Experimental Infections of the Reservoir Species *Peromyscus leucopus* with Diverse Strains of *Borrelia burgdorferi*, a Lyme Disease Agent

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ABSTRACT The rodent *Peromyscus leucopus* is a major natural reservoir for the Lyme disease agent *Borrelia burgdorferi* and a host for its vector *Ixodes scapularis*. At various locations in northeastern United States 10 to 15 *B. burgdorferi* strains coexist at different prevalences in tick populations. We asked whether representative strains of high or low prevalence differed in their infections of *P. leucopus*. After 5 weeks of experimental infection of groups with each of 6 isolates, distributions and burdens of bacteria in tissues were measured by quantitative PCR, and antibodies to *B. burgdorferi* were evaluated by immunoblotting and protein microarray. All groups of animals were infected in their joints, ears, tails, and hearts, but overall spirochete burdens were lower in animals infected with low-prevalence strains. Animals were similar regardless of the infecting isolate in their levels of antibodies to whole cells, FlaB, BmpA, and DbpB proteins, and the conserved N-terminal region of the serotype-defining OspC proteins. But there were strain-specific antibody responses to full-length OspC and to plasmid-encoded VlsE, BBK07, and BBK12 proteins. Sequencing of additional VlsE genes revealed substantial diversity within some pairs of strains but near-identical sequences within other pairs, which otherwise differed in their *ospC* alleles. The presence or absence of full-length *bbk07* and *bbk12* genes accounted for the differences in antibody responses. We propose that for *B. burgdorferi*, there is selection in reservoir species for (i) sequence diversity, as for OspC and VlsE, and (ii) the presence or absence of polymorphisms, as for BBK07 and BBK12.

IMPORTANCE Humans are dead-end hosts for *Borrelia* agents of Lyme disease (LD), and, thus, irrelevant for the pathogens' maintenance. Many reports of human cases and laboratory mouse infections exist, but less is known about infection and immunity in natural reservoirs, such as the rodent *Peromyscus leucopus*. We observed that high- and low-prevalence strains of *Borrelia burgdorferi* were capable of infecting *P. leucopus* but elicited different patterns of antibody responses. Antibody reactivities to the VlsE protein were as type-specific as previously characterized reactivities to serotype-defining OspC proteins. In addition, the low-prevalence strains lacked full-length genes for two proteins that (i) are encoded by a virulence-associated plasmid in some high-prevalence strains and (ii) LD patients and field-captured rodents commonly have antibodies to. Immune selection against these genes may have led to null phenotype lineages that can infect otherwise immune hosts but at the cost of reduced fitness and lower prevalence.

Lyme disease (LD) is a common infection in parts of the North America and Eurasia continents. But humans, aside from our collective effects on forestation, climate, and biodiversity, are irrelevant for maintaining the etiologic agents in nature. Unlike the plague agent *Yersinia pestis*, which can be transmitted between humans as well as from animals, *Borrelia burgdorferi* is obligately vector-borne and depends entirely on other vertebrates for its reservoirs. If humans disappeared from North America, *B. burgdorferi* would persist on this continent in a variety of mammals and birds, as it has for thousands of years (1).

If the research goal is understanding disease in humans, focusing on pathogenesis is rewarding. If the objective is a vaccine, results from the laboratory mouse, *Mus musculus*, are sufficiently predictive to justify clinical trials (2). But for questions on the ecology of this pathogen, predictions about its geographic spread, and field-based prevention strategies, insights gained from studies of human cases or laboratory rodents may be less informative than studies of the actual reservoirs of *B. burgdorferi*.

In regions of North America where LD has its highest incidences, *Peromyscus leucopus* is a common reservoir for *B. burgdorferi* and a preferred host for *Ixodes scapularis*, its tick vector (3). In these areas the majority of *P. leucopus* become infected with *B. burgdorferi* (4, 5). Its common name of "white-footed mouse" belies its phylogenetic distance from the house mouse, *Mus musculus*. *Peromyscus* belongs to the taxon Cricetidae, together with voles and hamsters, and not Muridae, the family for laboratory mice and rats (6). While *B. burgdorferi*-infected *M. musculus* mice exhibit arthritis and carditis (7), such lesions were not observed in *P. leucopus* (8, 9), *Peromyscus maniculatus* (10), or the related species *Mesocricetus auratus*, the Syrian hamster (11). *P. leucopus* and

TABLE 1 *Borrelia burgdorferi* strains, origins, prevalences, and genotypes

Strain or isolate	State of origin	Prevalence (%)[a]	OspC type[b]	IGS type[b,c]	MLST type[d]	Presence or absence of[e]:	
						bbe22	*bbf01*
SH2	NY	19.4	K	2	3	+	+
B31/BL206	NY	14.4	A	1	1	+	+
TBO2	NY	9.3	N	4	36	+	−
HB19	CT	4.8	I	7	16	+	+
27577	NY	4.5	F	17	8	+	+
WQR	RI	4.3	T	28	37	+	+
N40/B348	NY	4.0	E	9	19	+	+
IDS	NY	14.4, 9.3	A, N	1, 4	1, 36	+	+

[a] Prevalence in the northeastern United States (reference 24).
[b] Reference 27.
[c] IGS, 16S-23S intergenic spacer.
[d] MLST, multilocus sequence type, according to reference database at http://borrelia.mlst.net.
[e] Determined by mapping of sequence reads as described in the text (for results, see Table S2 in the supplemental material).

Syrian hamsters may be persistently infected with *B. burgdorferi* (4, 12–14) but, unlike *M. musculus* (7), without discernible evidence of disease or disability (9, 10, 15).

Differences in disease expression in the host may also be attributable to traits of the infecting microbe. The concept of strain differences in infectiousness or virulence was suggested by epidemiological studies of human cases whose *B. burgdorferi* isolates were genotyped (16–18). Similar differences in propensities to disseminate in *M. musculus* were reported for limited strain sets (19, 20). But in another study of different strains, bacterial densities in the blood were not predictive of tissue burdens or pathology in the laboratory mouse (21). Derdáková et al. and Hanincová et al. infected *P. leucopus* with two different strains and reported differences in durations of transmissibility to naive mice (22, 23), but tissue distributions of the bacteria and the antigen-specific immune responses were not assessed.

One basis for isolate selection for studies of strain differences in a natural reservoir is their relative prevalences, which can be estimated by counting genotypes of *B. burgdorferi* among nymphal *I. scapularis* ticks (1, 24, 25). Genotypes can be defined by sequences of 16S-23S rRNA intergenic spacers (IGS), the genes for the outer membrane protein OspC, and multilocus sequence typing (MLST). For a given geographic area, these different schemes are congruent (1, 25–27). Most genotype surveys have found a log-normal distribution of strains, which has suggested the action of balancing selection (28).

In previous studies of *P. leucopus* captured in the field, sera from infected animals had antibody reactivity profiles that were similar to those of LD patients (4, 29). This included a high frequency of reactivity to the OspC, VlsE, BBK07, and BBK12 proteins of a single strain, B31. The OspC proteins are highly polymorphic and largely strain-specific (24), but contain epitopes shared among the protein family (30). Magnarelli et al. also reported a high frequency of antibody to the VlsE protein among captured *P. leucopus* in Connecticut (31). The VlsE protein is associated with antigenic variation in B31 and other strains (32), but it or a component peptide is also a basis for immunodiagnostic assays with high sensitivity and specificity for LD (33). The BBK07 and BBK12 proteins are encoded by a virulence-associated linear plasmid, lp36, of strain B31 (34) and are commonly bound by antibodies from patients with LD (29, 35).

The present study of *P. leucopus* was carried out in a laboratory setting with quantitative PCR, immunoblotting, and protein microarray analysis. We found that six *B. burgdorferi* isolates, which represented strains with different prevalences in the northeastern United States, were infectious for *P. leucopus* and elicited similar antibody responses to conserved antigens. However, different isolates elicited different antibody reactivities to other antigens, notably the plasmid-encoded OspC, VlsE, BBK07, and BBK12 proteins. These strain-specific responses led to a deeper study of the heterogeneity of the genetic loci for these antigens.

RESULTS

Strains for infections. Table 1 summarizes nymphal prevalences and selected genetic characteristics of the isolates: 5 clonal populations (Sh-2-82, TBO2, HB19, 27577, and WQR) and one mixed-population isolate, IDS, which was discovered to be a mixture comprising strains B31 and TBO2 after experiments began (see Text S1 in the supplemental material). The strains represented in the study varied in prevalence from 4% to 19% among infected *I. scapularis* nymphs in a large survey of ticks in the northeastern United States (24, 27). For this study, Sh-2-82 (here abbreviated SH2), B31, and TBO2 were classified as high-prevalence strains, and HB19, 27577, and WQR as low-prevalence strains. We carried out next-generation sequencing of the genomes of SH2, TBO2, HB19, 27577, and WQR, as well as resequencing of the genome of strain B31. We had previously noted that the *ospC* and IGS types of strains SH2 and HB19 were identical to corresponding loci in strains 297 and WI91-23, respectively (25, 27). Additional sequence data revealed that strains SH2 and 297 had the same MLST type, but the MLST type of strain HB19, which was from the northeastern U. S, differed from that of WI91-23, which was from the north-central region. Two other strains, 27577, which has the type F *ospC* allele, and WQR, which has the type T allele, were characterized for this study (see Table S2 in the supplemental material). Strains BL206 and B348 have the same OspC type, IGS sequence, and MLST type as strains B31 and N40, respectively (http://borrelia.mlst.net), and for this study were considered to belong to the same lineages.

Experimental infections. *P. leucopus* animals were inoculated i.p. with 50 to 100 *B. burgdorferi* cells of each of 6 isolates; inoculum sizes were close to estimates of numbers of spirochetes transmitted by ticks (36). All 6 populations had been passed through immunodeficient mice before the *P. leucopus* infections. Each had the *pncA* gene of the lp25 plasmid and, with the exception of strain

TABLE 2 Burdens of spirochetes of different strains of *B. burgdorferi* in tissues of seropositive *P. leucopus* mice

Strain or isolate	Mean no. of 16S rRNA gene copies/μg total DNA^a			
	Ankle	Tail	Ear	Heart
SH2	346 (175–685) [5/5]	1,248 (64–24,419) [5/5]	370 (172–798) [5/5]	3 (2–6) [2/5]
TBO2	1,467 (575–3,738) [5/5]	196 (80–481) [5/5]	249 (75–832) [3/5]	27 (13–52) [5/5]
HB19	802 (350–1,836) [5/5]	47 (23–96) [5/5]	121 (23–636) [5/5]	19 (9–40) [2/5]
27577	1231 (540–2,804) [4/4]	551 (92–3,283) [4/4]	181 (61–533) [4/4]	4 (2–8) [4/4]
WQR	3,722 (2,037–6,800) [4/4]	86 (167–474) [3/4]	69 (17–280) [4/4]	3 (1–7) [3/4]
IDS	1,082 (535–2190) [5/5]	2,687 (1,371–5,266) [5/5]	193 (42–889) [5/5]	7 (3–17) [5/5]

^{a} Values in parentheses are antilogarithms of 95% confidence intervals for log-transformed values; values in brackets are numbers of mice with >10 gene copies in tissue/number of mice of that strain examined.

TBO2, the bbf01 gene of lp28-1 of strain B31 (Table 1; also, see Table S2 in the supplemental material) (37, 38).

At the end of 5 weeks of infection, tissue and blood were collected from animals, and *B. burgdorferi* cells were quantitated by qPCR. All 30 animals became infected by the criterion of ≥2 tissues out of 4 being positive by qPCR (see Table S3 in the supplemental material). Table 2 summarizes the burdens of *B. burgdorferi* in the ankle joints, distal tails, ear punch biopsy specimens, and heart tissues in the 28 (93%) of 30 animals that were also seropositive by immunoblot at 5 weeks (Fig. 1). Clear distinctions between strains with regard to their burdens in the animals were not discernible on a tissue-by-tissue basis. But intrastrain as well as interstrain variances between these outbred animals were high, with coefficients of variation ranging between 1 and 2 across all strains by tissue. By normalizing the burdens in tissues by z scores (Table S3 in the supplemental material), commensurate burdens in the four tissues could be determined for each mouse (Fig. 2). Aggregate z scores, which were the sum of z scores for each tissue,

for the 15 mice infected with high-prevalence strains were higher overall than those for 13 seropositive mice infected with low-prevalence strains (odds ratios [OR; 95% confidence intervals are in brackets], 2.15 [1.18 to 4.67]; $R^2 = 0.23$; Kruskal-Wallis rank test, $P = 0.018$; ANOVA, $P = 0.009$).

The two seronegative animals excluded from the preceding analysis were infected with the low-prevalence strains 27577 and WQR. All four tissues of these mice had markedly higher burdens than those of the seropositive animals infected with the same strain and of all but 3 animals infected with other strains (see Table S3 in the supplemental material). In the group infected with 27577, the seronegative animal had ~2,300 gene copies in the heart sample, while the mean number of gene copies for the four seropositive animals was 4.

Immunoblot analysis. Sera collected at 5 weeks were used in a whole-cell *B. burgdorferi* immunoblot. In the immunoblot assay, which is shown in Fig. 1, the antigens most commonly bound by the 28 sera were the BmpA at 39 kDa (82%), FlaB at 41 kDa (75%),
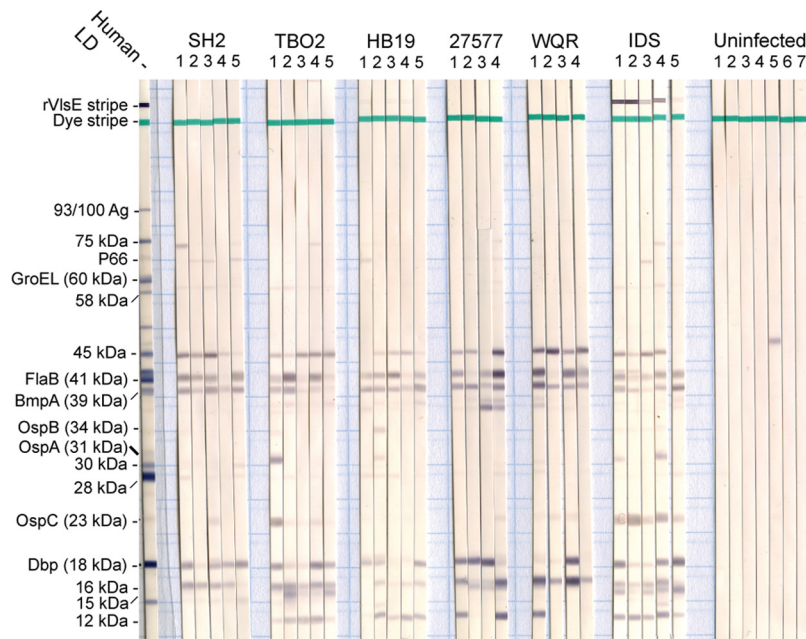

FIG 1 Immunoblot analysis of whole-cell lysates of *Borrelia burgdorferi* strain B31 with sera from *P. leucopus* mice that were uninfected or infected with isolate IDS or strain SH2, TBO2, HB19, 27577, or WQR. A stripe of recombinant strain B31 VlsE was present on the nitrocellulose strips. Antibody binding was detected with alkaline phosphatase-labeled secondary antibody to *P. leucopus* IgG. The migrations of selected antigens on a reference strip, supplied by the manufacturer, with pooled sera from patients with Lyme disease (LD), is shown on the left. The numbers at the top of the aligned strips indicate the individual animals in each group of infected animals or controls.
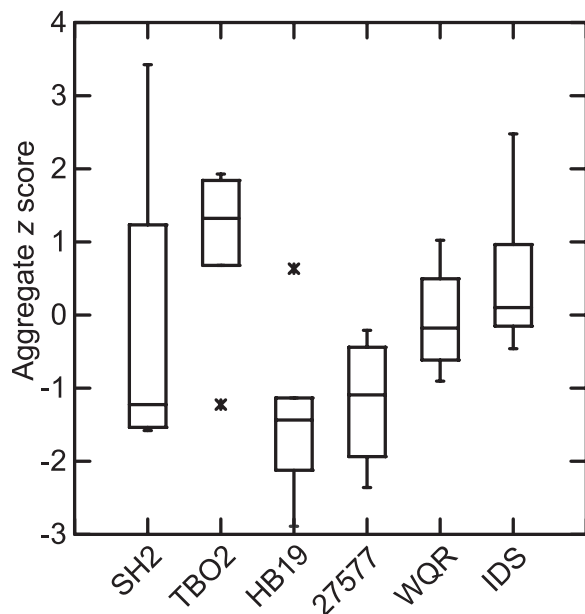
FIG 2 Adjusted overall tissue burdens of *B. burgdorferi* by isolate or strain in skin, tail, joint, and heart of *P. leucopus* mice after 5 weeks of infection. The box-whisker plots show aggregate *z* scores for all 4 tissues; their calculations are described in the text. Spirochete burdens in genome copies were estimated by quantitative PCR. Each box indicates the first and third quartiles, and the line inside the box is the median. The 1.5× interquartile range is indicated by the vertical line (whiskers) bisecting the box, and values outside this range are indicated by asterisks.

an unidentified 45-kDa protein (71%), either DbpA or DbpB at 18 kDa (64%), and an unidentified 16-kDa protein (64%). The binding to two other major antigens, OspC and VlsE, was more strain specific than anticipated from previous reports on field-collected *P. leucopus* (4, 29, 31). All 5 mice infected with the IDS isolate, which includes B31, had detectable antibody to the type A OspC of strain B31 cells this assay was based on, but only one other serum, a sample from an animal infected with strain TBO2, had discernible binding to this OspC protein. While all mice infected with IDS had detectable antibodies to recombinant VlsE of strain B31 on the blot, none of the sera from animals infected with other strains demonstrated this reactivity.

Protein microarray analysis. For the protein microarray, 26 seropositive and 12 control *P. leucopus* sera were studied. In addition, we examined the binding reactions of sera from the following sources: (i) 3 *P. leucopus* mice infected with the relapsing fever agent *Borrelia hermsii*, (ii) groups of 3 *M. musculus* mice infected with SH2 or IDS, and (iii) 5 uninfected *M. musculus* mice. The binding values for these specimens against a whole-cell lysate of strain B31, recombinant antigens FlaB, BmpA, DbpB, BBK07, BBK12, OspA, and VlsE of strain B31, and *B. hermsii*'s Vlp17 are given in Table S4 in the supplemental material.

In general, the reactivities with the whole-cell lysate, FlaB, BmpA, and DbpB were similar to what we observed with the immunoblots. That is, most of the infected *P. leucopus* and *M. musculus* mice had antibodies against these antigens, regardless of the infecting strain. There was no difference between animals infected with low- and high-prevalence strains with respect to antibody binding to the lysate, FlaB, BmpA, or DbpB.

We also examined by microarray the antibody responses to the

diverse set of OspC proteins represented by the infecting strains, as confirmed by mapping sequence reads to references (see Table S2 in the supplemental material): A (B31), K (SH2), N (TBO2), I (HB19), F (27577), and T (WQR). The array also included type E OspC, which is produced by strain B348. To assess the cross-reactivity attributable to the conserved N-terminal end of the processed protein and its first helix, we synthesized a peptide that included 54 amino acids of type A OspC ("first helix"). The corresponding regions of OspC types K, N, I, F, T, and E were 94 to 98% identical over this length to the reference. The results of the array analysis are given in Table S5 in the supplemental material.

Sera from all infected *P. leucopus* and *M. musculus* had antibody binding to the first helix that was significantly higher than that from uninfected controls. Antigenic cross-reactivity between the longer recombinant OspC proteins was also evident in the binding of antibodies to heterotypic proteins for both species. For the *P. leucopus* samples, the pairwise R^2 values averaged 0.69 (0.57 to 0.81) for the first helix versus OspC A, E, F, I, K, N, and T and 0.59 (0.50 to 0.68) for these OspC types against each other. But, as we observed with the immunoblot analysis, there was also evidence of type-specific antibody responses among the infected animals of both species (see Table S5 in the supplemental material). For all groups of animals except the *P. leucopus* mice infected with WQR, there was more antibody binding to the homotypic OspC than to the 6 heterotypic ones, by an overall ratio of 4.3 (2.0 to 5.6), including the WQR results (see Table S5 in the supplemental material).

Antibodies to VlsE. To identify and quantitate antibodies to specific antigens, we used a microarray with selected recombinant proteins. As with the immunoblotting, the microarray results were evidence of strain-specific responses of *P. leucopus* to the VlsE protein (see Table S4 in the supplemental material). Figure 3 summarizes these data and for comparison also shows the binding values for antibodies to FlaB. The binding of antibodies of strain WQR-infected animals was as negligible as that of control sera. The binding of antibodies of animals infected with 27577, TBO2, SH2, and HB19 was marginally greater than that of controls, but the values were still significantly lower than the binding shown by sera from IDS-infected animals.

While we cannot exclude a confounding effect of the mixed infection, we presume that the binding of antibodies from IDS-infected animals to VlsE of strain B31 is largely attributable to the B31 component of that mixture. Evidence for this was the lack of antibody binding to VlsE shown by animals infected with TBO2 alone. The strain specificity shown by *P. leucopus* for VlsE protein presented in the array format contrasts with the evidence of cross-reactivity of anti-VlsE antibodies in the case of *M. musculus* infected with strain SH2 (see Table S4 in the supplemental material).

P. leucopus did not have detectable cross-reactive antibodies to *B. hermsii*'s Vlp17 protein, which is homologous to VlsE (39) (see Table S4 in the supplemental material). A reciprocal pattern was observed with *B. hermsii* infections: little or no antibody to FlaB, BmpA, DbpB, and VlsE of *B. burgdorferi* but large amounts of binding to the Vlp17 protein.

Antibodies to BBK7 and BBK12. Another set of antigens of interest were the paralogous BBK07 and BBK12 proteins, because of their documented immunogenicity in natural infections of *P. leucopus* and humans (29), as well as the association of their encoding plasmid with infectiousness (40). As Fig. 3 and as Table S4 in the supplemental material show, there were marked dif-
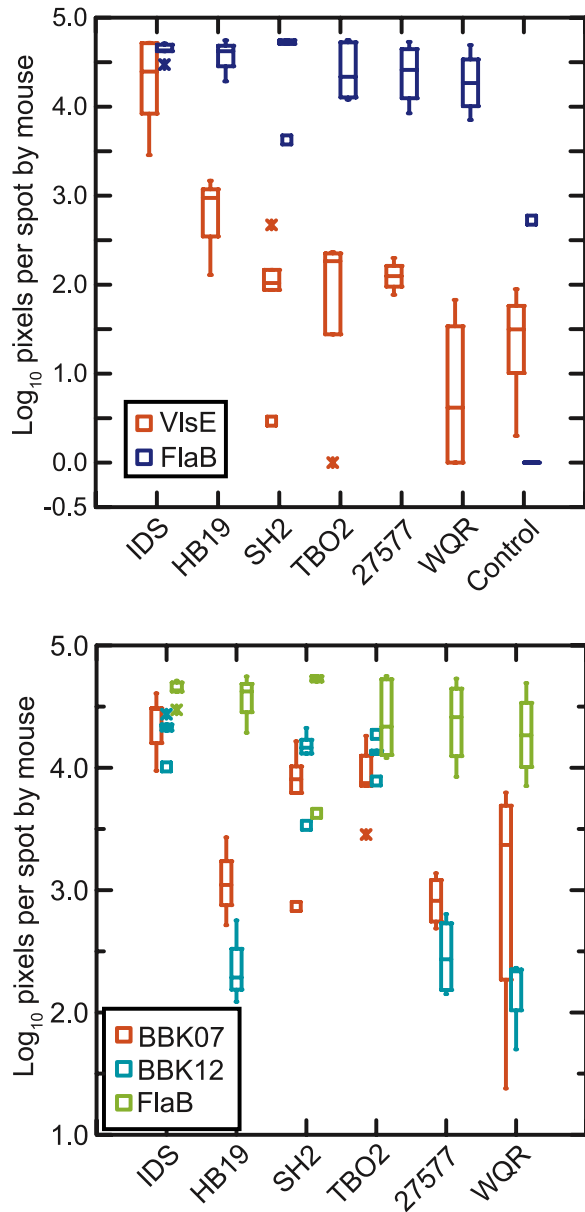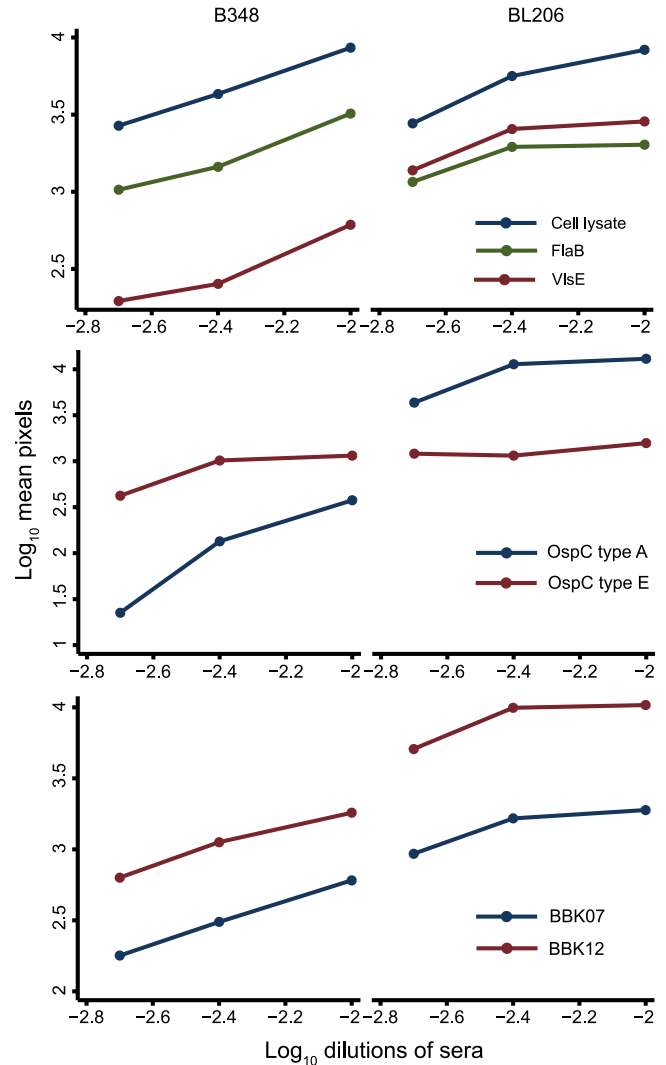
FIG 4 Binding of antibodies in sera from *P. leucopus* mice, infected by tick bites with *B. burgdorferi* strain B348 or BL206, to purified recombinant FlaB or VlsE (top), OspC type A or E (middle), and BBK07 or BBK12 (bottom) on arrays. The graphs are scatter plots, separated according to the infecting strain, with connecting lines of log-transformed intensity values in pixels against log-transformed serum dilutions.

FIG 3 Binding of antibodies in *P. leucopus* sera to selected proteins of *B. burgdorferi* strain B31 on a protein microarray. (Top) VlsE (red) and FlaB (blue) proteins; (bottom) BBK07 (red), BBK12 (blue), and FlaB (green) proteins. The box-whisker plots (Fig. 2) show log-transformed intensity values in pixels for the binding of antibodies from uninfected control animals (top) and infected animals (top and bottom).

ferences between strains in whether antibodies to these proteins were elicited during infection of *P. leucopus*. For comparison purposes antibodies to FlaB again are shown; there was little or no detectable antibodies to these proteins in control *P. leucopus* or *M. musculus* sera (Table S4 in the supplemental material). Mice infected with IDS or one of the other two high-prevalence strains had antibodies that bound to the BBK07 and BBK12 proteins of strain B31. There was little or no detectable binding to either protein by antibodies in sera from mice infected with the low-prevalence strains HB19, 27577, and WQR.

Sera of *P. leucopus* mice infected by tick bites. To assess whether the route of infection had an effect on some of the anti-

body responses, we examined sera from *P. leucopus* infected by tick bite with either strain BL206, which carries a type A *ospC* gene, or strain B348, which bears a type E *ospC* gene, for antibody binding to the whole-cell lysate, FlaB, VlsE, OspC type A, OspC type E, BBK07, and BBK12 on the array. The sera were diluted 1:100, 1:250, or 1:500 (Fig. 4). The sera were similar in antibody binding to the cell lysate and to FlaB but differed in binding to the other antigens. The binding of antibodies in the B348 sample to VlsE of strain B31 was severalfold lower at each serum dilution than that observed with BL206 sample. With the OspC proteins, there was evidence again of both cross-reactive and type-specific antibody responses; at each dilution, the amount of binding to the homotypic OspC was greater, up to 10-fold, than that to the heterotypic protein. With both samples there was more binding to BBK12 than to BBK07, but the amount of binding of antibodies in the B348 sample was smaller than the corresponding value for BL206 at each serum dilution.

VlsE genes. In a previous study of sera from infected *P. leuco-pus* captured in the field, we observed that the majority of the animals had antibodies that bound to the VlsE protein of strain B31 (29). Here, cross-reactive antibodies to B31's VlsE were noted in *M. musculus* infected with SH2, but not in *P. leucopus* infected with that strain or strains TBO2, HB19, 27577, or WQR (see Table S4 in the supplemental material). This finding led us to determine if these other strains had *vlsE* sequences.

VlsE genes were identified in TBO2, HB19, SH2, 27577, and WQR by a combination of *de novo* assembly of the sequence reads, mapping of reads to reference *vls* gene sequences in the public domain, and then confirmation by sequencing of PCR products amplified from genomic DNA (see Text S1 in the supplemental material). Strain B31 was resequenced for this study to validate the sequence assembly approach. Figure 5 is a distance phylogram of a codon-based alignment of these nucleotide sequences and selected reference sequences (available at https://webfiles.uci.edu/xythoswfs/webui/_xy-8429602_1). As expected from their chromosomal loci and *ospC* alleles, the SH2 VlsE sequence clusters with strain 297s, and HB19's VlsE sequence clusters with WI91-23's protein. Both clusters are distant from the B31 sequences. More distant still are the VlsE sequences of TBO2, 27577, and WQR strains, which share similarities in VlsE sequence with strains 80a and 94a of *B. burgdorferi* and strain PLi of *Borrelia garinii*. The pairwise percent identities at ungapped sites of the deduced protein sequences ranged from 39 to 53% between the strain B31 VlsE of the proteins (see Table S5 in the supplemental material).

We identified regions of these proteins, as well as of the Vlp17 protein, that aligned with the "C6 peptide" of immunodiagnostic applications (41) and noted that sequences of other strains differed at several positions from that of the canonical C6 peptide and corresponding sequence of strain B31 (see Fig. S2 in the supplemental material).

bbk07 and bbk12 genes. In strain B31, the bbk07 and bbk12 genes are located on lp36, a 36-kb linear plasmid which is associated with infectiousness (34). In other strains of *B. burgdorferi*, some of the genes of lp36 may be distributed among other replicons (42). Given this variability between strains, we investigated the bbk07 and bbk12 loci in the strains B31, SH2, and TBO2, which alone or in combination elicited high levels of antibodies to both BBK07 and BBK12 (Fig. 3), as well as in the strains HB19, 27577, and WQR, which did not. In strain B31, bbk07 and bbk12 are paralogous but are oppositely oriented and separated by ~2 kb on the plasmid. The near-identity of BBK07 and BBK12 in their N-terminal regions is sufficient for antigenic cross-reactivity (29).

We first used PCR with primers from within and outside the two genes (Fig. 6, left; also, see Table S1 in the supplemental material). With primers for positions that flank bbk07 in strain B31 (see Table S1), there was the expected PCR product of ~800 bp in IDS and also in strains SH2 and TBO2. No products were observed with total DNA from HB19, 27577, and WQR. Similar results were obtained with internal primers that spanned positions 1 to 418 of bbk07. With a forward primer starting from position 66, we observed a product with HB19 as well, but not with 27577 or WQR. PCR with primers comprising the 5′ and 3′ ends of the bbk12 gene revealed the expected products for full-length genes in the IDS, SH2, and TBO2 samples, a truncated product from HB19, and only faint bands at ~280 bp with the 27577 and WQR samples.

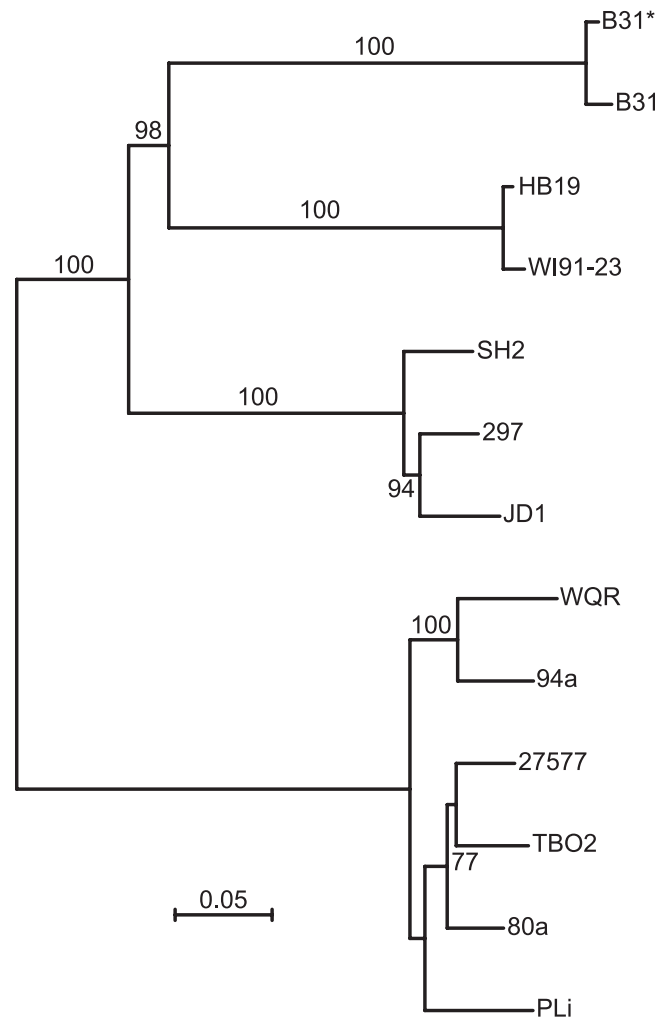Enumeration of matching reads from genome sequencing in-

FIG 5 Distance phylogram of partial *vlsE* nucleotide sequences deduced from assemblies of nucleotide sequences for strains SH2, HB19, WQR, TBO2, and 27577 of *B. burgdorferi* and reference sequences for strains B31, 297, JD1, WI91-23, 94a, and 80a of *B. burgdorferi* and strain PLi of *B. garinii*. The alignment was codon-based alignment. Nodes with support of ≥70% for 100 bootstrap iterations of the maximum-likelihood method, as carried out with the PhyML 3.0 algorithm (http://www.atgc-montpellier.fr/phyml) under the General Time Reversible model of substitution, are shown near the branches. B31* is derived from the assembly of reads from a resequencing of strain B31 by the same method as for the other strains. The scale bar indicates nucleotide distance.

dicated that the IDS preparation and the 5 strains of the other samples had genomes with complete or near-complete sequences for the bbk01, bbk13, bbk15, bbk17, bbk19, bbk32, and bbk52 genes of the lp36 plasmid (Fig. 6; also, see Table S2 in the supplemental material), but in strains 27577 and WQR there was not a detectable bbk07 gene sequence and there were significantly fewer reads matching the bbk12 gene (Fig. 6, right). When sequence reads were mapped to strain B31's lp36 plasmid as a reference, we obtained the full-length lp36 with the sample of B31 that was resequenced (see Fig. S2 in the supplemental material). The assemblies of SH2 and TBO2 reads mapped to the reference were only ~25 kb, but the B31 lp36 plasmid's left end with its oppositely oriented bbk07 and bbk12 genes was largely preserved in these strains. There was evidence of rearrangements or translocations at
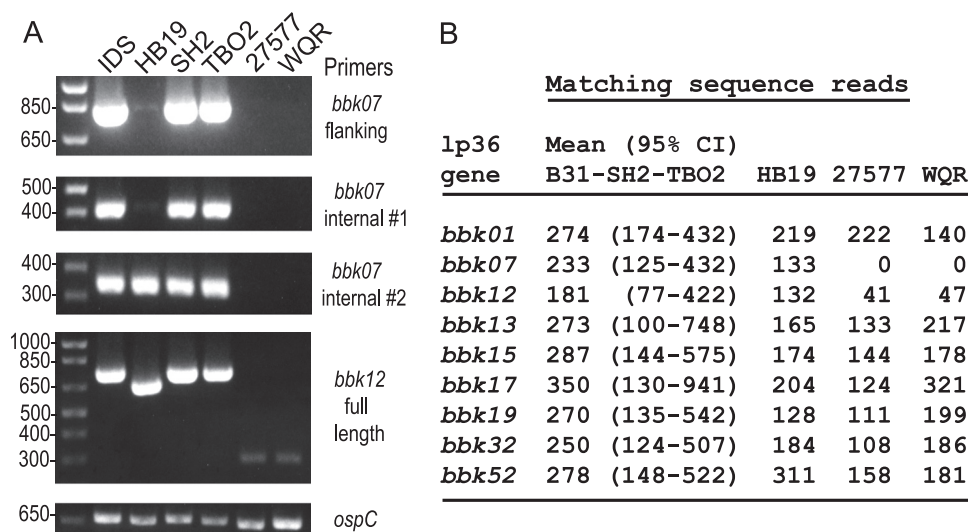
FIG 6 Analysis by PCR and genome-wide sequencing of bbk07 and bbk12 loci in different strains of *B. burgdorferi*. (Left) Ethidium bromide-stained agarose (1%) electrophoresis gels of PCR products with 3 primer sets for the bbk07 locus, one set for the bbk12 locus, and one set for the *ospC* locus of isolate IDS and strains HB19, SH2, TBO2, 27577, and WQR. Size markers (in base pairs) are shown on the left of the gels. (Right) Normalized counts of matching sequence reads for selected genes of the lp36 plasmid. Means with 95% confidence intervals (CI) for 3 strains (B31, SH2 and TBO2) with full-length bbk07 and bbk12 loci are compared with the individual counts for HB19, 27577, and WQR, which lacked full-length PCR products for bbk07 and bbk12.

both the 5′ and 3′ ends of what remained of the lp36 replicon in HB19 and of deletions in the region containing bbk07 and bbk12 in 27577 and WQR.

Sequencing of products of PCR with the flanking or internal primers for bbk07 confirmed the presence of full-length *bbk07* genes in SH2 and TBO2 (see Table S6 in the supplemental material). These alleles were identical in sequence to that of B31. As Fig 6 indicates, *bbk07* of HB19 lacked the 5′ end of the gene; it was identical in sequence to that of WI91-23. Analysis of PCR products for the bbk12 gene of SH2 revealed a sequence that was identical to that in strain 156a; in both, the first of several stop codons began at positions 481 to 483 (43). In strain TBO2 the homologous sequence also had a stop codon at positions 481 to 483 but differed as well at other positions from other sequences in the database. The nucleotide sequence of HB19's shortened bbk12 gene matched that of WI91-23 (see Table S6 in the supplemental material). Both sequences featured (i) an insertion of 7 nucleotides at position 112, which produced a frameshift and then a stop codon at positions 121 to 123, and (ii) a 99-nucleotide deletion in the middle of what otherwise was the open reading frame. In strains 27577 and WQR, the only detectable bbk07 or bbk12 sequence was a region of 231 nucleotides that was identical to part of the bbk12 ortholog in strain 156a. The length of this remnant of the gene was consistent with the PCR findings (Fig. 6).

Under the assumption that strain BL348 is in the same lineage as strain N40, we note that the lower antibody response to the BBK07 and BBK12 proteins for that tick-transmitted infection (Fig. 4) may be explained by 4 differences in the bbk07 locus of strain N40 from B31: (i) an extra A in the area of the −10 element of the σ^{70}-type promoter, (ii) a nonsense mutation at position 77 of the gene, and (iii) a nonsense mutation at position 640.

DISCUSSION

This study of experimental infection extends understanding of the biology of *B. burgdorferi* in *P. leucopus*, one of the major reservoirs

of this bacterium in North America. While all the strains infected this rodent species and disseminated from the inoculation site to other tissues, the strains differed in the extents of infection, as defined by burdens of spirochetes in selected tissues, and in the antibody responses to certain antigens, notably OspC, VlsE, and the BBK07 and BBK12 proteins. Some traits for strains clustered according their prevalences in ticks in the geographic area from which they were drawn. For instance, in comparison to strains with lower prevalence, higher-prevalence strains tended to have higher tissue burdens overall and were unique in having antibodies to BBK07 and BBK12 proteins. The latter result was unexpected, as was the decidedly strain-specific response to a VlsE protein, and these findings led to further characterization of the bbk07, bbk12, and *vlsE* loci of these strains.

The study had limitations. (i) The bacterial inocula were obtained from infected animals, not from culture medium, but they were delivered by needle, not by tick. This led us to confirm some of the findings with sera from *P. leucopus* mice experimentally infected by tick bite. (ii) The animals were reared in captivity, restricted by sex to female, and free from the traumas, nutritional deficiencies, and parasites that wild animals are exposed to. On the other hand, the outbred character of the study's animals was probably more representative of the diversity expected for host species than inbred mouse strains provide. (iii) The animals were all young adults, and the blood and tissues were sampled at one point, 5 weeks into infection. But these choices were probably not far from the average age at which *P. leucopus* first acquires the infection and the usual duration of infection at the height of transmission to larval *I. scapularis* (3). (iv) We did not initially intend to use a mixture of strains along with clonal populations. The mixed character of the IDS isolate was discovered during the course of the study. But we carried on with the isolate because coinfection by two or more different strains in ticks and *P. leucopus* is common in areas of high prevalence (27, 44, 45) and because both members of the mixture, strains B31 and TBO2, had been characterized.

Acknowledging these limits, we focus here on the findings that were not particularly dependent on the experimental conditions or predictable from previous reports. These findings include the strain-specific antibody response to the VlsE protein and the absent or low levels of antibodies to the BBK07 and BBK12 proteins in sera that were otherwise seropositive. Before discussing these results, we consider the antibody responses to the OspC proteins. Our findings confirm that there are antibodies to conserved regions (30), in particular the N-terminal region, which includes the first alpha-helix. This is consistent with the established sufficiency of a single type of OspC protein for sensitive immunoassays for sera from either patients with Lyme disease (46) or *P. leucopus* mice captured at field sites where several strains occur (4). But we also observed in the immunoblots and the microarrays evidence of the strain-specific antibody responses to OspC proteins that have been reported in vaccine studies in mice (47–49). On the basis of the array values for the different recombinant OspC proteins and the conserved first helix (see Table S5 in the supplemental material), we estimate that type-specific antibodies constituted at least half of the antibodies binding to a given OspC. This provides scope, in our opinion, for assays comprising multiple OspC types for discerning the strain that an animal in the field or a patient in a clinic was infected with.

In both our previous study and that of Magnarelli et al. of sera from field-captured *P. leucopus* mice, half or more of the animals in the samples had detectable antibody to a single type of VlsE protein (29, 31). These findings, along with the established diagnostic utility of immunoassays based on all or parts of a single VlsE (33), led us to anticipate that all or most of the *P. leucopus* mice infected with different strains would have antibodies to strain B31's VlsE. This could occur equivalently across strains or, more distinctly, from a combination of homotypic (specific) and heterotypic (cross-reactive) responses (50). While interstrain differences in VlsE protein sequences had been noted previously (51), this diversity did not prevent successful application of assays based on a single full-length VlsE or the conserved C6 peptide of VlsE for laboratory diagnosis *B. burgdorferi* infection (33, 52, 41). Cross-reactivity in VlsE binding by antibodies in human sera between different *Borrelia* species of LD agents has also been reported (53). The sera from infected *M. musculus* mice bore out this prediction: mice infected with the SH2 strain had cross-reactive antibodies to the VlsE of strain B31. But, to our surprise, cross-reactive antibodies to the B31 VlsE were not observed in either the immunoblots or the microarrays with sera from *P. leucopus* mice infected with strains other than B31.

We investigated whether the strains that did not elicit an anti-VlsE response had *vlsE*-like sequences and, if so, the extent to which these sequences differed from the B31 sequence. Identification and characterization of *vlsE* gene sequences are hampered by the telomeric location of the full-length expressed gene and the long tandem arrays of partial variant versions of the sequence (54). Through an iterative process of first counting the number of reads that mapped to each of a set of known *vlsE* sequences and then assembling the reads on a scaffold of the highest-ranking reference *vlsE* sequence, we identified *vlsE* sequences in all the strains. This approach was validated by resequencing of strain B31 and subsequent searching by the same method, as well as by concordances of the SH2 *vlsE* sequence with that of strain 297 and of the HB19 *vlsE* sequence with that of WI91-23.

Interstrain diversity between the assembled *vlsE* sequences was

high. In general, distances between *vlsE* sequences of some strains were as great as those for their *ospC* alleles. The lack of reaction to the VlsE of strain B31 of antibodies from *P. leucopus* infected with other strains is plausibly attributable to the distances between the VlsE proteins of these different strains. Although much of the work on VlsE proteins has justifiably emphasized the role of antigenic variation of VlsE once an infection is under way in an individual animal (32, 39), it is not obvious how this would account for such marked interstrain diversity, particularly when the heterogeneity is most marked in protein regions not involved in antigenic variation. The results here suggest that in addition to intrahost selection for antigenic variants, there may be balancing selection on VlsE proteins in a *B. burgdorferi* population comprising several strains. If this selection is carried out largely by adaptive immune systems, an animal with antibodies to a particular type VlsE protein, e.g., like that of B31, as the consequence of a prior exposure may be immune on that basis to infection by strains with the same or a similar type of VlsE. But, other things, like anti-OspC immunity, being equal, the animal may remain at risk of infection with strains with another type of VlsE protein, e.g., such as SH2's.

This account might also explain the more specific anti-VlsE antibody responses observed in *P. leucopus* in comparison to the more broadly cross-reactive responses that have been noted in humans, dogs, and *M. musculus* infected with *B. burgdorferi*. That is, in the coevolution between parasite, *B. burgdorferi*, and host, *P. leucopus*, there plausibly was selection against VlsE types that to the *P. leucopus* immune system were recognizably similar enough to extant VlsE types for there to be cross-protection. By this account, for species, such as humans, that are largely or entirely irrelevant for the parasite's maintenance in nature, whether or not their anti-VlsE antibodies functionally cross-neutralize strains with other VlsE proteins is of little or no consequence for *B. burgdorferi* evolution.

We consider a different possible outcome of selection for the case of the paralogous genes bbk07 and bbk12. The deduced proteins BBK07 and BBK12 of strain B31 have an overall sequence identity of 87%, differing only in the last third. There is evidence that one or both proteins are surface-exposed lipoproteins, expressed mainly in mice rather than ticks (35), and elicit antibodies during infections of humans, *P. leucopus*, and *M. musculus* (29, 35), but their functions are unknown. All or parts of the lp36 plasmid reportedly are associated with virulence and infectivity (34, 55), and there are DNA rearrangements or deletions involving this extrachromosomal replicon among different strains (see Fig. S2 in the supplemental material) (54–56). The genetic differences in the bbk07 and bbk12 loci between strains of this study plausibly explain the differences in the antibody reactivity in the sera of infected *P. leucopus* that we observed. Either the genes were undetectable or much truncated, e.g., as in WQR, or there were indels and/or nonsense mutations at the 5′ end of the gene that presumably would diminish protein expression, e.g., in HB19.

BBK07 and BBK12 are not essential for *B. burgdorferi*'s viability or growth in culture medium (37) and, as shown here, were not necessary for infection of either *P. leucopus* or immunodeficient *M. musculus*. But there was a tendency for the low-prevalence strains with missing or mutant bbk07/bbk12 loci to have lower tissue burdens during infection of *P. leucopus*. While the role of either of these proteins in dissemination or other aspects of virulence remains to be established, this study documents the occur-

Cy3-conjugated, goat anti-mouse heavy- and light-chain IgG (Jackson ImmunoResearch). Sera were diluted 1:100 in protein array blocking buffer (Whatman, Sanford, ME) that was supplemented with 10% (vol/vol) *Escherichia coli* lysate (MCLAB, San Francisco, CA). The array slides were incubated with sera, washed, incubated with secondary antisera, and washed again as described elsewhere (29). Slides were scanned in a ScanArray Express HT (PerkinElmer, Waltham, MA), and the bindings to spots were quantified with ProScanArray Express software (PerkinElmer) with spot-specific background correction. The mean pixel values for the two separate replicates for each antigen were calculated; values of ≤0 after background correction were assigned a value of 1. Replicate-to-replicate and pad-to-pad variation are described in the supplemental material (see Text S1). The results from two *P. leucopus* samples were excluded because of nonuniform exposure of the array pad. The cutoff for a positive interpretation of a value was 4 standard deviations above the mean for the control sera.

Statistics. For means and OR, 95% confidence intervals are given in parentheses or brackets. Significance tests were two sided. A z score for an assay result was the number of standard deviations above or below the mean for the entire sample of infected animals. The total z score was the sum of scores for each tissue or for each antibody binding value for an individual mouse. Asymptotic, exact, and nonparametric statistical analyses of continuous data were carried out with Stata/MP version 10.1 (StataCorp, College Station, TX) or Systat version 11 (Systat Software, Chicago, IL).

Nucleotide sequence accession numbers. The VlsE genes identified in TBO2, HB19, SH2, 27577, and WQR were assigned accession numbers JX406867 to JX406871, respectively.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org /lookup/suppl/doi:10.1128/mBio.00434-12/-/DCSupplemental.

  Text S1 , PDF file, 0.1 MB.
  Figure S1 , PDF file, 0.1 MB.
  Figure S2 , PDF file, 0.2 MB.
  Table S1, PDF file, 0.1 MB.
  Table S2, PDF file, 0.1 MB.
  Table S3, PDF file, 0.1 MB.
  Table S4, PDF file, 0.1 MB.
  Table S5, PDF file, 0.1 MB.
  Table S6, PDF file, 0.1 MB.
  Table S7, PDF file, 0.1 MB.

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