Characterization and genomic analysis of the Lyme disease spirochete bacteriophage ϕBB-1

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Abstract

Lyme disease is a tick-borne infection caused by the spirochete *Borrelia* (*Borreliella*) *burgdorferi*. *Borrelia* species have highly fragmented genomes composed of a linear chromosome and a constellation of linear and circular plasmids that encode diverse outer membrane lipoproteins, which facilitate movement of the spirochete between its tick vector and a vertebrate host in an enzootic cycle. The *B. burgdorferi* genome shows evidence of horizontal transfer between strains, but the mechanisms remain poorly defined. Almost all Lyme disease spirochetes are infected by 32-kp circular plasmid (cp32) prophages that undergo lytic replication and produce infectious virions called ϕBB-1. In the laboratory, ϕBB-1 transduces cp32s and shuttle vectors between spirochetes. However, the extent that ϕBB-1 participates in horizontal gene transfer between Lyme disease spirochetes is not known. Here, we use proteomics and long-read sequencing to characterize ϕBB-1 virions and the genetic material they package. Our studies reveal that ϕBB-1 packages linear cp32s via a headful mechanism. We identify the cp32 *pac* region and show that plasmids containing the cp32 *pac* region are preferentially packaged into ϕBB-1 virions. Additionally, we find ϕBB-1 packages fragments of the linear chromosome and other plasmids including lp54, cp26, and others. Furthermore, sequencing of ϕBB-1 packaged DNA allowed us to resolve the covalently closed hairpin telomeres for the linear *B. burgdorferi* chromosome and most linear plasmids in strain CA-11.2A. Collectively, our results shed light on the biology of the ubiquitous ϕBB-1 phage and further implicates ϕBB-1 in the generalized transduction of diverse genes between spirochetes and the maintenance of genetic diversity in Lyme disease spirochetes.

Introduction

The bacterium *Borrelia* (*Borreliella*) *burgdorferi* is the causative agent of Lyme disease, the most common tick-borne disease in the Northern Hemisphere [1-3]. Lyme disease spirochetes have complex and highly fragmented genomes composed of a ~900 kb linear chromosome and a constellation of linear and circular plasmids that are similar but not identical across the genospecies [4-6].

A key factor in the ability of *B. burgdorferi* to transmit from its tick vector to a vertebrate host is the differential expression of several outer membrane lipoproteins that assist *B. burgdorferi* in evading both vector and host immune responses [7]. As such, a large fraction of the *B. burgdorferi* genome encodes outer membrane lipoproteins, mostly carried on the plasmids [6, 8, 9].

In natural populations, the recombination rate between genomes of coexisting *B. burgdorferi* strains is exceeds the mutation rate and genetic variation in outer membrane lipoprotein alleles associated with species-level adaptations [6, 8-10]. Variation in outer membrane lipoprotein alleles across the genospecies is driven primarily by horizontal gene transfer [5, 11-17]. However, the mechanism(s) by which heterologous *B. burgdorferi* strains exchange genetic material are not well defined.

Viruses that infect bacteria (phages) are key drivers of horizontal gene transfer between bacteria [18]. The genomes of nearly all Lyme disease spirochetes include the 32-kb circular plasmid (cp32) prophages (**Fig 1A and B**) [4]. The cp32s carry several outer membrane lipoprotein gene families including *bdr, mlp*, and *ospE/ospF/elp* (*erps*), which are all involved in immune evasion [19-23] and exhibit sequence variation that is consistent with historical recombination amongst cp32 isoforms [24-26]. Recent work indicates that cp32 prophages are induced in the tick midgut during a bloodmeal [9, 27, 28]. When induced, cp32 prophages undergo lytic replication where they are packaged into infectious virions designated ϕBB-1 (**Fig 1C**) [29-31].

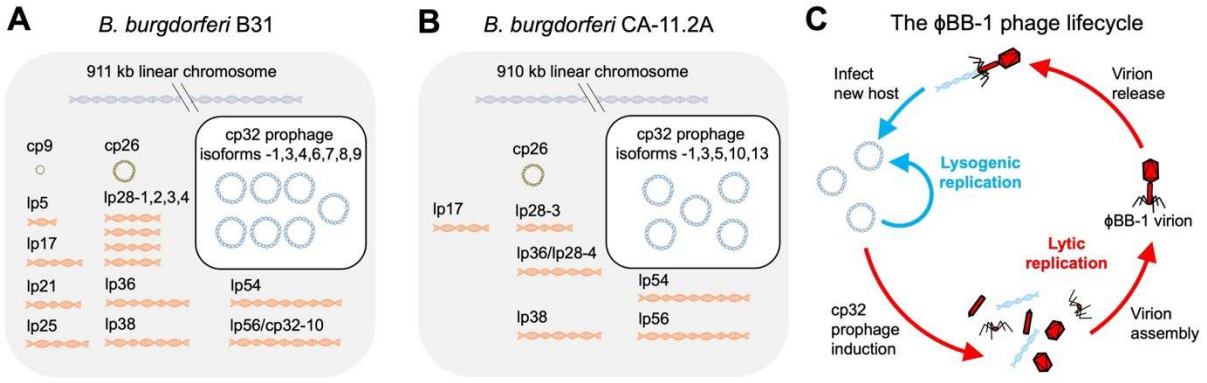


Figure 1. The *B. burgdorferi* genome is highly fragmented and is composed of a linear chromosome, linear and circular plasmids, and cp32 prophages. The genomes of *B. burgdorferi* strains **(A)** B31 and **(B)** CA-11.2A are shown. **(C)** The temperate ϕBB-1 phage lifecycle is depicted.

In addition to horizontally transferring phage genomes between bacterial hosts (transduction), phages frequently package and horizontally transfer pieces of the bacterial chromosome or other non-phage DNA (generalized transduction) [32]. Generalized

transduction was first observed in the *Salmonella* phage P22 in the 1950s [33]. Since then, generalized transduction has been observed in numerous other phage species [32, 34-37]. ϕBB-1 is a generalized transducing phage that can horizontally transfer shuttle vectors carrying antibiotic resistance cassettes between *B. burgdorferi* strains [29]. However, to our knowledge, generalized transduction of anything other than engineered plasmids by ϕBB-1 has not been observed.

Here, we define the genetic material packaged by ϕBB-1 virions isolated from *B. burgdorferi* strain CA-11.2A. Our proteomics studies confirm that ϕBB-1 virions are composed primarily of capsid and other phage structural proteins encoded by the cp32s; however, putative phage structural proteins encoded by lp54 were also detected. Long-read sequencing reveals that ϕBB-1 virions package a variety of genetic material including cp32 isoforms that are linearized at a region immediately upstream of the *erp* locus (o*spE/ospF/elp*) and packaged into ϕBB-1 capsids via a headful genome packaging mechanism at a packaging site (*pac*). When introduced to a shuttle vector, the *pac* region promotes the packaging of shuttle vectors into ϕBB-1 virions, demonstrating the utility of ϕBB-1 as a tool to genetically manipulate Lyme disease spirochetes. Additionally, full-length contigs of cp26, lp17, lp38, lp54, and lp56 are recovered from packaged reads as are fragments of the linear chromosome. Finally, long-read sequencing of packaged DNA allowed us to fully resolve most of the covalently closed hairpin telomeres in the *B. burgdorferi* CA-11.2A genome.

Overall, this study implicates ϕBB-1 in mobilizing large portions of the *B. burgdorferi* genome, which may explain certain aspects of genome stability and diversity observed in Lyme disease spirochetes.

Results

ϕBB-1 phage purification, virion morphology, and proteomic analysis

In the laboratory, lytic ϕBB-1 replication (**Fig 1C**) can be induced by fermentation products such as ethanol [38, 39]. We first measured ϕBB-1 titers in early stationary-phase cultures (~1 × 10^8 cells/mL) of *B. burgdorferi* B31 or CA-11.2A induced with 5% ethanol, as described by Eggers *et al.* [38]. Seventy-two hours after induction, bacteria were removed by centrifugation and filtering and virions were purified from supernatants by chloroform extraction and precipitation with ammonium sulfate. Purified virions were treated overnight with DNase to destroy DNA not protected within a capsid. Phage DNA was then extracted using a proteinase K/SDS/phenol-chloroform DNA extraction protocol [30] and qPCR was used to measure packaged cp32 copy numbers.

B. burgdorferi strain CA-11.2A consistently produced ~10 times more phage than B31 (**Fig 2A**) and was selected for further study. Imaging of purified virions collected from CA-11.2A by transmission electron microscopy reveals virions with an elongated capsid and contractile tail (**Fig 2B**), which is similar to the Myoviridae morphology of ϕBB-1 virions produced by strain B31 *in vitro* [9, 40, 41] and by a human *B. burgdorferi* isolate following ciprofloxacin treatment [42].

Mass spectrometry analysis of purified virions identified ten capsid and other

structural proteins encoded by the cp32s (**Fig 2C, Table S1**). We also detected highly conserved predicted phage capsid proteins encoded by lp54 (**Fig 2D**). While the virions we visualized all appear to have the same elongated capsid morphology, virions with a notably smaller capsid morphology have been isolated and imaged from *B. burgdorferi* CA-11.2A [29]. These observations raise the possibility that there are multiple intact phages inhabiting the CA-11.2A genome.

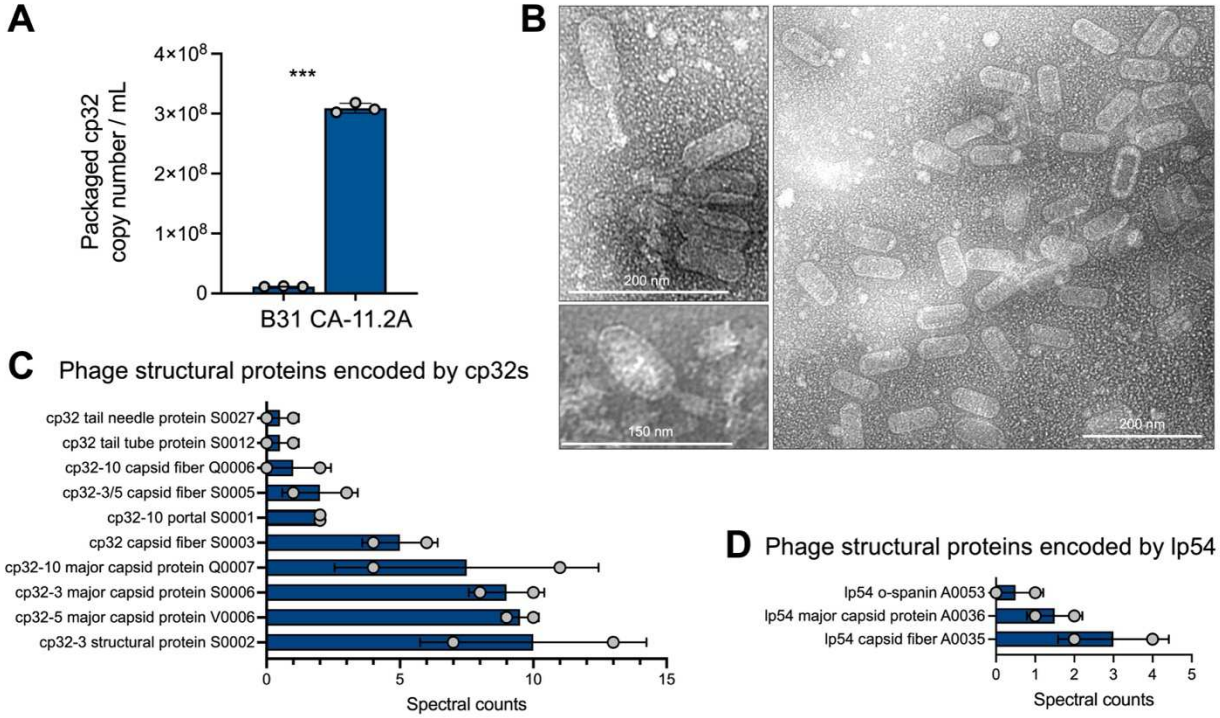


Figure 2. ϕBB-1 phage titer, virion morphology, and proteomic analysis. (A) Packaged, DNase-protected cp32 copy numbers in bacterial supernatants were measured by qPCR. Data are the SE of the mean of three experiments, ***P<0.001. **(B)** Virions were purified from 4-L cultures of *B. burgdorferi* CA-11.2A and imaged by transmission electron microscopy. Representative images from two independent preparations are shown. **(C and D)** HPLC-MS/MS-based proteomics was used to identify proteins in the two purified virion preparations. The SE of the mean of spectral counts for peptides associated with the indicated phage structural proteins are shown for each replicate. See also **Table S1** for the complete proteomics dataset.

ϕBB-1 virions package portions of the *B. burgdorferi* genome

We performed long-read sequencing on DNA packaged in purified ϕBB-1 virions, as outlined in **Figure 3**. There is concern that contaminating unpackaged *B. burgdorferi* chromosomal or plasmid DNA co-purifies with phage virions. To control for this, we spiked purified ϕBB-1 virions with high molecular weight (> 20 kb) salmon sperm DNA (**Fig 4A**) at 1.7 µg/mL, a concentration that approximates the amount of DNA released by 3×10^8 lysed bacterial cells into one milliliter of media [43]. Samples were then treated with DNase overnight followed by phage DNA extraction using a proteinase K/SDS/phenol-chloroform DNA extraction protocol [30]. Recovered DNA was directly sequenced using the Nanopore MinION (long read) platform.

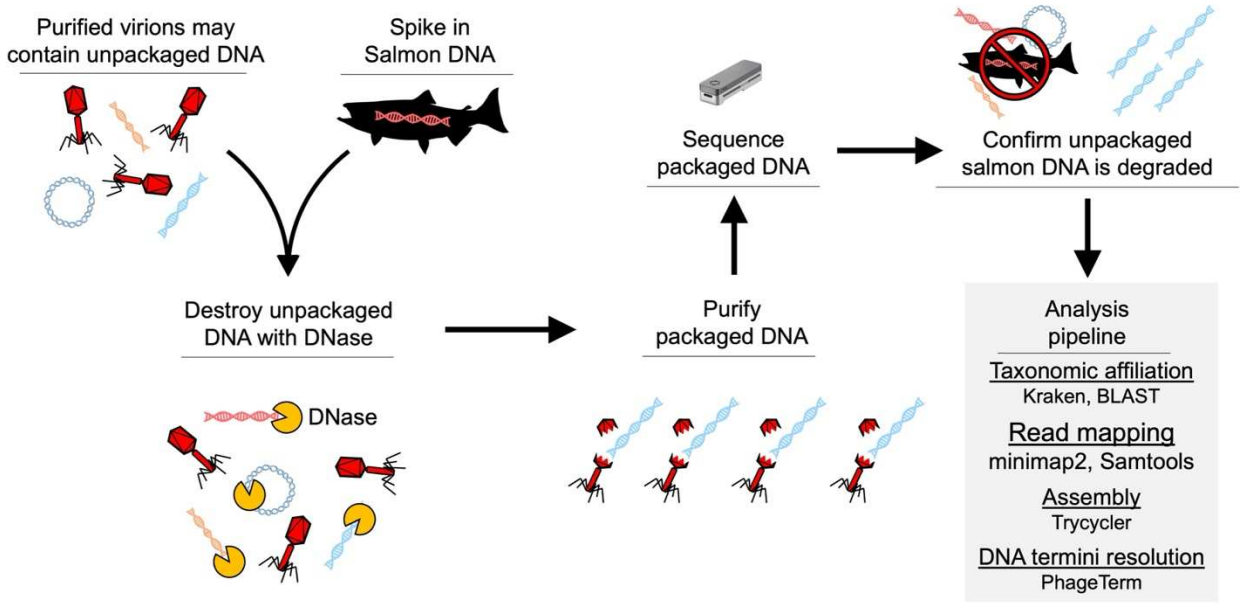


Figure 3. Workflow for sequencing packaged ϕBB-1 DNA.

Kraken [44] was used to classify prokaryotic and eukaryote sequences followed by BLAST to classify reads missed by Kraken (e-value <0.01, query coverage >10%). Reads less than 700 bp were discarded. Of 110,986 reads >700 bp, 0.14% were classified as matching salmon sequences (**Fig 4B**) with an average length of 1.2 kb (**Fig 4B**), indicating the DNase treatment step successfully degraded unpackaged DNA. To exclude reads that may be derived from unpackaged DNA, we removed all reads less than 5 kb from the dataset, leaving a total of 58,399 reads longer than 5 kb (**Fig 4C**) with a median length of 12.3 kb (**Fig 4D**). Note that cp32 prophages are approximately 32 kb in size and we detect a high number of ~32 kb reads in each replicate (**Fig 4D**, dashed line).

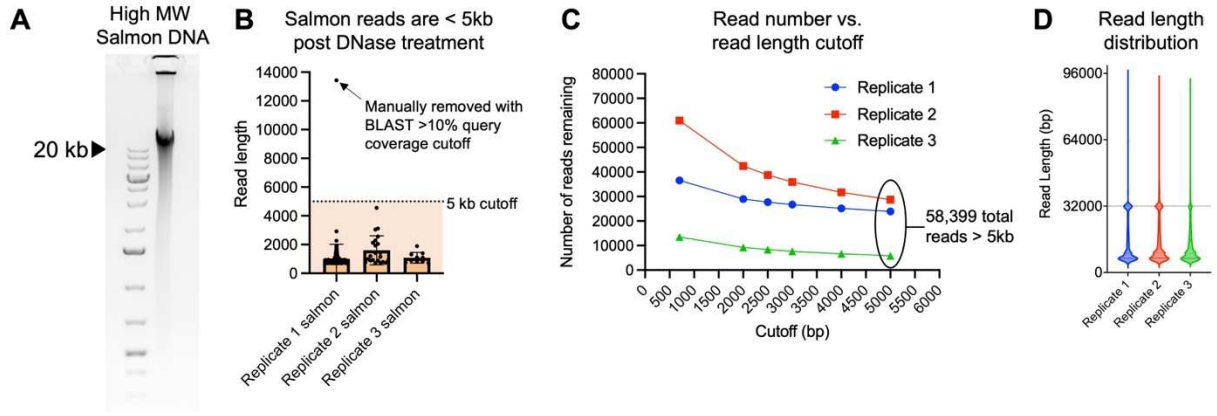


Figure 4. Establishing a 5kb read length cutoff to exclude unpackaged reads. (A) The salmon sperm DNA used to spike purified phages prior to DNase treatment was run on an agarose gel to estimate its size. Note that the majority of salmon DNA is larger than the 20-kb high molecular weight marker in the left lane. **(B)** 0.14% of 110,986 reads > 700 bp, 0.14% were classified as matching salmon sequences. Reads classified as salmon were plotted as a function of their length for each replicate. Error bars represent the SE of the mean of three replicate experiments. All reads except one (arrow) were below 5 kb in length (dashed line) with an average length of 1.2 kb. **(C)** Read length cutoff was plotted as a function of the number of reads remaining in each replicate dataset. In total, 58,399 reads remain after establishing a 5-kb cutoff. **(D)** Read length for all reads >5 kb in each replicate was plotted.

Overall, ~99.6% of packaged reads >5 kb were classified as *B. burgdorferi* (**Fig 5A**), the majority of which (~79%) were cp32 isoforms (**Fig 5B**). Cp32-10 and cp32-3 were preferentially packaged (~32% and ~25%, respectively) followed by cp32-13 and cp32-5 (each at ~10%) (**Fig 5B**). Reads mapping to cp32-3, cp32-5, cp32-10, and cp32-13 had a mean coverage of over 1,000× (**Fig 5C**). Cp32-1 reads accounted for only about one percent of all packaged reads (**Fig 5B**) and had lower mean coverage of approximately 36× (**Fig 5C**), suggesting that cp32-1 was not undergoing lytic replication. Read length distributions for the cp32s indicate that full-length ~32 kb molecules were frequently recovered for each cp32 isoform, except for cp32-10 (**Fig 5D**).

Additionally, 11.6% of reads > 5 kb mapped to the linear chromosome and ~6.3% of reads >5 kb mapped to lp54 (**Fig 5B**). The remaining reads mapped to *B. burgdorferi* plasmids cp26, lp17, lp36/lp28-4, lp38, lp56, and lp28-3 at 1–2% each (**Fig 5B**). *De novo* assembly of packaged reads produced full-length contigs of all cp32s, lp17, cp26, lp36, lp38, lp54, and lp56 (**Fig S1**), suggesting that full-length versions of these plasmids are packaged by ϕBB-1.

Of note, the CA-11.2A genome was reported to contain a unique plasmid, lp36/lp28-4, that is thought to have arisen from the fusion of lp36 with lp28-4 [45]. *De novo* assembly of packaged reads resolved lp36/lp28-4 into individual lp36 and lp28-4 contigs (**Fig S1E and F**). Additionally, whole genome sequencing of our CA-11.2A strain confirmed that lp36 and lp28-4 are separate as no reads that span the lp36-lp28-4 junction were observed and coverage depth was notably different between lp36 and lp28-4 (~200× vs. 25×, respectively, **Fig S2A**). Furthermore, PCR confirmed the sequencing results (**Fig S2B-D**). These data indicate that the lp36/lp28-4 plasmid is two distinct episomes in our CA-11.2A strain.

Collectively, these results indicate that in addition to cp32 molecules, ϕBB-1 is capable of packaging non-cp32 portions of the *B. burgdorferi* genome. We discuss the major packaged DNA species in the following sections.

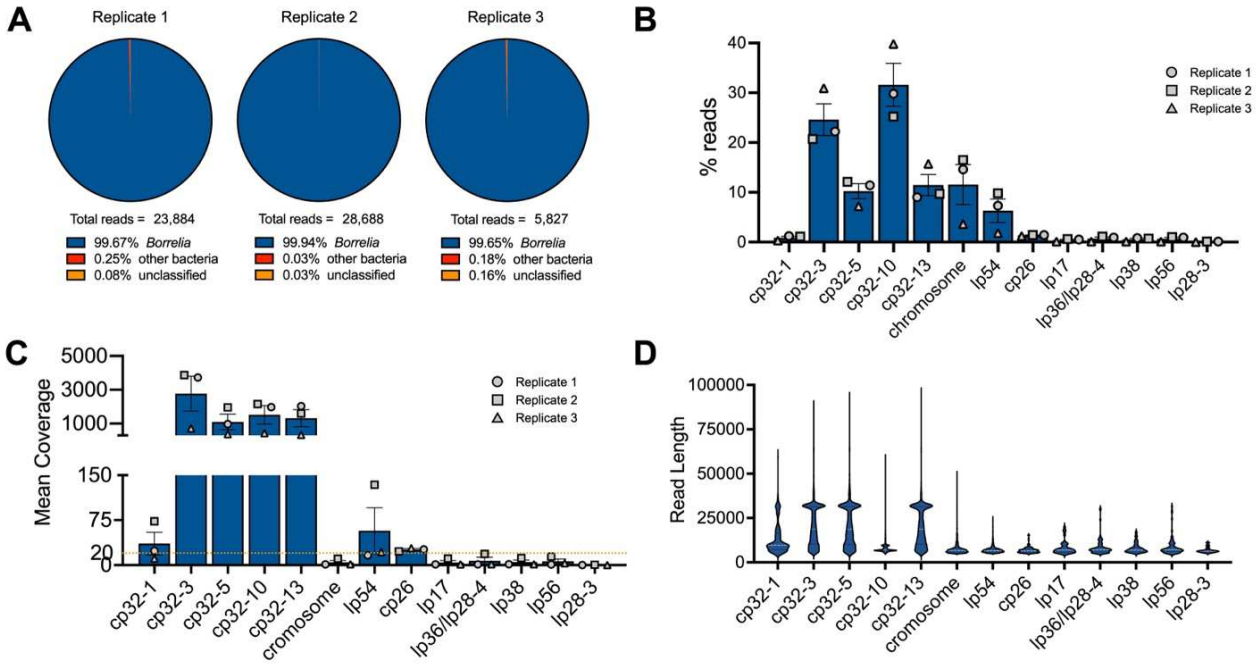


Figure 5. ϕBB-1 virions package cp32 isoforms, chromosome fragments, lp54, and other plasmids. **(A)** Kraken and BLAST were used to determine the taxonomic affiliation of reads >5kb. Note that no eukaryotic reads were identified. **(B and C)** The (B) percent and (C) mean coverage for reads affiliated with the indicated *B. burgdorferi* plasmid or linear chromosome are shown for each replicate. Error bars represent the SE of the mean. **(D)** Read length distributions for the indicated plasmids or chromosome are shown.

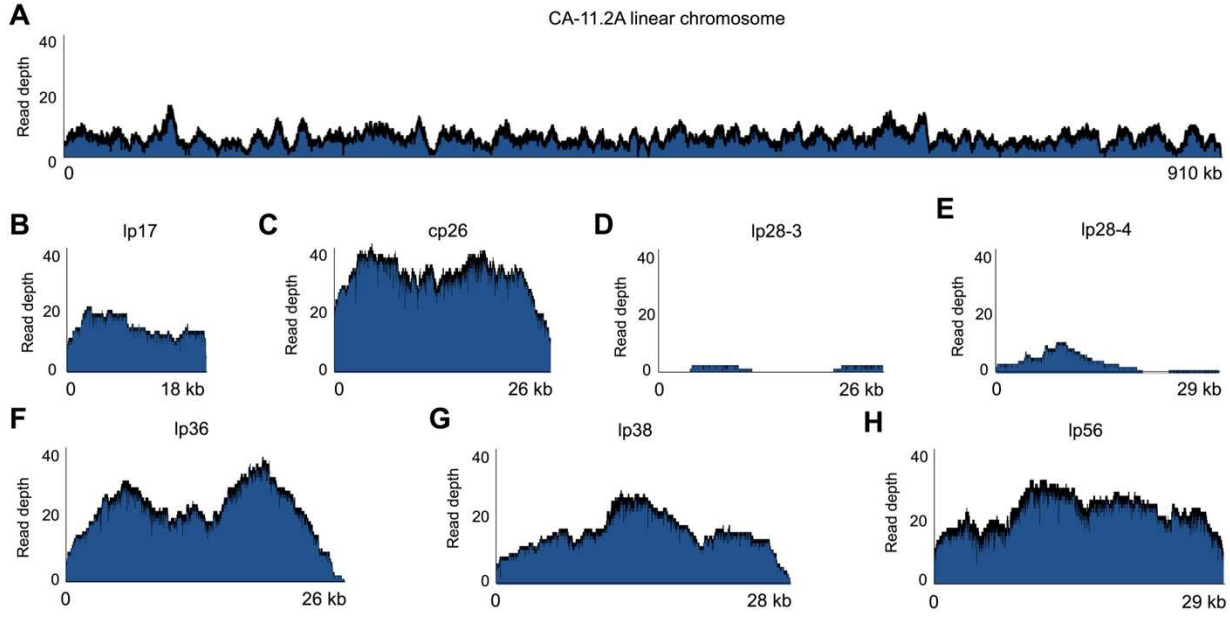


Figure S1. Packaged read depth across the *de novo* CA-11.2A genome assembly. *De novo* assembly of packaged reads >5kb produced the indicated contigs. Read coverage was then mapped to each contig. **(A–H)** Read coverage across the CA-11.2A chromosome or indicated plasmids are shown. Coverage maps for the cp32s and lp54 are shown in Figures 6 and 9, respectively.

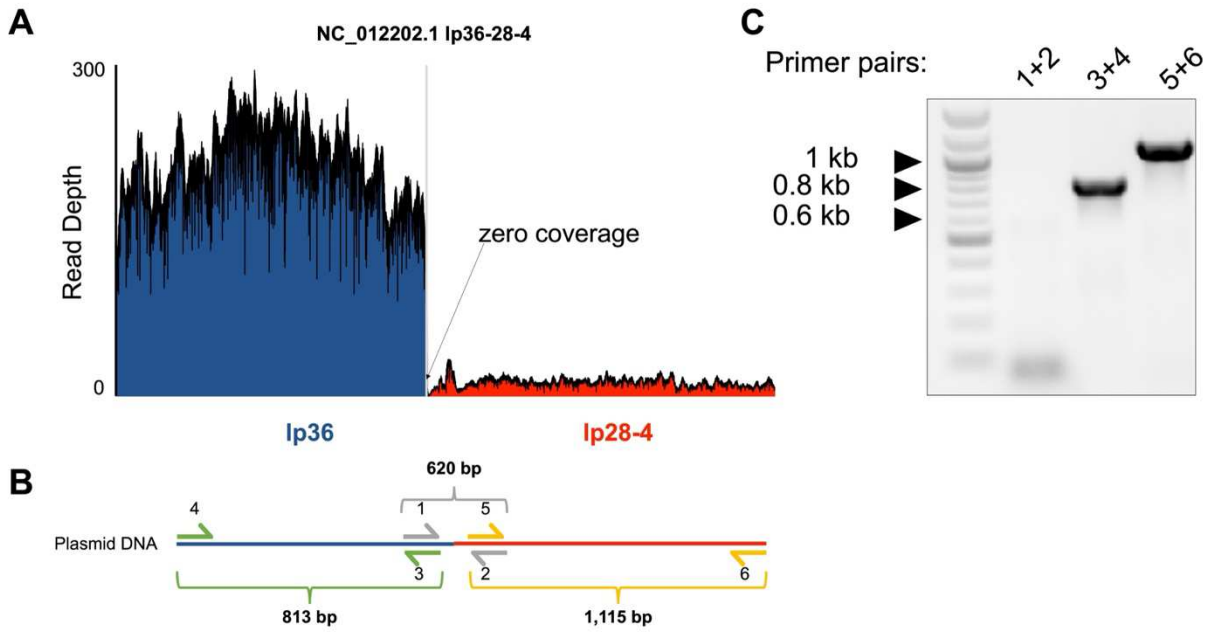
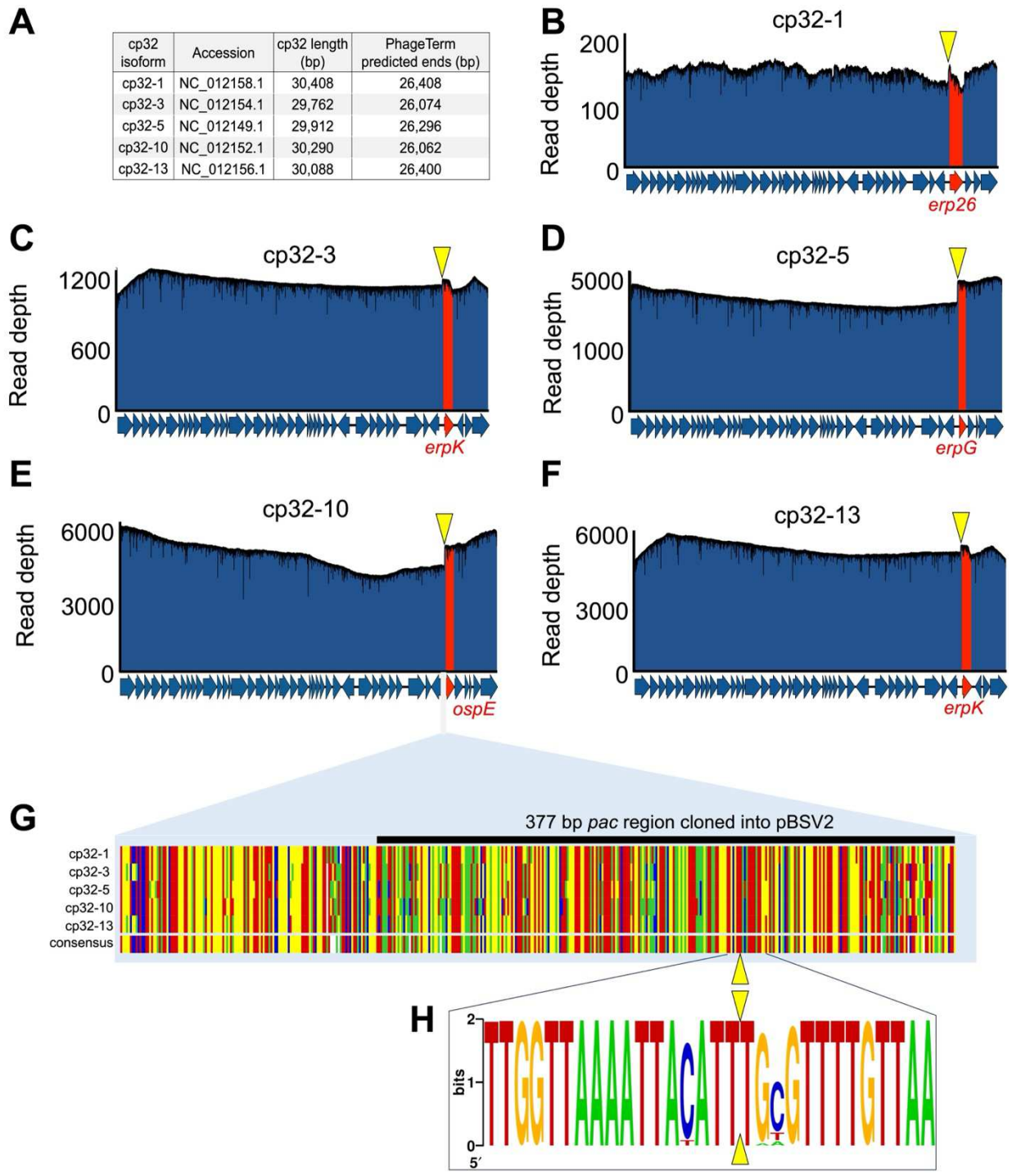


Figure S2. Whole genome sequencing of the CA-11.2A genome reveals that plasmid lp36/lp28-4 resolves into two separate episomes. (A) The CA-11.2A genome was sequenced using long-read technology. Reads were aligned to the lp36/lp28-4 reference sequence (NC_012202.1) and read depth plotted. **(B)** Schematic of PCR design. Primers 1 and 2 flank the lp36/lp28-4 junction, with primer 1 annealing to lp36 and primer 2 annealing to lp28-4, creating a 620 bp product if joined. Primers 3 and 4 anneal to lp36 DNA, creating an 813 bp product if present. Primers 5 and 6 anneal to lp28-4 DNA, creating a 1,115 bp product if present. **(C)** The presence or absence of lp36, lp28-4, or lp36/lp28-4 was confirmed by PCR.

cp32 molecules are linearized near the *erp* locus and packaged via a headful mechanism

Our sequencing data provide insight into how ϕBB-1 packages cp32 molecules. Many phage species package linear double-stranded DNA genomes that circularize after being injected into a host [46] and packaged cp32s are thought to be linearized [30]. We used PhageTerm [47] to predict the linear ends of packaged DNA. Native DNA termini are present once per linear DNA molecule, but non-native DNA ends produced during sequencing are distributed randomly along DNA molecules. Thus, reads that start at native DNA terminal positions occur more frequently than anywhere else in the genome. PhageTerm takes advantage of this to resolve DNA termini and predict phage packaging mechanisms [47]. PhageTerm identified the termini of packaged cp32 molecules at approximately 26 kb in a region lying immediately upstream of the *erp* (*ospE/ospF/elp*) loci (**Fig 6A**). In agreement with the PhageTerm results, when packaged reads were used to map the physical ends of packaged cp32 molecules, a sharp boundary in coverage depth is observed upstream of the *ospE/ospF/elp* loci in all cp32s (**Fig 6B–F**). Notably, the intergenic region upstream of the *erp* loci is conserved across the cp32 isoforms found in diverse strains of Lyme disease spirochetes (**Fig 6G**) [15] and the linear cp32 ends identified by long-read sequencing converge on the same terminal sequence motif (**Fig 6H**).



cp32 isoform	Accession	cp32 length (bp)	PhageTerm predicted ends (bp)
cp32-1	NC_012158.1	30,408	26,408
cp32-3	NC_012154.1	29,762	26,074
cp32-5	NC_012149.1	29,912	26,296
cp32-10	NC_012152.1	30,290	26,062
cp32-13	NC_012156.1	30,088	26,400

Fig 6. cp32s are linearized upstream of the *erp* loci. (A) PhageTerm was used to predict the linear ends of packaged cp32 molecules. **(B–F)** Nanopore reads were mapped to the indicated cp32s. Note the sharp boundary just upstream of the *erp* loci (highlighted in red). The yellow triangles indicate the PhageTerm predicted linear ends. **(G)** Alignments of the intergenic region upstream of the *erp* loci is shown for each cp32. The black line indicates the *pac* region that was cloned into a shuttle vector, as described in Figure 7. **(H)** A nucleic acid logo was constructed from 207 cp32 sequence alignments. Yellow triangles indicate the linear end of cp32 isoforms as predicted by PhageTerm and confirmed by long-read sequencing.

PhageTerm predicts that cp32s are packaged by a headful mechanism. This observation supports the previously proposed headful genome packaging mechanism for cp32s [39]. Phages that use the headful packaging mechanism generate a concatemer

containing several head-to-tail copies of their genome (**Fig 7A**). During headful packaging, a cut is made at a defined packaging site (*pac* site) and a headful (a little more than a full genome) of linear phage DNA is packaged. Once a headful is achieved, the phage genome is cut at non-defined sites, resulting in variable cut positions and size variation in packaged DNA, which we observe in packaged cp32 reads downstream of the initial cut site (**Fig 6B–F**).

Our results suggest that the cp32 *pac* site is upstream of the *erp* (*ospE/ospF/elp*) loci. If the cp32 *pac* site is in this region, then DNA molecules containing the *pac* sequence are expected to be packaged into ϕBB-1 virions. To test this, we cloned the putative cp32-3 *pac* site (**Fig 6G**, black bar) into the pBSV2 shuttle vector [48], transformed *B. burgdorferi* strain CA-11.2A, and induced lytic ϕBB-1 replication with 5% ethanol. Supernatants containing virions were collected, filtered, and DNase treated, and packaged DNA isolated. pBSV2 shuttle vector copy numbers were measured by qPCR using primers that target the pBSV2 kanamycin resistance (*kan*) cassette. To control for possible chromosomal DNA contamination, qPCR was also performed using primers targeting the *flaB* gene. Final packaged pBSV2 copy numbers were calculated by subtracting *flaB* copy numbers from pBSV2 (*kan* cassette) copy numbers.

Copy numbers of packaged pBSV2 encoding the cp32-3 *pac* site were significantly ($P<0.001$) higher compared to virions collected from cells either not carrying the pBSV2 vector or cells carrying an empty pBSV2 vector (**Fig 7B**), indicating that DNA molecules that contain the *pac* site are preferentially packaged by ϕBB-1 virions.

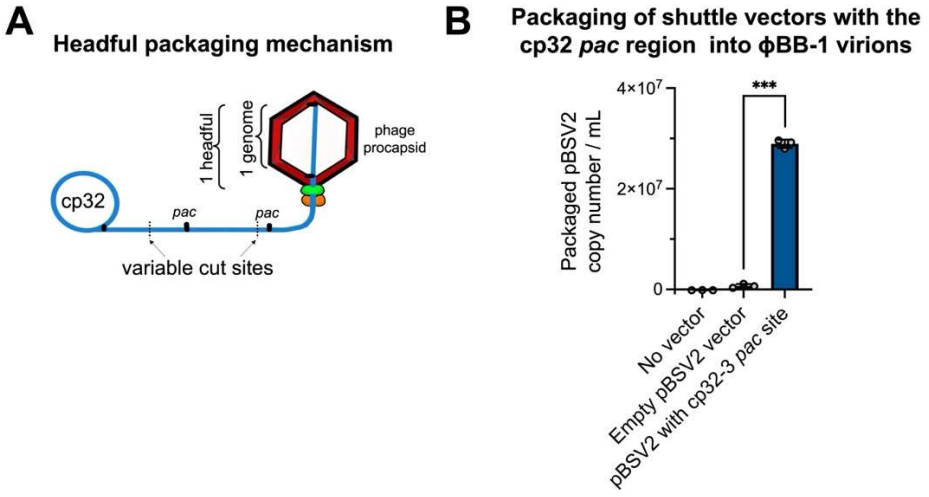


Figure 7. Shuttle vectors containing the cp32 *pac* region are preferentially packaged into ϕBB-1 virions. (A) Schematic depicting the headful genome packaging mechanism. **(B)** After ethanol induction, ϕBB-1 virions were collected from CA-11.2A cells not carrying plasmid pBSV2 (No vector), cells transformed with empty pBSV2, or cells transformed with pBSV2 with the cp32-3 *pac* site (see Fig 6G for the cloned *pac* region). Copy numbers of pBSV2 packaged into ϕBB-1 virions were measured by qPCR. Data are the SE of the mean of three experiments, ***$P<0.001$.

The cp32 prophages have conserved motifs that occur in a specific arrangement not found in other DNA sequences packaged by ϕBB-1 virions

To identify motif(s) that may be shared between the cp32s and other genomic

elements that are packaged into ϕBB-1 virions (*e.g.,* lp54), we first used an iterative BLAST search to identify distantly homologous DNA sequences (**Fig 8**). A non-redundant list of these diverse DNA sequences were then used as an input dataset for sequence motif discovery via MEME [49]. All five cp32 isoforms found in *B. burgdorferi* CA-11.2A have the same specific arrangement of conserved sequence motifs around the *pac* region (**Fig 8A and B**) and these are conserved in cp32 isoforms across *B. burgdorferi* (**Fig 8C**). However, significant matches to these motifs were not identified in other CA-11.2A genetic elements packaged by ϕBB-1 (**Supplementary Data file 1**), suggesting that packaging of non-cp32 DNA may occur spontaneously or through different mechanisms.

The complete or partial arrangement of motifs found around the *pac* site of *B. burgdorferi* cp32 isoforms is conserved in cp32 plasmids and some linear plasmids originating from several different *Borrelia* species (**Fig. 8C**). The iterative BLAST search also revealed that a diverse set of circular and linear plasmids in a broader set of *Borrelia* species share some of the motifs found in *B. burgdorferi* cp32 isoforms. In total, linear or circular plasmid sequences from 21 different *Borrelia* species (both Lyme disease and relapsing fever spirochetes) had homology to the *B. burgdorferi* cp32 *pac*-containing DNA sequences (**Fig 8C**). The motifs that are found most broadly, *e.g.,* blue triangle, and green square, may represent binding sites for conserved host factors that are present in all these *Borrelia* species whereas the other motifs may represent protein-binding sites or regulatory sequences that are specific to given prophage or plasmids.

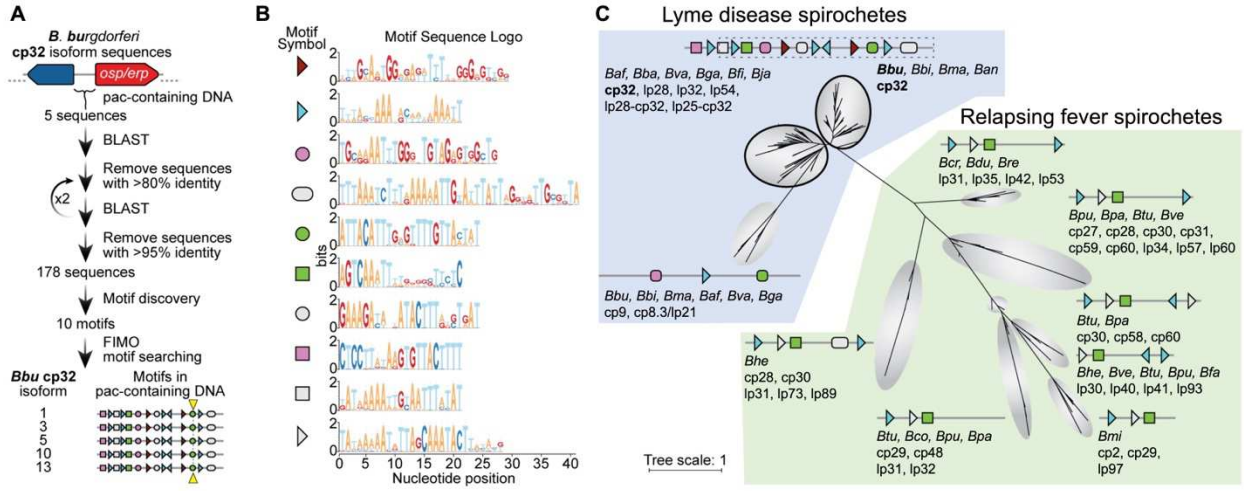


Figure 8. Cp32 prophages have conserved motifs that occur in a specific arrangement around the *pac* site. (A) Outline of bioinformatic strategy to identify motifs enriched in the *pac*-containing DNA sequence of cp32 isoforms. All *B. burgdorferi* cp32 isoforms have the same motifs in the *pac* region. The cp32 cut site is indicated by the yellow triangle. **(B)** Sequence logos of the motifs identified in panel A and schematized in panel C. Nine of the top ten motifs occur at least once in the *pac*-containing region of cp32 DNA sequences. Motifs represented with right or left facing triangles often occur as direct and/or indirect repeats. **(C)** Phylogenetic tree of non-redundant DNA sequences with homology to *B. burgdorferi* cp32 *pac*-region identified in panel A. For each clade, the bacterial species and type of plasmid are listed. For clarity in the figure, bacterial species names have been truncated to a three letter abbreviation consisting of the first letter of the genus and the first two letters of the species (*Borrelia afzelii, Baf; Borrelia andersonii, Ban; Borrelia bavariensis, Bba; Borrelia bissettiae, Bbi; Borrelia burgdorferi, Bbu; Borrelia coriaceae, Bco; Borrelia crocidurae, Bcr; Borrelia duttonii, Bdu; Borrelia fainii, Bfa; Borrelia finlandensis, Bfi; Borrelia garinii,*

Bga; Borrelia hemsii, Bhe; Borrelia japonica, Bja; Borrelia mayonii, Bma; Borrelia miyamotoi, Bmi; Borrelia parkeri, Bpa; Borrelia puertoricensis, Bpu; Borrelia recurrentis, Bre; Borrelia turicatae, Btu; Borrelia valaisiana, Bva; Borrelia venezuelensis, Bve). There is variability in the motif architecture between sequences within a single clade; however, for clarity, a representative motif architecture discovered by MEME is shown [49]. The top two clades of sequences (outlined in black) are dominated by cp32 isoforms and the cp32 motif architecture, therefore a single motif scheme is shown for these two clades. The region of DNA and motifs cloned into the pBSV2 shuttle vector is outlined in dashes.

Deciphering the structure of linear plasmids packaged by ϕBB-1

After the cp32s, lp54 is a major DNA species packaged by ϕBB-1 (**Fig 5C**). Lp54 is a linear plasmid with covalently closed telomeric ends [50]. *De novo* assembly of packaged lp54 reads produces a 67.4 kb contig consisting of full-length lp54 (54,021 bp, NC_012194.1) flanked by sequences containing tail-to-tail (7,310 bp) and head-to-head (6,074 bp) junctions (**Fig 9A**). Read depth for lp54 was >100 for most of the contig; however, read depth drops precipitously at both tail-to-tail and head-to-head junctions (**Fig 9A**), suggesting that the telomeres of lp54 interfere with sequencing.

B. burgdorferi telomeres contain inverted repeat sequences [51] and we identified the CA-11.2A lp54 inverted repeat sequence as 5′–TTTATTAGTATACTAATAAA (**Fig 9B and C**, boxed sequences). The 5′ inverted repeat of the lp54 reference sequence is missing seven nucleotides and our data extends and completes this sequence (**Fig 9B,** underlined). Further, compared to the lp54 reference sequence, the packaged 5′ and 3′ junction-spanning sequences each encode an additional 18 bp of sequence (**Fig 9B and C**). These sequences, although unique at each end (**Fig 9D**), form perfect hairpin structures (**Fig 9E and F**). Overall, these data suggest that lp54 molecules with complete telomere sequences are packaged into virions. However, whether linear lp54 with covalently closed telomeres or lp54 replication intermediates that contain head-to-head and tail-to-tail junctions are packaged is unclear.

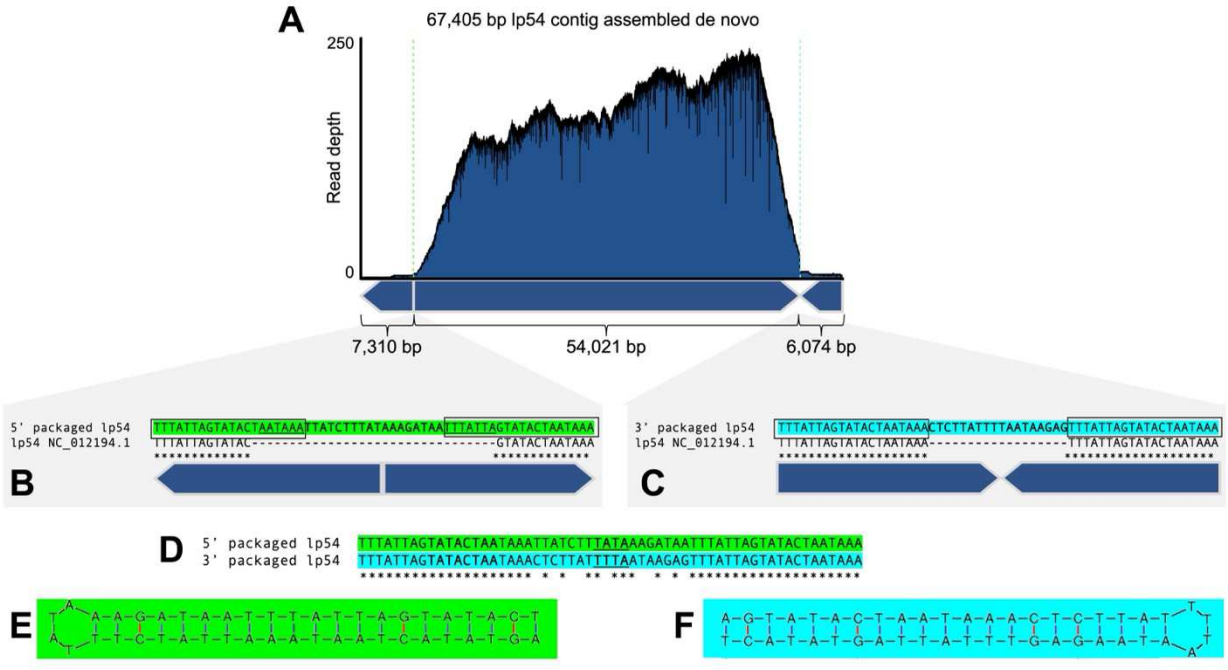


Figure 9. Full-length lp54 with fully resolved telomeres are recovered from ϕBB-1-packaged DNA. (A) *De novo* assembly of packaged reads produced a 67,405 bp contig with tail-to-tail and head-to-head

junctions. **(B and C)** Sequences at the packaged 5′ junction (green) or the 3′ junction (cyan) are compared to the lp54 reference sequence NC_012194.1. The conserved inverted repeat sequence 5′–TTTATTAGTATACTAATAAA is outlined. **(D)** Alignments of the tail-to-tail and head-to-head junctions reveals a variable 18-bp sequence in between the conserved inverted repeats. **(E and F)** Predicted hairpin structures are shown for each end of lp54. The loop sequence for each hairpin is underlined in panel D.

The *de novo* assembly approach applied to lp54 was also successful in resolving the telomeric ends of other linear elements of the CA-11.2A genome, including the linear chromosome and plasmids lp17, lp56, and lp38 (**Fig 10**). Additionally, we were able to resolve left and right telomeres for lp36 (**Fig 10**), providing yet further evidence that lp36 is not fused to lp28-4.

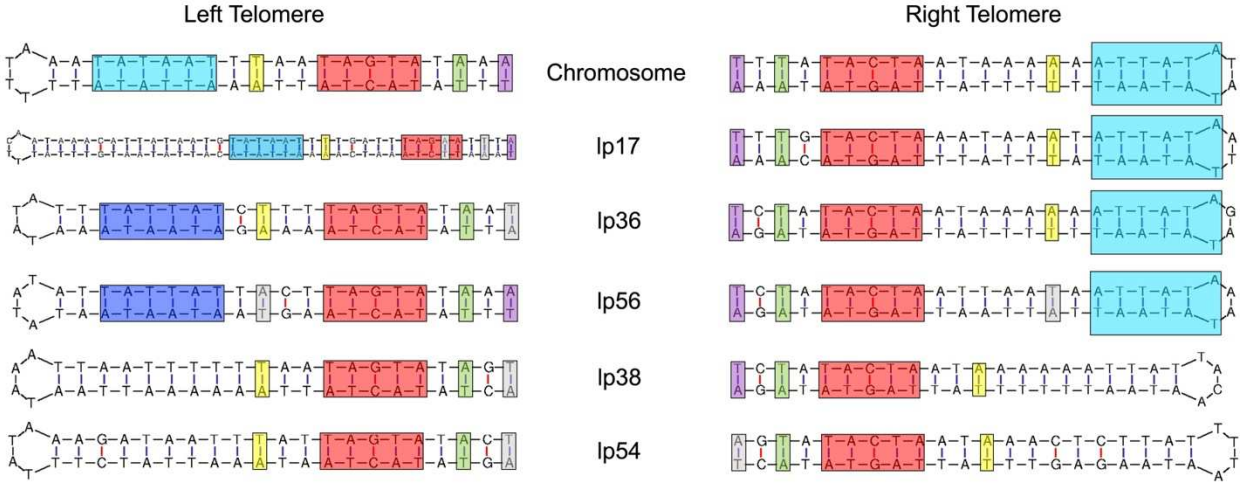


Figure 10. Packaged reads resolve the telomeric ends of the linear chromosome and most linear plasmids in the CA-11.2A genome. Reads spanning tail-to-tail or head-to-head junctions of the linear chromosome or the indicated linear plasmids form perfect hairpin structures. Conserved regulatory elements for each telomere are highlighted [52-57].

Discussion

In nature, Lyme disease spirochetes exist as diverse populations of closely related bacteria that possess sufficient antigenic variability to allow them to co-infect and reinfect non-naïve vertebrate hosts [58-69]. Moreover, horizontal gene transfer between Lyme disease spirochetes has been extensively documented [70-75]. Nevertheless, the mechanism underlying horizontal genetic exchange among Lyme disease spirochetes remains undefined.

Horizontal gene transfer between heterologous spirochetes likely occurs in the tick midgut during and immediately after a blood meal when spirochete replication rates and densities are at their highest. ϕBB-1 replication is also induced in the tick midgut during a bloodmeal [9, 27, 28] and homologous recombination between cp32 isoforms [15-17] and the horizontal transfer of cp32s between *Borrelia* strains has been documented [25]. These observations implicate ϕBB-1 in mediating horizontal gene transfer between Lyme disease spirochetes.

Our study further implicates ϕBB-1 in mediating horizontal gene transfer between Lyme disease spirochetes. Our sequencing data indicate that ϕBB-1 virions package portions of the entire *B. burgdorferi* genome, giving ϕBB-1 the potential to mobilize numerous beneficial alleles during the enzootic cycle via generalized transduction. For example, the circular cp32 prophages are highly conserved across the *Borrelia* genus [22]; however, cp32 isoforms contain variable regions that encode outer membrane lipoproteins such as Bdr, Mlp, and OspE/OspF/Elp, which are known to facilitate the *B. burgdorferi* lifecycle [20, 22, 23, 76]. The linear plasmid lp54 encodes the outer membrane lipoproteins OspA and OspB, which are required for *B. burgdorferi* to colonize the tick midgut [77-79]. The outer membrane lipoprotein OspC, which is required for *B. burgdorferi* to infect a vertebrate host, is encoded by the circular plasmid cp26 [58, 74, 80]. All these alleles (and many others) are packaged by ϕBB-1, which is consistent with a role for phage-mediated transduction of genes encoding essential membrane lipoproteins between heterologous spirochetes.

In *B. burgdorferi*, the linear chromosome is highly conserved as are the circular plasmids cp32 and cp26 and the linear plasmids lp17, lp38, lp54, and lp56 are evolutionarily stable [4-6, 16, 81]. However, other plasmids distributed across the genospecies show considerably more variation and encode mostly (87%) pseudogenes and are thought to be in a state of evolutionary decay [6]. The plasmids we identified as packaged by ϕBB-1 virions include the cp32s, cp26, lp17, lp38, lp54, and lp56 —the same plasmids that are evolutionarily stable across the genospecies [4-6, 16, 81]. These observations suggest that genes encoded on ϕBB-1-packaged plasmids are under positive selection, possibly as a result of transduction between Lyme disease spirochetes during the enzootic cycle.

In addition to providing evidence that ϕBB-1 virions package large portions of the *B. burgdorferi* genome, our study provides insight into ϕBB-1 virion structure and identifies virion proteins present in ϕBB-1. Using mass spectrometry-based proteomics, we confirm that putative capsid and other predicted structural genes encoded by cp32s such as the

major capsid protein P06 are indeed translated and assembled into mature ϕBB-1 virions.

Our long-read sequencing studies indicate that ϕBB-1 packages full-length linear cp32 molecules via a headful mechanism using *pac* sites. The headful packaging mechanism is used by numerous phages and was first described for *E. coli* phage T4 in 1967 [82]. After injecting linear DNA into a new host, the phage genome re-circularizes before continuing its replication cycle. Genes encoded near the ends of linear phage genomes are subject to copy number variation and recombination as the phage genome re-circularizes [83]. Our data suggest that the conversion of linear cp32 molecules into circular cp32 molecules occurs in the vicinity of the *erp* locus, which could facilitate recombination with polymorphic *erp* alleles encoded by other cp32 isoforms in diverse *B. burgdorferi* hosts.

In this study, the packaging of specific cp32 isoforms was biased: cp32-3, cp32-5, cp32-10, and cp32-13 were predominantly packaged while cp32-1 was rarely packaged. This result is consistent with observations by Wachter *et al.* where cp32 isoform copy number and transcriptional activity were not uniform across all cp32 isoforms in *B. burgdorferi* strain B31: cp32-1, cp32-3, and cp32-6 were predominantly induced (highest copy numbers) and had the highest transcriptional activity while cp32-9 was not induced and was transcriptionally inactive [9]. Variability in the *pac* region or other regulatory elements involved in cp32 induction may explain why different cp32 isoforms replicate and/or are packaged at different rates.

In the intergenic region upstream of the *erp* loci, we identified a 377-bp region that contains the cp32 *pac* signal. Introducing the cp32 *pac* region to a shuttle vector facilitated the packaging of the shuttle vector into ϕBB-1 virions. Identification of the cp32 *pac* site will be useful for the engineering of recombinant DNA that can be packaged into virions that infect spirochetes, giving ϕBB-1 the potential for use as a genetic tool.

After the cp32s, lp54 was the most frequently packaged plasmid. This may be related to the evolutionary origins of lp54: lp54 contains large blocks of homology with the cp32s and is thought to have emerged from an ancient recombination event between a cp32 and a linear plasmid [6]. In addition, lp54 encodes putative phage proteins including a porin (BBA74) [84] and phage capsid proteins that are highly conserved across the genospecies [85], which we detected in purified virions by mass spectrometry. While we observed virions with a distinct elongated capsid morphology, virions with a notably smaller capsid morphology have been observed after induction *in vitro* [9, 29, 30]. These observations raise the possibility that lp54 may be a prophage, although whether lp54 produces its own capsids or relies on cp32-encoded capsids, or if lp54 capsid proteins assemble with capsid proteins encoded by cp32 to produce chimeric capsids, is not clear.

Our long-read dataset contained reads that spanned head-to-head and tail-to-tail junctions in lp54. These reads allowed us to define the lp54 telomere sequences; however, whether full-length lp54 molecules are packaged or at which stage of the replication cycle lp54 is packaged is unknown. In *B. burgdorferi*, both the linear chromosome and linear plasmids have covalently closed hairpin telomeres and replicate via a telomere resolution mechanism [53, 55, 86, 87]. Examination of a naturally occurring

lp54 dimer in *B. valaisiana* isolate VS116 suggests that a circular head-to-head dimer is produced during lp54 replication prior to telomere resolution and replication completion [88]. Linear, covalently closed lp54 molecules may be packaged or lp54 replication intermediates may be packaged.

As obligate vector-borne bacteria, Lyme disease spirochetes live relatively restrictive lifestyles that might be expected to i) limit their exposure to novel gene pools, ii) enhance reductive evolution, and iii) favor the loss of mobile DNA elements. A role for ϕBB-1 in mediating the transduction of beneficial alleles between heterologous spirochetes in local vector and reservoir host populations may explain why cp32 prophages are ubiquitous not only among Lyme disease spirochetes, but also relapsing fever spirochetes.

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Methods

ϕBB-1 induction. *Borrelia burgdorferi* B31 or CA-11.2A was grown in BSK-II growth medium to 7 × 10^7/mL, transferred to 50 mL tubes and centrifuged at 6,000 × *g*, 10 min, 35°C. Supernatants were discarded and the pellet resuspended in fresh media to a density of 2 × 10^8 cells/mL. 100% EtOH was added to a final concentration of 5% (do not use 95% EtOH) and incubated at 35°C for 2 hours. The culture was centrifuged at 6,000 x g for 10 min., 35°C. and the pellet resuspended in fresh media to a density of 5 × 10^7 cells/mL. The sample was incubated at 35°C for 72 hours followed by filtration through 0.2 µm filter. Samples were stored at 4°C or phage were concentrated using a 10 kDa MW cutoff filter, replacing culture medium with SM buffer.

cp32 qPCR. Packaged cp32 molecules in DNase-treated supernatants were measured by qPCR using primers that amplify a conserved cp32 intergenic region between *bbp08* and *bbp09* (5′-CTTTACACATATCAAGACCTTAAC, 5′-CAAACCACCCAATTTCCAATTCC). Primers that amplify the *Bb flaB* gene were used to measure *B. burgdorferi* chromosomal DNA contamination (5′-TCTTTTCTCTGGTGAGGGAGCT, 5′- TCCTTCCTGTTGAACACCCTCT) [89]. Absolute cp32/ϕBB-1 copy numbers were determined using a standard curve generated with a cloned copy of the target sequence. Standard curves were performed in duplicate and ranged from 10^{10} copies/well down to 1 copy/well. The lower limit of detection of the assay is 175 copies/mL with a 1 µL input. qPCR was performed in 10 µl reaction volumes: 5 µL SYBR green supermix (BioRad), 1 µL primer 1 + primer 2 at 10 µM, 3 µL H_2O, 1 ul sample. Thermocycling parameters were as follows: Step 1: 95° C 1 min, Step 2: 95° C 15 sec., Step 3: 55° C 30 sec, Step 4: go to step 2, 45 times, Step 6: Melt curve from 65°C to 95°C in 0.5°C increments for 5 seconds.

ϕBB-1 virion purification. Phages were precipitated using an ammonium sulfate-based protocol [90] with some modifications. Briefly, cell-free supernatants were filtered and pH adjusted to 7.5 followed by the addition of 0.5M NaCl. Phages were precipitated by adding ammonium sulfate (35% w/vol, mix until completely dissolved) and incubated at 4°C for 18 hours. Samples were then centrifuged at 3,000 × *g* for 10 min at 4ºC. The pellicle on the surface was collected and resuspended in 1 mL PBS. Where appropriate, samples were dialyzed using 10-kDa MWCO cassettes against PBS at 4ºC with at least four buffer exchanges.

ϕBB-1 virion proteomics. Purified virions (200 µg total protein) were reduced, alkylated, and purified by chloroform/methanol extraction prior to digestion with sequencing grade modified porcine trypsin (Promega). Peptides were separated on an Acquity BEH C18 column (100 x 1.0 mm, Waters) using an UltiMate 3000 UHPLC system (Thermo). Peptides were eluted by a 50 min gradient from 99:1 to 60:40 buffer A:B ratio (Buffer A = 0.1% formic acid, 0.5% acetonitrile. Buffer B = 0.1% formic acid, 99.9% acetonitrile). Eluted peptides were ionized by electrospray (2.4 kV) followed by mass spectrometric analysis on an Orbitrap Eclipse Tribrid mass spectrometer (Thermo) using multi-notch MS3 parameters. MS data were acquired using the FTMS analyzer over a range of 375 to 1500 m/z. Up to 10 MS/MS precursors were selected for HCD activation with normalized collision energy of 65 kV, followed by acquisition of MS3 reporter ion data using the FTMS analyzer over a range of 100-500 m/z. Proteins were identified and

quantified using MaxQuant (Max Planck Institute) TMT MS3 reporter ion quantification with a parent ion tolerance of 2.5 ppm and a fragment ion tolerance of 0.5 Da.

Packaged ϕBB-1 DNA purification. Virions collected from 4-L cultures per replicate were purified and DNase treated as described above. 10X DNase I reaction buffer (100 mM Tris-HCl, pH 7.6, 25 mM $MgCl_2$, 5 mM $CaCl_2$) was added to each sample to a final concentration of 1×. DNase I was added to a final concentration of 0.1 U/1 µL sample, mixed gently, and incubated at 37°C for 18 h. EDTA was added to a final concentration of 100 mM followed by SDS to a final concentration of 0.3%. Proteinase K was added to a final concentration of 100 µg/mL and incubated at 65°C for 20 min. Samples were extracted twice with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) followed by a single extraction with an equal volume of chloroform-isoamyl alcohol (24:1). NaCl was added to 200 mM followed by 2.5 volumes of 100% EtOH. After mixing by inverting the tubes, samples were incubated at -20°C for >30 min. DNA was pelleted by centrifugation (14,000 × *g* for 20 min at 4°C), pellets washed with 70% EtOH, and re-spun for 20 min, at 14,000 × *g* 4°C. The ethanol supernatant was discarded, and the pellet gently air-dried followed by resuspension in in Tris-HCl, pH 8. Samples immediately proceeded to library preparation and sequencing.

Long-read sequencing library preparation. Full-length DNA molecules were ligated with multiplexing adapters and sequenced directly using the Nanopore MinION platform (FLO-MIN112 flowcell, sequencing kit SQK-LSK112 and barcoding kit SQK-NBD112.24). Quality control and adapter trimming was performed with bcl2fastq and porechop. Reads were deposited in the NCBI BioProject database accession PRJNA1059007 and in Supplementary Data File 2.

Sequence analysis pipeline. Adapter-trimmed long-reads with quality scores ≥7 were used to isolate ≥ 5kb reads using Filtlong (v0.2.1). ≥5kb reads were mapped to the reference *B. burgdorferi* CA-11.2A genome (RefSeq assembly: GCF_000172315.2) with minimap2 (v2.26-r1175) [91]. Primary mapping reads with MAPQ >20 were isolated by contig, filtered, and converted to final file formats using Samtools (v1.17) [92] and SeqKit (v2.5.1) [93]. Read statistics for each replicate were graphed and viewed using GraphPad Prism (v10.1.1). For each contig, *de novo* assemblies were created using Trycycler (v0.5.4) [94], which relied on input assemblies from Flye (v2.9.2-b1786) [95], Raven (v1.8.3) [96], and Minimap2/Miniasm/Minipolish (v2.26-r1175/v0.3-r179/v0.1.2) [91, 97]. The long-read *de novo* assemblies were then polished with short reads using Minipolish (v0.1.2) [97]. The telomeres of the linear chromosome and linear plasmids were manually identified in SnapGene (v5.3.3), and the hairpin structures were predicted by the Mfold webserver (http://www.unafold.org/mfold/applications/dna-folding-form.php) [98]. The terminal ends of the cp32 prophage genomes were predicted using PhageTerm through the Galaxy webserver (https://galaxy.pasteur.fr/) [47], via input of the ≥5kb long-read sequences. Coverage maps of the primary mapping or primary and supplementary mapping reads were created by mapping ≥5kb long-reads to the *de novo* assembled CA-11.2A genome or the reference *B. burgdorferi* CA-11.2A genome with Minimap2, converted to final file formats using Samtools, and viewed using R (v4.3.2) and ggplot2

(v3.4.4).

Pac site cloning and qPCR. The shuttle vector pBSV2 [48] was digested with NotI to remove the promoter region and re-ligated. The *pac* region from CA-11.2A genomic DNA was amplified using primers 5′-TGGGTTGTAGAGTGGCTGTG and 5′-TCACCACTTGCGTAATTCTTGC and cloned into the NotI site of digested pBSV2. All vectors were sequence verified by long-read sequencing (Plasmidsaurus) and transformed into CA-11.2A. Each clone was grown in triplicate to mid-log in BSK, phage replication was induced with ethanol, virions purified, and DNase-treated as described above, followed by qPCR in triplicate using primers that target the Kan resistance gene on pBSV2 (5′-CACCGGATTCAGTCGTCACT, 5′-GATCCTGGTATCGGTCTGCG, 120 bp product).

Identification of conserved motifs in *B. burgdorferi* cp32 isoforms. The roughly 430 nucleotides upstream of the *erp26*, *erpK*, *erpG*, *ospE* and *erpK* genes of the *B. burgdorferi* CA-11.2A cp32 isoforms cp32-1, cp32-3, cp32-5, cp32-10 and cp32-13 respectively were used as queries for a discontinuous MegaBLAST against the NCBI Nucleotide collection database. The results from these first five BLASTs were combined and sequence hits with more than 80% identity were removed with CD-HIT [99]. The resulting representative sequences were used as queries for discontinuous MegaBLAST against the NCBI Nucleotide collection (nt) database, and sequence hits with more than 80% identity were removed with CD-HIT [99], and this process was iterated twice more for a total of three MegaBLAST searches with a representative list of 80% identity query sequences. The sequence hits from the final MegaBLASTs were combined and sequences with more than 95% identity were removed with CD-HIT [99], generating a list of 178 sequences. These 178 sequences were used as an input dataset for the MEME webserver [49], with custom parameters of “Maximum Number of Motifs” set to “10”, and “Motif Site Distribution” set to “Any number of sites per sequence”. MEME identified motifs in 160 of the input sequences. The Position Weight Matrices (PWMs) of the 10 motifs identified by MEME were used as inputs for FIMO [100] to search for significant sequence matches (q-value < 0.001) in the *B. burgdorferi* chromosome and the *B. burgdorferi* cp32-1, cp32-3, cp32-5, cp32-10, cp32-13, cp26, lp17, lp54 plasmid DNA sequences. The cp32 isoforms had nine highly conserved sequence motifs, some motifs present in multiple copies and arranged in a conserved architecture. The cp26, lp17, lp54 and chromosome sequences did not contain this conserved architecture of nine motifs (see Supplementary Data file 1). The sequence logo of each motif was generated by taking the sequence fragments that MEME used to make each PWM, and submitting these sequence fragments to the WebLogo 3.0 webserver [101]. The iterative discontinuous MegaBLAST searches had introduced eukaryotic sequence fragments into the list of 178 non-redundant sequences, suggesting that the search likely reached an endpoint and found most of the related sequences in the NCBI database. To generate a phylogenetic tree, eukaryotic sequence fragments were first removed, and the remaining 149 non-redundant sequences were aligned using the MAFFT webserver [102], with custom parameters of “Direction of nucleotide sequences” set to “Adjust direction according to the first sequence”, and “Strategy” set to E-INS-2. The resulting aligned sequence fasta file was used as input for

the IQ-TREE webserver [103, 104], with the following command-line: path_to_iqtree -s 149_aligned_Borrelia__plasmid_sequences.fasta.fasta -st DNA -m TEST -bb 1000 -alrt 1000. TreeViewer was used to display the phylogenetic tree [105].

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