

Bacteriophages of Spirochetes

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Abstract

Historically, a number of bacteriophage-like particles have been observed in association with members of the bacterial order Spirochetales, the spirochetes. In the last decade, several spirochete bacteriophages have been isolated and characterized at the molecular level. We have recently characterized a bacteriophage of the Lyme disease agent, *Borrelia burgdorferi*, which we have designated ϕ BB-1. Here we review the history of the association between the spirochetes and their bacteriophages, with a particular emphasis on ϕ BB-1 and its prophage, the 32-kb circular plasmid family of *B. burgdorferi*.

Introduction

The Spirochetales order is an ancient one that contains species pathogenic for both humans and animals (Woese, 1987; Baranton and Old, 1995). Identified by the helical morphology that all spirochetes share, the many different spirochete species are nonetheless a rather heterogeneous group (Barbour and Hayes, 1986; Baranton and Old, 1995). Spirochetes that are pathogenic for humans include the causative agents of Lyme disease, relapsing fever, leptospirosis, and syphilis (Burgdorfer *et al.*, 1982; Holt *et al.*, 1984; Barbour and Hayes, 1986; Baranton and Old, 1995). With the consistent rise in the number of reported cases of Lyme disease in the United States within the last two decades (Steere, 1994; CDC, 1997), and other burgeoning and persistent foci of spirochete-caused diseases (Baranton and Old, 1995), the interest in individual spirochete species, including their evolution and epidemiology, has dramatically intensified.

The increased attention to the molecular nature of the spirochetes has been manifested in part by vigorous attempts to isolate and characterize bacteriophages that infect this group of bacteria. The study of bacteriophages has historically been a cornerstone of molecular biology (Hendrix *et al.*, 1983; Kornberg and Baker, 1992; Karam *et al.*, 1994; Judson, 1996). Because of their smaller genomes and inherent dependence upon at least some of the bacterial hosts' cellular machinery, bacteriophages have been used as essential tools for dissecting the macromolecular metabolism of DNA and other processes in many bacterial systems. Additionally, bacteriophages are naturally-occurring vectors for lateral gene transfer and may be used to shuttle genetic information between bacterial cells (Masters, 1996). Temperate phages, those that can integrate into a host's genome and establish a lysogenic state, have been used to study the mechanisms of recombination, stress response, transcriptional regulation, and replication (Hendrix *et al.*, 1983; Kornberg and Baker, 1992).

In addition to their use as molecular tools, bacteriophages and their hosts are intimately associated and they often share an evolutionary history (Cheetham and Katz, 1995; Ackerman, 1998). Some phages, particularly those that can package and transduce antibiotic-resistance markers, or those that encode virulence factors that contribute to the spread and maintenance of pathogenic bacteria, can have profound effects on bacterial physiology or epidemiology (reviewed by Cheetham and Katz, 1995; Hendrix *et al.*, 2000). The study of the relationship between a bacteriophage and its bacterial host can often provide valuable clues to the ecology and molecular biology of an organism.

Over the last several decades, research on bacteriophages that infect spirochete hosts has grown from the simple observation of an association between phage and bacterium to the molecular characterization of their interactions. In this review, we will survey the literature pertaining to bacteriophages of spirochetes and focus on bacteriophages of the Lyme disease agent, *B. burgdorferi*, particularly one that we have characterized (Eggers and Samuels, 1999) and now refer to as ϕ BB-1.

Spirochetes and their Bacteriophages

Bacteriophage-like particles have been observed in association with a number of spirochete genera, including *Leptonema*, *Leptospira*, *Brachyspira*, *Treponema*, and *Borrelia* (Ritchie and Ellinghausen, 1969; Ritchie and Brown, 1971; Saheb, 1974; Ritchie *et al.*, 1978; Berthiaume *et al.*, 1979; Masuda and Kawata, 1979; Hayes *et al.*, 1983; Davies and Bingham, 1985; Barbour and Hayes, 1986; Saint Girons *et al.*, 1990; Neubert *et al.*, 1993; Schaller and Neubert, 1994; Humphrey *et al.*, 1995; Humphrey *et al.*, 1997; Calderaro *et al.*, 1998; Eggers and Samuels, 1999). Almost all of these phages have polyhedral heads and in nearly all cases tails could also be observed. The majority of the tailed bacteriophages of spirochetes have contractile tails and so are members of the *Myoviridae*

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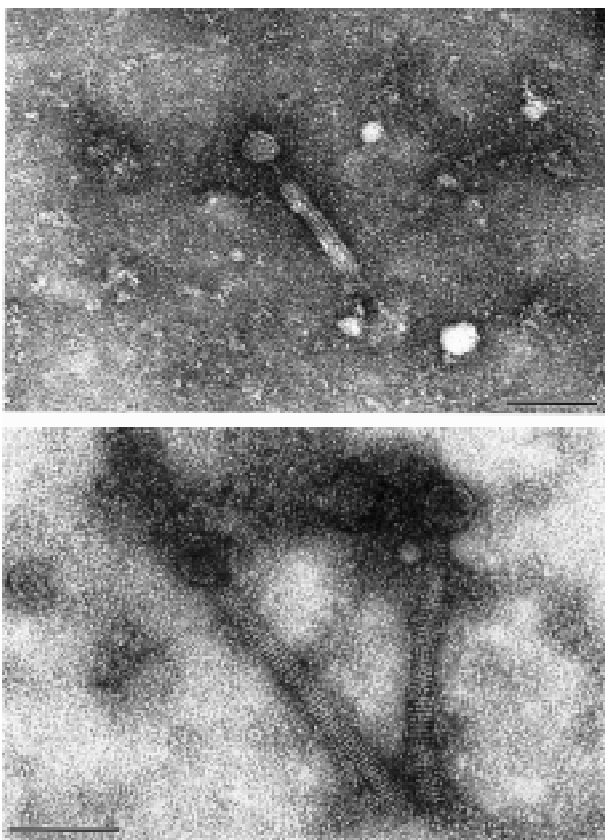


Figure 1. Bacteriophage-like particles from *B. burgdorferi*. Supernatant from the spontaneously lysed culture of *B. burgdorferi* strain CA-11.2A was collected and PEG-precipitated essentially as previously described (Eggers and Samuels, 1999). Three to 5 μ l were applied to a 3% Parlodion-coated 300-mesh copper grid (Ted Pella; Redding, CA) prepared as previously described (Garon, 1981). The particles were stained with 1% ammonium molybdate tetrahydrate (top panel; bar = 70 nm) or 0.5% aqueous uranyl acetate (pH 3.9) (bottom panel; bar = 60 nm) for 30 s and examined on a Hitachi HU-11E-1 transmission electron microscope at 75 kV.

family. VSH-1, a bacteriophage-like particle of *Brachyspira* (formerly *Serpulina*) *hyodysenteriae* (Humphrey *et al.*, 1995), and one of the ciprofloxacin-inducible phages of *Borrelia burgdorferi* (Neubert *et al.*, 1993) apparently have non-contractile tails.

We note that all bacteriophage-like particles observed in bacterial cultures may not be functional viruses that can be propagated in the expected sense. The defective phages, such as PBSX of *Bacillus subtilis* (Okamoto *et al.*, 1968; Krogh *et al.*, 1996), and the 'gene transfer agents' including GTA of *Rhodobacter capsulatus* (Lang and Beatty, 2000) and VSH-1 of *B. hyodysenteriae* (Humphrey *et al.*, 1995; Humphrey *et al.*, 1997) are phage-like particles and their heads contain fragments of the host's DNA rather than the virus chromosome. The gene transfer agents can, like true bacteriophages, bind to and inject their DNA into bacterial hosts with the appropriate surface receptors. Although such entities might not be considered true viruses, in this review we use the term 'bacteriophage' to include these bacteriophage-like particles.

The best characterized bacteriophages of spirochetes are LE1, LE3, and LE4, three apparently lytic phages of

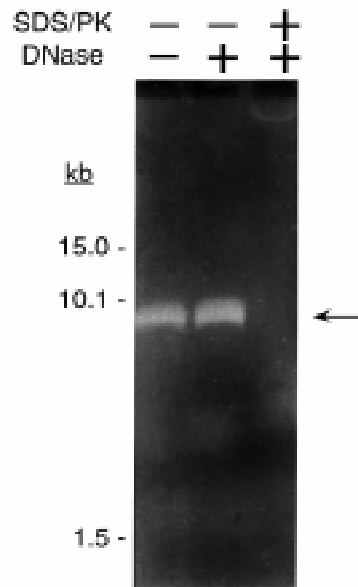


Figure 2. DNase-protection of extracellular bacteriophage-like particle DNA. One ml fractions of the filtered (0.2 μ m) and dialyzed supernatant from the spontaneously lysed culture of *B. burgdorferi* strain CA-11.2A were either not digested, digested with DNase I prior to DNA extraction, or digested with DNase I following DNA extraction utilizing sodium dodecyl sulfate and proteinase K (SDS/PK) essentially as previously described (Eggers and Samuels, 1999). The samples were resolved by electrophoresis on a 0.35% agarose gel, and stained with ethidium bromide. Molecular masses are indicated.

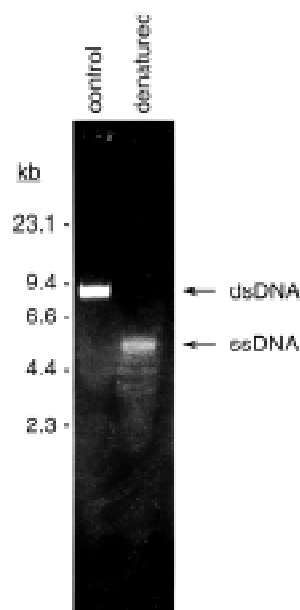


Figure 3. Denaturation of bacteriophage-like particle DNA. DNA isolated from the spontaneously lysed culture of *B. burgdorferi* strain CA-11.2A was either not treated (control) or denatured with 0.2 M NaOH and resolved on a 0.6% agarose gel as previously described (Eggers and Samuels, 1999). The denatured 8-kb DNA does not 'snapback' to regenerate double-stranded DNA. The arrows indicate double-stranded DNA (dsDNA) in the untreated sample and the single-stranded DNA (ssDNA) product generated by denaturation of non-covalently closed dsDNA. The gel was stained with ethidium bromide and molecular masses are indicated.

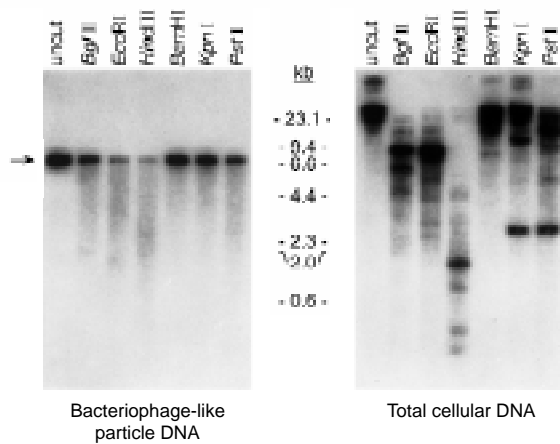


Figure 4. Composition and genomic source of the bacteriophage-like particle DNA. DNA isolated from either the spontaneously lysed culture of *B. burgdorferi* strain CA-11.2A (left panel) or total cellular DNA from *B. burgdorferi* CA-11.2A (right panel) was digested with a variety of restriction enzymes and resolved by electrophoresis on a 0.8% agarose gel. A Southern blot of the gel was probed with bacteriophage-like particle DNA that was extracted and radiolabeled essentially as previously described (Eggers and Samuels, 1999). The DNA from the bacteriophage-like particle (arrow) does not produce a distinct pattern following digestion with any of the restriction enzymes, suggesting that the DNA is a heterogeneous collection of 8-kb fragments (left panel). The bacteriophage-like particle DNA hybridizes to the host genomic DNA (right panel). Molecular masses are indicated.

the saprophyte *L. biflexa* (Saint Girons *et al.*, 1990), VSH-1 of *B. hyodysenteriae* (Humphrey *et al.*, 1995; Humphrey *et al.*, 1997), and ϕ BB-1 of *B. burgdorferi* (Eggers and Samuels, 1999). The three lytic phages of *L. biflexa* are morphologically identical with polyhedral heads

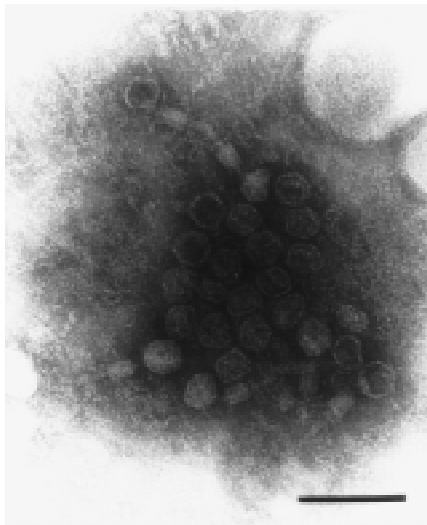


Figure 5. ϕ BB-1 particles. Supernatant from MNNG-treated *B. burgdorferi* CA-11.2A cells was collected and PEG-precipitated as described previously (Eggers and Samuels, 1999). The precipitated phage particles were ultracentrifuged at $100,000 \times g$ for 0.5 h, resuspended in 1 ml SM (50 mM Tris-Cl [pH8.0], 100 mM NaCl, 10 mM $MgSO_4$), and ultracentrifuged a second time at $40,000 \times g$ for 1 h. The pellet was resuspended in 100 μ l of SM and a drop of the suspension was applied to a 300-mesh carbon-coated copper grid (Ted Pella). The particles were stained with 1% phosphotungstic acid and examined on a Hitachi 7100 transmission electron microscope at 75 kV. (Bar = 115 nm)

approximately 85 nm in diameter and 100 nm long contractile tails (Saint Girons *et al.*, 1990). VSH-1 has a much smaller polyhedral head 45 nm in diameter with a short, non-contractile 64 nm tail (Humphrey *et al.*, 1995; Humphrey *et al.*, 1997). The size of the head of VSH-1 is similar to the capsid sizes of bacteriophages reported from *B. hyodysenteriae* and closely-related spirochetes by other investigators (Ritchie and Brown, 1971; Ritchie *et al.*, 1978; Calderaro *et al.*, 1998).

A number of bacteriophage-like particles, all having different structural features, have been reported from *Borrelia burgdorferi*. The first bacteriophage identified in association with *B. burgdorferi* had an elongated head 40 to 50 nm in diameter and a tail 50 to 70 nm in length (Hayes *et al.*, 1983). There were also two bacteriophages reported from a human isolate of *B. burgdorferi*, one with an isometric 30 nm head and a 50 to 64 nm contractile tail, and one with an isometric 30 nm head and a straight, non-contractile tail 115 to 130 nm in length (Neubert *et al.*, 1993; Schaller and Neubert, 1994).

During our biochemical, molecular, and genetic studies of the past decade, we have cultured more than 250 liters of *B. burgdorferi*, including a large amount of the CA-11.2A strain from which we have isolated and characterized ϕ BB-1 (Eggers and Samuels, 1999). One 0.5 liter culture of CA-11.2A (incubated at room temperature with three other cultures identically inoculated) unexpectedly lysed, a serendipitous phenomenon we have only observed on that singular occasion. The lysis was nearly complete, as no intact spirochetes could be seen by dark-field microscopy. The lysed culture supernatant was collected and precipitated using polyethylene glycol (PEG). Examination by electron microscopy revealed bacteriophage-like particles (Figure 1), similar in appearance to the defective bacteriophages PBSX (Okamoto *et al.*, 1968) and PBLB (Huang and Marmur, 1970) of *Bacillus* spp. The head had a diameter of 23 to 27 nm and the apparently contractile tail was 150 to 165 nm. These bacteriophage-like particles contained an ~8-kb double-stranded linear DNA molecule (Figures 2 and 3). The 8-kb DNA lacked covalently-closed hairpin loops at the ends (Figure 3), as does the 32-kb genome of ϕ BB-1 (Eggers and Samuels, 1999), distinguishing these molecules from the linear DNA of the host genome (Barbour and Garon, 1987; Hinnebusch and Barbour, 1991; Casjens *et al.*, 1997a; Fraser *et al.*, 1997). However, the DNA was not a single species from a discrete genomic source, but was a heterogeneous population of 8-kb fragments from the *B. burgdorferi* genome (Figure 4, left panel and D.S. Samuels, unpublished). Restriction mapping suggested that only a small region of the genome was predominantly packaged, as the bacteriophage-like particle DNA does not hybridize to all host restriction fragments (Figure 4, right panel and D.S. Samuels, unpublished). The rarely detected particle may be a defective bacteriophage, like PBSX and PBLB (Okamoto *et al.*, 1968; Garro and Marmur, 1970; Huang and Marmur, 1970), or a gene transfer agent, like VSH-1 (Humphrey *et al.*, 1997), although transduction has never been demonstrated. The bacteriophage-like particle packages 8-kb fragments of *B. burgdorferi* DNA that are 25% the size of the 32-kb ϕ BB-1 genome; this could possibly reflect a geometric constraint of defective capsid construction, as PBLB packages 13-kb fragments of *B. licheniformis* DNA that are 25% the size of the 53-kb PBLA genome (Huang

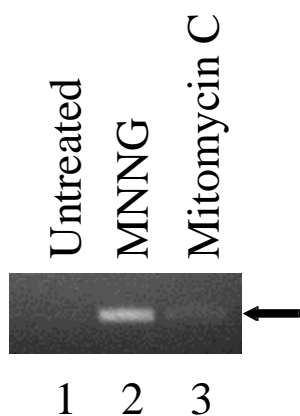


Figure 6. Induction of the lysogenic ϕ BB-1 prophage. Late log phase *B. burgdorferi* CA-11.2A cells were treated with 10 μ g/ml MNNG (lane 2) or 20 μ g/ml mitomycin C (lane 3). The treatment was performed for both agents as described previously for MNNG induction (Eggers and Samuels, 1999). The culture supernatants were PEG-precipitated and analyzed for phage DNA 60 h after recovery by electrophoresis on a 0.5% agarose gel followed by ethidium bromide staining. Supernatant from untreated *B. burgdorferi* cells was precipitated and analyzed as a negative control (lane 1). The black arrow indicates the double-stranded, linear 32-kb DNA. The presence and amount of ϕ BB-1 DNA remains the most efficient way of evaluating phage titer in culture supernatant. The amount of phage produced from the MNNG-treated culture was consistently higher than the amount produced from those cultures treated with mitomycin C.

and Marmur, 1970).

We had previously reported that ϕ BB-1 had a polyhedral head and an apparently non-contractile tail (Eggers and Samuels, 1999). Further analysis, however, indicates that the original appearance of the tail was an artifact of sample preparation and staining, and ϕ BB-1 has an isometric head 45 to 50 nm in diameter and a *contractile* tail 90 nm in length (Figure 5). Unlike other phage-like particles associated with *B. burgdorferi*, which were described solely by electron microscopy, ϕ BB-1 was initially identified by the presence of extracellular DNA that was protected from nuclease digestion (Eggers and Samuels, 1999). The protection persisted through two chloroform treatments, but was abolished if the samples were treated to remove the proteins first. The chloroform-resistance of the particle is consistent with a phage capsid that contains a protein coat and no lipid component (Ackerman *et al.*, 1978; Ackerman, 1998). Additionally, DNase-protection after chloroform treatment distinguishes the DNA packaged in bacteriophage particles from the nucleic acid incorporated into the membrane-bound vesicles that *B. burgdorferi* and other bacteria shed into the culture milieu (Dorward and Garon, 1990).

Bacteriophages of Spirochetes as Tools

Currently, the molecular tools available for the study of many pathogenic spirochetes are limited. Although several promising avenues are being explored, one of the most fruitful may be harnessing the power of bacteriophages for the study of DNA replication and other molecular processes in these bacteria.

The most successful attempt to date utilizing a spirochete bacteriophage has been VSH-1 in *B. hyodysenteriae* (Humphrey *et al.*, 1997). This gene transfer

agent has been used to transduce antibiotic-resistance markers efficiently between different strains, the first evidence of genetic transduction in a spirochete (Humphrey *et al.*, 1997). Because VSH-1 packages host chromosome DNA, and is readily inducible with mitomycin C (Humphrey *et al.*, 1995), this agent could be a very effective tool for studying molecular processes in this spirochete.

No other described spirochete phage has yet been used as effectively as VSH-1. However, many encouraging advances are being made in this area. A shuttle vector based on the genome of one of the *Leptospira* bacteriophages has been developed (I. Saint Girons, personal communication; see the review by Saint Girons, *et al.*, this volume). Recently, we have demonstrated transduction of an antibiotic-resistance marker by ϕ BB-1 between cells of different *B. burgdorferi* strains, the first direct evidence of lateral gene transfer in the Lyme disease spirochetes (C.H. Eggers and D.S. Samuels, unpublished).

Prophage Induction from Spirochetes

An important characteristic of any useful temperate bacteriophage is the ability to predict or control bacteriophage release. Although, putative temperate phages from spirochetes were observed after apparently spontaneous lysis (see Barbour and Hayes, 1986, and references therein; Ritchie and Ellinghausen, 1969; Ritchie and Brown, 1971; Saheb, 1974; Ritchie *et al.*, 1978; Berthiaume *et al.*, 1979; Hayes *et al.*, 1983; Davies and Bingham, 1985; Saint Girons *et al.*, 1990; Eggers and Samuels, 1999), bacteriophage-like particles have been more reliably detected in cultures of spirochetes treated with an inducing agent.

The two bacteriophages of the *B. burgdorferi* human isolate were observed after the cells were treated with the gyrase inhibitor ciprofloxacin (Neubert *et al.*, 1993). Bacteriophages have also been observed in association with *B. hyodysenteriae* (Humphrey *et al.*, 1995), other human and animal intestinal spirochetes (Calderaro *et al.*, 1998), and the Reiter treponeme (Masuda and Kawata, 1979) when cultures of these bacteria were treated with the DNA cross-linker mitomycin C.

We had previously reported that our initial attempts to induce the prophage of the *B. burgdorferi* bacteriophage ϕ BB-1 using mitomycin C were not successful (Eggers and Samuels, 1999). The most reliable inducer of the lysogenic prophage of ϕ BB-1 from *B. burgdorferi* strain CA-11.2A, and similar phages from strain B31 and *Borrelia bissettii* strain DN127, is the DNA alkylating agent 1-methyl-3-nitro-nitrosoguanidine (MNNG) (Eggers and Samuels, 1999). However, we have recently demonstrated that *B. burgdorferi* CA-11.2A cells treated with 20 μ g/ml mitomycin C in a manner similar to the protocol described for MNNG use (Eggers and Samuels, 1999), does induce the ϕ BB-1 prophage, although at lower levels than MNNG treatment (Figure 6). The number of DNA-containing particles of ϕ BB-1 released from an untreated culture of CA-11.2A approaches $\sim 10^6$ particles/ml culture, the number released from an MNNG-treated culture is $\sim 10^9$ particles/ml culture, and the number released from a mitomycin C-treated culture is somewhat less. The reliability of inducing prophage from other strains of *Borrelia* with mitomycin C has not yet been analyzed.

The mechanism for successful prophage induction

must be intimately related to the exit of the phage progeny from the cell. All well-studied tailed bacteriophages exit the cell by means of a lysis event in which the cell wall is destroyed (Young, 1992; Ackerman, 1998). There is no reason to speculate that bacteriophages of spirochetes perform this lytic event differently than the other known bacteriophages. In fact, there is a growing body of evidence that suggests that this phenomenon occurs in a similar manner. The three leptospire phages are apparently lytic (Saint Girons *et al.*, 1990). VSH-1 does not appear to grow on (as expected for a gene transfer agent), or lyse, plated *B. hyodysenteriae* cells, but the induction of the prophage can be monitored by a decrease in the density of the cells in liquid culture (Humphrey *et al.*, 1995; Humphrey *et al.*, 1997).

When describing the first bacteriophage-like particle that appeared spontaneously in *B. burgdorferi* cultures, Barbour and Hayes (1986) postulated that the periodicity observed during early attempts to cultivate *Borrelia* might be due to the induction of lysogenic bacteriophages. While inducing ϕ BB-1, we have never witnessed a culture-wide cell lysis event, even when inducing the prophage with MNNG, but we have observed a 16 to 25% relative decrease in cell density during the recovery period after treatment (C.H. Eggers and D.S. Samuels, unpublished). This decrease in cell density appears to correlate with phage release. Neubert and colleagues (1993) observed cellular plasmolysis in approximately 25% of the cells treated with sub-inhibitory concentrations of ciprofloxacin. These plasmolysed cells were associated with a large number of unassembled phage heads and tails, as well as apparently intact bacteriophage particles. The human skin isolate they described appeared to release two associated bacteriophages. Perhaps the large number of unassembled bacteriophage subunits observed still trapped in these plasmolysed cells (Neubert *et al.*, 1993) was due to the differences in timing of cell lysis by the two different phages. The ultrastructural effects of ciprofloxacin on *Borrelia* cells are very different from the effects observed under conditions that induce the lysogenic prophages, and the plasmolysis appears to correlate with bacteriophage release (Neubert *et al.*, 1993; Schaller and Neubert, 1994).

Bacteriophage release is mediated by a combination of endolysins and holins (Young, 1992; Young and Bläsi, 1995; Bläsi and Young, 1996). Every carefully studied tailed bacteriophage described has these two types of proteins, and usually these genes are clustered together on the phage genome (Young, 1992; Gasson, 1996). The endolysin hydrolyzes peptidoglycan and comprises four different enzymatic classes (Young and Bläsi, 1995). Holins are small proteins that cause lesions in the bacterial plasma membrane, allowing the endolysins access to the peptidoglycan. These proteins fall into at least 12 different families, but all share common characteristics (Young and Bläsi, 1995; Bläsi and Young, 1996).

To our knowledge, no holin or endolysin has been described for either the leptospire phages or the phage of *B. hyodysenteriae*. Recently, BlyA, a protein encoded by the prophage of ϕ BB-1 and initially identified as a hemolysin (Guina and Oliver, 1997), has instead been proposed to be a holin-like protein based on structural and functional characteristics (C.J. Damman and D.B. Oliver, unpublished). We have demonstrated that the synthesis of both this protein and BlyB, encoded for by the gene

immediately downstream of *blyA* on the same operon, increases dramatically under conditions of prophage induction as would be expected for a holin (C.J. Damman, C.H. Eggers, D.S. Samuels, and D.B. Oliver, unpublished).

Nucleic Acid Content of Spirochete Bacteriophages

The majority of the bacteriophage-like particles identified in association with spirochetes have gone uncharacterized at the molecular level. To our knowledge, only five bacteriophages of spirochetes have been examined for nucleic acid content: LE1, LE3 and LE4 from *L. biflexa* (Saint Girons *et al.*, 1990), VSH-1 from *B. hyodysenteriae* (Humphrey *et al.*, 1995; Humphrey *et al.*, 1997), and ϕ BB-1 from *Borrelia burgdorferi* (Eggers and Samuels, 1999).

All five of the bacteriophages package double-stranded, linear DNA. The leptophages package a 50-kb (LE3 and LE4) or 60-kb (LE1) molecule. The DNA found in the capsids of these lytic phages does not hybridize to cellular DNA, suggesting the phage DNA is not present as a prophage in the genome of the *Leptospira* strains analyzed (Saint Girons *et al.*, 1990). On the other hand, VSH-1 is a gene transfer agent and packages random ~7.5-kb pieces of *B. hyodysenteriae* host chromosomal DNA (Humphrey *et al.*, 1997).

ϕ BB-1 also packages linear, double-stranded DNA. The DNA is 32 kb and lacks the covalently closed hairpin loops that are found at the ends of all the linear DNA molecules in *B. burgdorferi* (Barbour and Garon, 1987; Hinnebusch and Barbour, 1991; Casjens *et al.*, 1997a; Fraser *et al.*, 1997; Eggers and Samuels, 1999). Unlike the genomes of the leptophages, the genome of ϕ BB-1 is resident in the *B. burgdorferi* cell, and, unlike VSH-1, ϕ BB-1 packages a discrete molecule as its genome. We have previously demonstrated that the lysogenic prophage is maintained in the *B. burgdorferi* cell as a 32-kb circular plasmid (cp32) (Eggers and Samuels, 1999).

cp32 (refer to Figure 7) has several related but distinct isoforms (Zückert *et al.*, 1994; Marconi *et al.*, 1996; Porcella *et al.*, 1996; Stevenson *et al.*, 1996; Casjens *et al.*, 1997b; Casjens *et al.*, 2000; summarized in Stevenson *et al.*, this volume) and more than one isoform can be stably maintained in an isolate (Stevenson *et al.*, 1996; Casjens *et al.*, 1997b; Casjens *et al.*, 2000). The cp32 plasmids are ubiquitous throughout the Lyme disease spirochetes (Marconi *et al.*, 1996; Stevenson *et al.*, 1996; Casjens *et al.*, 1997b; Fraser *et al.*, 1997; Casjens *et al.*, 2000), and all Lyme disease spirochetes analyzed carry multiple isoforms of cp32. All of the cp32s that are contained in the host strain (either *B. burgdorferi* CA-11.2A or B31) are present in the population of ϕ BB-1 virions released from that strain (C.H. Eggers and D.S. Samuels, unpublished).

The cp32s have large stretches of homologous DNA and three regions of significant variability that correspond to the possible partitioning region and two regions that encode different families of lipoproteins (Lam *et al.*, 1994; Zückert *et al.*, 1994; Porcella *et al.*, 1996; Stevenson *et al.*, 1996; Zückert and Meyer, 1996; Casjens *et al.*, 1997b; Stevenson *et al.*, 1997; Stevenson *et al.*, 1998; Akins *et al.*, 1999; Yang *et al.*, 1999; Caimano *et al.*, 2000; Casjens *et al.*, 2000; Sung *et al.*, 2000). The variability of the lipoproteins encoded by the different cp32s has been proposed as a possible mechanism of immune evasion and establishment of chronic infection, suggesting an

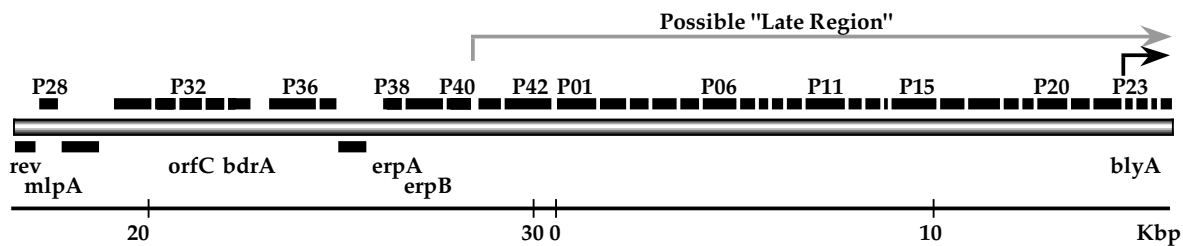


Figure 7. Map of the circular cp32-1 plasmid from *B. burgdorferi* B31 MI. A linearized physical map of the circular cp32-1 is shown opened between genes P26 and P27. Black rectangles above and below the shaded line representing the cp32-1 DNA are genes oriented for rightward and leftward transcription, respectively. Immediately above these are representative gene names and below are alternative names that are in use for some of them. The gray arrow above indicates the transcriptional direction across the possible ϕ BB-1 'late operon' that may encode the phage virion proteins as postulated in the text. The black arrow immediately 5' to gene P23 indicates the promoter and transcript identified by Porcella *et al.* (1996). A scale in kb is given below to indicate the nucleotide numbering system of Casjens *et al.* (2000).

evolutionary rationale for the physiological cost of maintaining several homologous replicons in a cell (Stevenson *et al.*, 1998; Yang *et al.*, 1999; Caimano *et al.*, 2000; Sung *et al.*, 2000).

In addition to the cp32 family, the smaller plasmids cp9 and cp18 are essentially truncated cp32s (Dunn *et al.*, 1994; Fraser *et al.*, 1997; Casjens *et al.*, 2000). Furthermore, lp54 appears to carry an ancient integration of a cp32-like plasmid and lp56 carries a recently integrated, nearly intact cp32 (Casjens *et al.*, 2000). These integrated cp32-like molecules are not packaged by ϕ BB-1 released from either *B. burgdorferi* strains CA-11.2A or B31 (C.H. Eggers and D.S. Samuels, unpublished). None of the *Borrelia* isolates we have assayed that release ϕ BB-1 contain the smaller, truncated cp32-like plasmids, and the ability of these molecules to be packaged into phage virions has not been investigated.

cp32 as the ϕ BB-1 Prophage

Casjens *et al.* (1995) had previously noted that the extreme conservation of the chromosome sizes and gene location in different isolates of *B. burgdorferi* and related species suggested that, unless all related species harbor the same integrated prophages (which seems very improbable given the world-wide distribution of the chromosomes studied), any prophages were likely contained in the plasmids. Besides the physical packaging of cp32 into phage capsids (Eggers and Samuels, 1999), there are several circumstantial arguments consistent with the cp32 plasmids being the ϕ BB-1 prophage.

The gene order and plasmid size are conserved among the cp32s (Casjens *et al.*, 1997b; Casjens *et al.*, 2000). Phage families, including the λ -like phages, have conserved gene orders with occasional replacement by non-homologous genes in different phages (Casjens *et al.*, 1992), as do the cp32s. The DNA packaged by tailed phages is limited by head size, and they can only successfully package DNA with somewhat narrow limits (Lane *et al.*, 1990; Moody, 1999), so if the cp32s have similar head proteins and packaging constraints, then the conservation of plasmid size is also consistent with the prophage model.

Most of the cp32 genes have no recognizable homologs in the current sequence database (other than paralogs on other cp32s), but this does not argue against

the cp32s as ϕ BB-1 genomes; the genes of tailed phages that encode virion structural proteins are notoriously variable, and many known phage structural genes have no sequence homologs even among analogous genes with identical functions in other tailed phages (Casjens *et al.*, 1992; Hendrix, 1999; Hendrix *et al.*, 1999).

Nearly all of the known temperate double-stranded DNA phages have a single operon (expressed late during lytic infection, but not expressed in a lysogen) containing 20 to 30 contiguous genes that function in virion assembly and lysis (Casjens *et al.*, 1992). Our current knowledge suggests that at least 15 gene products are required to build a long-tailed phage virion (Kikuchi and King, 1975; Casjens *et al.*, 1992). The analysis of the *B. burgdorferi* strain B31 genome sequence suggests that cp32s could carry a phage-like 'late operon.' In agreement with the postulate that there are no integrated prophage genomes in the chromosome, the nucleotide sequence of the chromosome shows that there are no regions of contiguous genes of unknown function that are likely to be long enough to constitute such a late operon (there are no known phage virion structural gene homologs on the chromosome, and no stretches of greater than ten contiguous genes with no predicted function) (Fraser *et al.*, 1997).

Temperate phage late operons nearly always have the lysis genes clustered at one or the other end of their late operon [for example, near the start in phage λ and its relatives, and near the distal end in a number of *Bacillus subtilis* and *Mycobacterium tuberculosis* phages (Casjens *et al.*, 1992; Young, 1992; Young and Bläsi, 1995; Ford *et al.*, 1998)]. In addition, the genes required for virion head assembly in essentially all studied dsDNA phages occur in a particular order, even in operons with analogous function that have no recognizable sequence similarity and in phages that infect phylogenetically distant hosts (Casjens *et al.*, 1992). These phages all contain a cluster of head genes in the following order (5'-3'): small subunit terminase, large subunit terminase, portal, and remaining structural genes (Casjens *et al.*, 1992; Hendrix, 1999). Terminase recognizes phage DNA for packaging into virions and then cuts it to virion length, and portal proteins form the hole through which the DNA enters the pre-formed head during packaging (Black, 1989; Fujisawa and Morita, 1997).

Two genes on cp32s are candidates for belonging to a phage late operon: P42 and *blyA* (P23) and their paralogs (using cp32-1 as an example of a typical cp32 plasmid).

Casjens *et al.* (2000) have noted that P42 (and its paralogs) are convincingly similar to a gene (*orf26*) on the *Streptococcus thermophilus* temperate phage ϕ O1205 genome (Stanley *et al.*, 1997). Although the function of the *orf26* gene has not been studied in ϕ O1205, it lies between genes with significant homology to the small terminase subunit and portal genes of the well-studied *B. subtilis* phage SPP1 (Chai *et al.*, 1992; Tavares *et al.*, 1992; Chai *et al.*, 1995; Droge and Tavares, 2000). Therefore, P42 and its paralogs are possible, if not likely, terminase genes based on sequence homology and gene position. Recently, *blyA* (P23) has been shown to be capable of substituting for phage λ 's gene *S* function [release of the phage-encoded endolysin into the periplasm (Young, 1992; Young and Bläsi, 1995)], strongly suggesting that it has a phage lysis-related function (C.J. Damman and D.B. Oliver, unpublished).

The longest universally present contiguous block of genes on the cp32s without tentatively assigned function (no sequence homology to known genes) is from P41 rightward through P26 (28 genes, all transcribed rightward, each of which has a paralog on every cp32) (Figure 7). P42 (the putative terminase gene) is the second most 5' gene in this cp32 gene cluster, exactly where it should be in a cluster of phage head genes, and *blyA* (P23; the putative holin gene) is near the 3' end of the gene cluster, a common location for lysis genes in other temperate phages (Casjens *et al.*, 1992; Young and Bläsi, 1995).

Borrelia hermsii harbors a circular plasmid that is similar in size and genetic content to the cp32s, but there are a number of substantial differences between the *B. burgdorferi* and *B. hermsii* plasmids, most of which lie outside the P41-P26 interval (Stevenson *et al.*, 2000; see Stevenson, *et al.*, this volume). This is reminiscent of other temperate phages in which the early regions seem to be substantially more variable than the virion assembly genes within phage structural types [as in phages HK97 and HK022 (Juhala *et al.*, 2000); L5 and D29 (Ford *et al.*, 1998); λ and N15 (Ravin *et al.*, 2000)].

This evidence, although circumstantial, is all consistent with a model in which genes P41 through P26 constitute a phage 'late operon', or, perhaps more accurately, a phage 'late regulon'. A final prediction is that if the P41-P26 region is a phage 'late regulon', then its transcripts should not be expressed in the lysogenic state. The very low expression of the P26 homolog in strain 297 has been previously reported (Porcella *et al.*, 1996). The P26 homolog is located within the same operon as the *blyA* homolog (P23) and is believed to be expressed on the same transcript (Porcella *et al.*, 1996). We have observed very low expression of *blyA* (P23) in strain B31 (Guina and Oliver, 1997), except following ϕ BB-1 prophage induction with MNNG (C.J. Damman, *et al.* unpublished).

Are the cp32 genes outside the P41-P26 interval consistent with cp32s being prophages? Several cp32 genes, including the *erp* loci (*ospE/ospF/elp*), *bdr*, *rev* and *mip* genes, appear to be expressed in culture (Lam *et al.*, 1994; Porcella *et al.*, 1996; Stevenson *et al.*, 1996; Casjens *et al.*, 1997b; Gilmore and Mbow, 1998; Stevenson *et al.*, 1998; Akins *et al.*, 1999; Yang *et al.*, 1999; Caimano *et al.*, 2000; Carlyon *et al.*, 2000; Zückert and Barbour, 2000). The *erp* loci, *rev* and *mip* genes encode surface lipoproteins (Akins *et al.*, 1995; Porcella *et al.*, 1996; Stevenson *et al.*, 1996; Gilmore and Mbow, 1998; Akins *et al.*, 1999; Yang

et al., 1999). Among the genes on the cp32s, only the *erp* loci and some of the *bdr* genes could be positioned within the putative 'late regulon' (Porcella *et al.*, 1996; Stevenson *et al.*, 1996; Akins *et al.*, 1999; Caimano *et al.*, 2000; Carlyon *et al.*, 2000; Zückert and Barbour, 2000). Prophages sometimes express genes that are involved in the interaction between a pathogenic host bacterium and its multicellular host (Cheetham and Katz, 1995; Hendrix *et al.*, 2000) and genes in this category can be expressed as late genes (Neely and Friedman, 1998a; Neely and Friedman, 1998b). Additionally, each cp32 carries a gene (P32 and paralogs) that is homologous to genes involved in the partitioning of some plasmids in other bacteria (Zückert and Meyer, 1996; Stevenson *et al.*, 1998; Caimano *et al.*, 2000; Casjens *et al.*, 2000), and the only two temperate phages with plasmid prophages to have been previously studied in detail, P1 and N15 (Abeles *et al.*, 1985; Ravin and Lane, 1999; Ravin *et al.*, 2000), each carry a homolog of P32. No known cp32 gene properties are inconsistent with the cp32s being the prophage of ϕ BB-1.

The cp32-4 and cp32-9 of *B. burgdorferi* have one and seven putative 'late operon' genes that contain frameshift mutations, respectively (Casjens *et al.*, 2000). Perhaps these B31 plasmids have not moved between cells in a long time and are now permanently associated with B31. For any prophage, one could ask why the virion structural genes have not decayed since they are not under selection in the prophage state. Perhaps a phage must move between hosts periodically to remain intact (in their ability to make virions). Alternatively, some of these plasmids may be 'satellite' prophages that do not encode all of their own required genes, but use paralogous 'late' genes from other cp32s to complement mutations.

As mentioned above, other *Borrelia* plasmids in the cell are homologous to the cp32s. cp18 is neatly missing the putative 'late operon'. Both cp18 and cp9 have the putative plasmid partition machinery intact (as might be expected), and they could be satellite phages. The integrated cp32 in lp54 has an intact P42 paralog but not a *blyA* paralog (for which there is a non-homologous replacement) (Casjens *et al.*, 2000). The putative 'late operon' of this integrated cp32 has a number of gene replacements (including a large insertion between P42 and P1 with the clearly non-phage structural gene *oppAV*, which encodes an ABC transporter component) relative to genuine cp32s and does not appear to be decaying. The site of apparent integration of the cp32-like molecule into the lp54 progenitor is between genes P18 and P20, the middle of the putative 'late operon'. We hypothesize that this is not the natural location of this cp32, and the fact that the disrupted 'late operon' has not begun to decay is mildly troubling. The recent integration of the cp32-like plasmid into lp56 took place in the middle of the cp32 P04 paralog in the putative 'late operon' (Casjens *et al.*, 2000). In this case, two of the 'late operon' genes have frameshift mutations, which is consistent with the idea that this 'late operon' may no longer be under selection.

Concluding Remarks

Many of the questions surrounding the role of cp32 as the ϕ BB-1 prophage will require a careful analysis of the expression and function of the paralogous cp32 proteins,

a task almost prohibitive in scope due to the large number of plasmids that *could* be a prophage. Complicating these analyses are the possible effects that paralogous cp32 proteins may have on other cp32s in *trans*. Some of the features of the ϕ BB-1 prophage will likely be elucidated as the research into the possible function of the cp32s in the epidemiology of the Lyme disease agents continues. Conversely, further characterizing ϕ BB-1 and its possible use for genetic transfer may play an important role in the study of plasmid maintenance, replication and segregation in *B. burgdorferi*.

Of more than a dozen bacteriophage particles observed in association with spirochetes, only a handful have been characterized at the molecular level to any degree. The number of spirochetes that harbor bacteriophages is likely to be greater even than what has been observed because many of these organisms are not cultivatable. Using the few bacteriophage that have been at least partially characterized, the available genomic sequences for the pathogenic spirochetes, and the number of tools that are becoming available for dissecting the molecular processes in some of these bacteria, we believe that the characterization of bacteriophages of spirochetes will play an increasingly conspicuous role in the future study of the Spirochetales order as a whole.

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