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# Cross-Immunity and Community Structure of a Multiple-Strain Pathogen in the Tick Vector

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**Many vector-borne pathogens consist of multiple strains that circulate in both the vertebrate host and the arthropod vector. Characterization of the community of pathogen strains in the arthropod vector is therefore important for understanding the epidemiology of mixed vector-borne infections. *Borrelia afzelii* and *B. garinii* are two species of tick-borne bacteria that cause Lyme disease in humans. These two sympatric pathogens use the same tick, *Ixodes ricinus*, but are adapted to different classes of vertebrate hosts. Both *Borrelia* species consist of multiple strains that are classified using the highly polymorphic *ospC* gene. Vertebrate cross-immunity against the OspC antigen is predicted to structure the community of multiple-strain *Borrelia* pathogens. *Borrelia* isolates were cultured from field-collected *I. ricinus* ticks over a period spanning 11 years. The *Borrelia* species of each isolate was identified using a reverse line blot (RLB) assay. Deep sequencing was used to characterize the *ospC* communities of 190 *B. afzelii* isolates and 193 *B. garinii* isolates. Infections with multiple *ospC* strains were common in ticks, but vertebrate cross-immunity did not influence the strain structure in the tick vector. The pattern of genetic variation at the *ospC* locus suggested that vertebrate cross-immunity exerts strong selection against intermediately divergent *ospC* alleles. Deep sequencing found that more than 50% of our isolates contained exotic *ospC* alleles derived from other *Borrelia* species. Two alternative explanations for these exotic *ospC* alleles are cryptic coinfections that were not detected by the RLB assay or horizontal transfer of the *ospC* gene between *Borrelia* species.**

Many vector-borne pathogens consist of multiple genetically distinct strains (1–4). The adaptive arm of the vertebrate immune system plays a key role in generating and maintaining this diversity of pathogen strains (5–7). Genetic diversity is often the highest at loci coding for surface-exposed pathogen molecules that function during the invasion and infection of host tissues (8, 9). The study of these highly polymorphic pathogen molecules is important for understanding how cross-reactive acquired immunity can mediate indirect competition and superinfection in the vertebrate host (10, 11). In addition, these pathogen outer surface proteins are often used to characterize pathogen strains because they provide an upper estimate of pathogen strain richness.

In vector-borne diseases, the community of pathogen strains can be studied in both the vertebrate host and the arthropod vector. The vertebrate immune system creates nonrandom associations between pathogen strains (1, 12) that are subsequently transmitted to the arthropod vector. Conversely, the study of mixed infections in the arthropod vector can provide information on the processes that structure the community of multiple pathogen strains in the vertebrate host (13, 14). In addition, estimates of strain richness in the arthropod vector are important for understanding the frequency with which vertebrate hosts are exposed to infections with multiple strains (13, 15). In summary, studying the diversity of pathogen strains in the arthropod vector is important for understanding the epidemiology of vector-borne diseases.

*Borrelia afzelii* and *B. garinii* are two species of tick-borne spirochete bacteria that cause Lyme borreliosis (LB) in Europe (16). These two sympatric pathogens use the same tick vector, *Ixodes ricinus*, but are adapted to different classes of vertebrate hosts (17–19). *Borrelia afzelii* cycles in rodents (20–25), whereas *B. garinii* cycles in birds (24, 26–29), and this host specificity is mediated by the vertebrate complement system (30, 31). Previous work has shown that the ecological separation is not 100% complete and that double infections with these two *Borrelia* species do occur inside ticks (14, 32–34). The tick vector, *I. ricinus*, has three stages, larva, nymph, and adult, that take a single blood meal to develop into the next stage. The reservoir hosts are infected by nymphal ticks, which acquired the spirochete in the previous year during the larval blood meal (vertical transmission is rare [35, 36]). In summary, *B. afzelii* and *B. garinii* occupy distinct ecological niches in the vertebrate reservoir host community, but this ecological separation is not 100% complete.

The three most-studied *Borrelia* species (*B. burgdorferi sensu stricto*, *B. afzelii*, and *B. garinii*) all contain a highly polymorphic, single-locus *ospC* gene that codes for outer surface protein C (OspC) (46, 47, 49, 72). The OspC protein is critical for establishing infection inside the vertebrate host (37, 38). This antigen induces a protective antibody response in the vertebrate host (39–41) that is highly specific for strains carrying that particular *ospC* allele (42–44). The *ospC* gene has a large amount of sequence variation that allows *ospC* alleles to be classified into discrete major *ospC* groups (45). A major *ospC* group is defined as a cluster of

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*ospC* alleles that is more than 8% divergent in DNA sequence from other such major groups and less than 2% divergent within the same major group (45). The absence of intermediately divergent *ospC* alleles (2% to 8%) suggests that cross-reactive acquired immunity has structured the community of *Borrelia* pathogens into discrete OspC serotypes (45–47). As *Borrelia* strains are essentially clonal (48–51), the *ospC* gene is a commonly used genetic marker for studying the ecology and evolution of multiple strains of *Borrelia* pathogens (1, 13, 45, 52–55).

In the present study, we used next-generation sequencing (NGS) methods to characterize the community of genetically distinct entities characterized by a particular major *ospC* group (referred to here as "*ospC* strains") of *B. afzelii* and *B. garinii* that were found in the same local community of *I. ricinus* ticks over a period of 11 years. As previous studies of the *ospC* polymorphism in European *Borrelia* species have used more traditional genotyping methods (46, 47, 49, 55), we expected that a larger sequencing effort by the NGS approach would change our understanding of the *ospC* gene polymorphism (52, 54). We predicted that infections with multiple *ospC* strains in *I. ricinus* nymphs would be common. We predicted that genetically similar major *ospC* groups would be less likely to occur in the same tick, as was previously demonstrated for *B. afzelii* in wild rodents (1). We predicted that our NGS approach would detect the intermediately divergent *ospC* alleles that must periodically appear, despite strong selection by the vertebrate immune system. Finally, we predicted that the genetic divergence between the *ospC* alleles of *B. garinii* and *B. afzelii* could take on intermediate divergence values (2% to 8%) because the two *Borrelia* species occur in different vertebrate hosts and their OspC antigens are therefore not subject to cross-immunity.

(This study is part of the Ph.D. thesis of Jonas Durand.)

## MATERIALS AND METHODS

**Tick collection and *Borrelia* detection.** *Ixodes ricinus* ticks were sampled monthly from the Bois de l'Hôpital site in a deciduous forest near Neuchâtel, Switzerland (47°00′55.6″N, 6°94′16.7″E; surface area of 1 ha), over a period of 11 years (2000 to 2010). We collected questing ticks by dragging a white flag (surface area of 1 $m^2$) over the vegetation along four transects of 100 m. After collection, the species and stage of each tick were identified. In the laboratory, each tick was cut into two halves using sterile scissors. One tick half was used to determine spirochete infection via immunofluorescence microscopy (56). The other tick half was used to grow *Borrelia* isolates as follows. Half-ticks were placed in culture tubes containing BSK II medium (57), incubated at 34°C, and examined by dark-field microscopy every 10 days for 2 months. DNA was extracted from all spirochete-positive BSK II medium cultures. Briefly, 1 ml of culture medium was centrifuged and washed two times with phosphate-buffered saline before the pellet was suspended in 50 ml of ultrafiltered water. Pellets were incubated at 100°C for 10 min, and the thermolysates were stored at −20°C. *Borrelia* species were identified using a PCR-reverse line blot (RLB) assay following the method of Schouls et al. (58). The PCR protocol amplifies the 23S-5S spacer gene of *B. burgdorferi sensu lato* and was previously described by Alekseev et al. (59), and the RLB assay probes were described by Burri et al. (60).

As of 2012, the isolate archive at the University of Neuchâtel contained ~3,000 tick-derived spirochete isolates for which the *Borrelia* species had been identified by RLB assay. In this region of Switzerland, *I. ricinus* ticks are commonly infected with the following *Borrelia* species: *B. afzelii*, *B. bavariensis*, *B. burgdorferi sensu stricto*, *B. garinii*, and *B. valaisiana* (14). In this study, we focused on *B. afzelii* and *B. garinii* because they are the two most common species in the isolate archive at the University of Neuchâtel and because they are the most common etiological agents of LB in Europe. We included only *Borrelia* isolates derived from questing nymphs because this stage takes only one blood meal during the previous larval stage. We included only those isolates that, according to the RLB assay, were infected with a single *Borrelia* species (either *B. afzelii* or *B. garinii*) so that we could assign major *ospC* groups with confidence to the correct *Borrelia* species. We established a database of all the nymph-derived spirochete isolates of either *B. afzelii* or *B. garinii* obtained from 2000 to 2010. For each of the two *Borrelia* species, we randomly sampled a maximum of 20 nymph-derived isolates for each of the 11 years. Using this sampling strategy, we obtained 193 isolates of *B. afzelii* and 190 isolates of *B. garinii* (Table 1).

**TABLE 1** Number of nymph-derived spirochete isolates used for each of the 22 combinations of *Borrelia* species and year[a]

| Yr | No. of nymph-derived isolates tested (total no.) | | | |
|---|---|---|---|---|
| | *B. afzelii* | | *B. garinii* | |
| | Infected nymphs | Uninfected nymphs | Infected nymphs | Uninfected nymphs |
| 2000 | 20 (51)[b] | 234 (599)[c] | 20 (41) | 297 (609) |
| 2001 | 17 (37)[d] | 295 (643) | 18 (37) | 312 (643) |
| 2002 | 16 (69) | 153 (661) | 19 (39) | 336 (691) |
| 2003 | 12 (21) | 261 (458) | 14 (19) | 338 (460) |
| 2004 | 13 (21) | 298 (448) | 14 (16) | 396 (453) |
| 2005 | 19 (88) | 104 (459) | 19 (29) | 339 (518) |
| 2006 | 20 (57) | 171 (490) | 15 (33) | 249 (514) |
| 2007 | 20 (41) | 275 (564) | 20 (62) | 175 (543) |
| 2008 | 18 (31) | 347 (598) | 20 (54) | 212 (575) |
| 2009 | 20 (51) | 211 (540) | 13 (14) | 535 (577) |
| 2010 | 18 (58) | 156 (503) | 18 (32) | 297 (529) |

[a] We randomly selected a maximum of 20 nymph-derived isolates for NGS (the total number of infected nymphs is shown in parentheses) for each combination of *Borrelia* species and year. To calculate the absolute frequency of each strain (F3 in Tables 2 and 3), we used a subsample of uninfected nymphs (the total number of uninfected nymphs is shown in parentheses) for each combination of *Borrelia* species and year. This subsample of uninfected nymphs is proportional to the subsample of infected nymphs.
[b] In the year 2000, 51 nymph-derived cultures tested positive for *B. afzelii*. We randomly selected 20 of these 51 isolates of *B. afzelii* and determined the community of *ospC* strains using NGS.
[c] In the year 2000, 599 nymph-derived cultures did not test positive for *B. afzelii*. We calculated the absolute frequencies of the *B. afzelii ospC* strains (F3 in Tables 2 and 3), using a subsample of 234 cultures [(20/51) × 599 = 234] not infected with *B. afzelii*.
[d] In the year 2001, 37 nymph-derived cultures tested positive for *B. afzelii*, but only 17 of these 37 isolates of *B. afzelii* had sufficient material for NGS.

**Deep sequencing of the *ospC* strain community in nymph-derived spirochete isolates.** The *ospC* gene was amplified using a nested PCR method consisting of two consecutive amplification steps that was described by Bunikis et al. (49). We modified the oligonucleotides for the second PCR to include 454 Life Science's A or B sequencing adapter, the key, and the template-specific primers (see the supplemental material). The forward primer was tagged for forward sequencing. The reaction was performed in an automatic thermocycler (Eppendorf, USA). We checked the success of the PCRs by visualizing the amplicons on a 1% agarose gel stained with Midori green (Labgene, Switzerland). The PCR products were purified using a DNA purification kit (Wizard SV Gel and PCR cleanup system; Promega, Switzerland). We created eight different pools of *Borrelia* isolates for the 454 sequencing run (40 to 50 isolates per pool). Isolates were randomly assigned to pools subject to the constraint that each pool contained a balanced number of isolates from each year. Sequencing was done on a 454 Roche GS FLX sequencing apparatus and was outsourced to Microsynth AG (Balgach, Switzerland).

**Bioinformatics. (i) Cleaning of *ospC* gene sequences.** The 454 sequencing run produced 352,882 sequences of the *ospC* gene. After cleaning the data set (see the supplemental material), we retained 240,410 *ospC* gene sequences that were each 521 bp long.

**(ii) Clustering analysis of major *ospC* groups.** We defined an *a priori* set of seed sequences prior to conducting the cluster analysis. Seed sequences were defined as those sequences that occurred at least 50 times in our cleaned data set. The seed sequences were selected using the cd-hit-dup function of CD-HIT software (61, 62) with an error rate of 1%, which produced a set of 331 seed sequences. These sequences were used as seeds to perform a cluster analysis of the data from the cleaned data set with similarity thresholds ranging from 98% to 91% using the software CD-HIT. In addition, we created a phylogenetic tree (1,000 bootstraps) of the 331 seed sequences using the PhyML program (63). This tree confirmed the reliability of the clustering of the seeds. To have a measure of genetic similarity of the major *ospC* groups, we calculated the mean pairwise genetic distances using the distmat program (64) with a Kimura model. Each mean pairwise genetic distance was based on 30 sequences of each major *ospC* group in the pair. We also calculated the focal pairwise genetic distance for each major *ospC* group. This coefficient measures the average genetic distance between a focal major *ospC* group and all other major *ospC* groups.

**(iii) Nature of genetic variation within major *ospC* groups.** The nature of the genetic variation observed within each major *ospC* group was investigated. Only the first 350 nucleotides of the *ospC* gene sequences were analyzed because the end of the forward sequences contained too much sequencing error. For each major *ospC* group, 30 sequences were selected from the seed sequences that had been used for the clustering (a total of 690 sequences). For major *ospC* groups with less than 30 seed sequences, nonseed sequences (sequences that occurred less than 50 times in our data set) were added. Sequences were aligned manually, and the numbers of synonymous and nonsynonymous substitutions, insertions, and deletions were recorded.

**Nomenclature of major *ospC* groups.** Several papers have dealt with the nomenclature of the major *ospC* groups in *B. burgdorferi sensu lato* (46, 47, 49, 54, 65, 66). In the United States, workers have developed a well-established system of names for the major *ospC* groups of *B. burgdorferi sensu stricto* (45). In Europe, in contrast, there is no universally adopted naming system for the major *ospC* groups of *B. afzelii* and *B. garinii* (47, 49). In the current study, we present a summary of all the different names that have been used depending on the author (see Tables S1 and S2 in the supplemental material). In addition, we suggest that future work on the major *ospC* groups of these two *Borrelia* species follow the nomenclature developed by Bunikis et al. (49), Hellgren et al. (50), and Strandh and Råberg (54) for *B. afzelii* and that developed by Lagal et al. (47) for *B. garinii*. Here, we use "*ospC* strain" to refer to a genetically distinct entity characterized by a particular major *ospC* group.

**Confirmation of *Borrelia* species classification by RLB assay.** To confirm the reliability of the RLB assay, we performed additional sequencing of a subset of 120 isolates. According to the RLB assay, 60 of these isolates were singly infected with *B. afzelii* and 60 were singly infected with *B. garinii*. For each of these 120 samples, we used PCR to amplify the *recA* gene and the *hbb* gene according to the protocol described by Richter et al. (67). The amplicons were purified using a Qiagen purification kit. Purified amplicons were sent to Microsynth AG (Balgach, Switzerland) for Sanger sequencing.

**Statistical methods.** All statistical analyses were performed using R (68).

**(i) Prevalence of *ospC* strains inside ticks.** The absolute prevalence of a pathogen strain is its frequency in the entire population (uninfected and infected individuals). The relative prevalence of a pathogen strain is its frequency in the subpopulation of infected individuals. In the present study, the absolute prevalence of infection was determined for each *Borrelia* species by the RLB assay. We then used 454 sequencing to estimate the relative prevalence of each major *ospC* group in a randomly selected subset of *Borrelia* isolates. Some analyses of multiple-strain pathogen communities require estimates of the absolute prevalence. We therefore converted our relative prevalences to absolute prevalences by including the fraction of uninfected ticks that corresponded to the fraction of infected ticks that had been processed by 454 sequencing for each of the 22 combinations of *Borrelia* species and year (Table 1). For example, if we subsampled half of the infected ticks for a particular combination of *Borrelia* species and year, then we included half of the uninfected ticks for that combination of species and year. In this way, our data set was a random and representative sample of the populations of infected and uninfected ticks (Table 1). All analyses described below were conducted separately for each *Borrelia* species.

**(ii) Aggregation of *ospC* strains inside ticks.** Strain richness refers to the number of different *ospC* strains that were found within a single tick (including uninfected ticks). To test whether strains were aggregated inside ticks, we compared the observed distribution of strain richness to the Poisson distribution, where the variance is equal to the mean (69). Aggregation occurs when the number of ticks with multiple strains is greater than the Poisson expectation (variance/mean $> 1$). We used a bootstrap analysis to calculate the 95% confidence limits of the variance-to-mean ratio.

**(iii) Association of *ospC* strains inside ticks.** The strength and the direction of each pairwise association were estimated using the association coefficient described by Pielou (70). We used the absolute frequencies to calculate this pairwise association coefficient, which has a range from $-1$ (a complete negative association) to $+1$ (a complete positive association) and is undefined if all hosts are uninfected or infected with both strains. For each pair of strains, the statistical significance of the pairwise association was assessed using a two-tailed Fisher exact test. The significance level was adjusted for multiple comparisons using the Bonferroni correction (for *B. afzelii*, $P = 0.05/171$ pairs $= 0.000292$; for *B. garinii*, $P = 0.05/153$ pairs $= 0.000327$).

We also calculated the focal pairwise association coefficient for each major *ospC* group. This coefficient is a measure of whether a focal major *ospC* group is more or less likely to co-occur with other *ospC* strains. Strains with high focal pairwise association coefficients ($+1$) are more likely to be found in multiple infections than strains with low focal pairwise association coefficients ($-1$).

Finally, we tested whether strains with genetically dissimilar major *ospC* groups were more likely to occur together inside the same tick. Specifically, we tested for a correlation between the pairwise associations and the pairwise genetic distances using the Mantel test in the vegan package in R (71).

**Nucleotide sequence accession numbers.** The *ospC* gene sequence data have been deposited in the Sequence Read Archive under BioProject PRJNA293785 with the accession number SRP063760. A type sequence of each major *ospC* group of *B. afzelii* and *B. garinii* is available in GenBank (see the supplemental material for the GenBank accession numbers). The GenBank accession numbers for major *ospC* groups V1 of *B. valaisiana* and Q of *B. burgdorferi sensu stricto* are AF093483 and JQ253799, respectively.

## RESULTS

**Clustering analysis.** For each of the two *Borrelia* species, we randomly sampled a maximum of 20 nymph-derived isolates for each of the 11 years. Using this sampling strategy, we obtained 193 isolates of *B. afzelii* and 190 isolates of *B. garinii* (Table 1). The 454 sequencing run produced 352,882 sequences of the *ospC* gene. After cleaning the data set (see the supplemental material), we retained 240,410 *ospC* gene sequences that were each 521 bp long. There were 23 different major *ospC* groups in our local population of *I. ricinus* ticks, and this number was stable across a range of similarity thresholds (93% to 98%; see Fig. S1 in the supplemental material). According to BLAST analysis of the 23 major *ospC* groups, 10 belonged to *B. afzelii* (*ospC* groups A1, A2, A3, A5, A7, A9, A10, A11, A12, and A14), 11 belonged to *B. garinii* (*ospC* groups G2, G4, G6, G7, G8, G9, G10, G11, G13, G14, and G15), 1 belonged to *B. valaisiana* (*ospC* group V1), and 1 belonged to *B. burgdorferi sensu stricto* (*ospC* group Q). All of the 23 major

**TABLE 2** Frequencies of major *ospC* groups in the *B. afzelii* isolates

| *ospC* allele | *ospC* status | Frequency (%) | | |
|---|---|---|---|---|
| | | F1[a] | F2[b] | F3[c] |
| A10 | Native[d] | 43.35 (49,601) | 54.40 (105) | 3.90 (105) |
| A9 | Native[d] | 14.31 (16,372) | 31.09 (60) | 2.23 (60) |
| A1 | Native[d] | 12.28 (14,056) | 24.35 (47) | 1.75 (47) |
| A14 | Native[d] | 11.02 (12,613) | 24.87 (48) | 1.78 (48) |
| A11 | Native[d] | 2.31 (2,642) | 10.36 (20) | 0.74 (20) |
| A12 | Native | 4.61 (5,270) | 10.36 (20) | 0.74 (20) |
| A3 | Native | 2.69 (3,080) | 4.66 (9) | 0.33 (9) |
| A5 | Native | 2.90 (3,318) | 7.77 (15) | 0.56 (15) |
| A2 | Native | 2.64 (3,023) | 8.81 (17) | 0.63 (17) |
| A7 | Native | 2.02 (2,307) | 7.25 (14) | 0.52 (14) |
| G9 | Exotic[e] | 0.49 (560) | 7.77 (15) | 0.56 (15) |
| G11 | Exotic[e] | 0.39 (449) | 4.15 (8) | 0.30 (8) |
| Q | Exotic | 0.36 (409) | 1.04 (2) | 0.07 (2) |
| V1 | Exotic | 0.27 (314) | 0.52 (1) | 0.04 (1) |
| G13 | Exotic[e] | 0.26 (302) | 10.88 (21) | 0.78 (21) |
| G8 | Exotic[e] | 0.04 (45) | 8.81 (17) | 0.63 (17) |
| G14 | Exotic[e] | 0.02 (27) | 7.77 (15) | 0.56 (15) |
| G2 | Exotic[e] | 0.02 (24) | 6.74 (13) | 0.48 (13) |
| G7 | Exotic[e] | 0.02 (20) | 5.70 (11) | 0.41 (11) |

[a] Frequency 1 (F1) refers to the frequency of the major *ospC* group in the sample of sequences ($n$ = 114,432 sequences), and values in parentheses are numbers of sequences.
[b] Frequency 2 (F2) refers to the frequency of the major *ospC* group in the sample of infected nymphs ($n$ = 193 nymphs), and values in parentheses are numbers of infected nymphs.
[c] Frequency 3 (F3) refers to the frequency of the major *ospC* group in the sample of infected and uninfected nymphs ($n$ = 2,500 nymphs), and values in parentheses are numbers of infected nymphs.
[d] These native major *ospC* groups were horizontally transferred from *B. afzelii* to *B. garinii*.
[e] These exotic major *ospC* groups were horizontally transferred from *B. garinii* to *B. afzelii*.

**TABLE 3** Frequencies of major *ospC* groups in the *B. garinii* isolates

| *ospC* allele | *ospC* status | Frequency (%) | | |
|---|---|---|---|---|
| | | F1[a] | F2[b] | F3[c] |
| G8 | Native[d] | 25.19 (31,737) | 42.11 (80) | 2.18 (80) |
| G14 | Native[d] | 16.85 (21,223) | 30.00 (57) | 1.55 (57) |
| G9 | Native[d] | 11.71 (14,748) | 22.63 (43) | 1.17 (43) |
| G13 | Native[d] | 10.34 (13,030) | 27.89 (53) | 1.44 (53) |
| G2 | Native[d] | 10.68 (13,457) | 21.05 (40) | 1.09 (40) |
| G7 | Native[d] | 9.57 (12,057) | 31.05 (59) | 1.61 (59) |
| G11 | Native[d] | 4.28 (5,389) | 14.74 (28) | 0.76 (28) |
| G4 | Native | 3.99 (5,025) | 14.74 (28) | 0.76 (28) |
| G6 | Native | 3.14 (3,952) | 11.58 (22) | 0.60 (22) |
| G15 | Native | 0.61 (774) | 3.68 (7) | 0.19 (7) |
| G10 | Native | 0.55 (689) | 1.58 (3) | 0.08 (3) |
| A10 | Exotic[e] | 1.75 (2,209) | 31.58 (60) | 1.63 (60) |
| A1 | Exotic[e] | 0.74 (930) | 12.11 (23) | 0.63 (23) |
| V1 | Exotic | 0.41 (512) | 1.58 (3) | 0.08 (3) |
| Q | Exotic | 0.10 (128) | 2.63 (5) | 0.14 (5) |
| A9 | Exotic[e] | 0.04 (50) | 11.05 (21) | 0.57 (21) |
| A14 | Exotic[e] | 0.03 (36) | 10.53 (20) | 0.54 (20) |
| A11 | Exotic[e] | 0.03 (32) | 5.26 (10) | 0.27 (10) |

[a] Frequency 1 (F1) refers to the frequency of the major *ospC* group in the sample of sequences ($n$ = 125,978 sequences), and values in parentheses are numbers of sequences.
[b] Frequency 2 (F2) refers to the frequency of the major *ospC* group in the sample of infected nymphs ($n$ = 190 nymphs), and values in parentheses are numbers of infected nymphs.
[c] Frequency 3 (F3) refers to the frequency of the major *ospC* group in the sample of infected and uninfected nymphs ($n$ = 3,486 nymphs), and values in parentheses are numbers of infected nymphs.
[d] These native major *ospC* groups were horizontally transferred from *B. garinii* to *B. afzelii*.
[e] These exotic major *ospC* groups were horizontally transferred from *B. afzelii* to *B. garinii*.

*ospC* groups had been previously described in the literature (1, 46, 47, 72).

There were a number of major *ospC* groups that were found in both *Borrelia* species. Five of the 10 *B. afzelii* major *ospC* groups (A1, A9, A10, A11, A14) were found in isolates that were singly infected with *B. garinii* according to the RLB assay. Conversely, 7 of the 11 *B. garinii* major *ospC* groups (G2, G7, G8, G9, G11, G13, G14) were found in isolates that were singly infected with *B. afzelii* according to the RLB assay. In what follows, we use the terms "native" and "exotic" to distinguish between these two types of major *ospC* groups. Thus, *B. afzelii* had 10 native and 9 exotic *ospC* groups (7 *B. garinii*-derived *ospC* groups and the V1 and Q *ospC* groups) for a total of 19 major *ospC* groups (Table 2), and *B. garinii* had 11 native and 7 exotic *ospC* groups (5 *B. afzelii*-derived *ospC* groups and the V1 and Q *ospC* groups) for a total of 18 major *ospC* groups (Table 3).

**Description of the *Borrelia ospC* strain communities in *I. ricinus*.** The major *ospC* groups were more evenly distributed in *B. garinii* than *B. afzelii*. In *B. afzelii*, the strain of *ospC* group A10 (referred to here as strain A10) was found in 54.40% of the infected nymphs (Table 2). Three strains (A1, A9, A14) were found in 24.35% to 31.09% of the infected nymphs, and the remaining 15 strains were found in less than 11% of the infected nymphs (Table 2). In *B. garinii*, strain G8 was found in 42.11% of the infected nymphs, and six other strains were found in 21.05% to 31.58% of the infected nymphs (Table 3). Six strains were found in 10.53% to 14.74% of the infected nymphs, whereas the five remaining strains were found in less than 6% of the infected nymphs. In *B. garinii*, the exotic major *ospC* group A10 was very common and occurred in 31.58% of the infected nymphs.

**Pairwise genetic distances within the native major *ospC* groups.** For *B. afzelii*, the mean pairwise genetic distance within each of the 10 native major *ospC* groups was 1.06% ($n$ = 44,250 pairwise comparisons, range = 0.42% to 2.16%). For *B. garinii*, the mean pairwise genetic distance within each of the 11 native major *ospC* groups was 1.14% ($n$ = 54,285 pairwise comparisons, range = 0.66% to 1.63%).

**Nature of genetic variation within major *ospC* groups.** Across the 690 sequences belonging to the 23 major *ospC* groups, there were 140 nucleotide substitutions, of which 58.9% were synonymous (83/141) and 41.1% were nonsynonymous (58/141). Single nucleotide insertions and deletions and codon deletions were common, whereas double nucleotide insertions or deletions were rare (see Table S3 in the supplemental material).

**Pairwise genetic distances between the native major *ospC* groups.** We calculated the pairwise genetic distances between the 21 native major *ospC* groups (i.e., the exotic major *ospC* groups were excluded). The conspecific or allospecific pairwise genetic distance refers to whether the two native major *ospC* groups belonged to the same *Borrelia* species or to different *Borrelia* species, respectively. The mean conspecific pairwise genetic distances of *B. afzelii* ($n$ = 45 pairwise comparisons, mean = 17.84%, range = 8.86% to 23.59%; Fig. 1) and *B. garinii* ($n$ = 55 pairwise compar-

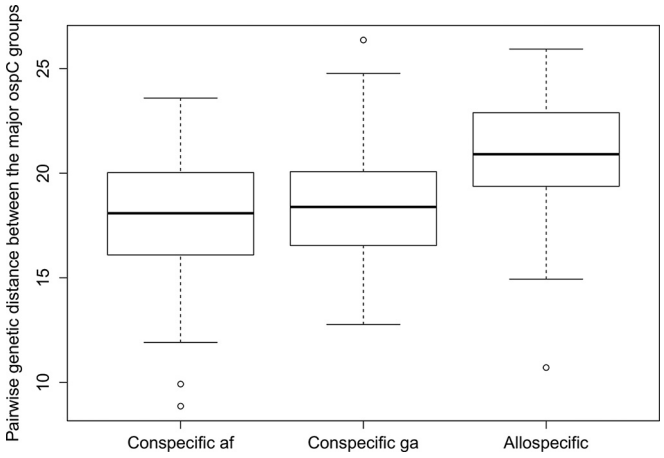


**FIG 1** Distribution of pairwise genetic distances between major *ospC* groups. The genetic distances for three different pairwise comparisons are shown: conspecific *B. afzelii* (af; $n = 55$), conspecific *B. garinii* (ga; $n = 55$), and allospecific (*B. afzelii* versus *B. garinii*; $n = 121$). Shown are the medians (heavy black lines), the 25th and 75th percentiles (edges of the boxes), the minimum and maximum values (whiskers), and the outliers (circles).

isons, mean = 18.54%, range = 12.77% to 26.36%; Fig. 1) were not significantly different ($t = -1.13$, degrees of freedom [df] = 98, $P = 0.261$). The mean allospecific pairwise genetic distance ($n = 110$ comparisons, mean = 20.94%, range = 10.71% to 25.93%; Fig. 1) was significantly larger than the mean conspecific pairwise genetic distance ($t = 6.921$, df = 208, $P < 0.001$).

**Strain richness in nymphal ticks.** The strain richness of the infected nymphal ticks was calculated using both the native and exotic major *ospC* groups for each *Borrelia* species. Most nymphal ticks were infected with multiple *ospC* strains. For *B. afzelii* (19 major *ospC* groups), 78.76% (152/193) of the infected nymphal ticks carried multiple *ospC* strains. For *B. garinii* (18 major *ospC* groups), 84.74% (161/190) of the infected nymphal ticks carried multiple *ospC* strains. The mean strain richness of *B. garinii* (2.96 strains per infected tick, range = 1 to 11 strains per tick) was 25% higher than that of *B. afzelii* (2.37 strains per infected tick, range = 1 to 7 strains per tick), and this difference was statistically significant ($t = -3.764$, df = 381, $P < 0.001$).

**Aggregation of strains in nymphal ticks.** The variance-to-mean ratio of strain richness was calculated using the absolute frequencies of both the native and exotic major *ospC* groups for each *Borrelia* species. The variance-to-mean ratio of strain richness was significantly greater than 1.0 for both *Borrelia* species, indicating that the *ospC* strains were highly aggregated in the nymphal ticks (Fig. 2). The variance-to-mean ratio of strain richness was 47.4% higher in *B. garinii* (mean = 3.98, 95% confidence interval [CI] = 3.44 to 4.51) than in *B. afzelii* (mean = 2.70, 95% CI = 2.50 to 2.91).

**Pairwise association index.** The pairwise association coefficients within each *Borrelia* species were calculated using the absolute frequencies of both the native and exotic major *ospC* groups. For *B. afzelii* (19 major *ospC* groups), 66.7% (114/171) of the pairwise association coefficients were positive (mean = 0.08, range = −0.01 to 0.35). All of the 39 statistically significant ($P < 0.05/171$) pairwise associations were positive (mean = 0.20, range = 0.11 to 0.35). For *B. garinii* (18 major *ospC* groups), 77.8% (119/153) of the pairwise association coefficients were positive (mean = 0.15, range = −0.004 to 0.48). Again, all of the 78 statistically significant ($P < 0.05/153$) pairwise associations were positive (mean = 0.25, range = 0.11 to 0.48). The mean pairwise association coefficient of *B. garinii* was significantly greater than that of *B. afzelii* ($t = 6.10$, df = 322, $P < 0.001$). The results were the same for the subset of 117 statistically significant pairwise association coefficients ($t = 3.54$, df = 115, $P < 0.001$).

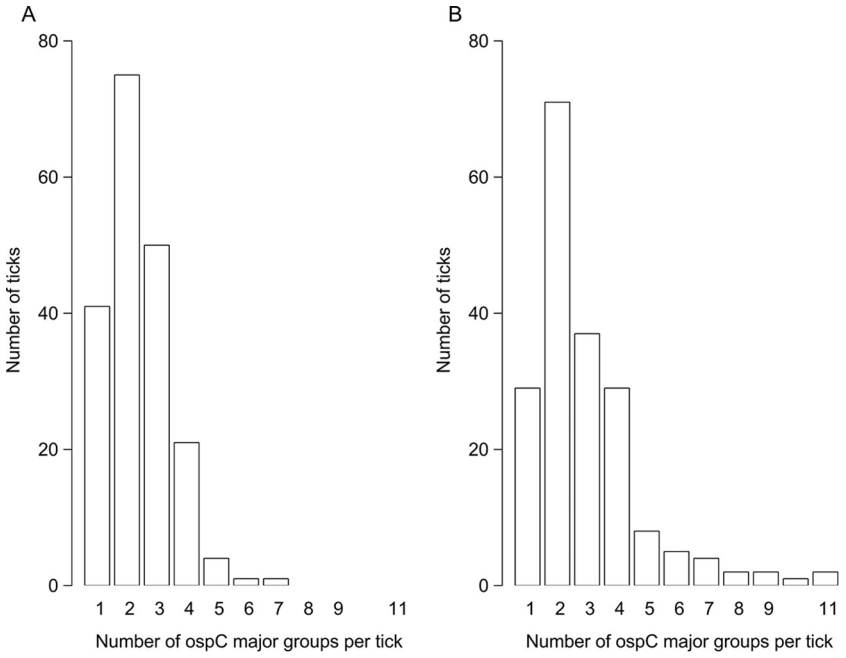


**FIG 2** Distribution of strain richness in nymphs infected with *B. afzelii* (A) and nymphs infected with *B. garinii* (B). The numbers of ticks infected with zero strains are not shown for scaling purposes (i.e., uninfected nymphs in Table 1).

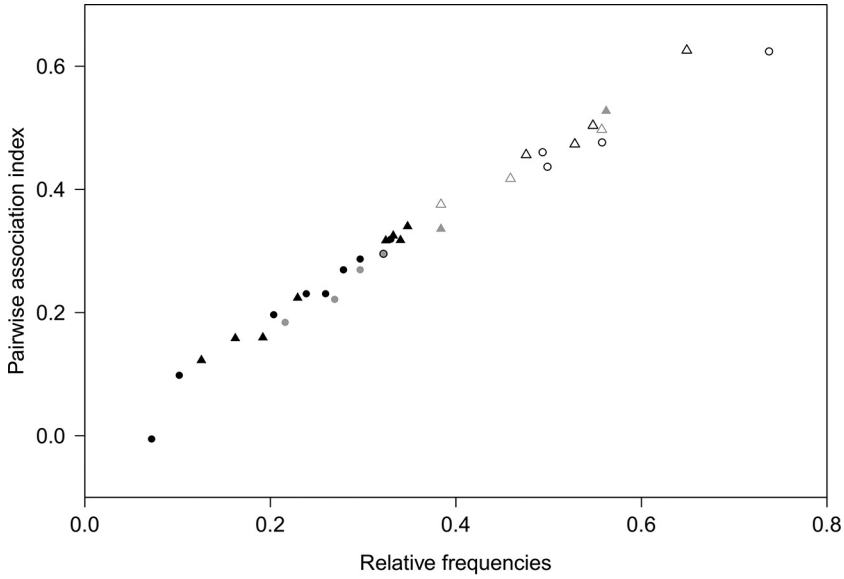


**FIG 3** Positive relationship between the focal pairwise association index and the square root-transformed relative frequency of each major *ospC* group inside the tick. Results are combined for *B. afzelii* (circles) and *B. garinii* (triangles). The native major *ospC* groups that were horizontally transferred to the other *Borrelia* species (white symbols; $n = 12$) had much higher relative frequencies and pairwise association indices in the native *Borrelia* species than the native major *ospC* groups that were not transferred (gray symbols; $n = 9$). The exotic major *ospC* groups ($n = 16$) are shown in black symbols.

The mean focal pairwise association index of *B. garinii* ($n =$ 18 major *ospC* groups, mean = 0.35, range = 0.12 to 0.63) was 20.7% higher than that of *B. afzelii* ($n = 19$ major *ospC* groups, mean = 0.29, range = −0.005 to 0.62), but this difference was not significant ($t = 1.35$, df = 35, $P = 0.187$). Some *ospC* strains, such as *B. afzelii ospC* strain A10 and *B. garinii ospC* strains G8, G14, and A10, had very high focal pairwise association coefficients (>0.50).

**Relationship between relative frequency and the focal pairwise association index.** The relative frequency (F2 in Tables 2 and 3) of each major *ospC* group (transformed by the square root) was a highly significant predictor of the focal pairwise association index in *B. afzelii* ($n = 19$ major *ospC* groups, $F_{1, 17} = 565.7$, $P < 0.001$, $r^2 = 0.971$; Fig. 3) and in *B. garinii* ($n = 18$ major *ospC* groups, $F_{1, 16} = 1{,}688.0$, $P < 0.001$, $r^2 = 0.991$; Fig. 3). An analysis of covariance found no significant interaction between *Borrelia* species and relative frequency on the pairwise association index ($F_{1, 33} = 0.9$, $P = 0.354$). The slope of the relationship between the focal pairwise association index and the square root-transformed relative frequency was therefore the same for each *Borrelia* species (0.90 ± 0.044). After controlling for the relative frequency, the focal pairwise association index remained significantly higher for *B. garinii* than *B. afzelii* ($F_{1, 34} = 4.5$, $P = 0.041$).

**Relationship between pairwise genetic distance and pairwise association index.** The correlation between the pairwise genetic distance between the major *ospC* groups and the pairwise association index of the major *ospC* groups inside the ticks was not statistically significant for either *B. afzelii* ($n = 171$ pairwise elements; Mantel test, $r = -0.041$, $P = 0.630$; Fig. 4) or *B. garinii* ($n = 153$ pairwise elements; Mantel test, $r = -0.053$, $P = 0.664$; Fig. 4). Similarly, the correlation between the focal pairwise genetic distance and the focal pairwise association index was not statistically significant for either *B. afzelii* ($n = 19$ major *ospC* groups; Pearson correlation test, $r = 0.032$, $t = 0.13$, df = 17, $P =$ 0.897; Fig. 5) or *B. garinii* ($n = 18$ major *ospC* groups; Pearson correlation test, $r = -0.175$, $t = -0.71$, df = 16, $P = 0.485$; Fig. 5).

**Exotic major *ospC* groups were widespread in both *Borrelia* species.** For the *B. afzelii*-infected nymphs (as determined by the RLB assay), 42.0% (81/193) of the infections contained exotic major *ospC* groups. Conversely, for the *B. garinii*-infected nymphs (as determined by the RLB assay), 55.3% (105/190) of the infections contained exotic major *ospC* groups. In *B. afzelii*, 1.88% of the *ospC* gene sequences (2,150/114,432) were of exotic origin. In *B. garinii*, 3.09% of the sequences (3,897/125,978) were of exotic origin.

**Exotic major *ospC* groups were common in their native *Borrelia* species.** The five native *B. afzelii* major *ospC* groups that had undergone horizontal transfer (to *B. garinii*) accounted for 84.62% of the native *ospC* gene sequences of *B. afzelii* (95,598/112,596). Similarly, the seven native *B. garinii* major *ospC* groups that had undergone horizontal transfer (to *B. afzelii*) accounted for 91.45% of the native *ospC* gene sequences of *B. garinii* (111,641/122,081). For the two *Borrelia* species combined, we compared the mean relative frequency between native major *ospC* groups that had experienced horizontal transfer or not (Tables 2 and 3). The 12 native major *ospC* groups that had experienced horizontal transfer had a significantly higher relative frequency ($t = 4.89$, df = 19, $P < 0.001$; Fig. 3) than the 9 native major *ospC* groups that had not been transferred. For the 12 major *ospC* groups that had experienced horizontal transfer, the relative frequency in the native species was always higher than the frequency in the exotic species (paired $t$ test, $t = 8.13$, df = 11, $P < 0.001$). There was a significant correlation between the relative frequencies of these 12 major *ospC* groups in their native species and in their exotic species (Pearson correlation test, $r = 0.783$, $t = 3.99$, df = 10, $P = 0.002$).

**Sanger sequencing of *Borrelia* genes to confirm *Borrelia* species classification by RLB assay.** For a subsample of 120 *Borrelia*

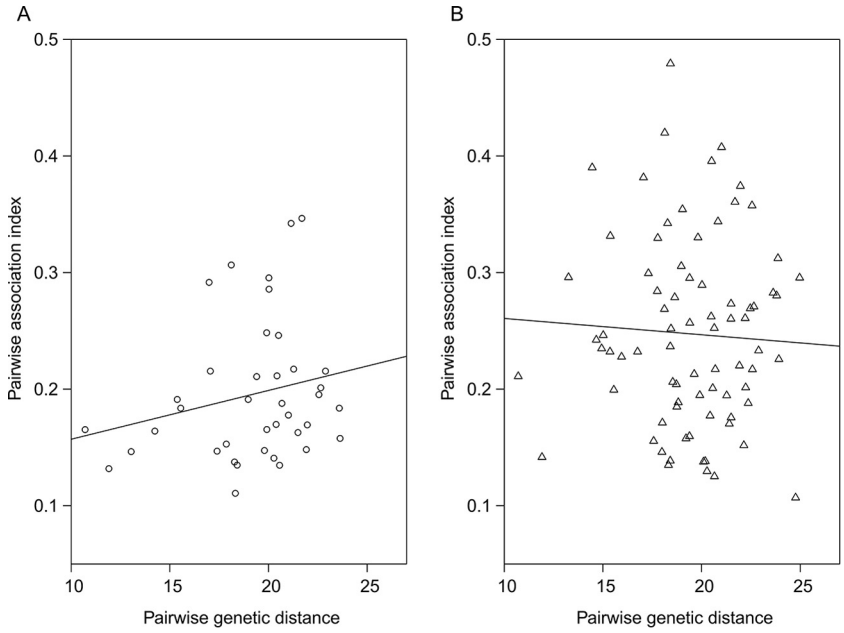


**FIG 4** Relationship between the pairwise association index of the major *ospC* groups inside the tick versus the pairwise genetic distance. The relationship is shown for *B. afzelii* (A) and *B. garinii* (B). The regression was done on the subset of the statistically significant pairwise associations indices but was not statistically significant for either *Borrelia* species.

isolates, we obtained high-quality sequences for both the *recA* gene and the *hbb* gene for 110 isolates. This genetic analysis confirmed the *Borrelia* species classification of the RLB assay in 95.5% (105/110) of our samples. In general, this additional analysis showed that the RLB assay provided a reliable classification of the *Borrelia* species.

## DISCUSSION

**Cross-immunity and co-occurrence of *Borrelia ospC* strains.** Cross-reactive acquired immunity should reduce the co-occurrence of antigenically similar strains in the same reservoir host (5, 73, 74). Assuming that the genetic similarity between major *ospC* groups predicts the probability of cross-reactive acquired immunity between their respective OspC antigens, we expected a positive relationship between the pairwise genetic distance and the pairwise association index. A study of multiple *B. afzelii* infections in a population of wild voles found that dissimilar major *ospC* groups were more likely to be found together in the same host than similar major *ospC* groups (1). In contrast, we found no relationship between the pairwise genetic distance and the pairwise association index inside the ticks. A simple explanation for the difference between these two studies is that one looked at the reservoir

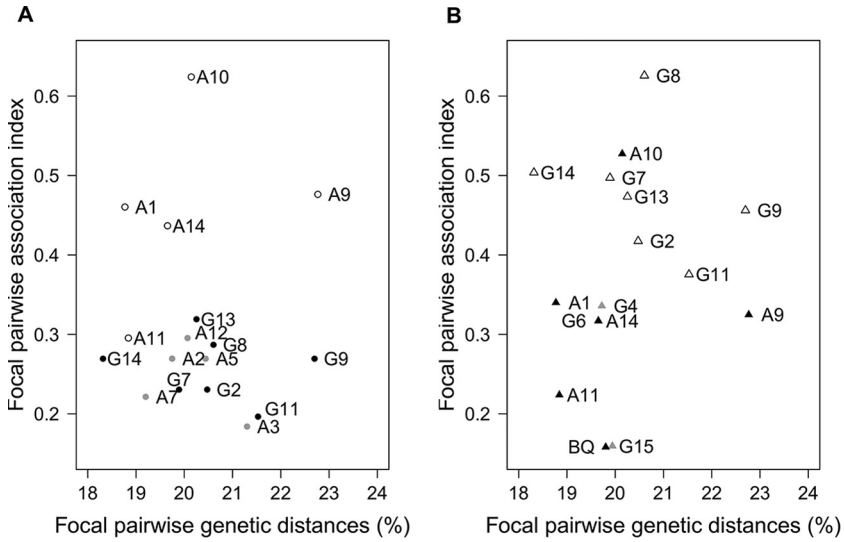


**FIG 5** Relationship between the focal pairwise association index and the focal pairwise genetic distance of each major *ospC* group inside the tick. The relationship is shown for *B. afzelii* (A) and *B. garinii* (B). All data points are labeled with the name of the major *ospC* group. White and gray symbols indicate native major *ospC* groups that had and that had not been transferred to the other *Borrelia* species, respectively; black symbols indicate exotic major *ospC* groups.

host, whereas the other looked at the tick vector. The vertebrate immune system structures the community of *ospC* strains inside the reservoir hosts, and the tick is expected to acquire a subsample of this strain community (13, 75). However, the community structure of the colonizing strains might be modified by subsequent events inside the tick. The spirochete load can change dramatically following the blood meal and during the larva-to-nymph molt, and these changes in abundance may alter the community of strains inside the tick (14, 76). There is currently very little information as to how *Borrelia* strains interact inside the tick over time (75).

**Strong selection against *ospC* alleles with intermediate divergence.** Despite a large sequencing effort (240,410 sequences from 389 ticks over a period of 11 years), our study did not find any *ospC* alleles that were intermediately divergent (2% to 8%) from the community of major *ospC* groups. The average pairwise genetic distance between conspecific major *ospC* groups was always greater than 8%. Thus, our study confirmed the highly discontinuous pattern of genetic variation at the *ospC* locus, as was reported in previous studies of *B. burgdorferi sensu lato* (45–47, 49). The original studies demonstrating the 8% divergence cutoff between the major *ospC* groups in *B. afzelii* and *B. garinii* were based on a total of 140 sequences (46, 47, 49). Thus, it was reasonable to expect that a larger sequencing effort would discover the missing intermediate major *ospC* groups. The present study suggests that strains carrying *ospC* alleles that are 2% to 8% divergent rarely coexist in the same *Borrelia* species. Interestingly, our results also suggest that such intermediately divergent *ospC* alleles rarely coexist in *Borrelia* species occupying different niches in the vertebrate host community. The allospecific pairwise genetic distances between *B. afzelii* and *B. garinii* had the same discontinuous pattern, suggesting that the evolution of the *ospC* allele is not independent in each *Borrelia* species. One explanation is that frequent horizontal transfer of the *ospC* gene (see below) essentially synchronizes the community of major *ospC* groups between these two *Borrelia* species. Thus, a new *ospC* mutant can rise to a detectable frequency only if it is at least 8% divergent from all the major *ospC* groups of all the *Borrelia* pathogens in the local community.

Purifying selection by cross-reactive antibodies is the most plausible explanation for the missing intermediate major *ospC* groups. Mutant major *ospC* groups that are genetically similar and thus antigenically similar to their conspecific counterparts are vulnerable to cross-reactive antibodies, which prevent these mutants from invading the local population (11, 74, 77). Indirect evidence for this hypothesis comes from studies of *B. burgdorferi sensu stricto* that have shown that antibodies against a given OspC antigen provide protection against strains belonging to the same major *ospC* group but not against strains belonging to a different major *ospC* group (42, 44). Thus, in *B. burgdorferi sensu stricto*, we see the end result of divergent selection on the *ospC* antigen to escape from the cross-reactive antibodies directed against its neighbors. However, immune escape between strains carrying different major *ospC* group alleles is not always perfect. A recent study of *B. afzelii* found that OspC antigens belonging to different major *ospC* groups (that were 20.68% divergent in DNA sequence) could still interfere with one another via the cross-reactive antibodies of the rodent host (43). In summary, cross-reactive antibodies are the most plausible source of purifying selection that removes intermediately divergent major *ospC* groups from the local population of *Borrelia* pathogens.

Each major *ospC* group contains a cluster of *ospC* alleles that are less than 2% divergent from each other with respect to their DNA sequence. An earlier study of the *ospC* polymorphism found that there was very little sequence variation within the major *ospC* groups (9 of the 11 major *ospC* groups were isosequential) and concluded that the effective population size of each group was very small (45). The present study found that strain richness within the major *ospC* groups was high (331 unique seed sequences clustered into 23 major *ospC* groups, giving a mean richness of 14.4 seed sequences per group). Examination of the sequence variation within the major *ospC* groups found that the majority (~60%) of the nucleotide substitutions were synonymous. Sequencing error was the likely cause of all insertions and deletions that resulted in a frameshift, as the downstream parts of the OspC protein were highly conserved. The present study suggests that most of the sequence variation observed within a major *ospC* group does not affect the amino acid sequence and/or the function of the OspC protein. Under this scenario, natural selection cannot discriminate among the different allelic variants, and the frequencies of these silent alleles depend on genetic drift alone (78). Thus, the observation that the major *ospC* groups contain allelic variants does not contradict the hypothesis that cross-immunity selects against insufficiently divergent *ospC* alleles.

**Richness of the major *ospC* groups.** We found that the *B. afzelii* and *B. garinii* isolates in our local area contained 10 and 11 native major *ospC* groups, respectively (strain richness increased to 19 and 18, respectively, after the exotic major *ospC* groups were included). These findings were similar to those of previous studies of the *ospC* polymorphism in local tick populations (45, 53, 55, 79). In the United States, local populations of *B. burgdorferi sensu stricto* have between 13 and 16 different major *ospC* groups (13, 45, 53, 79). In Europe, local populations of *B. afzelii* have 10 different major *ospC* groups (55). Why do populations of *B. burgdorferi sensu lato* contain 12 and not 120 different major *ospC* groups? The relatively small length of the *ospC* gene (~600 bp) in combination with the constraints of remaining functional and of avoiding cross-reactive acquired immunity with all of its neighbors must set an upper limit to the number of major *ospC* groups that can exist in a single, local population.

**Aggregation of *Borrelia ospC* strains in the tick vector.** *Ixodes ricinus* nymphs infected with *B. afzelii* or *B. garinii* contained a mean richness of 2.37 and 2.97 different *ospC* strains per nymph, respectively. Our estimates of the mean *ospC* strain richness in questing *I. ricinus* nymphs were considerably higher than those from previous studies (1.07 to 1.44 strains per nymph) of *B. burgdorferi sensu lato* infections of questing nymphs in *I. scapularis*, *I. pacificus*, and *I. ricinus* (53, 55, 80). However, our estimates of the mean *ospC* strain richness are similar to those from previous studies (1.0 to 3.7 strains per tick) that sampled larval ticks feeding on infected hosts (13, 55, 81, 82) or questing adults (45, 53). We found a very high prevalence of multiple *ospC* strains in both *B. afzelii* (78.76%) and *B. garinii* (84.74%). Our estimates of the prevalence of multiple *ospC* strain infections were much higher than those in previous studies (0.0% to 50.0%) (1, 45, 55, 81–85). These earlier studies estimated the mean *ospC* strain richness and the prevalence of multiple strain infections by using molecular methods, such as the RLB assay, cold single-strand conformation polymorphism (SSCP) analysis, and Sanger sequencing. A previous study showed that the RLB assay was more sensitive at detecting infections with multiple strains than SSCP analysis (45, 53).

Thus, differences in strain richness among studies are partially confounded by differences in the sensitivity of the molecular methods. In the present study, we used next-generation sequencing (NGS) to analyze an average of 618 *ospC* gene sequences per tick (240,410 sequences, 389 ticks). This high level of coverage for each tick allowed us to detect major *ospC* groups that represented a tiny fraction of that particular amplicon (<0.2%). Thus, NGS methods estimate higher levels of strain diversity than the older molecular methods because these approaches are more sensitive at detecting rare strains.

The *ospC* strains were highly aggregated in *I. ricinus* ticks. There are a number of explanations for this pattern of aggregation. First, vertebrate hosts vary in their competence in acquiring infections and transmitting spirochetes to vector ticks (18, 30, 86, 87). If a substantial proportion of ticks feed on incompetent hosts and the remaining ticks feed on infected reservoir hosts, the *Borrelia* strains will appear to be aggregated inside the ticks when these two subpopulations of ticks are analyzed together. Second, the aggregation of ticks on reservoir hosts can also cause the aggregation of *Borrelia* strains in the tick vector. Numerous studies have observed that a minority of wild rodent hosts feeds a majority of the *I. ricinus* ticks (88, 89). There are a variety of ecological factors that cause aggregation of ticks in wild rodents (90). Third, the immune system of the vertebrate host might have more difficulty controlling and/or clearing infections with multiple strains than infections with a single strain (74, 91). Under this hypothesis, infections with multiple strains have a higher probability of establishing infection in the reservoir host and/or have a higher host-to-tick transmission success than infections with a single strain.

**Differences between *B. garinii* and *B. afzelii*.** The mean *ospC* strain richness of *B. garinii* (2.96 strains per nymph) was 25% higher than that of *B. afzelii* (2.37 strains per nymph). *Borrelia garinii ospC* strains were more often found in multiple infections and had higher pairwise association indices than their *B. afzelii* counterparts. The higher mean *ospC* strain richness for *B. garinii* was not caused by differences in total strain richness, which was the same between *B. afzelii* ($n = 19$ *ospC* strains) and *B. garinii* ($n = 18$ *ospC* strains). *Borrelia garinii* is mostly found in avian hosts, whereas *B. afzelii* is mostly found in rodent hosts (16, 20–22, 24, 25). Thus, one explanation is that the richness of *B. garinii ospC* strains in birds is higher than the richness of *B. afzelii ospC* strains in rodents. To date, most of the studies investigating *ospC* strain richness in vertebrate reservoir hosts have focused on mammals (1, 13, 50, 52, 55, 66, 82, 83). The two studies that investigated birds (81, 85) found a mean *ospC* strain richness (1.00 to 1.76 strains per tick) that was low compared to that found in the studies with mammals, but this could be an artifact of the relatively inefficient molecular methods that were used in the bird studies (RLB assay and Sanger sequencing). A recent study characterized the spirochete loads of different *Borrelia* species inside questing *I. ricinus* nymphs (14). This study found that the median spirochete load of *B. garinii* (5,080 spirochetes per nymph) was 1.6 times higher than that of *B. afzelii* (3,140 spirochetes per nymph) (14). Thus, another explanation for the discrepancy in strain richness between the two species is that the tick vector has a higher carrying capacity for *B. garinii* than for *B. afzelii*.

**Presence of exotic major *ospC* group alleles in nymphs that, according to the RLB assay, were infected with a single *Borrelia* species.** In the present study, we used only nymph-derived isolates that, according to the RLB assay (which targeted the 23S-5S spacer gene), were singly infected with either *B. afzelii* or *B. garinii*, and we therefore excluded those isolates that, according to the RLB assay, were coinfected with multiple *Borrelia* species. A surprising result was therefore that 42% of the *B. afzelii*-infected ticks and 55% of the *B. garinii*-infected ticks contained exotic major *ospC* groups from other *Borrelia* species. While the exotic major *ospC* groups were widespread, they were not abundant. For *B. afzelii* and *B. garinii*, the exotic major *ospC* groups accounted for 1.88% and 3.09% of all the *ospC* sequences, respectively. Two alternative explanations for these results are the coinfection explanation and the horizontal gene transfer explanation.

**Coinfection explanation for the presence of exotic major *ospC* group alleles.** In the coinfection explanation, the presence of native and exotic *ospC* groups in the same isolate represents a true coinfection with *B. afzelii* and *B. garinii* that was not detected by the RLB assay. In this scenario, the RLB assay detected the highly abundant *Borrelia* species (corresponding to the abundant native *ospC* alleles from the 454 sequencing) but not the *Borrelia* species of low abundance (corresponding to the low-abundance exotic *ospC* alleles from the 454 sequencing). Although we have recently shown that the sensitivity of our RLB assay is similar to that of a widely used quantitative PCR assay for detecting *Borrelia* infections in ticks (14, 92), we cannot exclude this possibility. Previous studies have shown that nymphs and adult ticks carry coinfections with *B. afzelii* and *B. garinii* (14, 32–34). In our local *I. ricinus* population, the RLB assay found that 0.75% (13/1,731) of the nymphs are coinfected with *B. afzelii* and *B. garinii* (14). If the coinfection explanation is true, the implication is that the true frequency of coinfections with *B. afzelii* and *B. garinii* has been underestimated by a factor of 56 to 73 (42% to 55% in the present study versus 0.75% in the study by Herrmann et al. [14]). Interestingly, we recently showed that the spirochete load of *B. afzelii* and *B. garinii* coinfections was much lower than the additive expectation (14). We suggested that the complement system of the vertebrate host reduced the spirochete load of the maladapted *Borrelia* species inside the nymphal tick (14). Thus, the observation that the exotic *ospC* groups had low abundances (1.88% and 3.09%) is consistent with our understanding of how the vertebrate complement system would interact with coinfections containing bird- and rodent-adapted *Borrelia* species inside the same tick.

If the coinfection hypothesis is true, the observation that >50% of our nymphs had coinfections with *B. afzelii* and *B. garinii* requires an additional explanation, given that these two *Borrelia* species are adapted to different vertebrate hosts (24, 32). We recently presented four mechanisms by which *B. afzelii* and *B. garinii* could coinfect the same tick (14). The specificity of *B. afzelii* for rodents and of *B. garinii* for birds is not 100% complete. Studies in France (93) and England (94) found that introduced species of rodents were infected with the bird-adapted *B. garinii*. Similarly, other studies found that birds can transmit the rodent-adapted *B. afzelii* (95, 96). Rodents or birds that are coinfected with *B. afzelii* and *B. garinii* could transmit both species to feeding ticks. Larval ticks could also acquire coinfections of *B. afzelii* and *B. garinii* by taking multiple blood meals from different hosts (43, 44), by a combination of cofeeding and systemic transmission, or by a combination of vertical transmission and systemic transmission (14). For example, a larval tick cofeeding next to a *B. afzelii*-infected nymph on a *B. garinii*-infected bird would acquire both *Borrelia* species. Cofeeding transmission has been observed in both *B. afzelii* and *B.*

*garinii* (90). In contrast, vertical transmission of *Borrelia* pathogens is believed to be rare (36).

**Horizontal gene transfer explanation for the presence of exotic major *ospC* group alleles.** In the horizontal gene transfer explanation, the RLB assay result is correct (all nymphs were infected with a single *Borrelia* species) and the exotic major *ospC* group alleles were horizontally transferred into the recipient *Borrelia* species. Previous studies have found that major *ospC* group alleles can be transferred between *Borrelia* species (46, 97, 98). In the present study, we found that 12 of the 21 native *ospC* major groups occurred as exotic major *ospC* groups in the other *Borrelia* species. If the horizontal gene transfer explanation is true, we make the following three observations. First, the present study suggests that horizontal transfer of the *ospC* gene is much more common than previous reports in the literature would suggest (45, 72, 97–100). Second, horizontal gene transfer was more likely for major *ospC* groups that were common in the donor *Borrelia* species. Third, some of the exotic *ospC* alleles increased to an appreciable frequency in the recipient *Borrelia* species. For example, the major *ospC* group A10, which is native to *B. afzelii*, had a relative frequency of 31.58% (60/190 infected nymphs) in *B. garinii* (Table 3). If the horizontal gene transfer explanation is true, one puzzling observation is why the exotic major *ospC* groups are so much less abundant (1.88% to 3.09%) than the native *ospC* groups (98.12% and 96.91%) in the nymph-derived isolates. This observation suggests that the exotic strains are underperforming relative to the native strains. Future studies should perform deep sequencing of other *Borrelia* genes that differ between *B. afzelii* and *B. garinii* (e.g., *recA* or *hbb*) in order to differentiate between the coinfection hypothesis and the horizontal gene transfer hypothesis.

In conclusion, despite a large sequencing effort (240,410 sequences), our study was unable to find any *ospC* alleles that were intermediately divergent (>2% to <8%) from the community of major *ospC* groups. Our study suggests that cross-reactive antibodies prevent the invasion of intermediately divergent *ospC* alleles. In contrast, we found no evidence that cross-immunity in the vertebrate host was structuring the community of *ospC* strains in the tick vector. Deep sequencing found that more than 50% of our *Borrelia* isolates contained exotic major *ospC* group alleles that had a low abundance (1.88% to 3.09% of all *ospC* gene sequences) and that had been derived from other *Borrelia* species. Two alternative explanations for these exotic major *ospC* group alleles are coinfections, where only the numerically dominant *Borrelia* species was detected by the RLB assay, or horizontal transfer of the *ospC* gene between *Borrelia* species.

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## REFERENCES

1. **Andersson M, Scherman K, Raberg L.** 2013. Multiple-strain infections of *Borrelia afzelii*: a role for within-host interactions in the maintenance of antigenic diversity? Am Nat **181:**545–554. http://dx.doi.org/10.1086/669905.
2. **Futse JE, Brayton KA, Dark MJ, Knowles DP, Palmer GH.** 2008. Superinfection as a driver of genomic diversification in antigenically variant pathogens. Proc Natl Acad Sci U S A **105:**2123–2127. http://dx.doi.org/10.1073/pnas.0710333105.
3. **Juliano JJ, Porter K, Mwapasa V, Sem R, Rogers WO, Ariey F, Wongsrichanalai C, Read A, Meshnick SR.** 2010. Exposing malaria in-host diversity and estimating population diversity by capture-recapture using massively parallel pyrosequencing. Proc Natl Acad Sci U S A **107:**20138–20143. http://dx.doi.org/10.1073/pnas.1007068107.
4. **Lorono-Pino M, Cropp C, Farfan J, Vorndam A, Rodriguez-Angulo E, Rosado-Paredes E, Flores-Flores L, Beaty B, Gubler D.** 1999. Common occurrence of concurrent infections by multiple dengue virus serotypes. Am J Trop Med Hyg **61:**725–730.
5. **Frank SA.** 2002. Immunology and evolution of infectious disease. Princeton University Press, Princeton, NJ.
6. **Gog JR, Grenfell BT.** 2002. Dynamics and selection of many-strain pathogens. Proc Natl Acad Sci U S A **99:**17209–17214. http://dx.doi.org/10.1073/pnas.252512799.
7. **Gupta S, Anderson R.** 1999. Population structure of pathogens: the role of immune selection. Parasitol Today **15:**497–501. http://dx.doi.org/10.1016/S0169-4758(99)01559-8.
8. **Brunham RC, Plummer FA, Stephens RS.** 1993. Bacterial antigenic variation, host immune-response, and pathogen-host coevolution. Infect Immun **61:**2273–2276.
9. **Conway D, Polley S.** 2002. Measuring immune selection. Parasitology **125:**S3–S16.
10. **Fairlie-Clarke KJ, Shuker DM, Graham AL.** 2009. Why do adaptive immune responses cross-react? Evol Appl **2:**122–131. http://dx.doi.org/10.1111/j.1752-4571.2008.00052.x.
11. **Read AF, Taylor LH.** 2001. The ecology of genetically diverse infections. Science **292:**1099–1102. http://dx.doi.org/10.1126/science.1059410.
12. **Gilbert SC, Plebanski M, Gupta S, Morris J, Cox M, Aidoo M, Kwiatkowski D, Greenwood BM, Whittle HC, Hill AV.** 1998. Association of malaria parasite population structure, HLA, and immunological antagonism. Science **279:**1173–1177. http://dx.doi.org/10.1126/science.279.5354.1173.
13. **Brisson D, Dykhuizen DE.** 2004. *ospC* diversity in *Borrelia burgdorferi*: different hosts are different niches. Genetics **168:**713–722. http://dx.doi.org/10.1534/genetics.104.028738.
14. **Herrmann C, Gern L, Voordouw MJ.** 2013. Species co-occurrence patterns among Lyme borreliosis pathogens in the tick vector *Ixodes ricinus*. Appl Environ Microbiol **79:**7273–7280. http://dx.doi.org/10.1128/AEM.02158-13.
15. **Imwong M, Nakeesathit S, Day NP, White NJ.** 2011. A review of mixed malaria species infections in anopheline mosquitoes. Malar J **10:**253. http://dx.doi.org/10.1186/1475-2875-10-253.
16. **Piesman J, Gern L.** 2004. Lyme borreliosis in Europe and North America. Parasitology **129:**S191. http://dx.doi.org/10.1017/S0031182003004694.
17. **Gern L, Humair P.** 2002. Ecology of *Borrelia burgdorferi* sensu lato in Europe, p 149–174. *In* Lyme borreliosis biology, epidemiology and control. Centre for Agriculture and Biosciences International (CABI), Wallingford, United Kingdom.
18. **Gern L, Estrada-Peña A, Frandsen F, Gray JS, Jaenson TGT, Jongejan F, Kahl O, Korenberg E, Mehl R, Nuttall PA.** 1998. European reservoir hosts of *Borrelia burgdorferi* sensu lato. Zentralbl Bakteriol **287:**196–204. http://dx.doi.org/10.1016/S0934-8840(98)80121-7.
19. **Humair P-F, Gern L.** 2000. The wild hidden face of Lyme borreliosis in Europe. Microbes Infect **2:**915–922. http://dx.doi.org/10.1016/S1286-4579(00)00393-2.
20. **Hanincova K, Schäfer S, Etti S, Sewell H-S, Taragelova V, Ziak D, Labuda M, Kurtenbach K.** 2003. Association of *Borrelia afzelii* with rodents in Europe. Parasitology **126:**11–20. http://dx.doi.org/10.1017/S0031182002002548.
21. **Huegli D, Hu C, Humair P-F, Wilske B, Gern L.** 2002. *Apodemus* species mice are reservoir hosts of *Borrelia garinii* OspA serotype 4 in Switzerland. J Clin Microbiol **40:**4735–4737. http://dx.doi.org/10.1128/JCM.40.12.4735-4737.2002.
22. **Humair P, Gern L.** 1998. Relationship between *Borrelia burgdorferi* sensu lato species, red squirrels (*Sciurus vulgaris*) and *Ixodes ricinus* in enzootic areas in Switzerland. Acta Trop **69:**213–227. http://dx.doi.org/10.1016/S0001-706X(97)00126-5.
23. **Humair P-F, Peter O, Wallich R, Gern L.** 1995. Strain variation of Lyme disease spirochetes isolated from *Ixodes ricinus* ticks and rodents col-

lected in two endemic areas in Switzerland. J Med Entomol **32:**433–438. http://dx.doi.org/10.1093/jmedent/32.4.433.
24. **Kurtenbach K, Peacey M, Rijpkema SG, Hoodless AN, Nuttall PA, Randolph SE.** 1998. Differential transmission of the genospecies of *Borrelia burgdorferi* sensu lato by game birds and small rodents in England. Appl Environ Microbiol **64:**1169–1174.
25. **Humair P, Rais O, Gern L.** 1999. Transmission of *Borrelia afzelii* from *Apodemus* mice and *Clethrionomys* voles to *Ixodes ricinus* ticks: differential transmission pattern and overwintering maintenance. Parasitology **118:**33–42. http://dx.doi.org/10.1017/S0031182098003564.
26. **Hanincová K, Taragelová V, Koci J, Schäfer SM, Hails R, Ullmann AJ, Piesman J, Labuda M, Kurtenbach K.** 2003. Association of *Borrelia garinii* and *B. valaisiana* with songbirds in Slovakia. Appl Environ Microbiol **69:**2825–2830. http://dx.doi.org/10.1128/AEM.69.5.2825-2830.2003.
27. **Humair P, Postic D, Wallich R, Gern L.** 1998. An avian reservoir (*Turdus merula*) of the Lyme borreliosis spirochetes. Zentralbl Bakteriol **287:**521–538.
28. **Taragel'ová V, Koči J, Hanincová K, Kurtenbach K, Derdáková M, Ogden NH, Literák I, Kocianová E, Labuda M.** 2008. Blackbirds and song thrushes constitute a key reservoir of *Borrelia garinii*, the causative agent of borreliosis in Central Europe. Appl Environ Microbiol **74:**1289–1293. http://dx.doi.org/10.1128/AEM.01060-07.
29. **Olsén B, Jaenson TG, Noppa L, Bunikis J, Bergström S.** 1993. A Lyme borreliosis cycle in seabirds and *Ixodes uriae* ticks. Nature **362:**340–342. http://dx.doi.org/10.1038/362340a0.
30. **Kurtenbach K, De Michelis S, Etti S, Schäfer SM, Sewell H-S, Brade V, Kraiczy P.** 2002. Host association of *Borrelia burgdorferi* sensu lato—the key role of host complement. Trends Microbiol **10:**74–79. http://dx.doi.org/10.1016/S0966-842X(01)02298-3.
31. **Kurtenbach K, Sewell H-S, Ogden NH, Randolph SE, Nuttall PA.** 1998. Serum complement sensitivity as a key factor in Lyme disease ecology. Infect Immun **66:**1248–1251.
32. **Kurtenbach K, De Michelis S, Sewell H-S, Etti S, Schäfer SM, Hails R, Collares-Pereira M, Santos-Reis M, Hanincová K, Labuda M.** 2001. Distinct combinations of *Borrelia burgdorferi* sensu lato genospecies found in individual questing ticks from Europe. Appl Environ Microbiol **67:**4926–4929. http://dx.doi.org/10.1128/AEM.67.10.4926-4929.2001.
33. **Gern L, Douet V, López Z, Rais O, Cadenas FM.** 2010. Diversity of *Borrelia* genospecies in *Ixodes ricinus* ticks in a Lyme borreliosis endemic area in Switzerland identified by using new probes for reverse line blotting. Ticks Tick-Borne Dis **1:**23–29. http://dx.doi.org/10.1016/j.ttbdis.2009.11.001.
34. **Rauter C, Hartung T.** 2005. Prevalence of *Borrelia burgdorferi* sensu lato genospecies in *Ixodes ricinus* ticks in Europe: a metaanalysis. Appl Environ Microbiol **71:**7203–7216. http://dx.doi.org/10.1128/AEM.71.11.7203-7216.2005.
35. **Richter D, Debski A, Hubalek Z, Matuschka F-R.** 2012. Absence of Lyme disease spirochetes in larval *Ixodes ricinus* ticks. Vector-Borne Zoonotic Dis **12:**21–27. http://dx.doi.org/10.1089/vbz.2011.0668.
36. **Rollend L, Fish D, Childs JE.** 2013. Transovarial transmission of *Borrelia* spirochetes by *Ixodes scapularis*: a summary of the literature and recent observations. Ticks Tick-Borne Dis **4:**46–51. http://dx.doi.org/10.1016/j.ttbdis.2012.06.008.
37. **Grimm D, Tilly K, Byram R, Stewart PE, Krum JG, Bueschel DM, Schwan TG, Policastro PF, Elias AF, Rosa PA.** 2004. Outer-surface protein C of the Lyme disease spirochete: a protein induced in ticks for infection of mammals. Proc Natl Acad Sci U S A **101:**3142–3147. http://dx.doi.org/10.1073/pnas.0306845101.
38. **Tilly K, Krum JG, Bestor A, Jewett MW, Grimm D, Bueschel D, Byram R, Dorward D, VanRaden MJ, Stewart P.** 2006. *Borrelia burgdorferi* OspC protein required exclusively in a crucial early stage of mammalian infection. Infect Immun **74:**3554–3564. http://dx.doi.org/10.1128/IAI.01950-05.
39. **Gilmore RD, Kappel KJ, Dolan MC, Burkot TR, Johnson B.** 1996. Outer surface protein C (OspC), but not P39, is a protective immunogen against a tick-transmitted *Borrelia burgdorferi* challenge: evidence for a conformational protective epitope in OspC. Infect Immun **64:**2234–2239.
40. **Preac-Mursic V, Wilske B, Jauris S, Will G, Reinhardt S, Lehnert G, Patsouris E, Mehraein P, Soutschek E, Klockmann U.** 1992. Active immunization with OspC protein of *Borrelia burgdorferi* protects gerbils against *B. burgdorferi* infection. Infection **20:**342–349. http://dx.doi.org/10.1007/BF01710681.
41. **Probert WS, LeFebvre RB.** 1994. Protection of C3H/HeN mice from challenge with *Borrelia burgdorferi* through active immunization with OspA, OspB, or OspC, but not with OspD or the 83-kilodalton antigen. Infect Immun **62:**1920–1926.
42. **Probert WS, Crawford M, Cadiz RB, LeFebvre RB.** 1997. Immunization with outer surface protein (Osp) A, but not OspC, provides cross-protection of mice challenged with North American isolates of *Borrelia burgdorferi*. J Infect Dis **175:**400–405. http://dx.doi.org/10.1093/infdis/175.2.400.
43. **Jacquet M, Durand J, Rais O, Voordouw MJ.** 2015. Cross-reactive acquired immunity influences transmission success of the Lyme disease pathogen, *Borrelia afzelii*. Infect Genet Evol **36:**131–140. http://dx.doi.org/10.1016/j.meegid.2015.09.012.
44. **Earnhart CG, Buckles EL, Dumler JS, Marconi RT.** 2005. Demonstration of OspC type diversity in invasive human Lyme disease isolates and identification of previously uncharacterized epitopes that define the specificity of the OspC murine antibody response. Infect Immun **73:**7869–7877. http://dx.doi.org/10.1128/IAI.73.12.7869-7877.2005.
45. **Wang IN, Dykhuizen DE, Qin WG, Dunn JJ, Bosler EM, Luft BJ.** 1999. Genetic diversity of *ospC* in a local population of *Borrelia burgdorferi* sensu stricto. Genetics **151:**15–30.
46. **Baranton G, Seinost G, Theodore G, Postic D, Dykhuizen D.** 2001. Distinct levels of genetic diversity of *Borrelia burgdorferi* are associated with different aspects of pathogenicity. Res Microbiol **152:**149–156. http://dx.doi.org/10.1016/S0923-2508(01)01186-X.
47. **Lagal V, Postic D, Ruzic-Sabljic E, Baranton G.** 2003. Genetic diversity among *Borrelia* strains determined by single-strand conformation polymorphism analysis of the *ospC* gene and its association with invasiveness. J Clin Microbiol **41:**5059–5065. http://dx.doi.org/10.1128/JCM.41.11.5059-5065.2003.
48. **Brisson D, Drecktrah D, Eggers CH, Samuels DS.** 2012. Genetics of *Borrelia burgdorferi*. Annu Rev Genet **46:**515–536. http://dx.doi.org/10.1146/annurev-genet-011112-112140.
49. **Bunikis J, Garpmo U, Tsao J, Berglund J, Fish D, Barbour AG.** 2004. Sequence typing reveals extensive strain diversity of the Lyme borreliosis agents *Borrelia burgdorferi* in North America and *Borrelia afzelii* in Europe. Microbiology **150:**1741–1755. http://dx.doi.org/10.1099/mic.0.26944-0.
50. **Hellgren O, Andersson M, Råberg L.** 2011. The genetic structure of *Borrelia afzelii* varies with geographic but not ecological sampling scale. J Evol Biol **24:**159–167. http://dx.doi.org/10.1111/j.1420-9101.2010.02148.x.
51. **Qiu W-G, Schutzer SE, Bruno JF, Attie O, Xu Y, Dunn JJ, Fraser CM, Casjens SR, Luft BJ.** 2004. Genetic exchange and plasmid transfers in *Borrelia burgdorferi* sensu stricto revealed by three-way genome comparisons and multilocus sequence typing. Proc Natl Acad Sci U S A **101:**14150–14155. http://dx.doi.org/10.1073/pnas.0402745101.
52. **Jacquot M, Bisseux M, Abrial D, Marsot M, Ferquel E, Chapuis J-L, Vourc'h G, Bailly X.** 2014. High-throughput sequence typing reveals genetic differentiation and host specialization among populations of the *Borrelia burgdorferi* species complex that infect rodents. PLoS One **9:**e88581. http://dx.doi.org/10.1371/journal.pone.0088581.
53. **Qiu W-G, Dykhuizen DE, Acosta MS, Luft BJ.** 2002. Geographic uniformity of the Lyme disease spirochete (*Borrelia burgdorferi*) and its shared history with tick vector (*Ixodes scapularis*) in the northeastern United States. Genetics **160:**833–849.
54. **Strandh M, Råberg L.** 2015. Within-host competition between *Borrelia afzelii ospC* strains in wild hosts as revealed by massively parallel amplicon sequencing. Philos Trans R Soc Lond B Biol Sci **370:**20140293. http://dx.doi.org/10.1098/rstb.2014.0293.
55. **Pérez D, Kneubühler Y, Rais O, Jouda F, Gern L.** 2011. *Borrelia afzelii ospC* genotype diversity in *Ixodes ricinus* questing ticks and ticks from rodents in two Lyme borreliosis endemic areas: contribution of co-feeding ticks. Ticks Tick-Borne Dis **2:**137–142. http://dx.doi.org/10.1016/j.ttbdis.2011.06.003.
56. **Gern L, Hu CM, Kocianova E, Vyrostekova V, Rehacek J.** 1999. Genetic diversity of *Borrelia burgdorferi* sensu lato isolates obtained from *Ixodes ricinus* ticks collected in Slovakia. Eur J Epidemiol **15:**665–669. http://dx.doi.org/10.1023/A:1007660430664.
57. **Sinsky RJ, Piesman J.** 1989. Ear punch biopsy method for detection and

isolation of *Borrelia burgdorferi* from rodents. J Clin Microbiol **27:**1723–1727.
58. **Schouls LM, Van De Pol I, Rijpkema SG, Schot CS.** 1999. Detection and identification of *Ehrlichia*, *Borrelia burgdorferi* sensu lato, and *Bartonella* species in Dutch *Ixodes ricinus* ticks. J Clin Microbiol **37:**2215–2222.
59. **Alekseev AN, Dubinina HV, Van De Pol I, Schouls LM.** 2001. Identification of *Ehrlichia* spp. and *Borrelia burgdorferi* in *Ixodes* ticks in the Baltic regions of Russia. J Clin Microbiol **39:**2237–2242. http://dx.doi.org/10.1128/JCM.39.6.2237-2242.2001.
60. **Burri C, Cadenas FM, Douet V, Moret J, Gern L.** 2007. Ixodes ricinus density and infection prevalence of *Borrelia burgdorferi* sensu lato along a north-facing altitudinal gradient in the Rhône Valley (Switzerland). Vector-Borne Zoonotic Dis **7:**50–58. http://dx.doi.org/10.1089/vbz.2006.0569.
61. **Fu L, Niu B, Zhu Z, Wu S, Li W.** 2012. CD-HIT: accelerated for clustering the next-generation sequencing data. Bioinformatics **28:**3150–3152. http://dx.doi.org/10.1093/bioinformatics/bts565.
62. **Li W, Godzik A.** 2006. Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. Bioinformatics **22:**1658–1659. http://dx.doi.org/10.1093/bioinformatics/btl158.
63. **Guindon S, Dufayard J-F, Lefort V, Anisimova M, Hordijk W, Gascuel O.** 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst Biol **59:**307–321. http://dx.doi.org/10.1093/sysbio/syq010.
64. **Rice P, Longden I, Bleasby A.** 2000. EMBOSS: the European Molecular Biology Open Software Suite. Trends Genet **16:**276–277. http://dx.doi.org/10.1016/S0168-9525(00)02024-2.
65. **Theisen M, Borre M, Mathiesen MJ, Mikkelsen B, Lebech A-M, Hansen K.** 1995. Evolution of the *Borrelia burgdorferi* outer surface protein OspC. J Bacteriol **177:**3036–3044.
66. **Anderson JM, Norris DE.** 2006. Genetic diversity of *Borrelia burgdorferi* sensu stricto in *Peromyscus leucopus*, the primary reservoir of Lyme disease in a region of endemicity in southern Maryland. Appl Environ Microbiol **72:**5331–5341. http://dx.doi.org/10.1128/AEM.00014-06.
67. **Richter D, Postic D, Sertour N, Livey I, Matuschka FR, Baranton G.** 2006. Delineation of *Borrelia burgdorferi* sensu lato species by multilocus sequence analysis and confirmation of the delineation of *Borrelia spielmanii* sp. nov. Int J Syst Evol Microbiol **56:**873–881. http://dx.doi.org/10.1099/ijs.0.64050-0.
68. **Core Team R.** 2013. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/.
69. **Lord C, Barnard B, Day K, Hargrove J, McNamara J, Paul R, Trenholme K, Woolhouse M.** 1999. Aggregation and distribution of strains in microparasites. Philos Trans R Soc Lond B Biol Sci **354:**799–807. http://dx.doi.org/10.1098/rstb.1999.0432.
70. **Pielou EC.** 1977. Mathematical ecology. John Wiley & Sons, New York, NY.
71. **Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'Hara RB, Simpson GL, Solymos P, Stevens MHH, Wagner H.** 2013. Package 'vegan.' Community ecology package, version 2.0-10. https://cran.r-project.org/web/packages/vegan/vegan.pdf.
72. **Wang G, Dam AP, Dankert J.** 1999. Evidence for frequent OspC gene transfer between *Borrelia valaisiana* sp. nov. and other Lyme disease spirochetes. FEMS Microbiol Lett **177:**289–296. http://dx.doi.org/10.1111/j.1574-6968.1999.tb13745.x.
73. **Gupta S, Ferguson N, Anderson R.** 1998. Chaos, persistence, and evolution of strain structure in antigenically diverse infectious agents. Science **280:**912–915. http://dx.doi.org/10.1126/science.280.5365.912.
74. **Balmer O, Tanner M.** 2011. Prevalence and implications of multiple-strain infections. Lancet Infect Dis **11:**868–878. http://dx.doi.org/10.1016/S1473-3099(11)70241-9.
75. **Rego RO, Bestor A, Stefka J, Rosa PA.** 2014. Population bottlenecks during the infectious cycle of the Lyme disease spirochete *Borrelia burgdorferi*. PLoS One **9:**e101009. http://dx.doi.org/10.1371/journal.pone.0101009.
76. **Piesman J, Oliver JR, Sinsky RJ.** 1990. Growth kinetics of the Lyme disease spirochete (*Borrelia burgdorferi*) in vector ticks (*Ixodes dammini*). Am J Trop Med Hyg **42:**352–357.
77. **Gupta S, Maiden MCJ, Feavers IM, Nee S, May RM, Anderson RM.** 1996. The maintenance of strain structure in populations of recombining infectious agents. Nat Med **2:**437–442. http://dx.doi.org/10.1038/nm0496-437.
78. **Grenfell BT, Pybus OG, Gog JR, Wood JL, Daly JM, Mumford JA, Holmes EC.** 2004. Unifying the epidemiological and evolutionary dynamics of pathogens. Science **303:**327–332. http://dx.doi.org/10.1126/science.1090727.
79. **States SL, Brinkerhoff RJ, Carpi G, Steeves TK, Folsom-O'Keefe C, DeVeaux M, Diuk-Wasser MA.** 2014. Lyme disease risk not amplified in a species-poor vertebrate community: similar *Borrelia burgdorferi* tick infection prevalence and OspC genotype frequencies. Infect Genet Evol **27:**566–575. http://dx.doi.org/10.1016/j.meegid.2014.04.014.
80. **Girard YA, Travinsky B, Schotthoefer A, Fedorova N, Eisen RJ, Eisen L, Barbour AG, Lane RS.** 2009. Population structure of the Lyme borreliosis spirochete *Borrelia burgdorferi* in the western black-legged tick (*Ixodes pacificus*) in Northern California. Appl Environ Microbiol **75:**7243–7252. http://dx.doi.org/10.1128/AEM.01704-09.
81. **Ogden NH, Lindsay LR, Hanincova K, Barker IK, Bigras-Poulin M, Charron DF, Heagy A, Francis CM, O'Callaghan CJ, Schwartz I, Thompson RA.** 2008. Role of migratory birds in introduction and range expansion of *Ixodes scapularis* ticks and of *Borrelia burgdorferi* and *Anaplasma phagocytophilum* in Canada. Appl Environ Microbiol **74:**1780–1790. http://dx.doi.org/10.1128/AEM.01982-07.
82. **Ogden NH, Margos G, Aanensen DM, Drebot MA, Feil EJ, Hanincova K, Schwartz I, Tyler S, Lindsay LR.** 2011. Investigation of genotypes of *Borrelia burgdorferi* in *Ixodes scapularis* ticks collected during surveillance in Canada. Appl Environ Microbiol **77:**3244–3254. http://dx.doi.org/10.1128/AEM.02636-10.
83. **Fedorova N, Kleinjan JE, James D, Hui LT, Peeters H, Lane RS.** 2014. Remarkable diversity of tick or mammalian-associated borreliae in the metropolitan San Francisco Bay Area, California. Ticks Tick-Borne Dis **5:**951–961. http://dx.doi.org/10.1016/j.ttbdis.2014.07.015.
84. **Hamer SA, Hickling GJ, Walker ED, Tsao JI.** 2014. Increased diversity of zoonotic pathogens and *Borrelia burgdorferi* strains in established versus incipient *Ixodes scapularis* populations across the midwestern United States. Infect Genet Evol **27:**531–542. http://dx.doi.org/10.1016/j.meegid.2014.06.003.
85. **Mathers A, Smith RP, Cahill B, Lubelczyk C, Elias SP, Lacombe E, Morris SR, Vary CP, Parent CE, Rand PW.** 2011. Strain diversity of *Borrelia burgdorferi* in ticks dispersed in North America by migratory birds. J Vector Ecol **36:**24–29. http://dx.doi.org/10.1111/j.1948-7134.2011.00137.x.
86. **Ginsberg HS, Buckley P, Balmforth MG, Zhioua E, Mitra S, Buckley FG.** 2005. Reservoir competence of native North American birds for the Lyme disease spirochete, *Borrelia burgdorferi*. J Med Entomol **42:**445–449.
87. **LoGiudice K, Ostfeld RS, Schmidt KA, Keesing F.** 2003. The ecology of infectious disease: effects of host diversity and community composition on Lyme disease risk. Proc Natl Acad Sci U S A **100:**567–571. http://dx.doi.org/10.1073/pnas.0233733100.
88. **Perkins SE, Cattadori IM, Tagliapietra V, Rizzoli AP, Hudson PJ.** 2003. Empirical evidence for key hosts in persistence of a tick-borne disease. Int J Parasitol **33:**909–917. http://dx.doi.org/10.1016/S0020-7519(03)00128-0.
89. **Randolph S, Miklisova D, Lysy J, Rogers D, Labuda M.** 1999. Incidence from coincidence: patterns of tick infestations on rodents facilitate transmission of tick-borne encephalitis virus. Parasitology **118:**177–186. http://dx.doi.org/10.1017/S0031182098003643.
90. **Voordouw MJ.** 2015. Co-feeding transmission in Lyme disease pathogens. Parasitology **142:**290–302. http://dx.doi.org/10.1017/S0031182014001486.
91. **Read A, Taylor L.** 2000. Within-host ecology of infectious diseases: patterns and consequences, p 59–75. *In* Molecular epidemiology of infectious diseases. Arnold, London, United Kingdom.
92. **Berret J, Voordouw MJ.** 2015. Lyme disease bacterium does not affect attraction to rodent odour in the tick vector. Parasites Vectors **8:**249. http://dx.doi.org/10.1186/s13071-015-0856-8.
93. **Marsot M, Sigaud M, Chapuis J, Ferquel E, Cornet M, Vourc'h G.** 2011. Introduced Siberian chipmunks (*Tamias sibiricus barberi*) harbor more-diverse *Borrelia burgdorferi* sensu lato genospecies than native bank voles (*Myodes glareolus*). Appl Environ Microbiol **77:**5716–5721. http://dx.doi.org/10.1128/AEM.01846-10.
94. **Millins C, Magierecka A, Gilbert L, Edoff A, Brereton A, Kilbride E, Denwood M, Birtles R, Biek R.** 2015. An invasive mammal (the gray squirrel, *Sciurus carolinensis*) commonly hosts diverse and atypical genotypes of the zoonotic pathogen *Borrelia burgdorferi* sensu lato. Appl Environ Microbiol **81:**4236–4245. http://dx.doi.org/10.1128/AEM.00109-15.
95. **Dubska L, Literak I, Kocianova E, Taragelova V, Sychra O.** 2009.

Differential role of passerine birds in distribution of *Borrelia* spirochetes, based on data from ticks collected from birds during the postbreeding migration period in Central Europe. Appl Environ Microbiol **75:**596–602. http://dx.doi.org/10.1128/AEM.01674-08.

96. **Franke J, Moldenhauer A, Hildebrandt A, Dorn W.** 2010. Are birds reservoir hosts for *Borrelia afzelii*? Ticks Tick-Borne Dis **1:**109–112. http://dx.doi.org/10.1016/j.ttbdis.2010.03.001.
97. **Livey I, Gibbs C, Schuster R, Dorner F.** 1995. Evidence for lateral transfer and recombination in OspC variation in Lyme disease *Borrelia*. Mol Microbiol **18:**257–269. http://dx.doi.org/10.1111/j.1365-2958.1995.mmi_18020257.x.
98. **Barbour AG, Travinsky B.** 2010. Evolution and distribution of the *ospC* gene, a transferable serotype determinant of *Borrelia burgdorferi*. mBio **1:**e00153-10. http://dx.doi.org/10.1128/mBio.00153-10.
99. **Dykhuizen DE, Baranton G.** 2001. The implications of a low rate of horizontal transfer in *Borrelia*. Trends Microbiol **9:**344–350. http://dx.doi.org/10.1016/S0966-842X(01)02066-2.
100. **Jauris-Heipke S, Liegl G, Preac-Mursic V, Rössler D, Schwab E, Soutschek E, Will G, Wilske B.** 1995. Molecular analysis of genes encoding outer surface protein C (OspC) of *Borrelia burgdorferi* sensu lato: relationship to *ospA* genotype and evidence of lateral gene exchange of *ospC*. J Clin Microbiol **33:**1860–1866.