

Microviridae, a Family Divided: Isolation, Characterization, and Genome Sequence of ϕ MH2K, a Bacteriophage of the Obligate Intracellular Parasitic Bacterium *Bdellovibrio bacteriovorus*

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A novel single-stranded DNA phage, ϕ MH2K, of *Bdellovibrio bacteriovorus* was isolated, characterized, and sequenced. This phage is a member of the *Microviridae*, a family typified by bacteriophage ϕ X174. Although *B. bacteriovorus* and *Escherichia coli* are both classified as proteobacteria, ϕ MH2K is only distantly related to ϕ X174. Instead, ϕ MH2K exhibits an extremely close relationship to the *Microviridae* of *Chlamydia* in both genome organization and encoded proteins. Unlike the double-stranded DNA bacteriophages, for which a wide spectrum of diversity has been observed, the single-stranded icosahedral bacteriophages appear to fall into two distinct subfamilies. These observations suggest that the mechanisms driving single-stranded DNA bacteriophage evolution are inherently different from those driving the evolution of the double-stranded bacteriophages.

Bacteriophages have been isolated and characterized from a wide range of microorganisms for more than 80 years. The vast majority of these phages are large double-stranded DNA viruses, as typified by the lambdaoid, T-odd and -even families (1). Recent advances in DNA sequencing technologies and bioinformatics have facilitated the study of double-stranded DNA bacteriophage evolution (13, 14, 28). From these and other studies, a clear picture has emerged (13, 14). The prevalence of double-stranded DNA phages and prophages—cryptic, defective, and replication competent—creates an enormous pool of evolutionary material for horizontal exchange. Consequently, a mosaic spectrum of related phage species has arisen.

The icosahedral, single-stranded DNA phages, or *Microviridae*, appear to be less common than double-stranded DNA phages. The DNAs of nine members of the family, isolated from very diverse hosts (proteobacteria, *Spiroplasma*, and *Chlamydia*), have been sequenced (11, 16, 17, 18, 22, 24–26). The species falls into two distinct subfamilies, although the subfamilies are not officially recognized in the virus taxonomy. One is represented by ϕ X174 and contains the phages that propagate in proteobacteria. The other subfamily contains phages of *Chlamydia* and *Spiroplasma*. Although these two hosts are not closely related, their phages, Chp1, Chp2, and SpV4, respectively, are quite similar. The principal difference between the two subfamilies is the existence of two genes and the complexity of major coat protein. The *Chlamydia* and *Spiroplasma* phages do not encode major spike and external scaffolding proteins. Their more-complex coat proteins contain an insertion loop that forms large threefold-related protrusions (6). Protein homologies between the two subfamilies are ap-

proximately 20% or less (6), a typical value when comparing the most distantly related members of either the lambda or T4-like groups (14, 28). However, unlike tailed double-stranded DNA families, no mosaic species that bridge the evolutionary chasms have been isolated.

While the available DNA sequences suggest that the evolution of *Microviridae* may differ from the evolution of the more-prevalent, double-stranded DNA phages, the phages have been isolated from such distantly related hosts that only the extremes of a diversity spectrum may have been uncovered. To further elucidate the evolution of the *Microviridae*, we have isolated, characterized, and sequenced a new family member, ϕ MH2K, infecting *Bdellovibrio bacteriovorus*, a nonenterobacterial proteobacterium with an obligate intracellular parasitic life cycle similar to that of *Chlamydia*. The results suggest that ϕ MH2K is closely related to the phages of *Chlamydia* and *Spiroplasma*. In some instances, ϕ MH2K is more closely related to an individual chlamydiaophage than chlamydiaophages are related to each other. While the number of available *Microviridae* genomes is still limited, these results suggest that the forces driving single-stranded DNA phage evolution may differ from those driving double-stranded DNA phage evolution.

MATERIALS AND METHODS

Media and *Bdellovibrio* strains. The host-independent *B. bacteriovorus* strain HI 109 was grown in medium containing 1.0% peptone, 0.5% yeast extract, 3.0 mM MgCl₂, and 2.0 mM CaCl₂. Plates and soft agar were supplemented with 1.2 and 0.7% agar, respectively. Plaque assays were performed by mixing 0.3 ml of exponentially growing cells with phage in 2.0 ml of soft agar. Plates were incubated at 33°C for 48 to 72 h.

Isolation of phage ϕ MH2K. A 200-ml volume of raw sewage sludge was centrifuged at 5,000 \times g to remove the large debris. The supernatant was then passed through 0.45- and 0.22- μ m-pore-size filters. One milliliter of this solution was mixed with 10.0 ml of exponentially growing cells at 30°C and incubated for 6 h. Cultures were passed through a 0.45- μ m-pore-size filter and then concentrated to 1.0 ml in a Centricon filter. One hundred microliters was layered atop of a 5 to 30% sucrose gradient, along with wild-type ϕ X174 as an S value marker, and spun for 1.0 h at 240,000 \times g in an SW50.1 rotor. Gradients were fraction-

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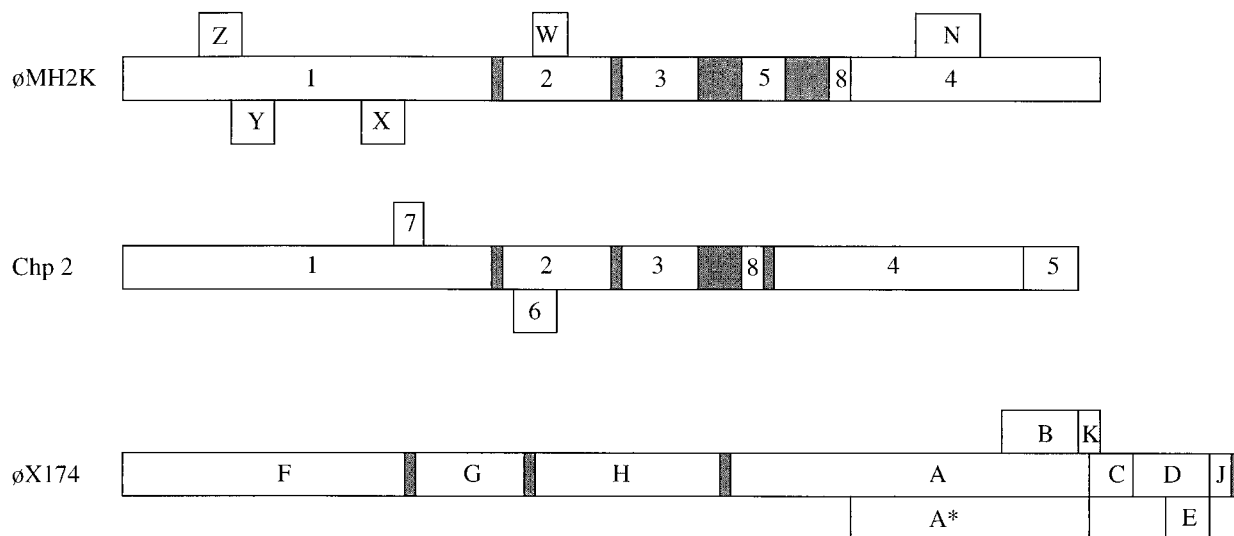


FIG. 1. Genetic maps of ϕ MH2K, Chp2 (18), and ϕ X174 (25). Reading frames that encode proteins with Chp2 homologues were designated with the same numbers used in the Chp2 genetic map (18). Reading frames that are not found in Chp2 were assigned letters. The numbering of the nucleotides commences with the start codon of gene 1.

ated, and the titer of infectious ϕ X174 on *Escherichia coli* C for each fraction was determined. Fractions containing material with S values less than 114 were pooled and reconcentrated to a final volume of 100 μ l, layered atop a second sucrose gradient, and treated as described above. Finally, fractions were plated on *Bdellovibrio* for the isolation of phage. Single plaques were picked and used to infect 1.0 ml of exponentially growing cells. Phage DNA was extracted and analyzed using ϕ X174 protocols as described below.

Isolation and purification of ϕ MH2K genomic and RF DNA. To isolate genomic DNA, 1.0 liter of exponentially growing cells was inoculated with ϕ MH2K at a multiplicity of infection (MOI) of 0.001. After an 18-h incubation at 33°C, infected cells were stored at 4°C for 12 h. Cellular debris, containing attached phage particles, was concentrated by centrifugation. Pellets were resuspended in 8.0 ml of borate EDTA buffer (10), and virus and DNA were extracted three times with 8.0 ml of ether. Virions were purified on CsCl gradients, and DNA was extracted as previously described (10). To determine the strandedness of the genomes, genomic DNA was digested with S1 nuclease (Promega) as specified by the manufacturer. Results were compared to those of similar digestions of genomic and replicative-form (RF) ϕ X174 DNA. To generate RF DNA, 100 ml of exponentially growing cells was inoculated with ϕ MH2K at an MOI of 5.0 and incubated for 6.0 h. Infected cells were concentrated by centrifugation. RF DNA was extracted and characterized by standard techniques (5).

For electron microscopy, particles were purified as described above and concentrated 10-fold with Centricon filters, stained with 2.0% uranyl acetate, and examined with a JEOL EM10A electron microscope.

Cloning and sequencing of ϕ MH2K RF DNA. RF DNA was digested with *Hind*III (1395), *Bgl*II (1515), *Msp*AI (350 and 2816), *Eco*RI (392, 1090, and 4107), or *Stu*I (1185 and 3541) endonucleases (nucleotide positions of restriction enzyme digestion sites given in parentheses). Fragments from single-enzyme digestions were cloned into a modified TOPO vector (Invitrogen) digested with enzymes yielding compatible ends. Clones were sequenced by the DNA sequencing facility at the University of Arizona. T7 and M13R primers were provided by the facility. The entire genome was sequenced twice. Overlapping clones were used to determine the order of the genes.

Sequence alignment. The amino acid sequences of the major capsid proteins of the coliphages α 3, G4, ϕ K, and ϕ X174; the chlamydiae Chp1 and Chp2; a *Spiroplasma* phage, SpV4; and ϕ MH2K were aligned using the program CLUSTAL W, version 1.4 (29). Default parameters were used. The colors of the resulting alignment were modified to give a better representation of the similarities and differences between the eight sequences. The secondary structure elements of ϕ X174, based on knowledge of the atomic resolution structure (19, 20, 21), are given under the sequence alignment.

Nucleotide sequence accession number. The nucleotide sequence of ϕ MH2K was deposited in GenBank under accession number AF306496.

RESULTS

Genome organization of ϕ MH2K. RF DNA was digested and cloned into a modified TOPO vector as described in Materials and Methods and sequenced. The genetic map of ϕ MH2K is depicted in Fig. 1. The organization and size of the 4,594-base DNA genome are very similar to those of chlamydiae phages (18, 22, 26). However, the location of gene 5 differs. In ϕ MH2K, it is located between genes 3 and 8, as opposed to after gene 4. The ϕ MH2K genome is approximately 20% smaller than the ϕ X174-like phages. This is due to the absence of genes encoding the major spike and external scaffolding proteins, as is seen in SpV4 and the chlamydiae phages (6, 18, 22, 24, 26). The relationship between ϕ MH2K and the previously isolated *Microviridae* phage MAC-1 (2) is unclear since no MAC-1 sequence data are available. Three other phages were isolated. Their morphologies were similar to those of previously isolated bacteriophages MAC-3, MAC-6, and VL1 (2, 30).

Computer analysis of the ϕ MH2K genome suggests the existence of two very strong promoters (23). They reside in the two largest noncoding regions, located between genes 5 and 8 (230 bases) and between genes 3 and 5 (247 bases). Genes 1, 2, 3, 4, 5, and 8 all have upstream ribosome binding sites and homologues within the chlamydiae phages. There are four additional open reading frames (ORFs) within the ϕ MH2K genome, ORFs N, W, X, Y, and Z. All five ORFs are contained within overlapping reading frames. The largest of these ORFs, ORF N, is the only one with a discernible ribosome binding site. The predicted protein is very hydrophobic and is similar to the ϕ X174 lysis protein in character.

Protein homologies. The ϕ MH2K proteins encoded by genes 1 to 5 and 8 were compared to homologous proteins found in Chp1, Chp2, SpV4, and ϕ X174-like phages. The ϕ MH2K proteins most closely resemble the chlamydiae phage

TABLE 1. Putative ϕ MH2K gene products and amino acid homologies

| Gene product ^a | Coding sequence ^b | % Amino acid identity between: | | | | |
|---------------------------|------------------------------|--------------------------------|-------------------------------|-------------------------------|------------------------|---|
| | | ϕ MH2K and Chp2 proteins | ϕ MH2K and Chp1 proteins | ϕ MH2K and SpV4 proteins | Chp2 and Chp1 proteins | ϕ MH2K and ϕ X174-like proteins |
| VP1 | 1–1602 | 46.9 | 40.4 | 38 | 49.6 | 19 (α 3 F) |
| VP2 | 1684–2283 | 26.5 | 21.3 | 25 | 29.9 | 20 (α 3 H) |
| VP3 | 2330–2785 | 32 | 27.6 | 18.4 | 27.3 | 18 (α 3 B) |
| VP4 | 3651–4598 | 27.9 | 22.5 | 27 | 22.2 | 18 (G4A) |
| VP5 | 3032–3286 | 39.5 | 26.8 | 18.4 | 30.2 | 20 (α 3 C) |
| VP8 | 3516–3632 | 32.6 | 33.3 | 31 | 54.5 | 21 (G4 J) |

^a Genes (and gene products) which are conserved between ϕ MH2K and the chlamydiaphages. The locations of the unconserved ORFs N, W, X, Y, and Z are nucleotides 3997 to 4326, 1836 to 1991, 917 to 1129, 425 to 616, and 267 to 461, respectively.

^b Location within the ϕ MH2K genome.

proteins, especially those of Chp2 (Table 1). Considering the evolutionary distance between their respective hosts, this result was unexpected (see Discussion). In some instances, comparisons of ϕ MH2K and Chp2 proteins reveal closer relationships than that seen between the Chp1 and Chp2 proteins. In addition, polyclonal antibodies raised against the Chp2 major coat protein cross-react with ϕ MH2K (I. Clarke, personal communication). Only weak homologies exist with the proteins of the ϕ X174-like phages (ϕ X174, G4, α 3, S13, and ϕ K). There does not appear to be significant homologies between the ϕ MH2K ORFs N, W, X, Y, and Z and the small Chp2 ORFs 6 and 7.

ϕ MH2K virion and virion proteins. The T=1 capsid is primarily composed of Vp1, the major coat protein, as assayed by polyacrylamide gel electrophoresis, and measures 27 ± 2 nm in diameter (Fig. 2). Vp2, with an amino acid composition similar to that of the ϕ X174 DNA pilot protein, protein H, is also a component of the virion. The exact nature of Vp3 remains somewhat obscure. Like the chlamydiaphage proteins (18, 22, 26), ϕ MH2K Vp3 most closely resembles the internal scaffolding proteins of the ϕ X174-like phages. Various amounts (depending on the purification protocol used) of Vp3 are associated with ϕ MH2K capsids. Whether this is caused by the selective loss of this protein due to particle instability or the

copurification of capsids and procapsids remains to be determined.

Figure 3 depicts an alignment of the sequences of eight *Microviridae* major coat proteins. Bacteriophages ϕ X174, G4, ϕ K, and α 3 are coliphages. However, host range variants which infect *Salmonella enterica* serovar Typhimurium and *Shigella* have been reported (3, 12). The aligned major coat protein sequences resulted in the identification of two distinct subclasses. ϕ MH2K, Chp1, Chp2, and SpV4 represented a class with large amino acid insertions between β -strands E and F of the core β -barrel motif; the ϕ X174-like phages formed the other class (Fig. 3). These additional amino acids are located between residues 210 and 280 in the ϕ MH2K sequence. The cryoelectron microscope image reconstruction of SpV4 (6) demonstrates that they form large protrusions at the threefold icosahedral axes of symmetry. The β -barrel core is conserved among all the phages.

DISCUSSION

Unlike the large double-stranded DNA bacteriophages for which a broad diversity spectrum has been observed (13, 14, 28), the sequenced members of *Microviridae* fall into two very distinctive subfamilies. However, the hosts of these phages, γ proteobacteria *Chlamydia* and *Spiroplasma*, are distantly related. To further study *Microviridae* diversity, a virus of the δ proteobacterium *B. bacteriovorus*, ϕ MH2K, was isolated. The data presented here indicate that ϕ MH2K, Chp2, Chp1, and SpV4 share a common ancestor not found in the ϕ X174-like lineage. For two of the gene products, Vp3 and Vp5, similarities between ϕ MH2K and the chlamydiaphage Chp2 were greater than between Chp2 and Chp1 (18, 26).

If phages reflect their host's phylogeny, this result is surprising. *B. bacteriovorus* is more closely related to the γ proteobacteria than to the chlamydia. However, the phylogenetic classification of *B. bacteriovorus*, based exclusively on 16S RNA sequences, may be incorrect. Considering the predatory nature of *Bdellovibrio*, which hunt and replicate inside proteobacteria, it is possible that its 16S RNA sequence was derived via a horizontal transfer, as has been seen in other bacteria (31). On the other hand, several *B. bacteriovorus* genomic sequences were characterized during the cloning of ϕ MH2K (GenBank accession numbers AF339026 to 339030). These sequences

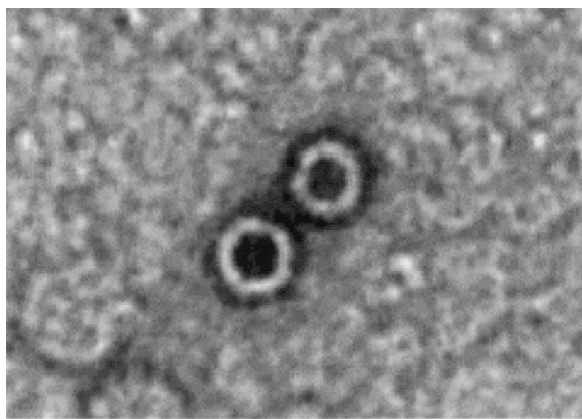


FIG. 2. Electron micrograph of ϕ MH2K. Magnification, $\times 31,500$. Bar, 100 nm.

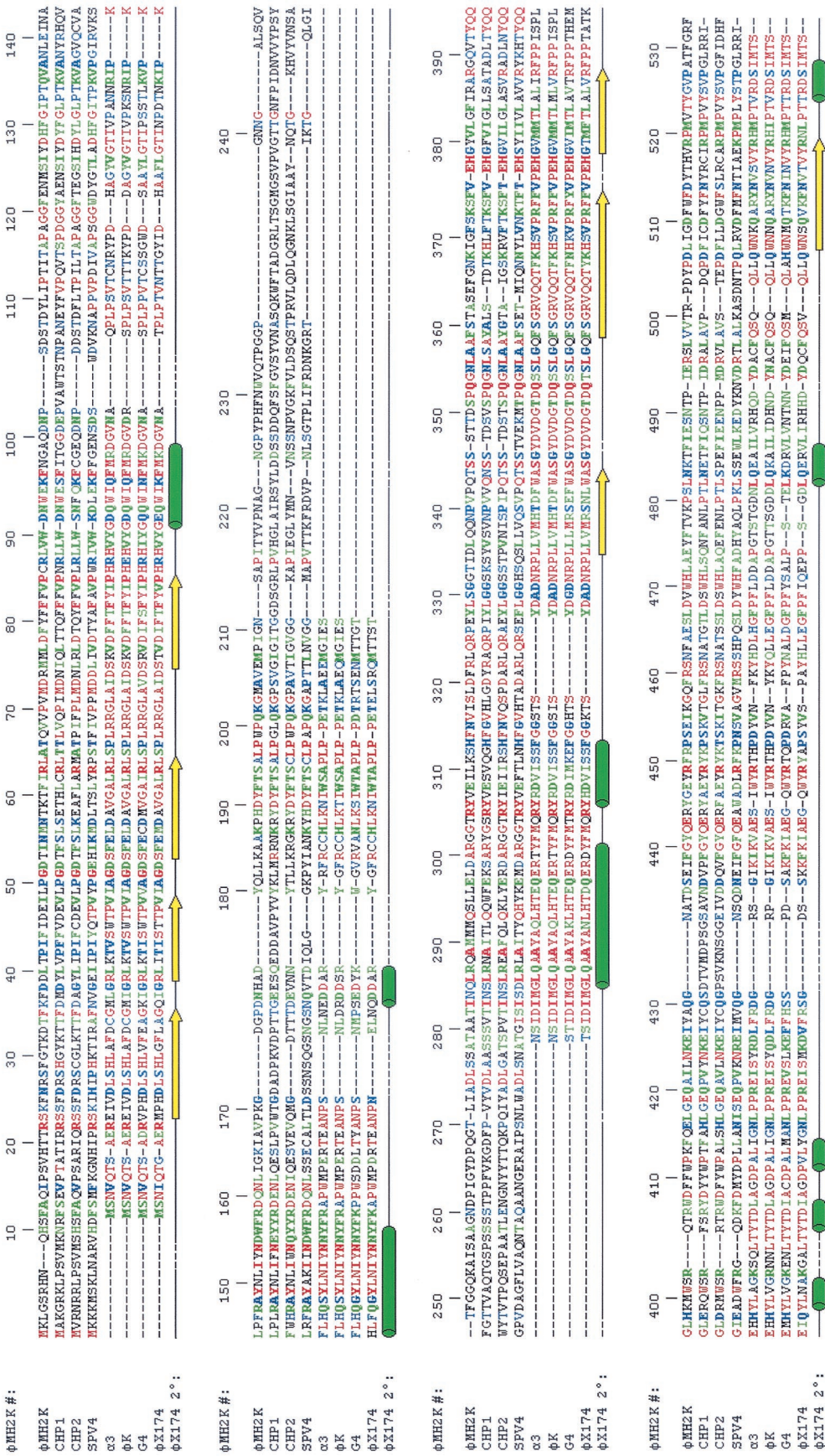


FIG. 3. Multiple-sequence alignment of coat proteins of *Microviridae* phages. Different colors were added to give a better representation of the similarities between the eight sequences. The four ϕ X174-like phages are grouped on the bottom; the smaller *Microviridae* are grouped at the top. Amino acids identical in all sequences (red boldface type) and conserved (green boldface type) and semiconserved (blue boldface type) substitutions in all sequences are indicated. Amino acids identical within the subgroups (red type) and conserved (green type) and semiconserved (blue type) substitutions within the subgroups are indicated. Dashes represent gaps introduced to maximize alignment of the sequences. The secondary structure (2°) of ϕ X174 is presented on the last line. The yellow arrows represent β -strands, and the green cylinders represent α -helices.

were most closely related to genes of proteobacteria and exhibit no discernible homologies with those of *Chlamydia*.

If the classification of *Bdellovibrio* is correct, then the evolution of single-stranded icosahedral bacteriophages may be fundamentally different from the evolution of the double-stranded phages, which is most likely driven by horizontal exchange (13, 14). There are several factors that may limit horizontal transfer in single-stranded DNA viruses. For example, *Microviridae* replication does not require recombination, and recombination is not prevalent. Double-stranded RF DNA is not abundant (20 to 50 copies per cell), and recombination frequencies for mutations separated by 2,500 bases range from 10^{-3} to 10^{-4} (B. A. Fane, unpublished results). Genomes are circular throughout the replication cycle, necessitating two recombination events. Lysogenic or latent life cycles have never been observed, therefore minimizing horizontal exchange with prophages. In addition, the small T=1 capsid may restrict the incorporation of exogenous DNA sequences, or morons (13). Although it is unlikely that these small viruses can acquire morons, all members of the *Microviridae* appear to have preserved ORFs, found mostly in overlapping genes. Mutations could accrete in these reading frames, termed cretins (for accrete in), until a gene encoding a beneficial function is produced. Examples of cretins may include the ϕ X174 lysis gene E, the putative ϕ MH2K lysis gene N, and the ϕ X174 genes A* and K, both unessential and of unclear function (7, 27). The close relationship between ϕ MH2K and the chlamydiae suggest that *Microviridae* evolution may be driven by cretins and species jumping. In light of these results, the present classification, dividing the *Microviridae* into four genera, based on host range (14), should be reexamined to reflect two distinct subfamilies.

Genetic maps of ϕ MH2K, Chp2 (18), and ϕ X174 (25) are given in Fig. 1. Neither Chp2 nor ϕ MH2K encodes an external scaffolding or major spike protein, ϕ X174 D or G protein, respectively. The external scaffolding protein has at least two known functions in ϕ X174 morphogenesis. It stabilizes the procapsids at the two- and threefold axes of symmetry (8, 9) and directs the placement of the major spike protein. These functions are either not required or performed by different proteins in ϕ MH2K-like phages. First, there is no major spike protein. The twofold stabilization function may be performed by Vp3, the internal scaffolding protein equivalent, which in ϕ X174 self-associates across twofold axes of symmetry. Finally, as seen in the cryoimage reconstruction of SpV4, a large coat protein insertion loop forms spikes at the threefold axis of symmetry. This large insertion loop may be a relic of the ancestral external scaffolding or major spike protein. Coding Vp3 in a normal reading frame, as opposed to a cretin, may be a related phenomenon. The B proteins of the ϕ X174-like phages are highly divergent, yet they cross-function, suggesting that interactions are primarily nonspecific and flexible (5). In addition, amino-terminal deletions are tolerated (4), probably because the external scaffolding protein performs a similar function. With the loss of the external scaffolding protein, internal scaffolding protein interactions may need to be more specific, requiring a reading frame unconstrained by other genes.

Regardless of their evolution, the similarity between ϕ MH2K and the chlamydiae is rather fortuitous. *Chla-*

mydia research has been stifled by the lack of a genetic transfer system, and it is hoped that the chlamydiae can serve as a basis for its establishment. To develop the in vitro packaging system necessary for fruition, the precise functions of the phage proteins and *cis*-acting packaging elements must be identified. These questions will be much easier to address in the ϕ MH2K system, in which host-independent *Bdellovibrio* mutants can be used in plaque assays to facilitate both genetic and biochemical analyses.

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