stricto stained with wheat germ agglutinin Alexa Fluor® 594.

Although the tempo of diversification has been a principal topic of studies in macroorganisms (137, 151, 153), much less is known about the rates of diversification in the exceedingly diverse and species-rich microbiota. Explosive species radiations appear to be a common pattern of diversification in plant and animal lineages, although the few existing analyses of

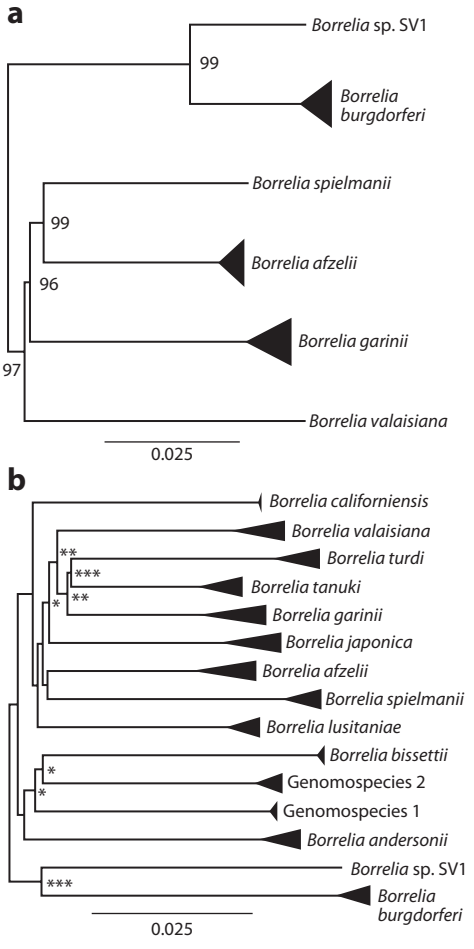

Figure 2

Bayesian phylogenies of *Borrelia burgdorferi* sensu lato; triangles represent the diversity within genospecies. The rapid burst of speciation that occurred early in the evolutionary history of the group can be inferred from both the (*a*) chromosome sequences and (*b*) the multilocus phylogenies. Nodes marked with asterisks are supported by posterior probabilities of more than 0.8 (*), 0.9 (**), and 0.95 (***). Figure adapted from Morlon et al. (112).

microbial lineages suggest that the tempo of diversification in prokaryotes may be fundamentally different (103). A recent study found that the Lyme disease group of bacteria, like plants and animals, underwent an explosive species radiation near the origin of the clade, which led to the current assemblage of species (**Figure 2**) (112).

In nature, *B. burgdorferi* is maintained in an enzootic cycle (**Figure 3**) between an *Ixodes* tick vector and a vertebrate host (89, 92, 121, 130). *Ixodes* larvae acquire *B. burgdorferi* from an infected animal during the first blood meal because transovarial transmission does not occur. The spirochetes persist in the tick midgut, weathering restricted nutritional conditions following digestion of the blood meal and austere environmental conditions as ticks overwinter. Transmission occurs during nymphal feeding in which the blood meal triggers *B. burgdorferi* replication, escape from the midgut to the hemocoel, and exit through the salivary glands into the mammalian host, thus completing the enzootic cycle. Nymphs are generally considered to be the relevant vector for human infection, although humans are a dead end host for *B. burgdorferi*. Transition through the vastly different environments of the enzootic cycle requires not only differential gene regulation (130, 144, 154) but has likely led to molecular adaptations reflected in its curious genome architecture (10, 30, 32, 33, 57).

The genome of *B. burgdorferi* is one of the most, if not the most, complex of any bacterium (30, 32, 33, 57). It consists of a ~950-kb linear chromosome and a variable complement of circular plasmids (cps) and linear plasmids (lps) that range in size from 9 to 62 kb. The linear replicons have covalently closed telomeres (10), whose replication requires the telomere resolvase ResT (33, 84). The genome has a low G+C content of ~28%. Most, but not all, housekeeping genes are on the chromosome, whereas the majority of genes encoding lipoproteins expressed on the bacterial outer membrane, presumably mediating transition through the enzootic cycle, are found on the plasmids. The importance of lipoproteins to *B. burgdorferi* is underscored by their abundance: They represent 7.8% of all open reading frames. In addition, the lipoproteins are differentially expressed during the enzootic cycle (130, 144, 154). Although much work has been done to elucidate the function of an increasing number of genes, many of the predicted open reading frames of the chromosome (~30%), and especially of the plasmids, share no significant homology with any previously annotated genes (30, 57). Each linear plasmid is distinct, but all contain multiple copies of paralogous genes. Pseudogenes and noncoding DNA constitute a significant amount of their sequence, suggesting a genome in flux (30, 32, 57, 85). Although different strains contain a discrete complement of plasmids, and the plasmid content may be shuffled between the linear components, the repertoire of genes remains relatively consistent (32). The plasticity of the linear replicons may be generated, at least in part, by telomere fusion in the reverse of the telomere resolution reaction catalyzed by ResT (85). Some circular and linear plasmids are essential for the enzootic cycle but not for propagation in vitro, such as lp28-1, lp25 and some members of the cp32 family (90, 125, 177); at least some cp32s are prophages that can be transduced, as discussed below. Loss of plasmids during in vitro manipulations represents one of several challenges for developing methodologies to genetically manipulate *B. burgdorferi*. Notably, cp26 is the only single-copy plasmid known to be essential for in vitro growth: It carries *resT*, which encodes the telomere resolvase required for replicating linear molecules (33, 84), and it also carries *ospC* (101, 141), which is required for transmission from tick to vertebrate and infectivity in vertebrates (65, 120, 162, 163).

B. burgdorferi lacks the capacity to synthesize amino acids, nucleotides, fatty acids, and enzyme cofactors, as the genes encoding the enzymes for these pathways were presumably lost during the coevolution with its tick vector and mammalian host (57, 62). Instead, *B. burgdorferi* is an accomplished importer and scavenger that has at least 52 genes encoding

Enzootic: maintenance of a microbe, without external inputs, in a geographically localized animal population or community

Transovarial transmission: transmission from the female tick through the eggs to the larvae of the next generation

Hemocoel: tick body cavity containing the hemolymph that delivers oxygen to the organs

Circular plasmid (cp) and linear plasmid (lp): components of the segmented genome that are smaller than the chromosome; the adjacent number is the approximate size in kilobase pairs in strain B31, e.g., cp32 is a 32-kb circular plasmid

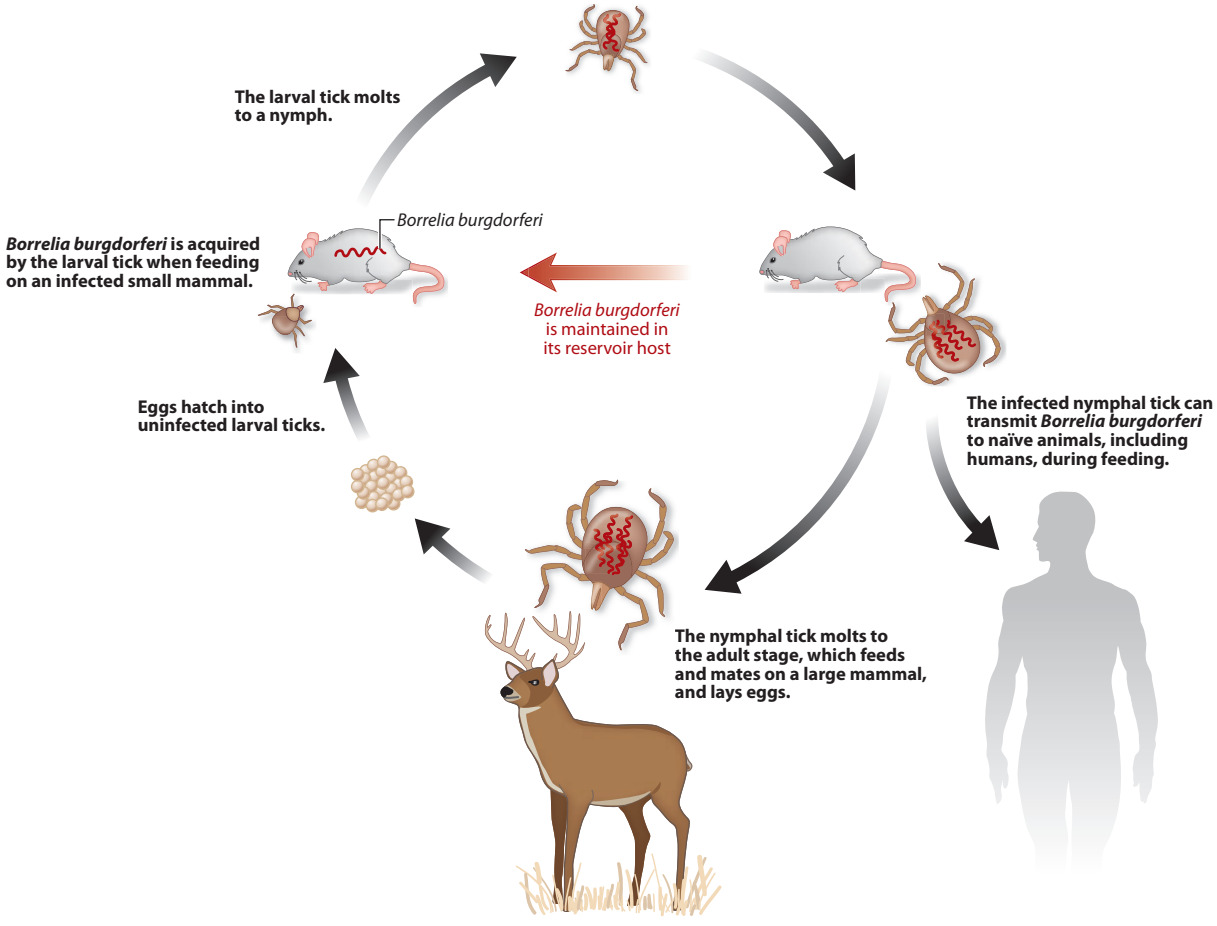

Figure 3

Enzootic cycle of *Borrelia burgdorferi*. Spirochetes are acquired when *Ixodes* spp. larvae feed on their first vertebrate host, usually a small mammal or bird. Larvae then molt to nymphs, which transmit the spirochetes when they feed on a second vertebrate host. Nymphs molt to adults, which feed on a third vertebrate host. All three stages of ticks feed on humans, which are thought to be incidental hosts, but *B. burgdorferi* transmission by nymphs is considered to cause most cases of Lyme disease.

transporters and/or binding proteins of carbohydrates, peptides, and amino acids (142). Additionally, energy is derived by glycolysis and the fermentation of sugars to lactic acid, as the genes encoding the components necessary for the citric acid cycle and oxidative phosphorylation are missing (57, 62). The prevalence of chemotaxis and motility genes, which represent approximately 6% of the genes on the chromosome, highlights the importance of identifying and moving to the correct niche in order to successfully navigate the enzootic cycle (37).

MOLECULAR GENETICS

Molecular genetics of *B. burgdorferi* commenced approximately ten years after the discovery of the spirochete (26) when the first genetically defined mutants were isolated (139, 140, 147) and the bacterium was first genetically transformed (146). Borreliologists utilized the awesome power of genetics in the fastidious microbe over the ensuing years, applying increasingly more sophisticated reverse genetics tools originally developed in model organisms (136). Forward genetic screens have

still been mostly limited to antibiotic resistance as a phenotype. However, the development of transposon mutagenesis (15, 97, 113, 116, 161) and inducible gene expression (13, 63, 171) will allow more complex phenotypes, such as infectivity, to be genetically dissected.

The natural exchange of DNA is mediated via transduction of cp32 prophage, at least in vitro (51). Transformation, which is relatively inefficient, has been demonstrated only using artificial conditions in the laboratory (135, 145, 146); however, the spirochete produces membrane blebs that contain DNA (61), which could theoretically be a mechanism of horizontal gene transfer (HGT). There has also been a report of heterologous conjugation in which erythromycin resistance was transferred from *B. burgdorferi* into two species of gram-positive bacteria, although the mechanism of resistance has not been defined, and the genetic element was not identified (77). In addition to HGT, genetic variation is effectively generated during mammalian infection by an intriguing recombination system at the *vlsE* locus (45, 97, 177), as discussed in detail below. The recombination machinery can, surprisingly, hop and skip over short sequences (41), and this was previously observed during the site-directed mutagenesis of the *gac* gene embedded in *gyrA* (82).

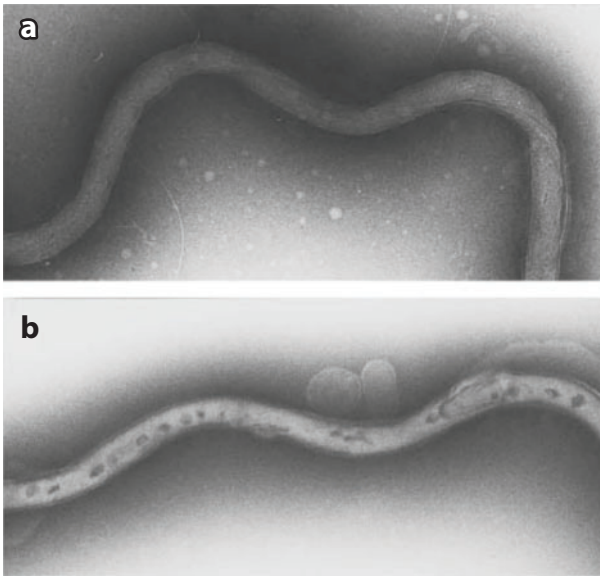

Figure 4

Electron micrograph of negatively stained *Borrelia burgdorferi* (*a*) before and (*b*) after electroporation. Following electroporation, *B. burgdorferi* have darkly stained regions, as visualized by transmission electron microscopy, that are thought to be transient pores through which the DNA enters (or leaves) the cell. Reprinted with permission from Samuels & Garon (145).

Mutations and Transformation

Reverse genetics has, not unexpectedly, proved to be a powerful approach to deciphering the enigmatic physiology of *B. burgdorferi*, including its fascinating mechanisms of motility (37), gene regulation (144), and linear DNA replication (33), as well as the pathogenesis of Lyme disease (130). Genetic manipulation required a system to transform the spirochete and to select for site-directed mutations, gene disruption by allelic exchange, shuttle vectors, and recombinant endogenous plasmids. *B. burgdorferi* is typically transformed by electroporation (**Figure 4**) using standard methodologies (143, 146).

Almost all *B. burgdorferi* mutants selected in a phenotypic screen have been those that are resistant to various antibiotics, and the mutations have been mapped to either a topoisomerase or the ribosome (42, 60, 147). However, the first isolated *B. burgdorferi* mutant lacked flagella and was nonmotile, although a specific mutation was not mapped to a defined locus (140). The first phenotypic selection of *B. burgdorferi* was resistance to a bacteriostatic antibody, which resulted in isolation of mutants in outer membrane lipoproteins (138, 139). Coumermycin A_1 (147), a coumarin that targets the B subunit of DNA gyrase (34, 40, 46, 71), was the first antibiotic used to isolate mutants in *B. burgdorferi* (147). Coumermycin A_1-resistant *gyrB* mutations map to a conserved residue (arginine 133), which is homologous to GyrB residues mutated in several other coumarin-resistant prokaryotes (147).

In addition to DNA gyrase, most bacteria encode topoisomerase IV, another type II topoisomerase (34, 73). Fluoroquinolone antibiotics, which are not clinically used to treat Lyme disease, target type II topoisomerases

Transduction: movement of genetic material from one bacterium to another by a bacteriophage

Horizontal (or lateral) gene transfer (HGT): the movement of genetic information between cells of the same generation

Electroporation: an electrical pulse used to transiently create holes in the cell membrane to introduce DNA

Restriction-modification system: used by bacteria to protect themselves from foreign DNA, such as that introduced by a bacteriophage

(46, 71, 114). Fluoroquinolone resistance maps, in most cases, to a small region in the A subunits of DNA gyrase (GyrA) in gram-negative bacteria or topoisomerase IV (ParC) in gram-positive bacteria (46, 71). Several fluoroquinolone-resistant mutants of *B. burgdorferi* were isolated with all mutations mapping to *parC*, strongly suggesting that topoisomerase IV is the primary target of fluoroquinolone antibiotics (60). The mutations are at conserved residues (threonine 69, which is a serine in most bacteria; serine 70; and glutamate 73) that are often mutated in other fluoroquinolone-resistant bacteria.

Spectinomycin targets the small subunit of the ribosome, and resistant mutations map to either the S5 protein or the 16S rRNA. In *B. burgdorferi*, mutations in the 16S rRNA gene *rrs* (at A1185 or C1186) confer high-level spectinomycin resistance and occur at a high frequency (42). The closely related aminoglycoside antibiotics kanamycin, gentamicin, and streptomycin also target the ribosome. Kanamycin-resistant and gentamicin-resistant *B. burgdorferi* have a mutation at A1402 of *rrs* that confers approximately 100-fold resistance to both antibiotics (42). Streptomycin-resistant mutants have mutations in *rpsL* (at lysine 88 of the S12 protein) that increase resistance tenfold (42). The frequency of the spectinomycin-resistant mutants is approximately 100-fold higher than the frequency of the aminoglycoside-resistant mutants. The high frequency is likely a result of a lower fitness cost for the spectinomycin-resistant *rrs* mutations. The spectinomycin-resistant mutants grow at the same rate as wild type without selection and are maintained in mixed cultures for up to 100 generations, whereas the aminoglycoside-resistant mutants are outcompeted at 50 generations and lost within 100 generations (42).

The coumermycin A_1-resistant *gyrB* allele was fashioned into the first selectable marker for genetic transformation (146), and the wild-type *rpsL* gene in a streptomycin-resistant background was harnessed as the first counterselectable marker (44). Several genes were mutated using *gyrB*, including *ospC* (164), *gac* (82), and others. Coumermycin A_1 resistance was also used to show that bacteria could be transformed with short oligonucleotides (145). However, *gyrB* is no longer utilized by molecular borreliologists as a selectable marker because of experimental limitations, including tedious screening of transformants due to homologous recombination into the chromosomal *gyrB* locus and pleiotropic effects presumably due to altered DNA topology. For example, *gyrB* mutants have abnormal expression of *groEL* (4) and *ospC* (3); the *gyrB* mutation is postulated to have suppressed the phenotype of the *gac* mutant (82). These experimental obstacles were overcome by fusing *B. burgdorferi* promoters to exogenous antibiotic resistance genes.

The chimeric markers allow for efficient selection of transformants and employ the *aphI* gene from Tn*903*, which confers resistance to kanamycin (14); the *aadA* gene from the *Shigella flexneri* plasmid R100, which confers resistance to spectinomycin and streptomycin (56); and the *aacC1* gene from Tn*1696*, which confers resistance to gentamicin (54). Note that selection with spectinomycin for transformants carrying the *flgBp-aadA* hybrid cassette fails because mutations in *rrs* confer high-level resistance and occur at a high frequency (42), necessitating the use of streptomycin for selection in *B. burgdorferi* (56). In addition, the *ermC* gene from the *Staphylococcus aureus* plasmid pE194, which confers erythromycin resistance (149), has been applied as a selectable marker.

Restriction-modification systems are considered to be transformation barriers (81, 95, 131). Two linear plasmids, lp25 and lp56, are correlated with low transformation efficiency, and many transformants have lost lp25 (95). These two plasmids carry genes *bbe02* and *bbq67*, respectively, encoding restriction-modification enzymes that methylate DNA (131). CpG methylation of plasmid DNA in vitro increases the efficiency of transformation into *B. burgdorferi* carrying lp56, suggesting that BBQ67 methylates CpG (38). A major reason that low-passage infectious strains are difficult to transform is that plasmid lp25 carries not only *bbe02*, which is selected against

during transformation, but also *pncA*, a gene essential for infectivity (125), so strains that are both readily transformable and highly infectious are not common.

Bacteriophage and Transduction

Casjens and colleagues have suggested that the genetic diversity found on plasmids may have arisen at least in part because of HGT (30, 32). One potential mechanism for HGT between *B. burgdorferi* cells is via a transducing bacteriophage (50, 51, 176). At least four different tailed bacteriophage-like particles have been observed in supernatants of *B. burgdorferi* cultures (11, 50, 52, 70, 115, 150). Of these, the best characterized is ϕBB-1 (**Figure 5**), which packages members of the ubiquitous cp32 family (52). Every *B. burgdorferi* strain examined to date includes multiple paralogous versions of cp32 (30–32, 157). The cp32s from all examined strains are largely homologous, with three regions of variability that appear independently assorted among the plasmids: (*a*) a region containing the replication and partitioning genes, including the unique PFam32 genes that determine compatibility with other plasmids; (*b*) a region in which the *mlp*, *rev*, and *bdr* genes can be found; and (*c*) a region in which the *ospE/ospF/elp* (also known as *erp*) and other alternative genes are located (30, 32, 49). In addition to putative phage assembly genes and plasmid maintenance genes, genes that modify the borrelial host (lysogenic conversion) are also encoded on the cp32s. Such genes include those that encode surface lipoproteins capable of binding mammalian host proteins, including fibronectin, plasminogen, laminin, and factor H complement regulatory factor binding protein (2, 16–18, 87, 111). Presumably, collecting cp32s and the variable genes they encode assists *B. burgdorferi* in surviving within a variety of host species (72, 159).

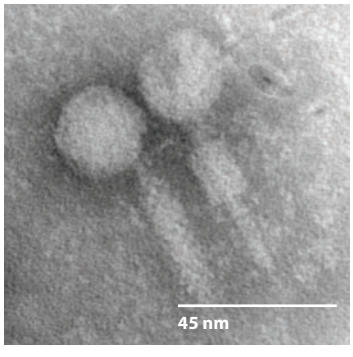

Figure 5

Bacteriophage ϕBB-1 of *Borrelia burgdorferi*. Virions were recovered from the supernatant of a culture that had been treated with 1-methyl-3-nitroso-nitroguanidine. The phage particles were negatively stained with phosphotungstic acid and observed by transmission electron microscopy. Reproduced with permission from Eggers et al. (51).

Eggers et al. (51) demonstrated that ϕBB-1 is capable of transducing a cp32 between *B. burgdorferi* cells both of the same strain and of different strains in vitro. Interestingly, phage from one strain more efficiently transduced DNA into cells of the same strain compared with cells of a different strain, which may be related to the restriction-modification systems described above. The overall effect that a restriction-modification system may have on ϕBB-1 as an agent of HGT of DNA has yet to be investigated. Indeed, the role of ϕBB-1 in moving DNA within or between strains is as yet undefined, although presumably transduction occurs in the tick vector, where the spirochete is at a relatively high density compared with the infection in a vertebrate. Stevenson & Miller have reported that *B. burgdorferi* strain Sh2-82, which seems to have diverged only recently from strain 297, has an extra cp32 that is apparently identical to the strain B31 cp32-8, suggesting the HGT of an entire cp32 between strains (160).

Although transduction of cp32s via ϕBB-1 may be the best-characterized mechanism for HGT thus far elucidated, it may not be the sole means. Several other plasmids contain cp32-derived sequences found within various *B. burgdorferi* strains, including lp56, lp54, and lp28-2, which were first characterized in strain B31, and two different cp18 plasmids from strains N40 and 297 (28, 30, 158). The cp32-like sequences on cp18, lp54, and lp56 are altered in ways that likely preclude their ability

Gene transfer agent (GTA): a bacteriophage-like particle that packages random pieces of host genomic DNA and can transfer this genetic material to other bacterial cells

to encode productive prophages; however, lp28-2, which is only distantly related to the cp32s, encodes a number of proteins with weak homology to known phage genes and could be a prophage. Although lp28-2 has not yet been observed packaged in phage heads recovered from culture supernatants, a number of different phage particles other than ϕBB-1 have been observed in association with *B. burgdorferi* (11, 50, 52, 70, 115, 150), and the genetic content of most of these has yet to be defined. One other potential phage-like mechanism for the transfer of smaller fragments of DNA is gene transfer agents (GTAs), such as those described for *Bacillus subtilis* (PBSX), *Rhodobacter capsulatus*, and the spirochete *Brachyspira hyodysenteriae* (VSH-1) (75, 93, 119, 174). These agents are phage particles that package genomic DNA of their bacterial hosts rather than a viral genome; this genetic material can then be introduced into new hosts (93, 155, 156). In at least one instance, a phage-like particle that packaged heterogenous regions of the borrelial genome rather than a distinct viral genome was observed in a lysed culture of strain CA-11.2A (50). Although transduction has never been demonstrated in *B. burgdorferi* using this rarely seen phage-like particle, the existence of a GTA in the Lyme disease spirochete could explain the observation that HGT throughout the *B. burgdorferi* genome involves primarily small regions of DNA, such as would be transduced by such an agent (69).

Antigenic Variation and Recombination

B. burgdorferi must evade the adaptive immune response to persist in the vertebrate host (130). Scrambling the exposed antigenic epitopes of a major surface protein during host infection is a strategy utilized by many other persistent pathogenic bacteria, including *Campylobacter jejuni*, *Neisseria gonorrhoeae*, *Treponema pallidum*, and *Borrelia hermsii*. However, the molecular mechanisms employed by *B. burgdorferi* to generate antigenic diversity are unusual.

Norris and colleagues (177), in a remarkable study published before the *B. burgdorferi* genome sequence was released (57), discovered the *vls* (variable major protein-like sequence) system. This antigenic variation system creates highly diverse epitopes of the outer membrane VlsE lipoprotein during mammalian infection. The *vls* system first described in strain B31 is located on lp28-1 and consists of the expression locus *vlsE* and a contiguous string of ~15 silent cassettes directly upstream of the expression locus that are highly homologous to the central 570 bp of *vlsE*. The central region of *vlsE* and each silent cassette are flanked by identical 17-bp direct repeats that likely facilitate gene conversion at the *vlsE* locus by the silent cassettes. Gene conversion, i.e., nonreciprocal homologous recombination, shuffles the DNA sequence at the *vlsE* expression locus while the sequence of the silent cassettes remains constant (178). Once activated during vertebrate infection, by as yet unknown signals, gene conversion at the *vlsE* locus alters the amino acid sequence of the surface exposed regions, thus conferring an adaptive advantage to these recombinant *B. burgdorferi* compared with the parental clone that retains the original *vlsE* sequence and antigenic profile.

vlsE recombination has been observed only in the mouse and rabbit models of infection; the recombination system does not seem to operate in culture or in the tick vector (55, 76, 117, 177). Antigenic variation begins four days after mouse infection, which results in the parental *vlsE* sequence disappearing from the population by 28 days postinfection (41, 179). In this manner, incredible VlsE antigenic diversity can be achieved (41). The variation rate is faster in immunocompetent mice compared with immunodeficient mice, which suggests that the VlsE sequence is a target for selection by the adaptive immune response. Furthermore, the mouse adaptive immune response recognizes VlsE as an antigen, and antibodies generated against the parental VlsE show reduced binding to the altered VlsE from *B. burgdorferi* isolated following mouse infection (110, 177). In strains that have lost lp28-1 or have had the *vls* locus deleted, infection of immunocompetent mice is not abolished but attenuated, lasting only three

weeks and restricted mainly to the joints (7, 90, 126, 177). Taken together, these data illustrate that *vlsE* recombination is essential for persistent mammalian infection and maintenance of the enzootic cycle.

The mechanism of *vlsE* recombination has not yet been fully elucidated. *B. burgdorferi* lacks many DNA repair genes implicated in antigenic variation in other bacteria, and the DNA repair and recombination genes *bbg32*, *mag*, *mfd*, *mutL*, *mutS*, *nth*, *priQ*, *nucA*, *recA*, *recG*, *recJ*, *rep*, *sbcC*, and *sbcD* are not required (45, 98). RuvA and RuvB, which catalyze Holliday junction migration, are the only gene products that have been shown to be required for efficient *vlsE* conversion (45, 97). Mutations in either of these genes dramatically reduce *vlsE* recombination and, correspondingly, infectivity in immunocompetent mice. The RuvABC Holliday junction branch migrase in *Escherichia coli* and other bacteria facilitates formation of heteroduplex DNA during homologous recombination and DNA repair (170). One can imagine that this enzyme complex could play a key role in bringing together the homologous regions of the silent cassettes and *vlsE* during gene conversion in *B. burgdorferi*, but the signals for activation and the details of the mechanism remain elusive.

While exhaustively sequencing *vlsE* gene conversion products in *B. burgdorferi* clones isolated from mice, Coutte et al. (41) observed what they term intermittent recombination events in which the *vlsE* sequences were mosaics of the parental and silent cassettes; this suggested that the crossover unexpectedly skipped back and forth. Knight et al. (82) also noticed this hop and skip recombination when introducing site-directed mutations into the *gyrA* gene to disrupt *gac*, which encodes a novel DNA-binding protein (83). Three sets of *gac* mutations (the Shine-Dalgarno sequence, the start methionine codon, and the second methionine codon) were synthesized on a transformation substrate linked to an upstream coumarin-resistant *gyrB* allele. One transformant had the upstream *gyrB* mutations and the downstream methionine mutations but lacked the Shine-Dalgarno mutations in between. A similar finding was made during fusion of an inducible promoter to *ospC* (63) when a heterologous *ospC* allele was inadvertently used, resulting in a variegated sequence (M.A. Gilbert & D.S. Samuels, unpublished data). Therefore, *B. burgdorferi* appears to have an unusual mechanism for recombination that may generate additional sequence diversity.

POPULATION GENETICS

Current and historical interactions with the external environment and with different species have left decipherable population genetics signatures on the genomes of living organisms. These signatures can be interpreted through analyses of the patterns of genetic and genomic variation within and among species. Recent evolutionary genetics studies of *B. burgdorferi* combine diverse scientific fields to infer the mutational processes, random drift, and population size as well as the selective pressures that have resulted in the genomic structure and the patterns of genetic variation within populations.

Recombination and Linkage Disequilibrium

The evolution of sexual reproduction, which is the exchange of genetic material, has been extensively explored in plants and animals because of both the potential selective benefits and the costs of the process (67, 86, 105). Interest in HGT among asexual prokaryotes has recently surged because of the rapid evolution of bacterial pathogens, which can be fueled by HGT (59, 94, 118). For example, the transfer of antibiotic resistance alleles among bacteria in human institutions, such as hospitals, has resulted in numerous multidrug-resistant human pathogens (5). Curiously, several pathogenic bacteria, including *B. burgdorferi*, appear to experience limited HGT (25, 47, 107). The relationship between HGT and the rate of adaptation by natural selection is described below, along with the empirical evidence of the rate of HGT in *B. burgdorferi* and its effect on

the observed and expected evolutionary rates of the species complex.

Genetic exchange between lineages can dramatically affect the rate of evolution (39, 133, 167). Classical theory indicates that HGT allows for the selective removal of deleterious mutations from populations, thus preventing their accumulation (also called Muller's ratchet) (36). In addition, the Fisher-Muller model posits that HGT brings beneficial mutations together into a single genome, which eliminates clonal interference and accelerates adaptation (167). HGT can also increase the realized strength of natural selection on new mutations so that the frequency of advantageous mutations increases and that of deleterious mutations decreases (133). Theory predicts that HGT increases the realized strength of selection because it prevents mutations from being trapped in their original genetic background and thereby reduces the dilution of direct selection by background selection (35, 132). HGT reduces the extent to which natural selection on new mutations is diluted by stochastic noise generated by collateral selection on genetic backgrounds. Mutations move between genetic backgrounds via HGT in each generation such that a favored mutation can be fixed and a harmful mutation can be purged in any genetic background. Therefore, HGT works equally well in the context of either the accumulation of beneficial mutations or the removal of deleterious mutations because both are a consequence of the same phenomenon: reduced interference between direct versus background selection (35, 132). Consequently, HGT can have a profound effect on the rate of adaptation in natural populations.

HGT is often investigated by assessing the degree to which genes are genetically linked across a genome (linkage disequilibrium). The rate and extent of HGT are negatively correlated with the degree of linkage disequilibrium observed between genes (109). In a purely clonal species, alleles at one locus are always observed in the same genomes with specific alleles at other loci as a result of common descent, whereas HGT disrupts these associations (106). Early analyses of genetic linkage disequilibrium suggested that *B. burgdorferi* sensu lato was one of the most clonal groups of bacteria (108). This conclusion has been consistently supported by multilocus studies (47). For example, no recombination was found between four genes [*p66*, intergenic spacer (IGS), *ospA*, and *ospC*] in 61 *B. burgdorferi* sensu stricto isolates from southern Connecticut and between IGS and *ospC* in 73 *B. afzelii* isolates from southern Sweden (25). However, the same *ospC* allele group can be found in different backgrounds in different geographical regions, implying HGT (12, 24). Interestingly, despite a genome that is extensively fragmented into many plasmids (30, 32, 57), some of which are expected to be easily transferred across lineages (30, 50, 52), alleles are almost always found in linkage disequilibrium with other loci, even when the loci are on different plasmids (6, 24, 25, 47, 109). Until recently, evidence of HGT events was limited to short DNA fragments in genes that were expected to be under strong selection for diversity, such as the *ospC* locus, implying that HGT would be apparent only when transferred fragments were favored by natural selection (78, 99, 169). The limited HGT in *B. burgdorferi* suggests that lineages are likely to carry extensive deleterious mutations, although this prediction is not apparent in recent genomic analyses (69, 129).

Strong linkage disequilibrium among genetic loci can result from several evolutionary and ecological forces, such as a lack of recombination machinery or limited opportunity for gene exchange (47), in addition to small population size (genetic drift). Genetically diverse strains of *B. burgdorferi* are found within the same tick vector or same vertebrate host, suggesting an opportunity for HGT (21, 74, 128), and recombination occurs within genomic lineages of *B. burgdorferi* (30, 32, 51, 160, 178), which suggests that *B. burgdorferi* has the recombination system needed for genetic exchange. Thus, the reasons for high levels of genetic linkage among alleles remained mysterious until recently (69). Furthermore, these data had indicated a severe weakening of the

Linkage disequilibrium: the occurrence of some alleles together more often than would be expected by chance

Intergenic spacer (IGS): located between the 16S and 23S ribosomal DNA and used as a neutral marker in bacterial population genetics

Genetic drift: the change in the frequency of alleles in a population due to random processes

strength of selection to purge deleterious mutations and favor beneficial mutations, both of which are inconsistent with observations from natural populations and genome sequence data, suggesting that strong selection occurs in natural populations (21, 23, 129).

Recent studies of whole genome sequences have found that HGT is much more common than originally thought (12, 69, 129). Gene trees are often inconsistent with the ribosomal spacer region sequence on the chromosome, which is generally used to construct strain phylogenies, strongly suggesting HGT. Unexpectedly, the phylogeny built from sequences of *ospC*, a gene known to undergo extensive HGT, was consistent with the phylogeny from the ribosomal spacer region sequence (6, 129). Collectively, 28 examples of homoplasy could be detected in the other gene trees analyzed (6). Interestingly, the rate of HGT of plasmid-borne loci is approximately 100-fold higher than in chromosomal loci (6, 129). Although *B. burgdorferi* is not strictly clonal, HGT events appear rare in general and are nearly nonexistent on the chromosome. The low rate of HGT in *Borrelia* implies that most or all of the genes showing allelic diversity are under balancing selection in which recombinants have a selective advantage or are genetically linked to such a system under balancing selection.

The DNA fragments transferred in the infrequent HGT events are generally small. Recent analyses of 23 genomes suggest that transferred fragments are generally less than 2,000 base pairs (12, 69). Currently, no data support the transfer of whole plasmids, except the cp32 prophage (51, 160), despite a genome extensively fragmented into many smaller, more easily transferable units. Importantly, each genome analyzed showed evidence of very few HGT events. Rare HGT events that incorporate small fragments would weaken linkage disequilibrium among local sites, as small fragment exchange would affect linkage combinations only on this scale. However, linkage combinations among distant sites would not be affected, resulting in strong linkage disequilibrium at distantly located sites. Analysis of more than 13,500 single-nucleotide polymorphisms from the 23 *B. burgdorferi* genomes showed this counterintuitive pattern of limited linkage disequilibrium among local sites but high genome-wide linkage disequilibrium (69). The small size of fragments transferred and the relative rarity of HGT events may explain why the early studies found near-perfect clonality.

Although HGT is relatively rare, the majority of the sequence diversity in *B. burgdorferi* results from reassortment of preexisting sequence polymorphisms through localized HGT, and only one quarter results from de novo point mutations (69, 129). Importantly, HGT events occur both within and among *B. burgdorferi* sensu lato genospecies (47, 69, 129). HGT of selectively advantageous DNA segments, such as all or part of the *ospC* locus, can quickly increase in frequency within populations. In fact, the data strongly suggest HGT of all or part of *ospC* among lineages within *B. burgdorferi* sensu stricto and among *B. burgdorferi* sensu lato genospecies (12, 69). Thus, despite limited HGT in *B. burgdorferi*, there is compelling evidence that HGT is pervasive enough to allow strong natural selection and adaptation in *B. burgdorferi* populations.

A glaring exception to the modest HGT and transfer of small-sized DNA fragments experienced by most *B. burgdorferi* loci are the cp32s (51, 160). cp32s are transferred among *B. burgdorferi* lineages at a high rate, presumably via transduction (51). Despite the strong linkage disequilibrium observed among most loci, alleles at the *ospE/ospF/elp* (*erp*) locus are in near-perfect linkage equilibrium in *B. burgdorferi* sensu stricto isolates cultured from human patients (48a). The overwhelming impression is that the cp32s transfer between strains much faster than do other loci, and consequently, cp32 genetic variation is not expected to be in linkage disequilibrium with the remainder of the genome.

Genetic Variation and Natural Selection

The patterns of genetic variation in natural populations of *B. burgdorferi* suggest that the

Homoplasy: similarity in DNA sequence or morphological characteristics due to convergent evolution to identical character states rather than due to common ancestry

Balancing selection: a type of natural selection in which genetic diversity is actively maintained in the gene pool of a population

majority of loci are under stabilizing selection or have experienced a recent population bottleneck (102, 127, 166). Both stabilizing selection and low effective population sizes result in very low genetic diversity, as seen in most *B. burgdorferi* loci, especially those located on the chromosome (24, 102, 166). Interestingly, loci with appreciable variation show patterns of diversity reminiscent of balancing selection (68, 69, 129). In other words, the allelic frequency distribution is even more than that expected by chance alone. Genome-wide linkage to a single locus under balancing selection, *ospC*, is likely responsible for maintaining genetic variation at linked loci (69).

ospC is probably the most genetically diverse locus in all *B. burgdorferi* genospecies. In the northeastern United States, 16 OspC major groups are defined as alleles that are less than 2% different in protein sequence and more than 8% different from alleles in other major groups (24, 169). OspC major groups differ by an average of 14%, with the lower end of this variation (8% to 11%) representing variation between three groups that share a segment homogenized by recombination (99, 169). Allele frequencies higher than expected under a neutral drift hypothesis imply that balancing selection preserves genetic variation (21, 128). Functionally, OspC major groups have different serotypes (64). A strong adaptive immune response to OspC occurs early in infection (173). Once infected, a vertebrate cannot be infected by another strain expressing an *ospC* allele from the same major group but can be infected by strains with alleles from other major groups (64). The surface-exposed regions of OspC display the highest amino acid variability, further supporting the serotypic difference hypothesis (53, 88).

The mechanism by which *ospC* diversity is selectively maintained is still debated. Two primary models of balancing selection apply to *B. burgdorferi* populations: negative frequency-dependent selection (NFDS) and multiple niche polymorphisms (MNPs), each of which can account for some, but not all, of the theoretical and empirical data. NFDS occurs when rare genotypes have a selective advantage over common genotypes (66). The strong immune response generated against OspC could give rare serotypes a selective advantage and maintain the *ospC* polymorphism (64, 69) because immune memory to specific OspC serotypes protects vertebrates from future infections with the same serotype but does not prevent infection with alternate OspC serotypes (64, 124). In nature, the number of susceptible hosts would be greater for rare OspC serotypes, and they should increase in frequency, suggesting that NFDS is a plausible hypothesis to explain the *ospC* diversity in natural populations. The NFDS hypothesis is also favored by a recent theoretical analysis showing that the observed diversity at OspC and at other genomic loci could be readily simulated using a simple NFDS model with very few parameters (69).

Alternatively, MNP may be the driving force maintaining genetic diversity at the *ospC* locus. MNP preserves diversity within populations when the environment is heterogeneous and no one serotype has the highest fitness in all environments. The environments, which are the different species of vertebrate hosts that *B. burgdorferi* encounter, are heterogeneous with respect to OspC serotype fitness. Serotype fitness differs dramatically among the examined vertebrate species from natural northeastern forests (21–23, 68). In fact, the majority of serotypes had a fitness of nearly zero in most vertebrate species. That is, only a subset of serotypes can infect and be acquired by ticks from each vertebrate species, and the subsets differ among species (21, 68). Thus, host species selectivity could create the conditions necessary to maintain *ospC* diversity by MNP.

MNP through host selectivity is further supported by analyses of *ospC* serotypes from human infections. Similar to natural host species, only five OspC serotypes (A, B, K, I, and N) commonly infect humans in the Northeast (48, 152, 175). Similar patterns are found in other Lyme disease hotspots and in other *B. burgdorferi* genospecies (9, 24, 91). The OspC serotypes that commonly infect humans are regularly isolated from skin lesions, blood, and the central nervous system of patients,

Stabilizing selection: a type of natural selection in which genetic diversity is removed from the gene pool of a population

Negative frequency-dependent selection (NFDS): a type of balancing selection that occurs when the fitness of a phenotype is inversely correlated with its frequency in the population

Multiple niche polymorphism (MNP): a type of balancing selection that occurs in heterogeneous environments where no one phenotype has the greatest fitness in all environments

whereas all other serotypes are less common in skin lesions, rarely disseminate to blood, and are absent from the central nervous system (1, 48, 91, 152). OspC serotypes that are commonly found in human infections also appear to be more infectious when scaled by the probability of exposure to that OspC serotype from a tick bite (48). Human selectivity, like many biological phenomena, is a bimodally distributed continuum.

Although much of the data support both the NFDS and the MNP hypotheses, several empirical and theoretical observations do not conform to each model. For example, NFDS cannot account for the apparent host species associations seen in natural systems. All hosts, regardless of species, should be infected by each of the OspC serotypes if NFDS maintains *ospC* diversity as implied by theory (66). The preponderance of data revealing that serotypes have differential evolutionary fitness in each host species demonstrates that NFDS cannot be the only selective force maintaining diversity at the *ospC* locus. Furthermore, NFDS should result in serotypes at approximately the same frequency, and the most frequent serotypes should change through time. However, serotypes in natural populations vary in frequency by 20-fold (169), and the dominant types are consistent through time in each location, again suggesting that NFDS by immune exclusion is not the major cause of balancing selection.

Theoretical and empirical observations are more concordant with the MNP hypothesis, although several observations remain unresolved. First, models of MNP are likely to require more parameters than NFDS (69), indicating they are a less parsimonious explanation, although this does not rule out MNP as the force maintaining *ospC* diversity. Second, the same OspC types are found in many geographic areas despite different host species compositions (12, 24, 74, 102, 123). However, even in single geographic areas, the majority of OspC serotypes infect multiple evolutionarily divergent vertebrate species, so they can likely infect species in multiple geographic regions (1, 21, 22, 68). Finally, and most importantly, there appear to be many more OspC serotypes than there are host species. A predominance of data demonstrates that most *B. burgdorferi* sensu stricto use only five vertebrate species as a host in the northeastern United States, whereas at least 16 OspC types are maintained (20, 23, 128, 169). No model of MNP can selectively maintain more alleles than there are ecologically distinct niches. However, there are many vertebrate species that have yet to be investigated that host at least 20% of the *B. burgdorferi* sensu stricto population and collectively could account for the remaining niche space (23). Furthermore, there is growing evidence of tissue specificity within hosts for different OspC serotypes (19), suggesting that host tissues within each species may further divide the niche space and thus account for the remaining niches. Although MNP may not be the only force maintaining *ospC* diversity, the current empirical evidence is more consistent with the MNP rather than the NFDS model.

SUMMARY POINTS

1. *B. burgdorferi*, a spirochete, is an extracellular pathogen whose lifestyle is restricted to cycling between *Ixodes* ticks and vertebrate hosts. Its unique genome is highly segmented and includes linear DNA molecules.
2. The taxonomic diversity of the Lyme disease *Borrelia*, of which only three genospecies cause human disease, resulted from an explosive species radiation near the origin of the species group.

3. Almost all mutants that have been selected for are resistant to antibiotics that target a topoisomerase or the ribosome. Systems to genetically manipulate *B. burgdorferi* have been developed based on electrotransformation and antibiotic-resistant selectable markers.
4. *B. burgdorferi* evades the adaptive immune response in the mammalian host by antigenic variation of a surface lipoprotein, VlsE, using an unusual system of gene conversion.
5. One likely mechanism of HGT in *B. burgdorferi* is via transduction. All *B. burgdorferi* isolates carry a family of prophages as 32-kb circular plasmids. The bacteriophage that packages these cp32s, designated ϕBB-1, can shuttle them between cells in vitro and potentially in vivo.
6. The modest HGT among lineages is sufficient to allow strong natural selection and adaptation in *B. burgdorferi* populations.
7. The polymorphism at the *ospC* locus is maintained by a combination of MNP and NFDS.

FUTURE ISSUES

1. Why is the *B. burgdorferi* genome so highly segmented?
2. What are the mammalian signals that induce *vlsE* recombination, and what are the molecular details of the mechanism?
3. What is the role of transduction by cp32 prophage in HGT throughout the enzootic cycle?
4. What cp32 genes encode phage structural elements? Are other plasmids, such as lp28-2, capable of being packaged into virions and transduced between cells?
5. What is the evolutionary history of *B. burgdorferi*? Multilocus studies have resulted in a much more robust understanding of the evolutionary, demographic, and migratory history of *B. burgdorferi* than the previous single-locus analyses. However, there is still very limited information at each locus, suggesting that genomic-level studies will dramatically increase analytical resolution.
6. To what degree do MNP and NFDS maintain polymorphisms? This would begin primarily as a modeling exercise but should identify areas that can be empirically investigated.
7. Are other polymorphic loci in linkage disequilibrium with OspC owing to selection (association of these alleles is selectively advantageous) or because of lack of HGT?

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RELATED RESOURCES

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