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Review

Freshwater Cyanophages

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Cyanophages are double-stranded DNA viruses that infect cyanobacteria, and they can be found in both freshwater and marine environments. They have a complex pattern of host ranges and play important roles in controlling cyanobacteria population. Unlike marine cyanophages, for which there have been a number of recent investigations, very little attention has been paid to freshwater cyanophages. This review summarizes the taxonomy and morphology, host range, distribution, seasonal dynamics, and complete genomes of freshwater cyanophages, as well as diagnostic markers that can be used to identify them.

Cyanophage; Freshwater; Morphology; Cyanobacteria

Introduction

Viruses-infecting cyanobacteria are referred to as cyanophages and are similar in morphology to bacteriophages. They typically have a head, a tail, and double-stranded DNA (dsDNA) (Martin E L, et al., 1999). Cyanophages are abundant in both freshwater and marine environments (Shane M S, 1971). During the past decade, the majority of cyanophage research has been on the cyanophages present in marine systems (Fuhrman J A, 1999; Suttle C A, 2005). Even though cyanophages were first isolated from freshwater environments, and these environments are important to humans, freshwater cyanophages have received less attention (Middelboe M, et al., 2008; Wilhelm S W, et al., 2008). The existence of freshwater cyanophages was first reported in 1963 by Safferman and Morris, who isolated podovirus LPP-1, which infects filamentous cyanobacteria. Cyanophages are abundant and widespread in freshwater and have been isolated from a variety of freshwater systems, including lakes, ponds, reservoirs, streams, and sewage outlets. They play important roles in modulating cyanobacterial populations and preserving water quality. This review focuses on freshwater cyanophages and summarizes the diversity of their structures, habitats, host ranges, and other properties.

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Taxonomy and morphology

The classification of cyanophages has changed in the past few decades (Safferman R S, 1973; International Committee on Taxonomy of Viruses [ICTV] current release). Generally, cyanophages are grouped within three families of bacterial phages: *Myoviridae*, *Siphoviridae*, and *Podoviridae* (Table 1). The family *Myoviridae* contains the majority of cyanophages isolated from marine water (Hambly E, et al., 2001; Shuttle C A, 2000), while *Podoviridae* and *Siphoviridae* contain cyanophages that are commonly isolated from fresh water. The myoviruses and siphoviruses tend to be lytic, while podoviruses are often lysogenic, with their genome integrated into the host genome (Suttle C A, 2005). Representative cyanophages from each of the families infect both unicellular and filamentous cyanobacteria. They are present in freshwater and are commonly isolated from waste stabilization ponds (Hu N T et al., 1981).

Cyanomyoviruses

Cyanophages of the *Myoviridae* have contractile tails that are separated from the head by the "neck." The G+C content of the cyanomyovirus varies from 37 to 55%, and the genome size is estimated as being 37 to 200 kb

Table 1. Examples of freshwater cyanophages

Family	Morphology	Phage species	Host	References
Myoviridae	Contractile tail	AS-1	*Aancystis nidulans*, *Synechococcus cedrorum*	Safferman R S, et al., 1972
		N-1	*Nostoc muscorum*	Adolph K W, et al., 1971
		Ma-LMM01	*Microcystis aeruginosa*	Yoshida T, et al., 2006
	Long, non-contractile tail	SM-2	*Synechococcus elongates*,*Microcystis aeruginosa*	Fox J A, et al., 1976
Siphoviridae		S-2L	*Synechococcus sp. 698*	Khudyakov I Y, et al., 1978
		S-4L	*Synechococcus. elongatus*	Khudyakov I Y, et al., 1982
		LPP-1	*Lyngbya*, *Plectonema*, *Phormidium*	Sherman L A, et al, 1970
Podoviridae	Short tail	SM-1	*Synechococcus elongatus*	Safferman R S, et al., 1969
		Ma-LBP	*Microcystis aeruginosa*	Tucker S, et al., 2005
Unassigned	Tailless	PaV-LD	*Planktothrix agardhii*	Gao E B, et al., 2009

(Safferman R S, et al., 1972; Suttle C A, 2000). Representatives of freshwater cyanophages in this family are AS-1, N-1, and Ma-LMM01. AS-1 was isolated from a waste stabilization pond and infects unicellular freshwater cyanobacteria originally assigned to the genera *Anacystis* and *Synechococcus*. The structure of AS-1 resembles the T-even bacteriophage that replicates in *Escherichia coli*. AS-1 has an isometric head 90 nm in diameter and a long contractile tail (22 nm × 244 nm) (Safferman R S, et al., 1972). N-1 can lyse or lysogenize some strains of the cyanobacterial genera *Anabaena* and *Nostoc* (Adolph K W, et al., 1971; Padhy R N, et al., 1978; Currier T C, et al, 1979) and has a head with 60 nm in diameter and a contractile tail that is 100 nm in length (Adolph K W, et al. 1973). Ma-LMM01 (*Microcystis aeruginosa* Lake Mikata Myoviridae 01) was first isolated from Lake Mikata in Japan, and specifically infects a toxic strain of *Microcystis aeruginosa* (Yoshida T, et al. 2006). It has a polyhedral head (86 nm in diameter), and a tail containing a central tube with a width of 9 nm, with a contractile sheath (24 nm × 209 nm) that contracts to a length of 90 nm. The genome of the virus is discussed in a later section of this review.

The ICTV formerly classified AS-1 and N-1 as unassigned species in the *Myoviridae* (ICTV release 1995 to 1998). However, only Ma-LMM01 is currently listed as a species in this family (ICTV current [2012] release).

Cyanosiphoviruses

Cyanophages of the *Siphoviridae* have long, non-contractile tails that can appear flexible on micrographs. SM-2, infecting the species *Synechococcus elongatus* and *M. aeruginosa* (which has since been found to be a *Synechococcus* species), has an isometric head 50 nm in diameter and a tail of 200 to 300 nm in length (Fox J A, et al., 1976; Suttle C A, et al., 1993; Sode K, et al., 1994).

S-2L, which lyses some strains of unicellular cyanobacteria of the genus Synechococcus, has a polyhedral head (56 nm in diameter) and a flexible non-contractile tail 120 nm in length (Khudyakov I Y, et al., 1978). The genome sizes of cyanosiphoviruses range from 40 to 100 kb, and the number of structural proteins also varies, from 13 to 23. The G+C contents of the cyanosiphophages are usually higher than that of cyanomyophages.

ICTV previously listed S-2L and S-4L (Kirons M D, et al., 1977) as species in the unassigned genera of *Siphoviridae* (ICTV 1998 release), but the current (2012) release does not list them as species in this family.

Cyanopodoviruses

Cyanophages of *Podoviridae* have short non-contractile tails. The representative species are LPP-1, SM-1, and Ma-LBP. LPP-1 infects cyanobacteria assigned to the genera *Lyngbya*, *Plectonema*, and *Phormidium* (Safferman R S, et al., 1963; Schneider I R, et al., 1964; Sherman L A, et al., 1970). LPP-1 has an isometric head 59 nm in diameter and a tail of 15 to 20 nm in length. There are about 10 structural proteins, and the genome is 27×10^6 Da in size. The reported G+C content of the LPP-1 genome is usually in the low 50s. The LPP-1 group has been found in numerous habitats, including lakes, fishponds, and sewage settling ponds (Safferman R S, et al., 1963). SM-1 infects cyanobacteria assigned to the genera *Synecbococcus and Microcystis* with an icosahedral head capsid. The average diameter of SM-1 is about 90 nm (Safferman R S, et al., 1969), and the G+C content of the genome is about 65%. Ma-LBP is a phage of *M. aeruginosa*, which was originally collected from Lake Baroon in Australia. It has a T7-like morphology, with a short geometric tail, and the head diameter ranges from 40 to 55 nm (Tucker S, et al., 2005).

ICTV previously placed LLP-1, SM-1, AC-1, and A-4(L) in the unassigned genera of the *Podoviridae* (ICTV 1998 release). However, the 2012 ICTV release lists only the *Phormidium* phages Pf-WMP3 and Pf-WMP4

as species in the unassigned genera of this family.

Other forms

Other morphological forms of cyanophages almost certainly exist, though few have been isolated. Examples are the three filamentous phages that infect *Microcystis*, *Anabaena*, and some potential *Planktothrix* strains (Deng L, et al. 2008), and a tailless cyanophage that infects *Planktothrix* (Gao E B, et al., 2012). The morphologies of these isolated cyanophages have not been reported previously.

The nomenclature of cyanophages is inconsistent. Previously, this nomenclature was based entirely on the cyanophage host, with the consequence being that it is impossible to tell the taxonomic group of a cyanophage from its nomenclature. Later, it was suggested that new cyanophage isolates should be named as Cyanophage Xx-YYZaa, where Xx are the first letters of the genus and species names of the host from which the virus was isolated, YY is a descriptor of the origin of the isolate, Z is the virus family (i.e., M = Myoviridae, S = Siphoviridae, P = Podoviridae), and aa is a reference number for the virus isolate (Suttle C A, 2000). However, this nomenclature system has not been fully adopted, and the current nomenclature of cyanophages is the result of a mixture of different systems.

Host range

Cyanobacteria are widespread in freshwater environments, and there are about 22 common genera of freshwater cyanobacteria. The most common toxic cyanobacteria in freshwater are *Microcystis spp., Cylindrospermopsis raciborskii, Planktothrix rubescens, Synechococcus spp.*, *Planktothrix agardhii*, *Gloeotrichia spp.*, *Anabaena spp.*, *Lyngbya spp.*, *Aphanizomenon spp.*, *Nostoc spp.*, *some Oscillatoria spp., Schizothrix spp.*, and *Synechocystis spp.* (WHO, 2009).

Unlike bacteriophages, which normally have a genus-specific host range, cyanophages commonly have hosts in more than one genus. Freshwater cyanophages can be classified into three major groups based on the taxonomy of their host organisms (Singh P, et al., 2012).

The LPP group, belonging to the cyanopodoviruses, can infect three different genera of non-heterocystous filamentous cyanobacterica: *Lyngbya*, *Phormidium*, and *Plectonema* (Pandan E, et al., 1973). The most abundant viruses in freshwater seem to be those that infect filamentous cyanobacteria, and cyanophages belonging to the LPP group are easy to isolate from the environment.

The second group is the A, AN, N, and NP group, classified on the basis of their ability to infect strains assigned to the genera *Anabaen*a, *Nostoc*, *and Plectonema* (Suttle C A, 2000).

Finally, the third group is the AS and SM group, known to infect *Anacystis*, *Synechococcus*, and *Microcystis*.

There has been some confusion regarding cyanophage nomenclature due to mischaracterization of their hosts. For example, SM-1, SM-2, and MA 1 were reported to be lytic for *M. aeruginosa*; however, the *M. aeruginosa* NRC-1 strain reported to be sensitive to SM-1 and SM-2 was later found to be a Synechococcus strain (Youshida T, et al., 2006). Therefore, grouping of cyanophages according to their host range should not be considered a taxonomically valid method.

Distribution abundance and seasonal dynamics

Cyanophages were first discovered in freshwater, and many studies have focused on understanding their role in the control of cyanobacterial populations in eutrophic freshwater environments, with the goal to use cyanophages as biological agents to prevent or treat cyanobacterial blooms.

The LPP group cyanophages have been found throughout the world in freshwater environments, including waste stabilization ponds, fish ponds, and rice fields (Safferman R A, et al., 1963; Majumdar A K, et al., 1973; Padan E, et al., 1969), and studies have reported that the abundance of these viruses shows seasonal variation. The numbers of LPP cyanophages in Israeli fish ponds were measured over a year, and the titers ranged from single figures to several tens of plaque-forming units (PFU) per mL, with the highest titers reaching several thousands of PFU/mL when intensive cyanobacterial blooms occurred (Padan E, et al., 1969).

The role that phages might play in the ecological dynamics of toxic bloom-forming *M. aeruginosa* was investigated recently (Yoshida M, et al., 2008). Changes in the populations of cyanophages and their host *M. aeruginosa* in Lake Mikata in Japan were monitored monthly from spring to early winter using real-time polymerase chain reaction (PCR). The cyanophgae *g91* (encoding viral sheath) copy numbers detected ranged from 10^2 to 10^4 copies/mL, and the phycocyanin intergenic spacer (PC) copy numbers of *M. aeruginosa* ranged from 10^1 to 10^5 copies/mL throughout the sampling period. Statistical analysis showed that cyanophage abundance was significantly negatively correlated with the numbers of *M. aeruginosa*, suggesting that cyanophages are an important factor in determining the seasonal changes in *M. aeruginosa* abundance (Yoshida T, et al., 2008).

Representatives of cyanophage in freshwater with reported complete genomes

The complete genome sequences of cyanophages can provide significant clues regarding their biological properties and ecological effects, as well as the evolutionary relationship between cyanophages and their hosts. Currently, six complete genomes of cyanophages have been reported, two (Ma-LMM01 and S-CRM01) belonging to *Myoviridae*, and three (Pf-WMP3, Pf-WMP4, and PP-1) to *Podoviridae*, while one (PaV-LD) is currently unassigned to any known family (Table 2).

Ma-LMM01 belongs to *Myoviridae* and apparently has distinct morphological features from Ma-LBP, which also inhibits *M. aeruginosa* growth (Tucker S, et al., 2005). Unlike the other myoviruses, which usually tend to exhibit broader host ranges, including *Prochlorococcus* and S*yechococcus* strains (Sullivan M B, et al, 2003), Ma-LMM01 has a narrow host range (Yoshida T, et al., 2006). Ma-LMM01 has a circularly permuted linear dsDNA that is 162,109 bp in length. The genome contains 184 predicted protein-coding genes and 2 tRNA genes, and only 28 open-reading frames (ORFs) have been assigned with putative functions. The Ma-LMM01 genome lacks homologs for photosynthetic genes, such as *psb*A, which are conserved in marine cyanophages, but it possesses a homolog of *nbl*A, which is essential for the degradation of the phycobilisomes (the major cyanobacteria light-harvesting complex). The genome codes for a site-specific recombinase and two prophage anti-repressors, suggesting that the virus may have a lysogenic life cycle (Yoshida T, et al., 2008).

Phage S-CRM01 was thought to be associated with toxic *M. aeruginosa* blooms because it was isolated from a surface sample taken from a *Microcystis*-dominated bloom in the Copco Reservoir in Northern California; however, its host is an endemic *Synechococcus* lineage. S-CRM01 is a T4-like phage with an isometric head (85 to 100 nm in diameter) and a contractile tail (15 to 20 nm × 140 to 170 nm long). It contains a circular permuted linear dsDNA genome 178,563 bp long that encodes 33 tRNA genes and 297 ORFs. Of these, 86 ORFs have homologs found in a group of myophages that lytically infect marine *Synechococcus* or *Prochloroccus*, but only 4 ORFs have homologs in Ma-LMM01. The genome of S-CRM01 is closely related to that of photosynthetic marine cyanomyophages, but a large part of its sequence has no significant homologs in the GenBank database. Marine cyanophages, particularly cyanomyoviruses and podoviruses, usually have several genes associated with photosynthesis, such as *psbA* and *psbD*. S-CRM01 is the first freshwater "photosynthetic" phage found to contain the photosystem gene *psbA* (Dreher T W, et al., 2011).

Pf-WMP3 and Pf-WMP4 were isolated from Lake Weiming in Beijing, China. Pf-WMP3 and Pf-WMP4 are both T7-like phages that infect the freshwater cyanobacterium *Phormidium floveolarum* Gom. (Liu X Y, et al., 2007; Liu X Y, et al., 2008). The virions are icosahedrons (55 nm in diameter) with short, stubby tails and belong to the family *Podoviridae*. The Pf-WMP3 genome is 43,294 bp long with 234-bp direct repeats. There are 41 potential ORFs in this genome, and on the left arm of the genome, several ORFs encoding highly conserved proteins, such as DNA polymerase (ORF12 and ORF14), DNA primase/helicase (ORF9), and endonuclease (ORF13), have been identified (Liu X Y, et al, 2008). The genome size of Pf-WMP4 was found to be 40,938 bp with 107-bp terminal repeats. No tRNA genes were found in its genome, but 54 ORFs were identified; 15 of these could be assigned functions based on homology, and 9 of these are T7-like core genes (Liu X Y, et al., 2007). The relationship with Pf-WMP3 and Pf-WMP4 is remote at the DNA level; however, they are closely related at the protein level and in their genomic architecture.

PP-1 was isolated from East Lake, Wuhan, China, and it can infects *Plectonema boryanum* and *Phormidium* foveolarum (Zhao Y J, et al., 2002). The genome of PP-1 is 42,480 bp long with 222 bp terminal repeats, and encodes for 41 putative ORFs. The genome can be divided into two parts: the first part encodes 23 ORFs in one direction and the second part encodes 18 ORFs in the opposite direction. There are 17 ORFs with assigned functions, of which 13

Table 2. Freshwater cyanophages with completely sequenced genomes

Name	Family	Host	Genome structure	Genome size (bp)	No. of ORFs	% G+C	Coding % of genome	GenBank Accession number
Ma-LMM01	*Myoviridae*	*Microcystis*	Circularly permuted linear	162109	184	45.0	92.0	AB231700
S-CRM01	*Myoviridae*	*Synechococcus*	Circularly permuted linear	178563	294	39.7	89.0	HQ615693
Pf-WMP4	*Podoviridae*	*Phormidium*	Linear	40938	41	51.8	89.9	DQ875742
Pf-WMP3	*Podoviridae*	*Phormidium*	Linear	43249	41	46.5	89.2	EF537008
PP-1	*Podoviridae*	*Plectonema*	Linear	42480	41	46.4	93.1	Unpublished
PaV-LD	unassigned	*Planktothrix*	Linear	95299	142	41.5	89.5	HQ683709

have been found to have homologs in Pf-WMP3 or Pf-WMP4, indicating that PP-1 is relatively closely related. However, the unique genes in the PP-1 genome do not have homologs in the current databases (Zhou Y R, et al., 2013 submitted).

PaV-LD was isolated from Lake Donghu, China, and it can infect the harmful filamentous cyanobacterium *Planktothrix agardhii* (Gao E B, et al., 2009). The PaV-LD particles are tailless, and the virus is quite different from other known cyanophages. The average diameter of the particles is approximately 70 to 85 nm. The genome of PaV-LD is a linear, dsDNA of 95,299 bp in length without terminal repeats. It encodes 142 potential ORFs with lengths of 35 to 1,584 amino acids. Of these, 53 ORFs can be matched with homologs in other phages or microbes, and 29 of them have been assigned to known functional proteins, such as replicative DNA helicase (007R), cytosine-5-methyltransferase (010R), thymidylate kinase (021L), acetyltransferase (032L), protein phosphatase (037R), protein kinase (044L), nuclease (098R), flavin-dependent thymidylate synthase (115L), N-6-adenine-methyltransferase (117R), and crossover junction endodeoxyribonuclease (119L). Moreover, the unique genome carries a non-bleaching protein A (NblA) gene (ORF022L), which is present in all phycobilisome-containing organisms and mediates phycobilisome degradation (Gao E B, et al., 2012).

Diagnostic markers to detect freshwater cyanophages

Marker genes for cyanophages are helpful to determine the presence, diversity, and abundance of cyanophages in the environment. Currently, only the structural genes of cyanomyoviruses have been used as markers. The common diagnostic marker primers used for freshwater cyanophages are summarized in Table 3.

The gene *g91*, which encodes sheath protein, is used as the diagnostic marker of cyanophages related to Ma-LMM01 in freshwater. The primer set RTF/RTR for sheath protein amplifies a 132-bp section of *gp91*, which is conserved among the Ma-LMM01-type phages, such as Ma-LMM02, Ma-LMM03, and Ma-HPM05 (Takashima Y, et al., 2007). The primer set G20-2/CPS4 can amplify a fragment of the capsid-assembling protein gene *g20* from myoviruses (marine or freshwater) infecting *Synechococcus spp.* (Short C M, et al., 2005). Similarly, the primer set CPS1/CPS8 amplifies the region (~826 to 1376) of the *g20* (Wilhelm S W, et al., 2006).

Freshwater cyanomyoviruses infecting filamentous cyanobacteria appear to be genetically very different from marine cyanomyoviruses. The CPS1/2, CPS1/8, and g20fD2/CPS2 primer sets designed for cyanophages could not be used to identify the freshwater cyanophages AN-15, A-1(L), or N-1, which infect the filamentous cyanobacteria genera *Anabaena* and *Nostoc* (Baker A C, et al, 2006). However, the major capsid protein (MCP) primer set AN15 MCPF5/AN15 MCPR5 was able to amplify the target MCP gene from a range of freshwater cyanophages, resulting in PCR products of approximately 350 bp (Baker A C, et al., 2006).

Study of freshwater cyanophages started in the 1960s and initially focused on the use of cyanophages to control cyanobacterial blooms. Freshwater cyanophages belonging to different families have been discovered, and to date, six viral genomes have been completely sequenced. With new techniques, such as metagenomics, it is expected that many more freshwater cyanophages will be discovered in the future. It is likely that functional genomics will help us to understand the common and specific features of freshwater and marine cyanophages, the interaction between cyanophages and their hosts, and the effects of cyanophages on fresh water environments.

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Table 3. Diagnostic markers for freshwater cyanophages

Family	Host	Gene	Gene product	Primer sequences (5′-3′)	References
Myoviridae	*Microcystis*	g91	Sheath protein	Sheath RTF: ACATCAGCCTTCGTTTCGG Sheath RTR: CAATCTGGTTAGGTAGGTCG	Takashima Y, et al., 2007
	Anabaena and *Nostoc*	MCP	Major capsid protein	AN15 MCPF5: GTTCCTGGCACACCTGAAGCGT AN15 MCPR5: CTTACCATCGCTTGTGTCGGCATC	Baker A C, et al., 2006
	Uncertain	g20	Viral capsid assembly protein	CPS1: GTAGWATTTTCTACATTGAYGTTGG CPS8: AAATAYTTDCCAACAWATGGA	Wilhelm S W, et al., 2006
	Synechococcus	g20	Viral capsid assembly protein	CPS4: CATWTCWTCCCAHTCTTC G20-2: SWRAAATAYTTICCRACRWAKGGATC	Short C M, et al., 2005

The codes for mixed bases are: R = A, G; Y = C, T; M = A, C; K = G, T; S = C, G; W = A, T; H = A, C, T; B = C, G, T; V = A, C, G; D = A, G, T; n = A, G, C, T; I = inosine.

Author Contributions

All authors carried out the work presented here. HX and ZH defined the theme of this review and wrote the paper, and TL and FD reviewed and edited the paper.

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