Multiple and Diverse \textit{vsp} and \textit{vlp} Sequences in \textit{Borrelia miyamotoi}, a Hard Tick-Borne Zoonotic Pathogen

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Abstract

Based on chromosome sequences, the human pathogen \textit{Borrelia miyamotoi} phylogenetically clusters with species that cause relapsing fever. But atypically for relapsing fever agents, \textit{B. miyamotoi} is transmitted not by soft ticks but by hard ticks, which also are vectors of Lyme disease \textit{Borrelia} species. To further assess the relationships of \textit{B. miyamotoi} to species that cause relapsing fever, I investigated extrachromosomal sequences of a North American strain with specific attention on plasmid-borne \textit{vsp} and \textit{vlp} genes, which are the underpinnings of antigenic variation during relapsing fever. For a hybrid approach to achieve assemblies that spanned more than one of the paralogous \textit{vsp} and \textit{vlp} genes, a database of short-reads from next-generation sequencing was supplemented with long-reads obtained with real-time DNA sequencing from single polymerase molecules. This yielded three contigs of 31, 16, and 11 kb, which each contained multiple and diverse sequences that were homologous to \textit{vsp} and \textit{vlp} genes of the relapsing fever agent \textit{B. hermsii}. Two plasmid fragments had coding sequences for plasmid partition proteins that differed from each other from paralogous proteins for the megaplasmid and a small plasmid of \textit{B. miyamotoi}. One of 4 \textit{vsp} genes, \textit{vsp1}, was present at two loci, one of which was downstream of a candidate prokaryotic promoter. A limited RNA-seq analysis of a population growing in the blood of mice indicated that of the 4 different \textit{vsp} genes \textit{vsp1} was the one that was expressed. The findings indicate that \textit{B. miyamotoi} has at least four types of plasmids, two or more of which bear \textit{vsp} and \textit{vlp} gene sequences that are as numerous and diverse as those of relapsing fever \textit{Borrelia}. The database and insights from these findings provide a foundation for further investigations of the immune responses to this pathogen and of the capability of \textit{B. miyamotoi} for antigenic variation.

\url{http://dx.doi.org/10.1371/journal.pone.0146283}
Introduction

Borrelia miyamotoi is a host-associated spirochete that is transmitted between its mammalian reservoirs by ticks of the genus Ixodes (reviewed in [1, 2]) Its vectors include I. scapularis in eastern North America, I. pacificus in far-western North America, I. ricinus in Europe, and I. persulcatus in Russia and Asia. These species are also the vectors of the Lyme disease agents of the genus Borrelia, as well as other zoonotic pathogens, like Anaplasma phagocytophilaum and Babesia microti, in the same areas where B. miyamotoi is enzootic. In most regions in North America where B. miyamotoi and B. burgdorferi are sympatric, infection prevalence of B. miyamotoi in nymphal ticks is about one-tenth that for B. burgdorferi [1, 3], but in some areas, such as in northern California, the nymphal infection prevalences of the two Borrelia species are near-equal [4, 5]. While a North American strain of B. miyamotoi has been cultivated in the laboratory [6, 7], the growth rate is slow, and the yields of cultures are low. Consequently, infection of laboratory mice remains an important means for propagating infectious organisms in sufficient amounts for genomic studies and when plasmid loss is a concern [8].

B. miyamotoi was first identified in 1994 [9], but two decades passed before its recognition as a human disease agent, first in a case series from Russia [10] and then subsequently in reports from United States, Europe, and Japan [11–13]. Seroepidemiologic surveys indicate that the incidence of B. miyamotoi infection in the northeastern United States is similar to that for two other I. scapularis-transmitted diseases: anaplasmosis and babesiosis [14]. While B. miyamotoi infection does not appear to have the long-term sequelae that can occur with untreated Lyme disease [1], the acute infection sometimes resembles sepsis [10, 15] and has justified hospitalization, an infrequent consequence of acute B. burgdorferi infection. B. miyamotoi’s disposition for neuroinvasion was illustrated by cases of meningoencephalitis in patients with pre-existing immunodeficiencies [16, 17].

By phylogenetic criteria B. miyamotoi is more closely related to Borrelia species that cause relapsing fever, such as B. hermsii and B. turicatae, than it is to B. burgdorferi and other Lyme disease agents [9, 18, 19]. But in its biological traits B. miyamotoi differs from most other species in the relapsing fever clade in its preference for hard (or ixodid) ticks instead of soft (or argasid) ticks as its vector [20]. This exception in its life cycle leads to the question of whether B. miyamotoi is properly considered a relapsing fever agent. Clinical accounts of human B. miyamotoi infection include a minority of cases that had relapses of illness before antibiotic treatment was initiated [10, 15], but the natural history of untreated infection has yet to be well-defined in an experimental animal model. If B. miyamotoi in fact has the capacity for antigenic variation, which is a hallmark of relapsing fever [21], it plausibly is based on possession of a repertoire of polymorphic genes, the singular expression of which determines the immunodominant antigen of the cells.

As first documented for B. hermsii and subsequently for other relapsing fever species [22], a genomic schema that fits this specification is a plasmid-borne set of genes for either variable small proteins (Vsp) of ~20 kDa and variable large proteins (Vlp) of ~40 kDa [23]. On the basis of their amino acid sequences the family of Vlp proteins is further categorized into four sub-families or clusters—alpha, beta, gamma, and delta [24, 25]. Examples are the alpha sub-family protein VlpA7 (accession number P21876), the beta sub-family protein VlpB10 (P70905), the gamma sub-family protein VlpC5 (P70898), and delta sub-family protein VlpD17 (P32777). In B. hermsii and B. turicatae there is a duplicate copy of one of this set of polymorphic vsp and vlp genes [26, 27]. Only this duplicate copy is transcribed at a single expression site [28], thereby providing the antigenic identity to the cell. As might be predicted if evasion of the immune system was a pathogen’s strategy, diversity among the Vsp and Vlp proteins of a given lineage is substantial [25, 29].
My objective was to search for and characterize \textit{vsp} and \textit{vlp} genes in the reference North American strain of \textit{B. miyamotoi}. Sequences homologous to \textit{vsp} genes had been identified in \textit{B. miyamotoi} strains from Japan \cite{30} and North America \cite{19}. But these studies were limited in scope, and the broader organization of these sequences was not revealed. Finding a variable gene repertoire of a scale possessed by \textit{B. hermsii} was not assured, though. \textit{B. anserina}, another species in the relapsing fever agent clade \cite{19}, appears to have no \textit{vlp} genes and only a single \textit{vsp} (accession number KJ136518), on the basis of publicly-available DNA sequences (BioProject 195596) of its genome (unpublished finding). Moreover, while identification of the variable regions of individual \textit{vsp} and \textit{vlp} sequences is achievable with high-coverage, short-read sequencing, the duplicated conserved sequences at their 5’ and 3’ ends makes unambiguous assembly of longer contigs with full-length genes a challenge \cite{25}. In addition, the genetic distance of \textit{B. miyamotoi} from other species precluded a resequencing procedure in which short reads were mapped to a reference. These considerations led to a hybrid approach with supplementation of the previously-acquired database of short-reads with the longer reads obtainable with real-time DNA sequencing from single polymerase molecules \cite{31}. This allowed not only identification of several complete or near-complete \textit{vsp} and \textit{vlp} sequences but also greater stretches of the plasmids bearing them.

\textbf{Materials and Methods}

\textbf{Ethics statement}

All animal work was conducted with approval of University of California Irvine’s Institutional Animal Care and Use Committee (protocol 2080–1999). Mice were housed under ABSL2 containment in an Association for Assessment and Accreditation of Laboratory Animal Care-approved facility. This study was carried out in strict accordance with the recommendations in the Guide for Care and Use of Laboratory Animals of the National Institutes of Health.

\textbf{Bacterial strain and propagation}

Strain LB-2001, which was isolated from \textit{I. scapularis} ticks collected in Connecticut in 2001 \cite{18}, was used. The isolate was provided to the author by Michele Papero and Durland Fish of Yale University in 2003 and was kept frozen in individual aliquots at -80°C. It had been passed three times in mice, and never under in vitro conditions, before further expansion for this study in adult CB17 severe-combined immunodeficient (SCID) mice from Charles River Laboratories (Wilmington, MA) as described \cite{32}. Infections of the mice were monitored by phase-microscopy of tail vein blood as described \cite{25}. When bacterial densities in blood reached 0.5–1.0 \times 10^7 cells per milliliter, mice were terminally exsanguinated under anesthesia; blood was collected in heparinized tubes. The isolate was registered as BioSample SAMN02604147 as part of BioProject PRJNA19262 (http://www.ncbi.nlm.nih.gov).

\textbf{Short-read sequences}

For our previous study of the chromosome of \textit{B. miyamotoi} LB-2001, we obtained \~1.7 million Ion Torrent (Life Technologies, Carlsbad, CA) reads of 50–250 nucleotides from a total DNA extract of whole blood of \textit{Mus musculus} mice infected with this strain \cite{19,32}. For the present study, I examined reads that did not map to the LB-2001 chromosome under moderately stringent conditions. The 1,135,420 unmapped reads, which were presumed to be mainly extrachromosomal DNA of \textit{B. miyamotoi} as well as some residual mouse DNA, were used for a de novo assembly using the Assembly Cell algorithm of CLC Genomics Workbench v. 8.1 (Qiagen, Valencia, CA). The resultant assembly had 101 contigs of \(\geq 2000\) nt, for a total of 392,484 nt.
from 256,835 reads. These contigs ranged in size from 2016 to 29,162 nt, the average size was 3886 nt, and the average coverage was 99X.

Long-read sequencing and assembly

For this experiment another harvest of strain LB-2001 was again produced by propagating in SCID mice as described [19, 32]. Total DNA was extracted from heparinized whole blood with Qiagen’s DNeasy Blood/Tissue Kit and then treated with RNase I. Library preparation and sequencing using the Single Molecule, Real-Time (SMRT) DNA approach on a PacBio RS I instrument (Pacific Biosciences, Menlo Park, CA) was performed in University of California Irvine’s Genomics High-Throughput Facility (http://ghtf.biochem.uci.edu). In brief, genomic DNA was purified with Genomic Tip 500/G columns (Qiagen) and was sheared using the g-Tube (Covaris, Woburn, MA), following the Pacific Biosciences protocol for low-input (10 kb) preparation and sequencing. Agencourt AMPure magnetic beads (Beckman Coulter, Brea, CA) were used to remove salt according to the PacBio template and preparation sequencing instructions. A SMRTbell Template Preparation kit (Pacific Biosciences) was used to construct a 3–10 kb library, and the size was determined using a 2100 Bioanalyzer (Agilent, Santa Clara, CA). The Pacific Biosciences calculator (v. 2.0) was used to determine the amount of primer and polymerase (DNA/Polymerase binding kit 2.0), and samples were sequenced with the MagBead Seq v1 protocol (Pacific Biosciences). Default filters removed reads of less than 50 nt and 0.75 accuracy.

Two SMRT cells yielded a total of 250,161 one-pass reads of 50 to 22,451 nt for a total of 540,499,213 bases and average length of 2237 nt. Error correction of the long PacBio reads with the total Ion Torrent reads was carried out with the default parameter settings of the pacBio-ToCA module of the Celera Assembler v. 8.1, and the output was provided as fastq and fasta files [33]. These were imported for analysis by the suite of programs of CLC Genomics Workbench v. 8 (Qiagen) and for the database of a local stand-alone WWW Blast Server v. 2.2 [34]. Thirty-eight percent of the reads mapped to a Mus musculus genome, but for all mouse chromosomes except the mitochondrial the coverage was <0.05X. Using the extrachromosomal de novo contigs as the reference, the coverage for putative plasmid sequences ranged between 5X and 10X.

De novo Ion Torrent contigs of ≥500 nt and the corrected PacBio reads with possible vsp and vlp genes were identified with TBLASTN searches of de novo Ion Torrent contigs and error-corrected PacBio reads with selected Vsp and Vlp protein sequences of B. hermsii strain HSI and Vsp1 of B. miyamotoi LB-2001. Candidate vsp or vlp-bearing sequences were used in turn for BLASTN searches on the local blast server to identify PacBio reads that overlapped the probe sequence by >200 bases with neither mismatches nor gaps. The resultant set of overlapping reads was then edited to produce a single continuous sequence.

Accession numbers

GenBank/EMBL/DDBJ accession numbers for the large fragments of plasmids lpB, lpD, and lpE are CP010328, KR869094, and KU041636, respectively. The coding sequences for plasmid replication and partition proteins PF-32, PF-49, and PF-50 of the megaplasmid of B. miyamotoi LB-2001 are given in accession number KU145468. The 6035 bp of the sequence of the small plasmid cpD of B. miyamotoi LB-2001 has accession number KT355574.

Other sequences

These included FR64b chromosome (CP004217) and its plasmid fragments (GenBank assembly number GCA_000568695), specifically, plasmid fragments 17 (CP004234), 36 (CP004253),
and 39 (CP004256), and *B. hermsii* strain HS1 coding sequences for Vsp6 (DQ166207), Vsp22 (EF156411), and Vsp24 (EF187445), a *B. hermsii* strain YBT vsp gene (CP005724), *B. turicatae* strain Oz1 coding sequences for VspA (AF129434) and VspB (AF049852), and coding sequence for *B. miyamotoi* LB-2001 flaB gene (AGT27144). The following ParA-type proteins of strain B31 of *B. burgdorferi* were used: BBA20 (AAC66247), BBB12 (AAC66318), and BBN32 (AAF07673). The 2.6 Gb *Mus musculus* sequence of Genome Reference Consortium Mouse Build 38 (GCA_000001635.2) served as the mouse reference. The Shuffle DNA algorithm (www.bioinformatics.org/sms2/shuffle_dna.html) was used to randomly mix the order of bases of a sequence while retaining the original base composition.

**Sequence analysis**

Open reading frames (ORFs) of $\geq 150$ bp with any codon as a start were identified and searched against the National Center for Biotechnology Information (NCBI) nonredundant protein database using BLASTP [34]. The BLAST search criteria for considering an ORF as homologous to a deduced protein of another organism were the following: $E$ values of $<10^{-5}$, pairwise amino acid identities of $>40\%$, and $>50\%$ coverage of the smaller protein. Further annotation took into account relationships with proteins whose functions have been identified or which are commonly known in the literature.

The convention for naming *vsp* and *vlp* genes and their deduced proteins was to arbitrarily assign a numeric suffix to a *vsp* or *vlp* ORF, e.g. *vsp1*, according to their position in a contig, proceeding from left to right, and then moving to the next contig once all the putative *vsp* and *vlp* sequences of one contig had been designated. The *vlp* coding sequences, either complete or partial at the 5' end, were further differentiated by sub-family membership, e.g. "*vlpA1*", "*vlpC2*", and "*vlpD6*" in the case of alpha, gamma, and delta sub-families. Some other ORFs were assigned designations that indicated their homology with specific proteins or a paralogous family (PF) protein of *B. burgdorferi* strain B31 [35], the source for the first genome sequence in the genus *Borrelia* [36].

Alignment of DNA and protein sequences was carried out with ClustalX v. 2 [37], and were codon-aligned manually with MacClade v. 4.10 (Sinauer Associates, Inc., Sunderland, MA). Distance-based clustering algorithms for ungapped alignments of proteins were neighbor joining with the BioNJ protocol and Poisson distances. Phylogenetic inference was carried out by maximum likelihood estimation, as implemented by PhyML v. 3.0 (Guindon et al., 2010) in the SeaView suite version 4.5.4 [38]. SplitsTree4 (http://www.splitstree.org) was used for distance-based clustering of ungapped nucleotide alignments and computation of an unrooted phylogenetic network [39].

**RNA-seq**

Total RNA was isolated from $\sim 1$ ml of heparinized whole blood from each of 3 infected male SCID mice using a RNeasy Mini Kit (Qiagen, Valencia, CA). There were $\sim 5 \times 10^6$ spirochetes per milliliter of blood at the time of collection. After RNA was eluted from the columns in RNase-free water, residual DNA was removed by treatment with DNase I (Thermo Fischer Scientific) during further processing with a RNA Clean & Concentrator-5 kit (Zymo Research). The sample was depleted of ribosomal RNA by incubation with the beads of Ribo-Zero Magnetic Kit for Gram-positive bacteria (Epicentre, Madison, WI) followed by ethanol precipitation. Synthesis of cDNA with random hexamer primers was carried out with a Maxima First Strand cDNA Synthesis Kit (Thermo Fischer Scientific). The cDNA was then treated with RNase I before processing with a DNA Clean & Concentrator-5 kit (Zymo Research). The resultant cDNA was sheared enzymatically, and adapters were ligated with an Ion Express Plus.
Fragment Library Kit (Life Technologies, Carlsbad, CA). The products were size-selected with the E-Gel electrophoresis system (Life Technologies), attached to Ion Sphere Particles using an OneTouch 200 Template kit (Life Technologies), subjected to emulsion PCR on an Ion Torrent OneTouch apparatus, and then sequenced on an Ion Torrent Personal Genome Machine with Ion 316 chips and Ion PGM 200 Sequencing Kit (Life Technologies). The RNA-Seq program in the CLC Genomics Workbench, version 8.1 (Qiagen) was used to enumerate reads mapping to reference sequences with these criteria: minimum length fraction of 0.5, minimum similarity fraction of 0.9, and costs of 2, 3, and 3 (out of 3) for mismatches, insertions, and deletions, respectively.

**Results and Discussion**

**Three plasmid fragments bearing vsp and vlp sequences**

The assembled contigs of extrachromosomal DNA included one of 37.5 kb, which contained the *nrdEFI* and *thy* genes (KJ141201) of the 150–180 kb megaplasmids of *relapsing fever Borrelia* species [40], and another of 29 kb, which contained the plasmid partition proteins of the megaplasmids [41, 42]. There was also a 6 kb plasmid (accession number KT355574), which corresponded to small plasmids that are found in relapsing fever species but not Lyme disease species (unpublished findings). Neither the megaplasmid sequences nor the small plasmid sequence contained a discernible *vsp* or *vlp* gene sequence.

As detailed below, iterative TBLASTN and BLASTP searches revealed three contigs of >10 kb that contained coding sequences homologous to *vsp* and *vlp* genes of other *Borrelia* species. These plasmid fragments were 30,673 bp, 11,152 bp, and 16,042 bp in length and were designated lpB, lpD, and lpE, respectively (Fig 1). The lpB and lpE contigs had ORFs that were orthologous to plasmid replication and partition proteins of plasmids of the genus *Borrelia*. These included coding sequences for PF32 or ParA proteins, PF49 proteins, and PF50 proteins, as well as “ORF-A” coding sequences that were commonly found in association with genes for partitioning proteins of plasmids [35, 42]. The aligned ParA proteins of lpB and lpE were only 48% identical over their lengths. Fig 2 is a phylogram of the PF32 proteins of the megaplasmid, lpB, lpE, and the small plasmid cpD, as well as orthologous sequences of *B. burgdorferi*. The lpB ParA protein’s ortholog in *B. burgdorferi*, BBB12, is found on the cp26 plasmid, while lpE’s PF32 protein is more similar to those, such as BBN32, of the cp32 plasmids of *B. burgdorferi*. The *B. miyamotoi* protein found on the megaplasmid fragment is most similar to the PF32 protein BBA20 of the lp54-type (“A”) plasmids [35, 42]. By BLASTP and TBLASTN searches, there was no detectable ortholog of the cpD ParA protein among the published genomes of Lyme disease group *Borrelia* species.

The observed diversity of four PF32/ParA proteins and their correspondences to distinctly different replicons in *B. burgdorferi* indicate the presence of at least four different types of plasmids in *B. miyamotoi*. This is consistent with Hamase et al.’s report of 7–9 plasmids in strains of *B. miyamotoi* from *I. persulcatus* ticks in Japan [30]. The lpD contig did not include ORFs with a discernible replication or partition function, so a separate plasmid status cannot be inferred. But two of its ORFs for hypothetical proteins were homologous to proteins of the lp36 (or “K”) class of plasmids of Lyme disease *Borrelia* spp. [43].

The lpB fragment is largely homologous to the cp26 plasmid of *B. burgdorferi*

A shared ancestry between the 31 kb lpB sequence and the 26 kb cp26 circular plasmids of Lyme disease *Borrelia* species was further documented by discovery on lpB of coding sequences...
for the following proteins (with gene name) that typify cp26-type (denoted by "B" in ORF name) plasmids [44] (Fig 1): inosine 5-monophosphate dehydrogenase (guaB), GMP synthase (guaA), uracil-xanthine permease (BBB22), PTS glucose transporter subunit IIB (ptsG), and, what has been essential for other Borrelia spp., the telomere resolvase (resT) [45]. Other ORFs were orthologous to cp26 genes BBB24 through BBB28 [35]. The exceptions to the overall similarity between lpB and cp26, e.g. a BBB02-like ORF follows the BBB29-like ORF on lpB, may be attributable to retention in the lineage of Borrelia miyamotoi and other relapsing fever species of a linear structure for a plasmid whose counterpart in the divergent lineage of Borrelia burgdorferi and other Lyme disease species is circular, as suggested by Chaconas [47]. I found no PacBio reads that spanned the 5' and 3' ends of the lpB fragment, which would be an expected finding if lpB had a circular topology. However, since comparatively little is known about the telomeric sequences of the linear plasmids of relapsing fever Borrelia species, it was not possible to assign definite termini on the basis of sequence.

Vsp and Vlp genes

Hamase et al. reported that multiple restriction fragments of the Asian strain HT31 of B. miyamotoi hybridized with a probe incorporating a vsp sequence they had identified [30]. These findings were consistent with the presence of more than one vsp in the genome. Subsequently, I identified a vsp gene in strain LB-2001 and designated this vsp1 (KF031441) [19]. Further sequencing placed this sequence with its particular 5' flanking region on the 31 kb lpB fragment (Fig 1) and revealed another Vsp coding sequence, vsp2, and four Vlp coding sequences—vlpD1, vlpD2, vlpA1, as well as a pseudogene of a delta sub-family vlp—downstream of and in the same orientation as vsp1.
The fragments of plasmids lpD and lpE had additional sequences for different vsp, vlpA, and vlpD alleles. Fragment lpE contained coding sequences for three different gamma sub-family
Vlp proteins—vlpC1, vlpC2, and vlpC3—besides Vsp and delta sub-family VlpD proteins. No beta sub-family sequences were identified by TBLASTN with VlpB9, VlpB10, VlpB12, and VlpB14 sequences of B. hermsii [29] in either the de novo assemblies or the PacBio reads. This is in contrast to B. turicatae, B. parkeri, B. persica, B. coriaceae, B. hispanica, and B. duttonii, all of which have beta sub-family Vlp genes, as exemplified by the protein sequences with accession numbers AF130429, AHH10139, WP_051374011, AHH11256, WP_038359110, and ACH94076, respectively.

Six of the 19 vsp and vlp sequences of the lpB, lpD, and lpE plasmids would encode a full signal peptide for these lipoproteins. This proportion is similar to what was found for the silent vsp and vlp sequences in the B. hermsii genome [25]. Another 10 of the B. miyamotoi vsp and vlp genes had at least part the coding sequence for peptide sequence, including a consensus four amino acid site for the signal peptidase for lipoprotein processing. The remaining three ORFs began within a few residues of where the signal peptidase site would be expected. Adjacent to these vsp and vlp sequences there was not discernibly a repetitive sequence that was analogous to the Downstream Homology Sequence of B. hermsii [25].

The various pairs of aligned amino acid sequences of the Vlp proteins differed at ≥10% of positions within a sub-family and ≥30% of positions between proteins of different sub-families. The diversity among the four identified vsp genes of B. miyamotoi LB-2001 was examined in more depth by adding to a codon-based nucleotide alignment the following: 3 vsp sequences of strain FR64b of B. miyamotoi, 4 vsp sequences of strains HS1 and YBT of B. hermsii, and 2 vsp sequences of strain Oz1 of B. turicatae (S1 File). The 3 vsp sequences of strain FR64b were the only ones identified by BLASTN searches with each of the 4 vsp LB-2001 sequences of all strain FR64b contigs deposited with GenBank. Fig 3 is an unrooted phylogram that shows diversity of the B. miyamotoi vsp genes, both within and between strains, that is as great as for two species, B. hermsii and B. turicatae, that have documented antigenic variation of Vsp proteins for immune evasion [29, 48]. The splits-decomposition analysis revealed a recombination network among the B. miyamotoi vsp genes that appears as deep as that previously demonstrated for B. hermsii’s vsp repertoire [49].

Two loci with vsp1 sequences

As noted, the lpB plasmid fragment bears the vsp1 gene, but a 99.4% identical vsp sequence exists among a group of tandemly-arrayed vsp and vlp genes of lpD (Fig 1). Upstream of the two vsp1 sequences are a BBB14-like pseudogene on lpB and a delta sub-family Vlp gene, vlpD4, on the lpD fragment. Downstream of both vsp1 versions are sequences for delta sub-family Vlp proteins; these were designated vlpD1 on lpB and vlpD5 on lpD. There are at least 6 kb of vsp and vlp sequences 3’ to vsp1 on lpB. The version of vsp1 on lpD is followed over a length of 8 kb by 3 vsp and vlp sequences and beyond those by four other ORFs, including a homolog of the B. burgdorferi fibronectin-binding protein BBK32 (AAC66134).

Fig 4 provides sequence-level comparisons of the 5’ and 3’ flanking regions for lpB and lpD. At the 5’ end (section A), both the lpB and lpD sequences have a consensus ribosomal binding sequences and what is compatible with a “-10” element of a RpoD-type promoter. Further upstream the sequences diverge, and notably there is a consensus RpoD “-35” element on the lpB sequence but not on the lpD sequence. The GGT trinucleotide of the initial coding sequence for the lpD vsp1 replaces the AAA trinucleotide in the lpB vsp1 and thereby creates a stop within 2 codons of the start. A stop codon at the beginning of some putative “silent” genes was also noted in our studies of B. hermsii and B. turicatae [25, 50, 51]. These substitutions may serve to further limit adventitious expression of vsp and vlp genes at loci other than a preferred expression site.
The lpD sequence also lacks the inverted repeat just in front of the putative “-35” element. A similarly-positioned inverted repeat before the promoter for the ospC gene on the cp26 plasmid of *B. burgdorferi* serves as the gene’s operator [52, 53]. OspC is homologous to the Vsp proteins [54]. The first ORF of the 6 kb cpD plasmid (KT355574) of *B. miyamotoi* is homologous to *B. burgdorferi*’s BBD18 protein, which is the repressor for the ospC operator [53]. Plausibly, this *B. miyamotoi* protein has a similar function in that species.

![Unrooted distance phylogram with recombination network for codon-aligned partial vsp genes of selected *Borrelia* species, as implemented by the SplitsTree algorithm.](image)
Section B of Fig 4 shows the post-\( vsp1 \) region of each locus where the sequences diverge again downstream. This occurs after the codon for amino acid residue 37 of the following VlpD. Thereafter the deduced VlpD1 and VlpD5 proteins differed at 34 (\( 11\% \)) of 315 positions.

The sequences in common between the two loci, even after they begin to differ, are notable for the frequency of TCGA/AGCT palindromes.

RNA-seq

Of 2,763,166 total reads of 50–200 nt and trimmed of low quality regions, 2,386 (0.1\%) mapped to the 907,293 bp of LB-2001 chromosome (CP006647) or plasmid fragments lpB, lpD, and lpE, and 2,557,069 (92.5\%) mapped to chromosomes 1–19, chromosomes X and Y, and the mitochondrial of \( Mus \) musculus. The mean number of the reads that uniquely mapped were 7.1 per 1000 bp for the \( B. \) miyamotoi sequences overall and 0.8 per 1000 bp of mouse genome, excluding the mitochondrial sequence. Though the number of \( B. \) miyamotoi-mapped reads was low, there appeared to be a sufficient quantity to evaluate expression of \( vsp1 \) without resort to further PCR amplification with the risk of unrepresentative amplification. This was done by using as the target set for the analysis the 4 \( vsp \) genes for strain LB-2001, 3 \( vsp \) genes for strain FR64b, 4 \( vsp \) genes for \( B. \) hermsii, and 2 \( vsp \) genes for \( B. \) turicatae that were used for the phylogenetic analysis shown in Fig 3. The sequence of the \( flaB \) gene for the major component of the spirochete flagella served as a positive control, as it was anticipated to be one of the more highly
expressed genes in vivo [55, 56]. As additional negative controls to the vsp sequences from B. hermsii and B. turicatae, the LB-2001 vsp1 and flaB sequences were randomly shuffled. Fig 5 shows the length-adjusted values for reads uniquely mapping to the various sequences. Among the 4 known vsp sequences for LB-2001, vsp1 stood out from vsp2, vsp3, and vsp4 in the number of reads mapping to its sequence. This value exceeded that of reads mapping to the flaB sequence. A Poisson distribution described the counts of mapped reads by individual gene among the great excess of total reads. Under a null hypothesis of statistically indistinguishable frequencies of reads mapping to the different vsp sequences, the observed number for vsp1 was
highly improbable ($p < 0.0001$). This was evidence but not proof that vsp1 of lpB was the active vsp allele in the majority of the spirochetes growing in the blood of the mice.

Conclusions

Did this study establish that antigen variation occurs during B. miyamotoi infection? No. This will likely require the demonstration of evasion of adaptive immunity by a switch of surface proteins during an experimental infection with a minimum infectious inoculum of a clonal population and then attribution of that switch to a reversible DNA rearrangement [57]. But the finding of full- or near-full length sequences for a minimum of 19 diverse vsp and vlp indicates that B. miyamotoi has the genetic building blocks for the type multi-phasic antigenic variation that characterizes relapsing fever [22]. In addition, the presence of two copies of a vsp gene, with one copy next to a candidate promoter, is consistent with a mechanism for antigenic variation that has been demonstrated in other species in the relapsing fever group. The identification of a putative expression site and the various vsp and vlp sequences provides the database for design of primers and nucleotide and protein microarrays for further studies of expression in the organism during infection and the immune responses of infected hosts.

The diversity of the partition proteins, like the ParA/PF32 homologs, indicates that there at least four different types of plasmids in B. miyamotoi. Are these the complete sequences of the plasmids represented here? This is undoubtedly not the case for the lpE fragment, which lacks the aforementioned plasmid replication and partition ORFs. And it is probably not the case for lpD, which encodes unique PF32, PF49, and PF50 proteins, but is shorter in length than the complete plasmids that bear vsp and vlp genes in B. hermsii and B. turicatae [27, 58]. The lpB sequence may be close to complete; it is similar in size to a vsp-bearing plasmid in strain FR64b [30]. But confirmation of that plasmid’s structure—and what the ends are, if linear [42]—require further study. While a circular topology for the lpB plasmid cannot be excluded at this point, the absence of telomeres could be incompatible with a mechanism of antigenic variation dependent on duplicative translocations between linear plasmids [27, 59].

Supporting Information

S1 File. NEXUS format codon-based alignment of partial nucleotide sequences of vsp genes of selected Borrelia species as described in legends for Figs 3 and 5. (NXS)

Acknowledgments

I thank Arash Ghalyanchi Langeroudi for refining the method for producing cDNA from bacterial RNA extracted from whole blood, Fong Hue for technical assistance, and Jie Wu for implementing the protocol for error correction of PacBio reads with Ion Torrent reads.

Author Contributions

Conceived and designed the experiments: AGB. Performed the experiments: AGB. Analyzed the data: AGB. Contributed reagents/materials/analysis tools: AGB. Wrote the paper: AGB.

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