

ospC Diversity in *Borrelia burgdorferi*: Different Hosts Are Different Niches

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ABSTRACT

The outer surface protein C (*ospC*) locus of the Lyme disease bacterium, *Borrelia burgdorferi*, is at least an order of magnitude more variable than other genes in the species. This variation is classified into 22 *ospC* major groups, 15 of which are found in the northeastern United States. The frequency distributions of *ospC* within populations suggest that this locus is under balancing selection. In multiple-niche polymorphism, a type of balancing selection, diversity within a population can be maintained when the environment is heterogeneous and no one genotype has the highest fitness in all environments. Genetically different individuals within vertebrate species and different vertebrate species constitute diverse environments for *B. burgdorferi*. We examined four important host species of *B. burgdorferi* and found that the strains that infected each species had different sets of *ospC* major groups. We found no variation among conspecific hosts in the *ospC* major groups of their infecting strains. These results suggest multiple niches create balancing selection at the *ospC* locus.

BALANCED polymorphisms within populations can be used to understand the dynamics of natural selection. Most of the genetic polymorphisms observed in natural populations are neutral or nearly neutral alleles that are dominated by genetic drift and carry little information about the ecological interactions or the selection pressures a population faces (KIMURA and OHTA 1971; OHTA 1992). However, the few loci with multiple alleles and long coalescence times are generally maintained by balancing selection (CHARLESWORTH *et al.* 1997; MAY *et al.* 1999). Investigations of these loci can increase our understanding of the dynamics of natural selection and the ecological interactions driving selection in natural populations. We propose that the polymorphism at the outer surface protein C (*ospC*) locus of *Borrelia burgdorferi sensu stricto*, the etiological agent of Lyme disease, will provide insight into the mechanisms of selection in natural populations.

Lyme disease is the most prevalent vector-borne disease in the United States and Europe (JOHNS *et al.* 2001). *B. burgdorferi s.s.* (Spirochaetaceae) is an obligate internal parasite that is vectored between vertebrate hosts by hard-bodied ticks of the *Ixodes ricinus* complex (BURGDORFER *et al.* 1989; LANE *et al.* 1991; BOSLER 1993; DE SILVA and FIKRIG 1997; SHIH and CHAO 2002). To persist in nature, *B. burgdorferi s.s.* must remain in this zoonotic cycle because neither the vertebrates nor the vectors can transmit *B. burgdorferi s.s.* from mother to offspring (MAGNARELLI *et al.* 1987; BURGDORFER *et al.*

1988). *I. scapularis*, the exclusive vector for *B. burgdorferi s.s.* in the northeastern United States, feeds a maximum of three times during its two-year life cycle, once at each of the three non-egg life stages (WILSON and SPIELMAN 1985; ANDERSON 1989; BOSLER 1993). The proliferation of *B. burgdorferi s.s.* depends strongly on when, during the year, each *I. scapularis* subadult stage actively seeks a bloodmeal. Small mammals and ground-foraging birds become infected with *B. burgdorferi s.s.* in the early summer when infected nymphs, the second non-egg life stage, feed on them. Larvae, the first non-egg life stage, of the succeeding tick cohort, then feed on these infected hosts in the late summer and become infected before they molt to nymphs, renewing the cycle (ANDERSON 1989; BOSLER 1993). Transmission of *B. burgdorferi s.s.* is enhanced because the nymphal season occurs before the larval season, increasing the probability a larva feeds upon a *B. burgdorferi s.s.*-infected animal (LANE *et al.* 1991). Adult *I. scapularis* need not be considered in the life history strategy of *B. burgdorferi s.s.* because the adults generally parasitize a different set of vertebrate species than do the subadults, effectively removing adult ticks from the zoonotic cycle (ANDERSON 1989).

B. burgdorferi s.s. expresses *ospC* when it migrates from the midgut of the tick to the salivary glands during tick feeding and for the first 10 days after it enters the vertebrate host (FINGERLE *et al.* 1995; SCHWAN *et al.* 1995; MONTGOMERY *et al.* 1996; SCHWAN and PIESMAN 2000). *OspC* is one of the first and most heavily targeted borrelial antigens by the vertebrate immune system (WILSKE *et al.* 1986, 1993; DRESSLER *et al.* 1993; FUNG *et al.* 1994; MARGOLIS *et al.* 1994; STEVENSON and BARTHOLD 1994; ENGSTROM *et al.* 1995). The immune re-

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sponse poses a strong selective pressure on *ospC*, as the immune system quickly eliminates *B. burgdorferi* s.s. with antigenic OspC epitopes (PREAC-MURISIC *et al.* 1992; GILMORE *et al.* 1996). It has been suggested that the selection pressure from the vertebrate immune response is responsible for the *ospC* polymorphism (QIU *et al.* 1997; WANG *et al.* 1999).

ospC is a highly polymorphic single-copy gene with a long coalescence time compared to neutrally evolving genes (DYKHUIZEN *et al.* 1993; JAURISHEIPKE *et al.* 1993; MASUZAWA *et al.* 1997; WANG *et al.* 1999; BARANTON *et al.* 2001). The genetic diversity at the *ospC* locus has been classified into major groups of alleles. An *ospC* major group is defined as a group of alleles that are different in >8% of their nucleotide sequence from alleles in other major groups (average difference of ~20%) and <2% different from other alleles in the same major group (average difference of <1%; WANG *et al.* 1999). No reported sequences are between 2 and 8% different from any other sequence. Of the 22 *ospC* major groups found worldwide in *B. burgdorferi* s.s., four are found only in Europe (SEINOST *et al.* 1999) and one has recently been discovered in the southeastern United States (LIN *et al.* 2002). Of the remaining *ospC* major groups, 15 occur at fairly even frequencies in every well-sampled population in the northeastern United States (SEINOST *et al.* 1999; WANG *et al.* 1999; QIU *et al.* 2002). For the sake of brevity and clarity, we refer to all strains of *B. burgdorferi* s.s. with the same *ospC* major group as an oMG. For example, all strains with an allele from *ospC* major group A are called oMG A. We reserve the term *ospC* major group for discussions of the alleles at the genetic locus.

Loci that are dominated by neutral processes are expected to have few alleles at relatively high frequencies within a population. The *ospC* major groups violate this expectation, suggesting that the *ospC* polymorphism is not neutral (WANG *et al.* 1999). The polymorphism is probably not maintained by a balance between local selection and high migration between populations, as local tick populations are differentiated at the 16S rRNA mitochondrial gene, suggesting a very low migration rate of ticks between populations (QIU *et al.* 2002). Balancing selection, on the other hand, is expected to preserve multiple alleles at intermediate frequencies with high sequence diversity in a population (VEKEMANS and SLATKIN 1994), giving them deeper coalescence than neutrally evolving alleles (MAY *et al.* 1999; SCHIERUP *et al.* 2000), as seen at the *ospC* locus (WANG *et al.* 1999; LIN *et al.* 2002; QIU *et al.* 2002).

In haploid organisms such as *B. burgdorferi* s.s., the most likely forms of balancing selection are negative frequency-dependent selection and multiple niche polymorphism. Negative frequency-dependent selection, which occurs in populations when rare genotypes have a selective advantage over common genotypes (GROMKO 1977), has been shown to maintain polymorphisms in

natural populations, including many host-parasite systems (THRALL *et al.* 1995; BRUNET and MUNDT 2000; UYENOYAMA 2000; UYENOYAMA *et al.* 2001). Negative frequency-dependent selection caused by a strong secondary immune response to OspC could maintain the *ospC* polymorphism. Animals cannot clear a *B. burgdorferi* s.s. infection with an immune response, but the immune memory to OspC protects them from future infections of the same strain (BARTHOLD 1999). Therefore, an animal previously infected by one strain of *B. burgdorferi* s.s. cannot be reinfected by another strain with the same *ospC* major group due to a secondary immune response, or immune memory, to OspC. However, those animals are still susceptible to infection by strains with different *ospC* major groups (PREAC-MURISIC *et al.* 1992; GILMORE *et al.* 1996; PROBERT *et al.* 1997; BARTHOLD 1999). Rare oMG's could therefore have a selective advantage in natural systems. With simple negative frequency dependence, all of the oMG's would be at approximately the same frequency at equilibrium. In natural populations, however, oMG's vary ~20-fold in frequencies (QIU *et al.* 1997; WANG *et al.* 1999). If a secondary immune response to OspC is the only factor preventing *B. burgdorferi* s.s. from infecting an animal, every host would be infected by all of the oMG's that they had been challenged with during the nymphal season.

Multiple-niche polymorphism, also referred to as diversifying selection, is a conceptually possible model with a long history in the theoretical literature (LEVENE 1953; GLIDDON and STROBECK 1975; STROBECK 1979; CZOCHOR and LEONARD 1982). Yet there is very little uncontroversial evidence that this type of selection has maintained polymorphisms in natural systems (HEDRICK 1986). Multiple-niche polymorphism can maintain diversity within populations when the environment is heterogeneous and no one genotype has the highest fitness in all environments. The environments *B. burgdorferi* s.s. may encounter, the different species of vertebrate hosts, may be very heterogeneous. If the fitness of a strain depends upon an interaction between the *ospC* major group and a factor unique to the host species, the frequency distribution of the oMG's should differ significantly among vertebrate species. Conspecific individual vertebrates, regardless of species, may also be heterogeneous environments for *B. burgdorferi* s.s. due to variability among individuals and might also vary significantly in the frequency distribution of oMG's. Differences between species and variability within species are not mutually exclusive and both could be operating in this system to maintain the polymorphism at *ospC*.

In this study we present evidence that the diversity observed at the outer surface protein C locus of *B. burgdorferi* s.s. is a multiple-niche polymorphism due to differences among host species. The frequency distributions of oMG's are not different among individuals, suggesting that variability among individuals within a species is not creating a heterogeneous environment for *B.*

burgdorferi s.s. These data suggest that interspecies multiple-niche polymorphism is necessary and sufficient to maintain the polymorphism at the *ospC* locus. However, our data do not reject the possibility that negative frequency-dependent selection is also occurring in this system, but it is neither necessary nor sufficient to explain our data. Negative frequency-dependent selection and multiple-niche polymorphism are not mutually exclusive forces and may both contribute to the creation and maintenance of this polymorphism.

MATERIALS AND METHODS

We collected larval ticks that had fed on four species of vertebrates trapped in a natural system to determine the oMG's that had infected each animal. Because *B. burgdorferi* s.s. is not transmitted from mother to offspring in *I. scapularis*, we assumed that every oMG detected in a larva had also infected the host that larva had fed on. We chose to examine engorged larvae, as opposed to testing animal tissues directly, for two reasons. Testing several larvae that have fed upon a vertebrate can provide a quantitative assessment of the variability among conspecifics. In addition, we do not know if all the oMG's that a host is infected with can be detected in a small skin sample. We were able to account for all of the oMG diversity within a host and test for variation among conspecifics and among species by sampling engorged larvae from each host. In most instances, all of the variability within a host was detected by sampling five larvae from that host.

Collection: Four important host species for *B. burgdorferi* s.s., *Peromyscus leucopus* (white-footed mouse), *Tamias striatus* (eastern chipmunk), *Blarina brevicauda* (short-tailed shrew), and *Sciurus carolinensis* (gray squirrels), were trapped at the Institute of Ecosystem Studies in Millbrook, New York, in August 2002 (SCHMIDT *et al.* 1999). Captured animals were housed in the Institute of Ecosystem Studies rearing facility for 72 hr in wire mesh cages suspended over shallow water to allow the larval *I. scapularis* to feed to repletion and drop off. The engorged larvae were collected from the water twice daily into microfuge tubes. Microfuge tubes were stored at -20° until the DNA was extracted. In addition, we collected 188 host-seeking nymphs at the Institute of Ecosystem Studies in July 2002 to assess the frequencies of oMG's in the cohort of nymphs that infected the individuals trapped in this study.

Extraction: One tick was placed in a microfuge tube and homogenized with a sterile 200- μ l pipet tip in 100 μ l of a 5% Chelex resin (Bio-Rad, Richmond, CA) solution in water. A total of 900 μ l of 5% Chelex was added to each tube (no extra 5% Chelex was added to preparations of the foraging nymphs), which was subsequently vortexed for 30 sec. The tubes were then rocked gently overnight at 56° , vortexed for 30 sec, heated to 95° for 15 min, vortexed for 30 sec, and finally centrifuged at 10,000 rpm. We found that 1 ml of 5% Chelex solution was necessary to reduce the concentration of inhibitors found in engorged larvae to permit successful PCR.

Semi-nested PCR: A 594-bp fragment of the *ospC* locus was amplified using semi-nested PCR. The semi-nested PCR protocol is more sensitive than the PCR-based method previously reported (QIU *et al.* 2002), although more prone to contamination. PCR contamination was carefully avoided by using barrier pipet tips and negative controls. First, a 617-bp fragment was amplified using primers OC6(+) and OC623(-) (primer sequences are reported in QIU *et al.* 2002). Each 50- μ l PCR reaction contained 10 μ l of the DNA extraction, 50 mM KCl, 10 mM Tris (pH 8.3), 2.5 mM MgCl, 0.5 μ M of each primer,

TABLE 1
Oligonucleotide probes

Designation	Length	Sequence (5'-3')
OC-C182 + 2	20	TGCAAGTAAGTCTCAACTT
OC-C - 7	25	TCCGTTGTTATCTGCCTCATTATCT
OC-G + 3	19	GGTGTGTGATTCCGCATCA
OC-I + 1	24	GTTTGAATTAATATGCTCCTGA
OC-J + 7 - 6	17	TTGACCCACTTCAGCAC

dNTPs at 0.2 mM per nucleotide and 1 unit of recombinant Taq. After an initial denaturation at 95° for 1 min, the mixture was run for 30 cycles at 95° for 40 sec, 54° for 35 sec, and 72° for 1 min. The second round of PCR amplifies a 594-bp fragment using OC6(+Fluo) (a fluorescein label was commercially added to the 5' end) and OC602(-). The reaction conditions for the second round of PCR were identical to the first except 1 μ l of first-round PCR product was used as template and the reaction was run for 40 cycles. A total of 5 μ l of the second PCR reaction was run on a 1.5% agarose gel with ethidium bromide to assess the presence or absence of *B. burgdorferi* s.s. in the sample tick. Amplified fragments of different sizes were never detected, suggesting that these reaction conditions are specific for *ospC*. The PCR products from 66 infected host-seeking nymphs and five infected larvae from each of 15 individuals from each species (10 for *S. carolinensis*) tested were used in the subsequent reverse line blot procedure.

Reverse line blot: The reverse line blot is a very effective, high-throughput procedure that is well suited for systems where the alleles are defined and sequencing is not feasible because multiple alleles are present in a sample. As opposed to standard Southern blots, reverse line blot attaches the probe to the membrane, and a labeled test sample hybridizes to the probes. Many of the previously designed probes, including the positive control probe, work well (QIU *et al.* 2002) and are used in this study. Probes for *ospC* major groups G, I, and J were modified to increase signal strength and reduce potential cross-specificity (Table 1). Two new probes that bind *ospC* major group C were added to include group C in the analysis. *ospC* major group C appears to be a recombinant of alleles from *ospC* major groups I, B, and E, negating the possibility of using a probe unique to major group C. We designed two probes for major group C, one that has dual specificity for *ospC* major groups I and C (OC-C - 7) and one that is specific for major groups E and C (OC-C 182 + 2). With this combination, *ospC* major group C can be distinguished from all other *ospC* major groups and its frequency can be estimated. However, ticks that are infected by both *ospC* major groups I and E will have the same pattern on the reverse line blot membrane as a tick infected with oMG's I, E, and C. The frequencies of oMG's I and E are not affected by this discrepancy, as alleles from major group C do not bind to probes for either *ospC* major groups I or E. *ospC* major group C was not present in our samples from the Institute for Ecosystem Studies nor was any tick infected by both oMG's I and E. Every PCR product run on the reverse line blot hybridized to at least one of our *ospC* major group probes, suggesting that novel oMG's are either not present or in very low frequencies at the Institute for Ecosystem Studies.

The method for reverse line blotting is as follows: 400–600 thymine residues were added to the 3' end of all probes using terminal transferase (Roche Molecular Biochemicals, Indianapolis) to enhance sticking to the nylon membrane. Tailed probes were fixed to a 15- \times 15-cm nylon membrane (Bio-

Rad Zeta-Probe membranes), using a MiniSlot 30 manifold (Immunelect, Cambridge, MA). Forty picomoles of each probe was diluted in 2 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and evenly applied to individual slots (0.8 mm × 13 cm) created by the manifold. Each slot was subsequently washed twice with 2 ml TE buffer, removed from the manifold, and immediately UV-crosslinked (125 mJ). Checkerboard hybridization followed the protocol described in QIU *et al.* (2002). Chemiluminescent detection was performed using the procedure suggested by the manufacturer [digoxigenin (DIG) luminescent detection kit; Roche Molecular Biochemicals], substituting anti-fluorescein-AP, Fab fragments (Roche Molecular Biochemicals) at a 1:1000 dilution for the DIG antibodies. The membrane can be used up to 10 times by stripping the membrane after each use. To reuse a membrane, a boiling 0.1× SSC/0.5% SDS solution was poured over a used membrane and rocked for 15 min. This procedure was repeated two to three times. Membranes were exposed to X-ray film overnight to determine the extent the membrane was stripped. Once prepared, the membrane should be stored at 4° in 2× SSC (0.3 M NaCl, 30 mM sodium citrate, pH 7.0).

Statistical methods: Differences in the frequency distribution of oMG's among species were assessed using hierarchical log-linear analysis of frequencies (SOKAL and ROHLF 1995). If one larva from an individual tested positive for an oMG, that individual was considered infected by that oMG in the statistical analysis. This hierarchical log-linear analysis of frequencies is a three-dimensional contingency table with species, oMG, and counts of presence or absence as the factors. To test for intraspecific variation, we used the number of infected ticks that tested positive for each oMG from each individual in the analysis. In this analysis, the frequency distributions of the oMG's from each individual host are compared in a similar three-dimensional contingency table with conspecific individuals, oMG, and counts of presence or absence as the factors. Intraspecific differences in the number of oMG's that tested positive per infected larva were evaluated using a single-classification ANOVA (SOKAL and ROHLF 1995).

RESULTS

Using PCR and reverse line blotting, we assayed 66 infected host-seeking nymphs from the Institute for Ecosystem Studies and five infected larvae from each of 15 individuals from each vertebrate species except *S. carolinensis*, of which 10 individuals were assayed, for the presence of each oMG. We determined the percentage of host-seeking nymphs that test positive for each oMG, which is a measure of the total oMG diversity at the Institute for Ecosystem Studies. By testing larvae from animals, we determined both the number of larval ticks from each individual that tested positive for each oMG and the number of conspecific individuals that tested positive for each oMG. Differences in the frequency distributions of oMG's among individuals, among species, and between each species and the host-seeking nymphs were tested statistically.

Host-seeking nymphs: A total of 188 host-seeking nymphs collected from the Institute for Ecosystem Studies were processed by PCR of the *ospC* gene to determine the percentage of nymphs infected by *B. burgdorferi s.s.* Of these, 66 (35%) tested positive for *B. burgdorferi s.s.* by PCR, similar to what was found in previous studies

using a direct immunofluorescence antibody-staining assay (Table 2; LoGIUDICE *et al.* 2003). The PCR products from the 66 infected nymphs were subsequently run on a reverse line blot to determine the frequency distribution of oMG's in the cohort of nymphs that infected the animals examined in this study. We detected 15 oMG's at frequencies more even than expected by chance (Table 3), similar to what was observed previously at the Institute for Ecosystem Studies and in other localities in the northeastern United States (QIU *et al.* 2002).

Interspecific differences in oMG frequencies: As reported in previous studies that used a different method to detect *B. burgdorferi s.s.*, we found that many larvae are not infected with *B. burgdorferi s.s.* after feeding on infected animals (Table 2). Moreover, we found that a larva does not carry all the *B. burgdorferi s.s.* oMG's that infect the host on which the larvae had fed. Therefore, we tested five infected ticks from each of 15 individuals from each host species, except *S. carolinensis* where 10 individuals were examined, to determine which oMG's had infected each animal. An individual was considered infected by an oMG if any of the larvae that had fed on that individual tested positive for that oMG. In all vertebrate species except *S. carolinensis*, each oMG was found in either >80% or <15% of the individuals from each species. However, the oMG's that were found in high frequencies differed among the vertebrate species [$P < 0.0001$, d.f. = 42 (4 × 15 × 2), hierarchical log-linear analysis of frequencies; Table 3].

oMG's A, D, F, and K were found at very high frequencies in all four species. oMG G was found at high frequency in three of the species examined; oMG's E, I, and T were in two species; and oMG's B and U were each found at high frequency in only one species. Five oMG's (H, J, L, M, and N) were found in very low frequencies in all of the species examined. It is highly unlikely that these oMG's were not detected due to methodological problems, because all were found in the host-seeking nymphs. oMG's C and O were found in neither the host-seeking nymphs nor any of the vertebrate species.

Low-frequency oMG's: Some oMG's were detected in <3 of the 75 larvae examined from a species. We assessed the possibility that these oMG's were falsely detected by repeating the PCR and reverse line blot from these DNA extractions. Of 21 samples retested, 19 of these rare oMG's were absent in a follow-up test. From 232 samples we experimentally determined that the 100-fold dilution of an engorged larva during the DNA extraction reduces the concentration of different oMG's such that 24% are not detected in a single follow-up test. This may be expected, as the number of *B. burgdorferi s.s.* transmitted from infected vertebrates to feeding larvae is likely to be quite small (BENACH *et al.* 1987). However, low-frequency oMG's were absent in the follow-up PCR products at a much greater rate than expected at the

TABLE 2
Percentages of infected ticks

	<i>P. leucopus</i> (%)	<i>T. striatus</i> (%)	<i>B. brevicauda</i> (%)	<i>S. carolinensis</i> (%)	Nymphs (%)
Semi-nested PCR	78 (± 2.7)	55 (± 3.5)	42.6 (± 3.1)	19.2 (± 2.5)	35
LoGIUDICE <i>et al.</i> (2003)	92.1 (± 1.7)	55 (± 2.5)	41.8 (± 2.6)	14.7 (± 2.3)	37.6 ^a

^a From SCHMIDT and OSTFELD (2001).

DNA dilution we used ($P < 0.0001$, d.f. = 1, chi-square). Thus, we conclude either that many of these low-frequency oMG's were false positives or that they are in a much lower density in the engorged larvae than oMG's that commonly infect a vertebrate species. We discuss possible biological causes of these low-frequency oMG's later in this article.

Distribution of oMG's among conspecific hosts: All 5 larvae from some animals tested negative for oMG's for which most of their conspecific hosts tested positive. For two reasons all 5 larvae may test negative for an oMG even if the host carries it. First, the larvae are infected by only a subset of the oMG's that have infected the animal the larva had fed upon. Every animal, regardless of species, was infected by six to eight oMG's. However, infected larvae carried between one and seven oMG's. On average, larvae that had fed on *P. leucopus* tested positive for 3.19 (± 1.05) oMG's, 3.65 (± 1.23) oMG's were found in larvae from *T. striatus*, 2.73 (± 1.05) were in larvae from *B. brevicauda*, and 1.75 (± 0.83) were in larvae from *S. carolinensis*. Second, the dilution of the larvae for the DNA extraction reduces the density of spirochetes in the PCR reaction so that some oMG's that are in that larva are not amplified. Because an animal may falsely test negative for an oMG by our methods, we calculated the probability that 5 randomly sampled larvae will test negative for an oMG when the host they fed upon is actually infected by that oMG. If all of the individuals in a species are actually infected by an oMG, the probability a randomly sampled larva will test negative for that oMG is equal to the percentage of larvae from that species that actually tested negative for that oMG. For example, 43 of the 75 infected larvae (57%) that had fed on *P. leucopus* tested negative for oMG A (Table 3). The probability that 5 larvae randomly sampled from *P. leucopus* will test negative for oMG A is approximately the fraction of larvae testing negative raised to the fifth power (0.57^5), or 0.06. Thus, we expect that 0.9 ($= 0.06 \times 15$) of the 15 *P. leucopus* examined will test negative for oMG A, even though they are truly infected by this oMG. The number of individuals from each species expected to test negative for each of the oMG's coincides well with the number of individuals that actually tested negative in our sample (Table 4). Sampling too few larvae can account for the oMG's that were not found in a small percentage of the individuals of a species. However, this does not

exclude the possibility of intraspecific variation in resistance to oMG's.

Intraspecific variation: Most of the animals examined were infected by every oMG that commonly infected that animal's conspecifics. However, differences among individuals could be quantitative, as opposed to qualitative. Thus, the frequency in which each oMG tested positive in the larvae from each individual within a species was examined to quantitatively assess the intraspecific differences within each species. We found that there was no significant difference among conspecifics in any of the species examined [$P > 0.85$, d.f. = 196 (*S. carolinensis* d.f. = 126), hierarchical log-linear analysis of frequencies]. In addition, the frequency distributions of the number of oMG's found per infected tick were not significantly different among conspecifics [$P > 0.05$, d.f. = 14 (*S. carolinensis* d.f. = 9), single-classification ANOVA].

An animal may not be infected by an oMG simply because it was never fed upon by a nymph that was infected by that oMG. To control for this possibility, we calculated the probability that any individual we trapped in August 2002 had been exposed to each of the oMG's during the nymphal season. The probability an animal has not been exposed to an oMG is equal to the frequency of that oMG in the host-seeking nymphal population raised to the number of nymphs that have bitten that individual. The oMG frequencies were estimated from the cohort of host-seeking nymphal ticks that infected the small mammals sampled in this study (Table 3). We calculated the probability that an individual *T. striatus* or an individual *P. leucopus* we caught had been exposed to each of the oMG's using the average number of nymphs per individual. We estimated that an average of 60 nymphs fed on an individual *P. leucopus*, and 250 nymphs fed on *T. striatus*, during the nymphal host-seeking season. These data were collected in May, June, and July 2002 by counting the number of nymphs on the head of a live animal (R. S. OSTFELD, unpublished data). Visual counts are well correlated with, and are a conservative estimate of, the actual number of nymphs on an individual (R. S. OSTFELD, personal communication), leading to a conservative estimate of the probability of exposure. Nymphal burden data are lacking for *S. carolinensis* and *B. brevicauda*, although *S. carolinensis* are parasitized by a greater number of nymphs than *T. striatus* in August, and *B. brevicauda* are parasit-

TABLE 3

Frequency distribution of oMG's from four species of reservoir host

	A ^a	B	D	E	F	G	H	I	J	K	L	M	N	T	U
<i>P. leucopus</i> (N = 15) 5 ticks each	<u>15</u> ^b (32) ^c	14 (27)	15 (43)	2 (3)	15 (26)	14 (31)	1 (1)	<u>12</u> (20)	0 (0)	<u>15</u> (51)	0 (0)	1 (2)	1 (1)	2 (2)	0 (0)
<i>T. striatus</i> (N = 15) 5 ticks each	15 (28)	<u>1</u> (1)	15 (48)	2 (3)	13 (31)	13 (32)	1 (1)	<u>13</u> (20)	0 (0)	<u>15</u> (49)	2 (3)	2 (2)	1 (1)	14 (33)	12 (17)
<i>B. brevicauda</i> (N = 15) 5 ticks each	<u>15</u> (24)	<u>1</u> (1)	14 (36)	13 (30)	14 (19)	2 (4)	0 (0)	<u>1</u> (1)	0 (0)	<u>15</u> (63)	1 (1)	0 (0)	1 (1)	13 (22)	0 (0)
<i>S. carolinensis</i> (N = 10) 5 ticks each	<u>9</u> (19)	<u>1</u> (2)	6 (8)	5 (10)	7 (9)	5 (6)	1 (1)	<u>0</u> (0)	0 (0)	<u>10</u> (26)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Nymph ^d	14.9	9.6	13.8	8	8.5	9	6.4	5.9	3.7	23.9	1.1	9.6	2.7	4.8	2.1

Numbers in regular type represent oMG's where >80% of the individuals of a species tested positive. Numbers on shaded background represent oMG's where <15% of the individuals tested positive. Italic numbers represent oMG's where 15–80% of the individuals tested positive.

^a Underlined entries are the four oMG's (A, B, I, and K) that cause disseminated Lyme disease in humans (SEINOST *et al.* 1999).

^b The number of individuals where at least one larva tested positive for the oMG.

^c The number of larvae that tested positive for that oMG in the species.

^d The percentage of each oMG in the total host-seeking nymph population (both infected and noninfected nymphs).

ized by a fewer number than *P. leucopus* (Table 5). These data suggest that almost all animals had been exposed to all oMG's, so that lack of exposure is not the cause of individuals testing negative (Table 6).

DISCUSSION

The frequency distributions of oMG's differ significantly among vertebrate species, suggesting species-specific rejection of certain oMG's. Each oMG was detected in either >80% or <15% of the individuals from a species (Table 3). The individuals that tested negative for common oMG's likely tested negative because too few larvae were examined. Low-frequency oMG's were usually found in only 1 or 2 of the 75 larvae sampled from the entire species and many can be attributed to false positives. Members of a host species appear to be uniformly resistant or susceptible to any given oMG. Similarly, only four oMG's, A, B, I, and K, can establish an infection in humans (SEINOST *et al.* 1999). We believe that these other vertebrate species, like humans, can be infected only by certain oMG's.

Interspecies multiple-niche polymorphism: Our results demonstrate that none of the supposed principal reservoir host species at our study site (SCHMIDT *et al.* 1999) were infected by all of the 15 oMG's present in host-seeking nymphs. Each vertebrate species was infected by different subsets of oMG's (Table 3). Thus, the strain that an individual can and cannot reject is determined by an interaction between the *ospC* major group, or a closely linked gene, and features unique to each species. We propose that variation in the ability of oMG's to infect different vertebrate species is the primary selective force that maintains the *ospC* polymorphism.

The inability to infect a vertebrate species due to *ospC*

may have at least two possible causes. Differences in the function of the OspC protein among oMG's may determine whether a spirochete can establish an infection. The function of OspC is not known, but it has been speculated that it is an adhesin that binds to vertebrate connective tissue and may be important in establishing an infection. Alternatively, the interspecific variation in the vertebrate immune system may provide resistance to infection by certain oMG's. A vertebrate immune system that quickly eliminates one amino acid sequence may not recognize a highly divergent sequence in the early stages of *B. burgdorferi s.s.* infection, allowing the later oMG to establish an infection. Further investigations of the interaction between OspC and the vertebrate hosts are necessary to test these hypotheses.

Conceivably, balancing selection acts not on the *ospC* locus, but on a closely linked locus. With the possible exception of genes on a prophage-like plasmid (STEVENSON *et al.* 1998; EGGERS and SAMUELS 1999; EGGERS *et al.* 2000; STEVENSON and MILLER 2003), linkage disequilibrium is very high in *B. burgdorferi s.s.* (DYKHUIZEN *et al.* 1993; BALMELLI and PIFFARETTI 1996; DYKHUIZEN and BARANTON 2001), lending support to this hypothesis. Such a linked gene would have to be at least as variable as *ospC* to produce the patterns seen in this and previous studies (WANG *et al.* 1999; QIU *et al.* 2002). However, in a recent study of 17 highly polymorphic loci in 22 *B. burgdorferi s.s.* isolates, only two to four alleles were detected at all loci except *ospC*, of which 11 major groups were found (QIU *et al.* 2004). Thus, we believe that *ospC* is most likely the locus under balancing selection. Regardless of the target of selection, our data suggest that variation among host species is maintaining the polymorphism at the *ospC* locus.

Variation among individuals within species: There is no evidence of individual variation in resistance to dif-

TABLE 4
The actual and expected number of individuals testing negative for each oMG

	<u>A</u>	<u>B</u>	<u>D</u>	<u>E</u>	<u>F</u>	<u>G</u>	<u>I</u>	<u>K</u>	<u>T</u>	<u>U</u>
<i>P. leucopus</i>	0 ^a <u>0.93</u> ^b	1 <u>1.6</u>	0 <u>0.21</u>	–	0 <u>1.8</u>	1 <u>1</u>	<u>3</u> <u>3.2</u>	<u>0</u> <u>0.05</u>	–	–
<i>T. striatus</i>	0 <u>1.5</u>	–	0 <u>0.1</u>	–	2 <u>1</u>	2 <u>0.9</u>	2 <u>3.2</u>	0 <u>0.1</u>	1 <u>0.8</u>	3 <u>4.2</u>
<i>B. brevicauda</i>	0 <u>2.2</u>	–	1 <u>0.6</u>	2 <u>1.2</u>	1 <u>3.5</u>	–	–	0 <u>0</u>	2 <u>2.6</u>	–
<i>S. carolinensis</i>	1 <u>2.3</u>	–	4 <u>5.7</u>	5 <u>4.9</u>	3 <u>5.3</u>	5 <u>6.6</u>	–	0 <u>1.2</u>	–	–

–, oMG's that do not infect the species.

^a Regular type, number of individuals testing negative in this sample.

^b Underlined type, number of individuals expected to test negative.

ferent oMG's. Every individual examined tested positive for nearly all of the oMG's that are commonly found in that species (Table 3). Exceptions are likely due to sampling too few larvae (Table 4). However, it is possible that some of the individuals may be resistant to oMG's that commonly infect its conspecifics. The current data suggest that variation among individuals within a species is insignificant and does not affect our conclusions.

Although most apparent occurrences of very rare oMG's appear to result from experimental error, larvae do seem, rarely, to carry oMG's to which the host species they fed upon is seemingly resistant. Three biological processes could be responsible for these observations.

1. oMG's that do not cause a disseminated infection in humans are found at a low frequency in the skin near the site of nymphal attachment (SEINOST *et al.* 1999). Therefore, larvae that are feeding on a vertebrate host near a feeding nymph, a process known as cofeeding, can become infected by *B. burgdorferi* s.s. that have not disseminated in the host (RANDOLPH *et al.* 1996; PATRICAN 1997; PIESMAN and HAPP 2001; RANDOLPH and GERN 2003).
2. Larvae that do not complete a bloodmeal will seek a new host (our personal observation), carrying with them the *B. burgdorferi* s.s. acquired during the first meal, perhaps including strains that are not present in the second host.
3. Stochastic events such as depression of the host's

immune system might account for the rare oMG occurrence.

Because the data suggest that very few larvae are infected by any of these events, none are likely to significantly affect the frequency of the oMG's.

oMG's found in very low frequencies in all vertebrate species examined: The vertebrate species examined in this study are believed to be the principal hosts for *B. burgdorferi* s.s. because they support large numbers of larvae (Table 5) that have a high frequency of infection (Table 2; ANDERSON 1988; LANE *et al.* 1991; OSTFELD *et al.* 1996; SCHMIDT *et al.* 1999). However, our data suggest that a significant proportion of the infected host-seeking nymphs obtained *B. burgdorferi* s.s. from other vertebrate host species. For example, oMG M, which does not infect any of these four species, is often detected in host-seeking nymphs. Four other oMG's are not observed in any of these species, but are observed in host-seeking nymphs, albeit at a relatively low frequencies. These oMG's are likely to infect at least one of the many other vertebrate species that *I. scapularis* subadults parasitize (ANDERSON 1988).

Negative frequency-dependent selection: Previous investigators hypothesized that the polymorphism at the *ospC* locus is maintained by negative frequency-dependent selection caused by a strong secondary immune response to OspC (WANG *et al.* 1999). A strong secondary immune response to OspC prevents an animal from being infected by an oMG that previously infected the animal (PREAC-MURSIC *et al.* 1992; GILMORE *et al.* 1996; BARTHOLD 1999), but does not prevent infection by strains with other *ospC* major groups (PROBERT *et al.* 1997). This process gives rare oMG's a selective advantage over common oMG's. If the secondary immune response to OspC were the only force rejecting *B. burgdorferi* s.s., every oMG would be present in most of the animals (Table 6). The data from this study are not consistent with this model, suggesting that negative frequency-dependent selection is neither necessary nor suf-

TABLE 5

Average tick burden per individual when trapped

	Larvae	Nymphs
<i>P. leucopus</i>	38.97	0.58
<i>T. striatus</i>	34.8	1.22
<i>B. brevicauda</i>	57.47	0.06
<i>S. carolinensis</i>	49.03	2.56

TABLE 6

The probability an individual has been challenged by each of the oMG's

	A (%)	B (%)	D (%)	E (%)	F (%)	G (%)	H (%)	I (%)	J (%)	K (%)	L (%)	M (%)	N (%)	T (%)	U (%)
<i>P. leucopus</i> (60/yr) ^a	>99	>99	>99	99	99	>99	98	97	89	>99	47	>99	79	94	72
<i>T. striatus</i> (250/yr) ^a	>99	>99	>99	>99	>99	>99	>99	>99	>99	>99	93	>99	>99	>99	99

^a An estimation of the number of nymphs per year on each individual.

ficient to maintain the polymorphism at *ospC*. Negative frequency-dependent selection could play a role within host species. However, a model incorporating only inter-species multiple-niche polymorphism is sufficient to explain the maintenance of the polymorphism.

Multiple-niche polymorphism model: A model consisting of 15 “*ospC* niches,” one niche for each oMG, can sufficiently maintain the observed polymorphism in *B. burgdorferi s.s.* populations. An *ospC* niche is defined as the assemblage of vertebrate species that are susceptible to an oMG. For instance, *P. leucopus* is part of the niche for oMG B, while *T. striatus* is part of the niche for oMG U (Table 3). The fitness of oMG B is much greater than that of oMG U in *P. leucopus*, while the opposite is true in *T. striatus*. In fact, the fitness of oMG's U and B are approximately zero in *P. leucopus* and *T. striatus*, respectively. The *ospC* polymorphism persists because no oMG has the greatest fitness in all *ospC* niches. This model is sufficient to maintain the polymorphism and is the most parsimonious explanation of the data collected in this study and previous studies (WANG *et al.* 1999; QIU *et al.* 2002), but it does not exclude negative frequency-dependent selection as a subsidiary factor.

In localities where *B. burgdorferi s.s.* is prevalent in host-seeking nymphs, it is likely that every individual from each host species has been challenged by, and is infected with, every oMG that can infect that species (Table 6). Our model suggests that in these areas, the frequency distribution of oMG's in the nymph population is determined by the relative densities of each vertebrate species, the relative number of larvae that feed on individuals from each species, and the percentage of larvae that become infected with each oMG from feeding on that species. We are assuming that other host species not examined are part of some, but not all, of the *ospC* niches, similar to the four species examined in this study. These species could include some of the 31 species of mammals from seven orders, 49 species of birds from 17 families, and several species of reptiles that *I. scapularis* has been reported to feed on (ANDERSON 1988, 1989; LANE *et al.* 1991).

Annual *ospC* frequency distribution fluctuations: The frequency distributions of oMG's in host-seeking tick populations are homogeneous across space within years, but are significantly different among years (QIU *et al.*

1997). The data in this study suggest that these dramatic changes in the frequency distributions arise from fluctuations in the population densities of the reservoir hosts, many of which are known to fluctuate dramatically (GOODWIN *et al.* 2001; OSTFELD *et al.* 2001). Long-term observations on both the frequency distribution of oMG's and the densities of small mammals and birds could be used to further investigate this possibility.

Risk of human Lyme disease: The data presented in this study support previous claims that humans, like other species, can be infected by only certain oMG's (SEINOST *et al.* 1999). Thus, the risk of human Lyme disease could be reduced by decreasing the percentage of larvae that are infected by human infectious oMG's. *P. leucopus* is the only species investigated that is infected by all four of the human infectious oMG's. In addition, the density of *P. leucopus* is high in most areas of the northeastern United States (ALLAN *et al.* 2003) and the proportion of larvae infected from *P. leucopus* is greater than that from any other species investigated (Table 2; LOGIUDICE *et al.* 2003). Thus, measures aimed at reducing the relative contribution of *B. burgdorferi s.s.* from *P. leucopus* to *I. scapularis* larvae may significantly reduce the human Lyme disease risk. Reducing the contribution from *B. brevicauda* should also reduce the human Lyme disease risk, as the larval burden on *B. brevicauda* is much greater than that on the other species examined (Table 5). Further theoretical and empirical studies are necessary to determine the efficacy of any control measure.

According to the dilution-effect model, maintaining high vertebrate host diversity within a forest community reduces the proportion of infected ticks, as most reservoir hosts infect fewer of the larval ticks that feed on them than do mice (OSTFELD and KEESING 2000a,b). Fragmented and disturbed habitats, such as suburban communities, have low densities of non-mouse reservoir hosts, effectively increasing the proportion of ticks that feed on *P. leucopus* (NUPP and SWIHART 1996; ALLAN *et al.* 2003). According to the dilution-effect model, habitat fragmentation will therefore increase the proportion of ticks carrying *B. burgdorferi s.s.* and will coincidentally increase the proportion of nymphs carrying human infectious strains, both of which increase the risk of human Lyme disease. Thus, maintaining high species di-

versity by appropriate land management may reduce the risk of human exposure to Lyme bacteria.

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