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Molecular Evidence for a New Bacteriophage of *Borrelia burgdorferi*

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We have recovered a DNase-protected, chloroform-resistant molecule of DNA from the cell-free supernatant of a *Borrelia burgdorferi* culture. The DNA is a 32-kb double-stranded linear molecule that is derived from the 32-kb circular plasmids (cp32s) of the *B. burgdorferi* genome. Electron microscopy of samples from which the 32-kb DNA molecule was purified revealed bacteriophage particles. The bacteriophage has a polyhedral head with a diameter of 55 nm and appears to have a simple 100-nm-long tail. The phage is produced constitutively at low levels from growing cultures of some *B. burgdorferi* strains and is inducible to higher levels with 10 μg of 1-methyl-3-nitroso-nitroguanidine (MNNG) ml⁻¹. In addition, the prophage can be induced with MNNG from some *Borrelia* isolates that do not naturally produce phage. We have isolated and partially characterized the phage associated with *B. burgdorferi* CA-11.2A. To our knowledge, this is the first molecular characterization of a bacteriophage of *B. burgdorferi*.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** *B. burgdorferi* sensu stricto strain CA-11.2A (28), a clone of CA-11, was kindly provided by P. Rosa (Rocky Mountain Laboratories, Hamilton, Mont.). All other isolates used in this study were kindly provided by R. Marconi (Medical College of Virginia at Virginia Commonwealth University, Richmond) except for *B. burgdorferi* sensu stricto strains B31, HB19 (41), and CA-11 (39) (the latter were kindly provided by T. Schwan, Rocky Mountain Laboratories). Bacterial isolates were routinely cultivated in Barbour-Stoenner-Kelly (BSK) complete medium (Sigma) at 34°C with a 5% CO₂ atmosphere. Culture density was determined by spectrophotometry as described previously (36), except that 1 ml of culture was used and the A₆₀₀ was multiplied by 1.4 × 10⁻² to calculate the number of cells ml⁻¹.

**Bacteriophage recovery.** CA-11.2A cells were cultured in volumes of 10 to 250 ml, as described above, until they reached log phase (>10⁷ cells ml⁻¹; A₆₀₀ ≥ 0.05), approximately 3 to 5 days after an inoculation with a 1:100 dilution. All subsequent steps in the recovery of phage were performed at 4°C. The cultures were centrifuged at 6,000 × g for 10 min, and the supernatant was collected. A polyethylene glycol (PEG) precipitation of the culture supernatant was done in aliquots of up to 250 ml by a modification of a previously described protocol for phage concentration (35). NaCl was added to a 1 M final concentration and the sample was treated with a final concentration of 0.3% sodium dodecyl sulfate (SDS) and 100 μg ml⁻¹ proteinase K at 65°C for 10 min. The supernatant was retained, and 10% (wt/vol) PEG 8000 (Sigma) was added. The culture was rotated for 1 h, and the precipitate was recovered by centrifugation at 6,000 × g for 10 min. The supernatant was decanted, and the precipitate was resuspended in suspension medium (100 mM NaCl, 10 mM MgSO₄, 50 mM Tris-HCl [pH 7.5]; no gelatin). The suspension volume was 400 μl of suspension medium per 10 ml of original culture supernatant. The resuspended material was extracted once with an equal volume of chloroform, and the aqueous layer was recovered. The sample was extracted a second time with a 10% volume of chloroform, and the aqueous layer, which contained the phage, was recovered a second time. Samples were stored at 4°C.

**DNA extraction.** Total chromosomal DNA was extracted from *B. burgdorferi* cells based on a protocol described previously (36). To purify the small linear plasmid of *B. burgdorferi*, plasmid DNA was extracted from *B. burgdorferi* B31 cells with the Wizard Plus Miniprep DNA purification system (Promega) as instructed by the manufacturer. Plasmids were resolved by electrophoresis (see below) and sized with the λ mononuc marker (New England Biolabs). The linear 17-kb plasmid was excised from the gel and extracted with the Qiagen® Plasmid I gel extraction kit (QIAGEN) as instructed by the manufacturer.

Prior to the extraction of phage DNA, samples were treated with RQI-DNase (Promega) as instructed by the manufacturer. After DNase treatment, 100 mM EDTA was added and the sample was treated with a final concentration of 0.3% sodium dodecyl sulfate (SDS) and 100 μg of proteinase K ml⁻¹ at 65°C for 10 min (45). The sample was extracted twice, once with an equal volume of phenol-chloroform and a second time with an equal volume of chloroform. The aqueous layer was recovered, and the DNA was precipitated with NaCl and absolute ethanol as described previously for cellular DNA (36). The DNA pellet was resuspended in 20 μl of TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) per 100 μl of original PEG precipitate.

To denature the phage DNA, 10 μl of sample was treated with an equal volume of 0.2 N NaOH and incubated at 25°C for 10 min. Four microliters of 1 M Tris-HCl (pH 8.0) was added, and the sample was incubated at 25°C for 5 min.

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Agarose gel electrophoresis. DNA samples were heated for 3 to 5 min at 65°C in 1% N-laurylsarcosine, 10 mM EDTA, 3% Ficoll 400, 0.05 mg of bromophenol blue ml⁻¹, and 0.05 mg of xylene cyanol ml⁻¹, cooled briefly, and resolved on 0.5% agarose gels (SeaKem LE; FMC Bioproducts) in TAE (40 mM Tris-acetate, 1 mM EDTA) at 30 V (3 V cm⁻¹) for 5 h. Gels were stained with 0.5 μg of ethidium bromide (EtBr) ml⁻¹ for 0.5 to 1 h and destained in water for 1 to 2 h. The DNA was visualized on a UV transilluminator, and images were captured on a Gel Doc 1000 system (Bio-Rad). For field inversion gel electrophoresis, DNA samples were prepared as described above and resolved on 0.8% agarose gels (SeaKem GTG; FMC Bioproducts) in TBE (45 mM Tris-borate, 2 mM EDTA) at 80 V (5 V cm⁻¹) for 16 h with program 2 on the PPI-200 programmable power inverter (MJ Research) per the manufacturer’s instructions. Gels were stained with EtBr and visualized as described above.

Two-dimensional gel electrophoresis was performed as described previously (36). A 20-μl sample of total B. burgdorferi DNA was fractionated on a 0.35% agarose gel in TAE at 20 V (1.25 V cm⁻¹) for 16 h. After 16 h, the gel was rotated 90° and equilibrated with 15 μM chloroquine for 5 h. Electrophoresis was continued in the second dimension in the presence of 15 μM chloroquine at 20 V for another 16 h. The gel was soaked in three changes of water (>1 h each) to remove the chloroquine before staining with EtBr as described above.

Southern hybridization. Gels were vacuum blotted to Hybond N⁺ membranes (Amersham Pharmacia) and cross-linked as described previously (25). Probes used were either total phage DNA, prepared as described above, or small B. burgdorferi cp32-specific probes designated probe 2 and probe 4 (13). Probe 4 (300 bp) and probe 2 (250 bp) were generated from total phage DNA by PCR (25 cycles of 92°C for 1 min, 50°C for 30 s, and 72°C for 1 min; diluted probe at 1:100; and repeat PCR) with primer pairs CP-4–CP-5 and erp177–erp178 (13), respectively. Additionally, a probe (408 bp) encompassing the blyB gene on cp32 was generated by PCR as above, using rev8 (5’-CCCAAGATTAGTGTG-3’) and rev06 (5’-GATCTATGTTGGTATC-3’) kindly provided by Don Oliver (University of New England, Biddeford, Maine) (18). One hundred nanograms of DNA to be used as a probe was labeled with [α-32P]dATP with a random primer kit (Prime-it II; Stratagene) as instructed by the manufacturer. Radiolabeled probes were purified from unincorporated label by passage through G50 spin columns as instructed by the manufacturer (Boehringer Mannheim). The blots were hybridized in 15 to 20 ml of QuikHyb (Stratagene) supplemented with 1 mg of salmon sperm DNA for 15 to 20 min at 68°C. After prehybridization, the radioactive probe was added directly to the hybridization buffer and hybridization was conducted for 1 to 2 h at 68°C. The blots were washed twice in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS at 25°C (15 min each) and once at 50°C in 0.1× SSC-0.1% SDS (30 min), wrapped in cellophane, and exposed to Hyperfilm ECL (Amersham Pharmacia) for 16 to 24 h at 80°C with intensifying screens.

Induction of the bacteriophage. Various isolates were cultured in 10 ml of BSK complete medium as described above, until a density of approximately 5 × 10⁸ cells ml⁻¹ was reached. Cells were pelleted at 6,000 × g, and the supernatant was collected for PEG precipitation. The cell pellet was resuspended in an equal volume of BSK complete medium as described above, and the supernatant was discarded as waste. The cells were resuspended in an equal volume of BSK complete medium with 1 mg of salmon sperm DNA for 15 to 20 min at 68°C. After prehybridization, the radioactive probe was added directly to the hybridization buffer and hybridization was conducted for 1 to 2 h at 68°C. The blots were washed twice in 2× SSC–0.1% SDS (15 min each) and once at 50°C in 0.1× SSC–0.1% SDS (30 min), wrapped in cellophane, and exposed to Hyperfilm ECL (Amersham Pharmacia) for 16 to 24 h at 80°C with intensifying screens.

SDS and proteinase K, extracted with organic solvents, and subsequently treated with DNase (Fig. 1, lane 2). The nucleic acid is resistant to RNase treatment at every step (data not shown). The nucleic acid migrates as a 32-kb molecule with both field inversion (Fig. 1, lane 1) and conventional (Fig. 2, lane 1) gel electrophoresis, indicating the nucleic acid is a double-stranded, linear DNA molecule. When observed by electron microscopy, the phage nucleic acid also appears to be covalently closed double-stranded DNA. The gel was stained with EtBr, followed by more sensitive GelStar nucleic acid gel stain (FMC Bioproducts) as instructed by the manufacturer. After visualization, the gel was rinsed in water for >1 h to remove excess GelStar and then blotted and probed with a cp32-specific probe as described above.

Microscopy of phage particles. A culture of B. burgdorferi CA-11.2A was induced, and the phage particles were precipitated as described above. A drop of precipitated phage suspension was applied to a grid (copper 300-mesh, carbon coated; Ted Pella). The sample was stained with 2% phosphotungstic acid and examined on a Hitachi 7100 transmission electron microscope (TEM).

RESULTS

Identification of bacteriophage DNA. During a biochemical analysis of protein extracts of B. burgdorferi CA-11.2A, we serendipitously discovered a molecule of DNase-resistant nucleic acid in cell samples that had been sonicated to disrupt cell membranes. We found that this nucleic acid could also be recovered from the cell-free supernatants of late-log-phase B. burgdorferi CA-11.2A cultures (Fig. 1). The DNase protection persists throughout the PEG precipitation protocol, which involves two chloroform treatments (Fig. 1, lane 1). The protection is alleviated when a sample of phage is first treated with SDS and proteinase K, extracted with organic solvents, and subsequently treated with DNase (Fig. 1, lane 2). The nucleic acid is resistant to RNase treatment at every step (data not shown). The nucleic acid migrates as a 32-kb molecule with both field inversion (Fig. 1, lane 1) and conventional (Fig. 2, lane 1) gel electrophoresis, indicating the nucleic acid is a double-stranded, linear DNA molecule. When observed by electron microscopy, the phage nucleic acid also appears to be covalently closed double-stranded DNA. The gel was stained with EtBr, followed by more sensitive GelStar nucleic acid gel stain (FMC Bioproducts) as instructed by the manufacturer. After visualization, the gel was rinsed in water for >1 h to remove excess GelStar and then blotted and probed with a cp32-specific probe as described above.
linearized (middle band) forms of cp32 that were generated during DNA extraction. Molecular sizes are in kilobase pairs.

Additionally, the phage DNA hybridized to the nicked (upper band) and linear plasmids migrated on the diagonal. A Southern blot of the gel was probed with total phage DNA that was extracted and radio-labeled (right). The phage DNA hybridized to the circular 32-kb plasmid (black arrow), the linearity of phage DNA hybridized to the nicked (upper band) and linearized (middle band) forms of cp32 that were generated during DNA extraction. Molecular sizes are in kilobase pairs.

a double-stranded, linear ~32-kb DNA molecule with no gross secondary structure (24).

B. burgdorferi cells can be grown in solid medium, but they do not readily form a lawn (16), thus making plaque assays infeasible. The most efficient way of evaluating phage production and presence in a sample is DNA extraction and agarose gel electrophoresis. From an uninduced culture of B. burgdorferi CA-11.2A, phage DNA is usually visible by EtBr staining (approximately 200 ng) when extracted from 100 to 400 μl of phage precipitate (equivalent to 2.5 to 10 ml of original culture supernatant).

The ends of the linear DNA molecules of the B. burgdorferi genome are covalently closed hairpin loops, similar to the ends of the vaccinia virus (4). To characterize the nature of the ends of the linear phage DNA, a sample was denatured with NaOH, producing single-stranded products (Fig. 2). When DNA lacks covalently closed ends, the single-stranded molecules cannot reanneal rapidly (Fig. 2, left). As a control, the small linear plasmid of the B. burgdorferi genome, lp17, was exposed to the same conditions (Fig. 2, right). lp17 has covalently closed ends, and reannealing occurred rapidly during a brief recovery period after denaturation. The phage DNA did not rapidly reanneal, indicating both its double-stranded nature and its lack of covalently closed ends.

Identifying the prophage DNA. To locate the prophage in the B. burgdorferi genome, total cellular B. burgdorferi CA-11.2A DNA was resolved by two-dimensional gel electrophoresis (left). The large circular plasmid (white arrow) was retarded in its migration in the second dimension, and the linear plasmids migrated on the diagonal. A Southern blot of the gel was probed with total phage DNA that was extracted and radio-labeled (right). The phage DNA hybridized to the circular 32-kb plasmid (black arrow), the linearity of phage DNA hybridized to the nicked (upper band) and linearized (middle band) forms of cp32 that were generated during DNA extraction. Molecular sizes are in kilobase pairs.
suggesting that the putative bacteriophage released from this species and the bacteriophage that packages the 32-kb circular plasmid of *B. burgdorferi* are different. Furthermore, the DNA isolated from the supernatants of *B. anserina* cultures has been sized at approximately 42 kb (data not shown). Neither *B. burgdorferi* phage DNA nor probe 4, highly conserved among Lyme disease spirochetes (13), hybridizes to the DNA released from either *B. anserina* or *B. coriaceae*. The DNA isolated from the supernatants of these two species is detected by EtBr or GelStar staining. The presumptive bacteriophages produced from these two isolates appear to be unrelated to the *B. burgdorferi* phage that packages cp32.

**Electron microscopy of bacteriophages.** Phage ultrastructure was examined by TEM. Samples were prepared from uninduced (data not shown) or MNNG-induced (Fig. 5) cultures. The phage heads are apparently polyhedral with a diameter of 50 to 60 nm. The tails are approximately 100 nm without neck or baseplate. The tails do not appear to be contractile, although our analysis does not rule out that possibility. In the PEG-precipitated preparation, both empty (no DNA packaged) and full, electron-dense (DNA packaged) heads were observed (Fig. 5). The bacteriophage we report here has not yet been seen in association with *B. burgdorferi* cells. Previously, other researchers reported a cubic *Borrelia* phage (5, 19), and still others have described two isometric phages with contractile and noncontractile tails (30, 38).

**DISCUSSION**

Although bacteriophages have been occasionally observed in cultures of *B. burgdorferi* (19, 30, 38) and one of the circular plasmids has features of a possible prophage (13), until this study no phage of *B. burgdorferi* had been isolated and characterized. We now report the isolation of a bacteriophage of *B. burgdorferi* that has a polyhedral head of 50 to 60 nm and a simple, noncontractile tail of 100 nm. This phage is structurally different from the cubic phages (19) and one of the ciprofloxacin-inducible phages (30, 38) described previously. The phage that we isolated may be structurally similar to the phage with B-1 morphology reported by Neubert and colleagues, but the capsid size of the phage we have described is larger than that of the phage from previous work (30 nm) (30, 38). Additionally, the phage described by these investigators was inducible with ciprofloxacin (30, 38), while we have seen no evidence of phage induction with this antibiotic. These differences lead us to conclude that we have isolated a new bacteriophage of *B. burgdorferi*.

Based on the highly conserved size of the chromosome of widely distributed members of the Lyme disease complex, Casjens et al. have suggested that a prophage of *B. burgdorferi* would likely replicate as an extrachromosomal element (12, 13). Because of the ubiquitous nature of the cp32s and the highly conserved size of these different, but related, molecules, cp32 seemed to be a likely candidate for a prophage (13, 44). The phage we describe here packages a linear 32-kb molecule that lacks covalently closed ends and hybridizes under high-stringency conditions to the cp32 family of the *B. burgdorferi* genome. Additionally, several cp32-specific probes hybridize under high-stringency conditions with phage DNA and partial sequences from several cloned phage DNA fragments are nearly identical to known *B. burgdorferi* B31 cp32 sequences.

No phage-specific proteins have been purified to date, despite intensive efforts. *B. burgdorferi* is grown in a protein-rich, serum-based medium, and extensive purification attempts have failed to remove protein contaminants from phage prepara-
tions. The structural gene products of tailed phages usually lack similarity at the amino acid sequence level (1), hindering a sequence comparison of the predicted cp32 open reading frames to those of known structural proteins of other tailed phages. Two proteins encoded by cp32, BlyA and BlyB, were initially identified as hemolytic proteins with a possible role in *B. burgdorferi* pathogenesis (18). However, more recently, BlyA and BlyB have been proposed to constitute a holin-like system (14). Bacteriophage-encoded holins, which promote cell lysis for the release of bacteriophages, have been identified in almost all known tailed phages (47).

One of the most well-characterized, extrachromosomally replicating temperate phages is *Escherichia coli* phage P1, a bacteriophage that replicates autonomously during the lysogenic cycle (8, 22, 42). P1 is known to package via a processive “headful” packaging mechanism, in which a circular concatemer of viral units is generated by rolling circle replication late in the lytic cycle. The large circular intermediate is then cleaved at a specific site (the pac site), and four to five phage heads are filled processively (42). This process generates a cyclically permuted linear phage genome with terminal redundancy (22, 48). Preliminary efforts to label and identify the ends of the phage genome suggest that the *B. burgdorferi* CA-11.2A phage is also cyclically permuted (16), but efforts to identify concatemers in *B. burgdorferi* CA-11.2A have not been fruitful.

Previously, both mitomycin C and ciprofloxacin have been used to induce bacteriophages from spirochetes (11, 20, 30). The induction of prophage from *B. burgdorferi* with MNNG is presumably through the same well-characterized mechanisms of *E. coli* prophage induction, which involves the damage of DNA, the subsequent activation of the RecA protein, and the reversal of the repressed state of the prophage (8). Previously, MNNG has been used to generate mutant cyanophage (2, 37), induce prophage λ from recA mutants of *E. coli* (46), and induce prophages through mutation of *Haemophilus influenzae* (3, 9).

The natural or induced release of a temperate prophage is often associated with a decrease in cell density during the “lytic burst” (8). Barbour and Hayes suggested that this phenomenon might account for the periodicity seen during early attempts at cultivating borreliae (5). We have never witnessed a dramatic decrease in cell density associated with inducing bacteriophages from *B. burgdorferi* CA-11.2A. Preliminary time course studies have shown that during the recovery period following MNNG treatment, a treated culture grows at the same rate as an untreated culture for 24 h before growth levels at a cell density about 2.5 times lower than that of the untreated culture. There is no dramatic decrease in cell density at 60 h, which is the time of highest phage production (data not shown). This is presumably due to the small population of cells that is releasing phage, even after induction. The decrease in culture density due to phage release may be masked by the increase in viability and density of the MNNG-treated culture as a whole. Alternatively, the phage may be exiting the cell by means other than lysis.

Considerable work remains to be done on the similarities and differences of the bacteriophages shed from the different *Borrelia* isolates. Only a limited number of *Borrelia* species and strains produce phage constitutively or can be induced to produce phage by our methods. The degree of variability of phage production between even CA-11.2A and its parent strain, CA-11, is remarkable. We have performed multiple assays but have observed CA-11 to produce 32-kb extracellular DNA only once, even when treated with MNNG, whereas CA-11.2A, a clone selected based on its outer surface protein profile (28), releases phage continuously with and without induction.

Little is known about the way that *B. burgdorferi* replicates and partitions its plasmids. Different *E. coli* plasmids that have the same replication mechanisms and partition machinery are known to be incompatible (6). Some strains of *B. burgdorferi*, though, are able to maintain five or more homologous cp32 plasmids within a single cell (13). A detailed analysis of the replication and partitioning mechanisms of any of the plasmids of *B. burgdorferi* has yet to be done. Because of their small size and inherent similarities in the metabolisms of phage DNA and host DNA, phages have long been considered important tools for studying cellular replication mechanisms (23). Additionally, phages have been developed into manipulable genetic systems for many bacteria. Bacteriophages have been used successfully to transduce genetic markers between bacteria, including the spirochete *S. hyodysenteriae* (21). With the identification of cp32 as a prophage genome, we may now begin to dissect the phage requirements for replication, partitioning, induction, and packaging. We believe that with further characterization, the bacteriophage described here may be useful for analyzing the molecular mechanisms of DNA metabolism in *B. burgdorferi*.

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