AN ABSTRACT OF THE THESIS OF

Erin W. Riscoe for the degree of Honors Baccalaureate of Science in Microbiology presented on March 9th, 2011. Title: Survey of Cyanophages: A Study of the Bacteriophages associated with Pacific Northwest Cyanobacterial blooms

Abstract approved:

Dr. Theo Dreher

Cyanobacterial blooms are a growing concern in the Pacific Northwest because of their threat to watershed health and drinking water supplies. Bacteriophages are pathogens of bacteria and may contribute to the growth and decline of seasonal blooms. Bacteriophage S-CRM01, a lytic T4-type myophage, has been isolated from a freshwater strain of *Synechococcus* (Dreher et al., 2011). This phage was isolated from a cyanobacterial bloom of primarily toxic *Microcystis aeruginosa*, but an endemic *Synechococcus* lineage, that was a minor constituent of the bloom, is the actual host for this phage. This survey was conducted to assess the distribution of phage S-CRM01 in Oregon and Northern California in bodies of water with cyanobacterial blooms. On the basis of PCR identification of plaques on LC16 cyanobacteria plates, S-CRM01 was present across a length of about 250 km along the Klamath River valley, from the Williamson River Delta at the northern end of Upper Klamath Lake as far downstream as Seiad Valley in 2009. Although this phage has not been shown to infect toxic cyanobacteria, it is still valuable in the freshwater phage community where few genomes have been sequenced.

Key words: *Synechococcus,* freshwater bacteriophage, *Myoviridae*, harmful algal blooms, Klamath Basin, T4-like phage

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Survey of Cyanophages: A Study of the Bacteriophages associated with Pacific Northwest Cyanobacterial blooms

By

Erin W. Riscoe

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I understand that my project will become a part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

Erin W. Riscoe, Author

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Introduction

Cyanobacterial blooms are a growing concern in the Pacific Northwest because of their threat to watershed health and drinking water supplies. Also, future climatic change scenarios predict rising temperatures, enhanced vertical stratification of aquatic ecosystems, and alterations in seasonal and interannual weather patterns; these changes all favor harmful cyanobacterial blooms in eutrophic waters (Paerl and Huisman, 2009).

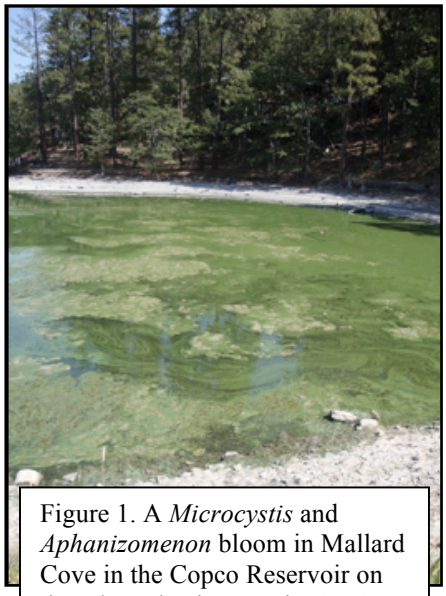


Figure 1. A *Microcystis* and *Aphanizomenon* bloom in Mallard Cove in the Copco Reservoir on the Klamath River Basin, 2008.

The phylum Cyanobacteria makes up the largest and most diverse group of photoautotrophic bacteria. Although cyanobacteria are gram-negative bacteria, their photosynthetic system is similar to that of the eukaryotes because they use chlorophyll *a* and photosystems I/II to carry out oxygenic photosynthesis. The electron transport chain and photosynthetic pigments are housed in the thylakoid membranes. These membranes are lined with phycobilisomes that contain phycocyanin and phycoerythrin: pigments that transfer energy to photosystem II. Carbon dioxide is fixed through the Calvin Cycle and the enzymes needed for this process are held in carboxysomes. Because cyanobacteria lack the enzyme alpha-ketoglutarate dehydrogenase, the pentose phosphate pathway is the route for carbohydrate synthesis, rather than the citric acid cycle that is used in plants.

Also known as "blue-green algae," cyanobacteria are very diverse in their appearance and shape. They may be unicellular, form filaments, or live as colonies, and can range in diameter from 1 to 10 μm. Some cyanobacteria can utilize a process known as phototaxis to help them thrive in fresh and marine waters; they use gas vacuoles to position themselves in optimum light intensity in the water column.

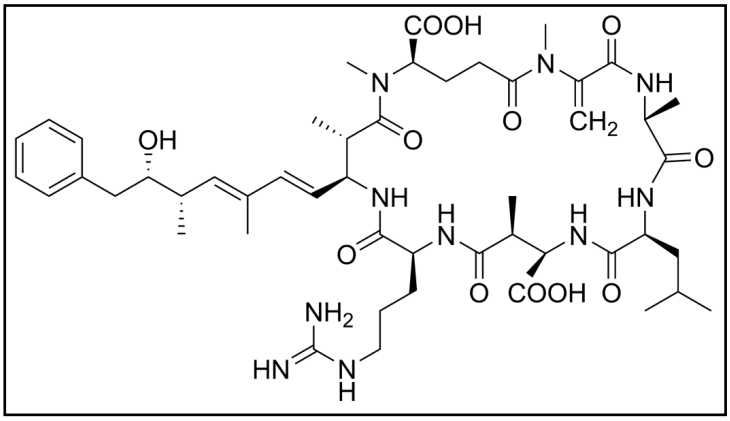
In eutrophic bodies of water, cyanobacteria can reproduce rapidly to form blooms. These organisms pose a challenge for those living nearby to lakes or reservoirs with blooms, mainly because of the threat they pose to health. Humans who drink or swim in water that contains high concentrations of cyanobacteria or cyanobacterial toxins may experience gastroenteritis, skin irritation, allergic responses, or liver damage (http://www.cdc.gov/hab/). These impacts stress the importance of understanding harmful algal blooms and developing tools to help predict, control, and prevent the events.

The release of copious amounts of organic matter upon their death fuels the growth of other microbes that deplete available oxygen as a result of their respiratory activity. For this reason, and also the fact that some species can produce toxins, blooms are harmful to fish and other organisms that come into contact with the water. *Microcystis* is a genus of cyanobacteria common to the Pacific Northwest that produces microcystin, a cyclic peptide hepatotoxin, which is harmful to humans and other mammals (Campos and Vasconcelos, 2010). The preferential location of microcystins to the cell's thylakoids suggests that the molecule may play an important role in light harvesting and adaptation responses (Young et al,. 2005). Another possible function of these secondary metabolites relates to metal chelating. Microcystins have an affinity to metal cations due to their

multiple carbonyl and amine groups on the cyclic ring. Complexes of microcystins and metal ions have been detected with mass spectrometry in a recent study. This study showed that microcystins might play a role in metal ion uptake and accumulation in the algal cells (Saito et al., 2008).

Outside of the cyanobacterial cells, microcystins have toxic effects on mammalian cells. These bioactive secondary metabolites of *Microcystis* mainly affect hepatocytes and macrophages. In hepatocytes, the toxin acts as an inhibitor of protein phosphatase (1 and 2A) and an activator of phospholipase A_2 and cyclooxygenase. The hyperphosphorylation (because of lack of phosphatase activity) of cytokeratins induces changes in cell shape and rearrangement of intermediate filaments in the cytoplasm. On the other hand, in macrophages, the toxin induces tumor necrosis factor-alpha and interleukin-1. These cytokines induce the production of platelet activating factor, which causes the activation of cyclooxygenase (Devlin, 2006). Thromboxanes and prostaglandins, as chemical mediators of inflammation, are then produced by cyclooxygenase (Campos and Vasconcelos, 2010; Devlin, 2006). This increases blood clot formation, pain, inflammation, and fever (Devlin, 2006).

Figure 2. Microcystin-LR chemical structure



There are many factors that influence bloom growth and decline over a season. These factors include but are not limited to: eutrophication, stratification, and loss of population control derived from predation or competition with other organisms. Bacteriophages are one factor capable of controlling cyanobacterial blooms, though the extent and precise influence is not well understood yet. Cyanophages are bacteriophages that specifically infect cyanobacteria. It has been established that the seasonal changes in the abundance of cyanophages and cyanobacteria suggest that infection by a cyanophage can be an important factor for the collapse of a bloom (Manage et al., 1999). The only currently available genome sequence from a phage infecting freshwater *Microcystis areuginosa* is that of the myophage Ma-LMM01 from a Japanese lake (Yoshida et al., 2008). Additionally, the Dreher team recently described the genome sequence and other properties of a cyanomyophage (S-CRM01) isolated from Copco Reservoir on the Klamath River in Northern California in September 2008. The phage was isolated from a toxic *M. aeruginosa* bloom, but an endemic *Synechococcus* lineage is the actual host for

this phage (Dreher et al., 2011). *Synechococcus* is a widespread, non-toxic, prokaryotic, autotrophic, marine and freshwater cyanobacterium.

Bacteriophages are pathogens of bacteria. They are significant members of terrestrial and aquatic ecosystems and are proposed to be the most abundant form of life of the biosphere (Kwan et al., 2005). Some prokaryotic viruses are virulent viruses; shortly after infecting their host, they begin reproduction. This is known as the lytic life cycle. To begin their life cycle, bacteriophages fasten to specific surface receptors (Yu and Mizushima, 1982). For example, these receptors can be cell wall lipopolysaccharides, teichoic acids, pili and flagella. At the end of their life cycle, they lyse their host cell to release new virions.

Until recently, capsid structure was a critical phenotypic trait used to classify viruses of prokaryotes; taxonomists now focus more on molecular and genomic data. Analysis of viral genomes provides insight into the evolution of viruses and their hosts. Phages demonstrate a wide degree of diversity in terms of genome structure. Although most possess double stranded DNA genomes, phage with single stranded DNA, single stranded RNA, and double stranded RNA genomes have been identified (Miller et al., 2003). The DNA genome serves as the template for mRNA synthesis, and the mRNA molecules made are translated to yield viral proteins. Then, DNA replication ensues and more viral genomes are made. The well-studied T-even phages of *E. coli* are some of the most intricate bacteriophages known; they contain dsDNA with contractile tails and are in the family *Myoviridae*. The formation of concatemers during replication of the T4 genome is an important step in phage reproduction. During assembly of the virions, the phage head is filled with DNA cleaved from one concatemer. Each virion contains a

different DNA fragment because slightly more than one full set of T4 genes is packaged into each head. However, if and when each genome is circularized, the sequence of genes would be the same (Miller et al., 2003). Glances into bacteriophage genomes have provided new paradigms for the evolution of viruses and their hosts. As more viral genomes are analyzed and catalogued, a greater understanding of their evolution and of their host's evolution will ensue.

Based on previously described marine and freshwater phages, there are expectations of the types of viruses that will be found associated with cyanobacterial blooms. More than 5000 phages have been observed and described, and 96% are tailed (Ackerman, 2001). T4-type myophages, a family within the tailed phages group, is extraordinarily widespread and diverse throughout the aquatic biosphere (Filee et al., 2005). Bacteriophage S-CRM01, a lytic T4-type myophage, has been isolated from a freshwater strain of *Synechococcus* and shown to be present in the upper Klamath River valley in northern California and Oregon (Dreher et al., 2011). The phage was isolated from a toxic *M. aeruginosa* bloom, but an endemic *Synechococcus* lineage is the actual host for this phage (Dreher et al., 2011). *Microcystis* has been shown to be difficult to cultivate on agar plates, therefore, it can be surmised that the *Synechococcus* has a lawn growth advantage in the top agar plaque assays. A possible explanation for this phenomenon is that *Microcystis* preferentially grows in liquid culture, while Synechococcus is more flexible with its cultivation methods.

Ma-LMM01, another lytic myophage, was isolated from freshwater in Japan and found to infect toxic *M. aeruginosa* (Yoshida et al., 2007). Both of these phages are

examples of the T4-type myophages that can be expected to be associated with freshwater cyanobacterial blooms.

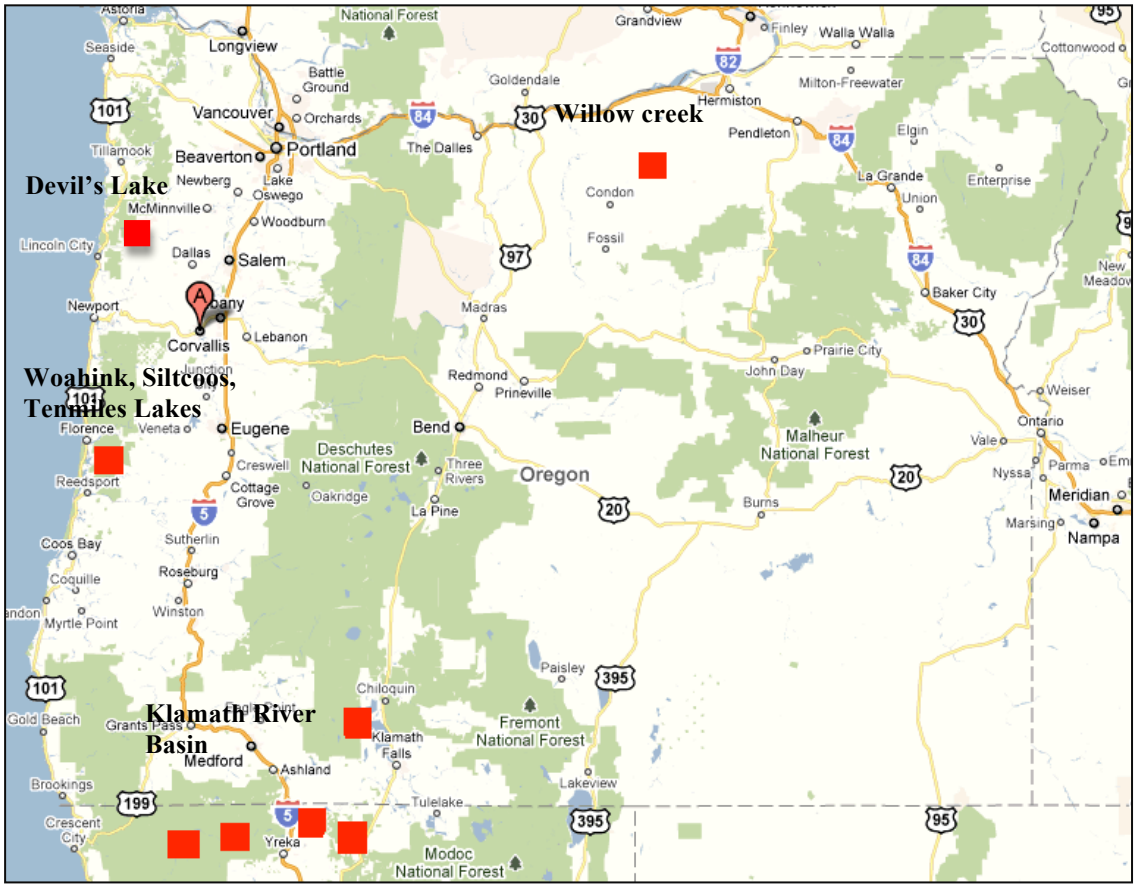
In order to determine possible strain differences and geographic distribution, genetic comparison of novel Pacific Northwest bacteriophages to previously isolated phages of the families *Myoviridae* and *Siphoviridae* was undertaken. This study's explorative efforts will contribute to understanding the distribution of *Synechococcus*-associated bacteriophage throughout the Pacific Northwest and may provide insights into the possible role they play in the cyanobacterial evolution and bloom growth cycle. This view is based on the fact that the previous discovery of phages has led to insights about host-phage relationships, such as the Ma-LMM01 phage that uses diverse genetic strategies to control its host's photosynthesis system (Yoshida et al., 2007).

Materials and Methods

Isolation of phage from freshwater sources through surface grab samples

The freshwater sampling sites from the 2009 bloom season were as follows: Copco Reservoir, Iron Gate Reservoir, Upper Klamath Lake, three Klamath River sites (Brown Bear, Seiad Valley and Happy Camp), Klamath River Estuary, and Williamson River delta in the area straddling the California-Oregon border. Additional samples were taken from Oregon's coastal region in Lincoln City's Devil's Lake and south of Florence, specifically Siltcoos Lake, Tenmile Lake, and Woahink Lake. Lastly, samples were also taken from Willow Creek Reservoir near Heppner, northeastern Oregon. These sites were chosen based on frequency of past harmful algal blooms (HABs) according to the NOAA and the Oregon Department of Health Services. A 200 mL sample was taken from the top six inches of water and refrigerated. This sample was then filtered through 47mm diameter, 0.2 μm pore-size disc filters (Supor-200; Pall Life Sciences). For long-term storage, one part of chloroform to ninety-nine parts of sample was added (chloroform destroys bacterial viability by disrupting the cell membrane, but most bacteriophages are unaffected).

Figure 3. Map of sampling sites in January through October 2009, Google Maps



Laboratory growth and storage parameters for cyanobacterial cultures

Cyanobacterial cultures for uses in top agar plaque assays for lawn growth were kept in sterile glassware at 25°C in BG11 medium (Rippka et al., 1979). Cultures were transferred into sterile flasks and diluted 1:10 in fresh sterile BG11 medium once every seven days to continue exponential growth. Strain LC16 was kept at 20 µE/m^2/sec of light (measured as photosynthetically active radiation), and LC18 at 10 µE/m^2/sec. One

Einstein unit is the energy per mole of photons carried by a beam of monochromatic light.

Putative phage amplification in liquid culture and concentration through ultracentrifugation

Amplification of putative bacteriophage in host strains of cyanobacteria is a vital step because, while viral abundance in many aquatic environments exceeds 10^6 viruses per ml, the frequency of even the most abundant strain within the assemblage is less than 1%. Therefore, a greater than 100-fold concentration of virus particles is necessary to obtain sufficient material for downstream analyses (Clokie et al., 2009)

Any chloroform that was added to the filtered lake water was removed with a pipet and the water was warmed to room temperature. Sterile 500 mL flasks containing sterile 1X BG11 nutrients were used as growth media for the cyanobacteria. One mL of each of the two exponential strains of cyanobacteria kept in liquid culture was added to 200 mL of filtered lake water/phage suspension, in order to select for cyanophages that infect any one of those particular strains. LC16 and LC18 were names given to the cyanobacterial strains used in this study. Both strains were derived originally from different blooms comprised mainly of *Microcystis* and are sustained in the lab year-round for use in a myriad of biological experiments.

Near September 2009, it was noted that the LC16 and LC18 liquid culture strains that were isolated from blooms primarily comprised of *Microcystis* had become outgrown by a strain later identified as *Synechococcus*. *Synechococcus* is a common non-toxic cyanobacterium that is thought to be almost ubiquitously present in freshwater bodies of

water. The morphology change was such that the colonies became similarly diffuse and lighter green in color. Also, it was obvious that the individual cells were significantly smaller by microscope observation. PCR directed at the ribosomal operon internal transcribed spacer (ITS) locus was used to identify both LC16 and LC18 cultures as belonging to the *Cyanobium gracile* cluster of freshwater *Synechococcus* {Ernst, 2003 #1962} {Chen, 2006 #1963} (Connie Bozarth, pers. commun.). It could not be determined exactly when this contamination occurred, but after extensive PCR investigation, it was found that *Synechococcus* was the predominant host present in top agar plaque assays throughout the 2009 bloom season and used in this study (Connie Bozarth, pers. commun.).

This suspension was allowed to incubate in the culture room for seven days. After incubation, chloroform was added again, in the same proportion, to lyse the cyanobacteria and release maximum amounts of cyanophage from inside their host cell. This suspension was centrifuged at 4,000 g, at 4°C, for 15 minutes, in a Beckman-Coulter centrifuge with a fixed angle rotor to pellet the bacterial debris. If the cyanobacterial debris did not pellet, then the suspension was chilled in the dark for 2 hours, to ensure that any intact cyanobacteria did pellet (cyanobacteria have gas vesicles that enable them to float and they empty those vesicles when they are in the dark). The phage-containing supernatant was filtered through a 47 mm diameter, 0.2 μm-pore-size disc filter and stored in the dark at 4°C until the next step. Using a Ti-60 fixed angle rotor, each viral preparation was ultracentrifuged at 50,000 rpm at 4°C for 90 minutes. Afterwards, the viral pellet was resuspended in 50 μL of SM bacteriophage dilution buffer.

Ultracentrifugation of the post-enrichment viral preparation served to concentrate the sample for further analyses.

Top - agar plaque assay

Top-agar plaque assays were performed to estimate viral titer, to purify phage, and to evaluate phage host range. These assays were scored as plaques or areas of clearing in cellular lawns caused by viral lysis of bacteria. It is assumed that each plaque represents one viral clonal isolate. BG11 agar plates were used to test combinations of concentrated phage preparation against each individual lab strain of cyanobacteria*:* LC16 and LC18. The amounts of viral preparation, liquid culture cyanobacteria, and BG11 agar were 10 µL, 2 mL and 2.5 mL, respectively. Plates were incubated in a light and temperature controlled room (25ºC) until plaques were visible. The amount of time for plaques to become clearly visible ranged from 7 to 14 days. Plaques were counted, picked, and resuspended in 50 µL of SM bacteriophage dilution buffer, and stored in 4ºC for future analysis.

Polymerase chain reaction for genetic characterization of environmental sample phages

In order to directly search for closely related phage from different blooms and seasons, the resuspended plaque-isolated phage were used as template DNA in PCR reactions. Each plaque-isolated phage sample was used in two separate reactions with primer pairs designed from two 2008 *Synechococcus*-associated bacteriophages (Families *Myoviridae* and *Siphoviridae*) isolated from the Klamath Basin area.

These phages are named CRM01 and CRS01. CRM01 is a myophage; the first primer pair designed for it was directed at gp34, which is uniquely homologous to g163 in the Ma-LMM01 phage genome. Other related cyanomyophages do not possess this gene. The nucleotide sequences are 5'-GTCAAATAGAATCCAGGATGAATTA and TACCATAGTCTCCACCGTTTC. The second set of primer pairs is directed towards the gp23 capsid structural protein. The nucleotide sequences are 5'-GACGTATGTGGCGTTCAGCCAATGA and CGGTTGATTTCTGC AAGGATTTC. These primer pairs give expected product sizes of 350 base pairs and 498 base pairs.

CRS01 is a siphophage and the two primer pairs designed for its detection correspond to a tail tape measure protein and a helicase. The putative tail tape measure protein's respective primer sequences are 5'-TTGTTGAGGGCCTTGTTGTACT for the upper primer and AGATCGTGGTCGCCGAATAC for the lower primer. The putative helicase protein's respective primer sequences are 5'-CCGACGTGATCCAACACGAT for the upper primer and ACAAGCGGATGGGCGACTAC. These primer pairs give expected product sizes of 394 base pairs and 424 base pairs, respectively.

Each reaction was in a total volume of 25 µL, and contained the following: 2.5 µL 10X PCR reaction buffer (200 mM Tris pH 8.4, 500 mM KCl), 500 µM dNTPs, 2.5 mM $MgCl_2$, 500 µM upper primer, 500 µM lower primer, 0.4 µL of 2.5 U/µL homemade Taq polymerase, and 1 µL template plaque-isolated DNA. The plaque-isolated phage samples (used as template DNA) were heated at 37ºC for 10 minutes prior to addition to reaction cocktail to melt the top-agar plug. All samples were subjected to an initial denaturing step at 95ºC for 5 minutes, then the following 30 cycles consisted of a 30 second denaturing step at 95ºC, a 30 second annealing temperature between 55 and 60ºC, and a 60 second

extension step at 72ºC. A final 10 minutes extension step was carried out at 72ºC. Amplifications of the target region were checked by electrophoresis on a 1.0% agarose gel stained with ethidium bromide.

Results

Bacteriophages thought to be specific to the LC16 strain of cyanobacteria were isolated from the Brown Bear Lower Klamath River site (8/6/09, 9/15/09, 10/1/09), Irongate Reservoir (8/18/09, 9/15/09, 10/13/09), Willow Creek Reservoir (10/4/09), Klamath River Estuary (10/8/09), and the Williamson River delta (10/15/09). Table 1 describes the sampling raw data from these locations, while Table 2 reports the PCR results.

Each plaque-isolated phage sample was used in two separate PCR reactions with primer pairs designed from two 2008 *Microcystis* bloom-associated bacteriophages (Families *Myoviridae* and *Siphoviridae*) isolated from the Klamath Basin area, in order to directly search for closely related phage across different blooms and seasons. PCR products were visualized by gel electrophoresis, stained with ethidium bromide. S-CRM01 is a myophage; the first primer pair designed for it is directed at gp34, a region that is similar to Ma-LMM01 phage genome. This coding region is also quite unique to S-CRM01 and not to others within the *Myoviridae* family. The second set of primer pairs is directed towards the gp23 structural capsid protein, common to all cyanomyophages. These primer pairs give expected product sizes of 350 base pairs and 498 base pairs. CRS01 is a siphophage and the two primer pairs designed for its detection correspond to a tail tape measure protein and a helicase. None of the environmental samples from the 2009 bloom season gave products with the CRS01 primer pairs. Figure 4 is the electrophoresis gel from the PCR reaction of ten Klamath River plaque samples late in the bloom season. This gel showed the expected PCR products with the two different

primer pairs derived from phage S-CRM01. Overall, the products given to all ten plaque samples in this gel show that this specific bacteriophage from the Upper Klamath River was very closely related to S-CRM01.

Bacteriophages specific to the LC18 strain of *Microcystis* were isolated from the Willow Creek Reservoir enclosures (7/6/09, 8/18/09, 9/4/09, 10/15/09), Irongate Reservoir (8/18/09, 10/13/09), Devil's Lake (8/18/09, 10/1/09), Black Bear Lower Klamath River site (9/3/09, 9/15/09), Upper Klamath Lake (8/18/09, 10/7/09), Happy Camp site on the Lower Klamath River (9/15/09), Klamath River Estuary (10/8/09), and the Williamson River Delta (10/15/09). It is important to note that the Willow Creek Reservoir enclosures were three sampling sites near the banks of the reservoir that were pooled together as one sample when returned to the lab. Table 1 describes the sampling raw data from these locations, while Table 2 organizes the PCR results.

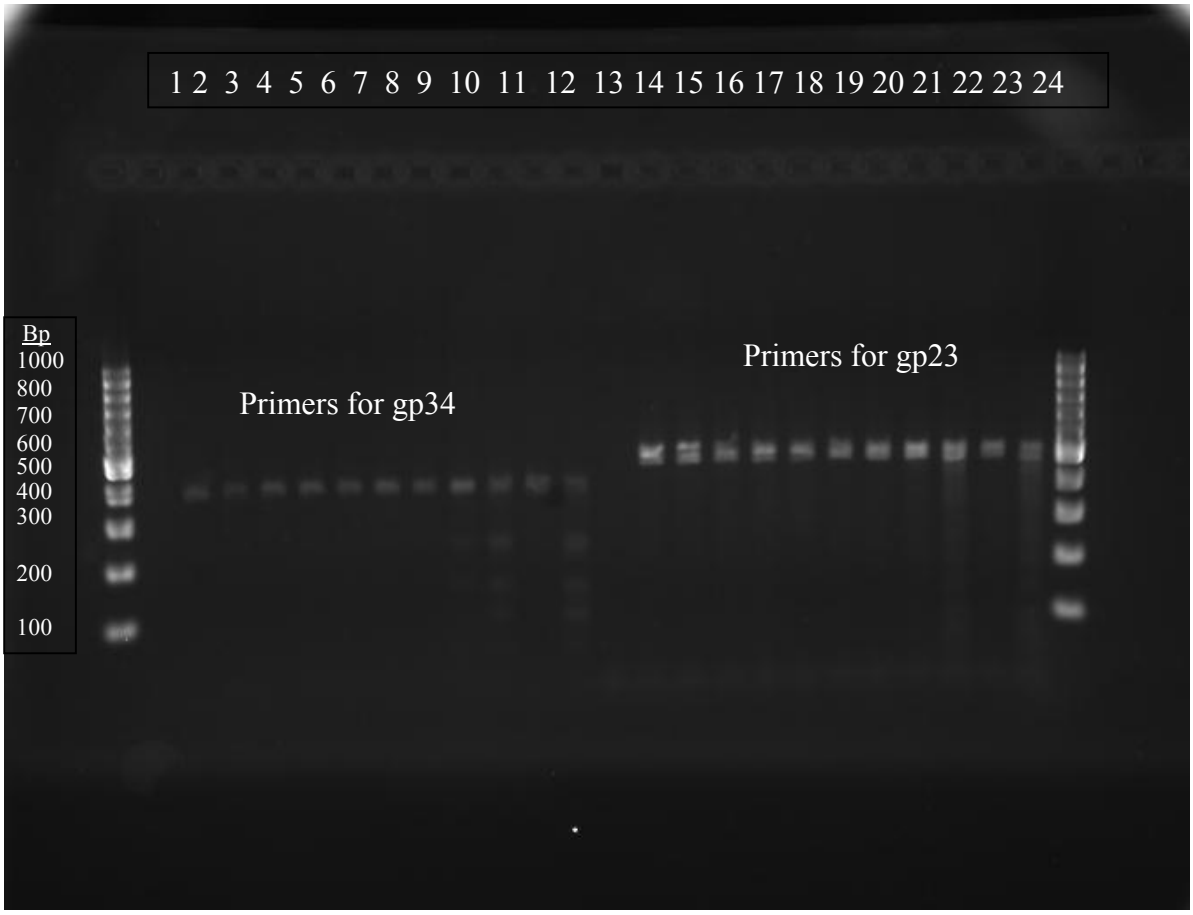
None of the environmental samples from the 2009 bloom season gave products with the CRS01 primers, which were specific to the siphophage previously isolated in the lab from the Copco Reservoir in 2008. As can be observed from Figure 5, the expected base pair size was 494 bp. The band in this gel was a PCR product from stock of CRS01 primed with putative helicase primers.

Table 1. Summary of initial isolation of bacteriophage in top agar plaque assays

Date	Lab Name	Source	Plaque Assay (Algal culture used, # of plaques, morphology, diameter)
1/14/09	CL 120	Tahkenitch Lake	-
	CL 122	Woahink Lake	-
	CL 124	Siltcoos Lake	-
	CL 126	Tenmile Lake	-
4/29/09	CL 131	Woahink Lake	-
	CL 134	Siltcoos Lake	-
	CL 138	Tenmile Lake	-
6/8/09	CL 142	Devil's Lake	-
6/12/09	WC 21	Willow Creek Lake	-
	WC 22-24	Willow Creek	-
6/17/09	CL 143	Woahink Lake	-
	CL 145	Siltcoos Lake	-
	CL 147	Tenmile Lake	-
	CL 149	Devil's Lake	-
6/21/09	WC 26	Willow Creek Lake	-
	WC 27-29	Willow Creek	-
7/6/09	WC 30	Willow Creek Lake	-
	WC 31-33	Willow Creek	LC18, 13, 0.5 cm diameter, even edges
7/8/09	CL 152	Devil's Lake	-
7/27/09	CL 154	Woahink Lake	-
	CL 156	Siltcoos Lake	-
	CL 158	Tenmile Lake	-
8/4/09	WC 36	Willow Creek Lake	-
	WC 37-39	Willow Creek	LC18, 1, 2 cm diameter even edges
8/6/09	CR 74	Brown Bear	LC16, ~100, very small plaques .25 cm diameter
8/11/09	CL 160	Devil's Lake	-
8/18/09	CR 77	Irongate	LC16, 50-100, very small plaques .20 cm diameter LC18, 200+, very small plaques .1 cm diameter
	CR 78	Copco	-
	WC 40	Willow Creek Lake	-
	WC 41-43	Willow Creek	LC18, 16, evenly distributed and 1cm diameter
8/19/09	CL 163	Devil's Lake	LC18, 6, some large and some small diameter
9/3/09	CR 82	Brown Bear	LC18, 70, very small, 1mm diameter
9/4/09	WC 45-47	Willow Creek	LC18, full plate lysis
	WC 48	Willow Creek Lake	-
9/14/09	UKL 26	Upper Klamath Lake	LC18, 7, 0.3-0.5 cm diameter

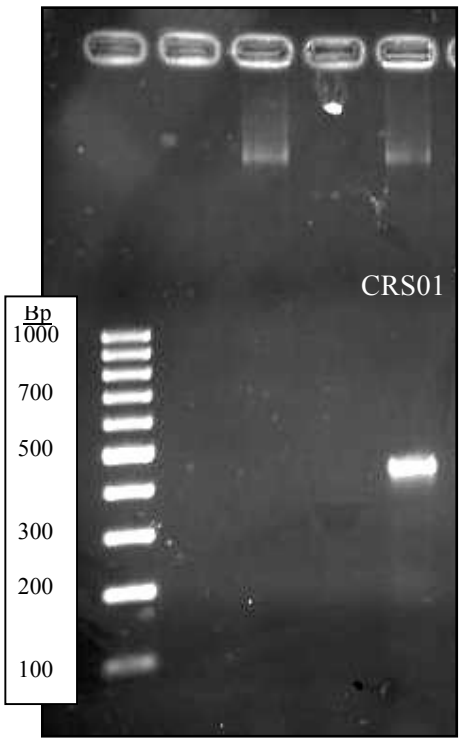
Date	Lab Name	Source	Plaque Assay (Strain, # of plaques, morphology)
9/15/09	CR 85	Irongate	LC16, 100, diameter 0.2 cm
	CR 87	Brown Bear	LC16, 15, defined and diameter 0.3 cm
	CR 89	Happy Camp	LC18, TNTC, tiny but defined
10/1/09	CR 90-92	Klamath River	LC16, 40, 0.2 cm diameter
	CL 164	Devil's Lake	-
10/4/09	WC 51	Willow Creek Lake	LC16, 1, irregular edges LC18, 100, 0.3 cm diameter
	WC 52-54	Willow Creek	-
10/7/09	UKL 27	Upper Klamath Lake	-
10/8/09	KES 1-2	Klamath River Estuary	LC16, TNTC, tiny LC18, TNTC, tiny
10/13/09	CR 93	Klamath River	-
	CR 94	Klamath River	LC16, 5, 0.2-0.4 cm diameter LC18, TNTC, tiny
10/15/09	NC 1-2	Williamson River Delta	LC16, 2, 0.2 cm diameter LC18, full plate lysis

Figure 4. PCR products of an environmental sample using two primer pairs from CRM01 Myophage



This sample was from the Klamath River during October 2009. The primer pair used in the first 12 lanes were derived from Contig 33F. The last 12 lanes in this gel contained products primed with a sequence specific to the gp23 structural protein. The numbers above designate the lane numbers. Lanes 1 and 13 are blanks. 100 base pair ladder was used.

Figure 5. PCR product of CRS01 Siphophage using primer pair directed at putative helicase sequence



As can be observed above, the expected base pair size was 424 bp with this primer pair. The lane on the far right of this figure above was a PCR product from stock of CRS01 phage primed with the putative helicase primer pair.

Table 2. PCR Results of the 2009 HAB season bacteriophage plaque samples

A summary of phages isolated during the 2009 Pacific Northwest bloom season from top agar plaque assays (recorded in Table 1). The *Microcystis* host strain refers to the strain of *Microcystis* (from the Dreher Lab collection) that the specific phage infected during its primary top agar plaque assay. The last two columns describe whether or not the phage isolated from the top agar plaque assays gave a PCR product with the given primers specific to two phages isolated from the Klamath Area in 2008: CRM01 (family *Myoviridae*) and CRS01 (family *Siphoviridae*).

Sample Date	Source	*Microcystis* host strain	PCR products against 2008 Myophage Primers?	PCR products against 2008 Siphophage Primers?
7/6/09	Willow Creek	LC18	No	No
8/4/09	Willow Creek	LC18	No	No
8/6/09	Klamath River BB	LC16	Yes	No
8/18/09	Irongate Reservoir	LC16	Yes	No
8/18/09	Willow Creek	LC18	No	No
8/19/09	Devil's Lake	LC18	No	No
9/3/09	Klamath River BB	LC18	No	No
9/4/09	Willow Creek	LC18	No	No
9/14/09	Upper Klamath Lake	LC18	No	No
9/15/09	Irongate Reservoir	LC16	Yes	No
9/15/09	Klamath River BB	LC16	Yes	No
9/15/09	Klamath River SV	LC16	Yes	No
10/1/09	Klamath River (pooled)	LC16	Yes	No
10/13/09	Klamath River site 9228	LC16	Yes	No
10/15/09	Williamson River delta	LC16	Yes	No

Conclusions

This survey was conducted to assess the distribution of S-CRM01 between Upper Klamath Lake and the Klamath River estuary. On the basis of PCR identification of plaques on LC16 cyanobacteria plates, S-CRM01 was present across a length of about 150 miles along the Klamath River valley, from the Williamson River Delta at the northern end of Upper Klamath Lake as far downstream as Seiad Valley. This phage was not present at the Willow Creek reservoir or any of the coastal lake samples, although further analysis would be needed to characterize the *Synechococcus*-associated phage found at these sites.

The isolation and biological characterization of this newly identified group comprised of freshwater bacteriophages, coupled with the sequencing of their genomes, knowledge of host range and geographic prevalence, may be informative in better understanding phage evolution and bloom growth regulation. This view is based on the fact that the previous discovery of phages has led to insights about host-phage relationships, such as Ma-LMM01 phage, which uses diverse genetic strategies to control their host's photosynthesis system (Yoshida et. al, 2007). It is interesting to note some of the patterns seen throughout the isolation process: many more phages were isolated late in the bloom season (August-October), and phages were consistently isolated from certain sample sites, such as the Willow Creek Reservoir and the Lower Klamath River. The phages in this study were isolated from areas where a *Microcystis aeruginosa*-predominated bloom was present, but an endemic *Synechococcus* lineage was found to be

the actual host after extensive scrutiny. *Synechococcus* is a common freshwater and marine cyanobacterium that is usually found in conjunction with other species, but is not the main species contributing to the bloom. We observed, through microscopy, that small amounts of *Synechococcus* were attached to the aggregate colonies of *Microcystis* being maintained in the lab. It is surmised that over time, the *Synechococcus* out grew (or was accidently selected for) the *Microcystis* in liquid culture. It is clear that all of the phages in this study infect *Synechococcus*, and not *Microcystis*, even though they were isolated from blooms predominated by *Microcystis*.

Harmful algal blooms deplete the oxygen and block the sunlight that other aquatic organisms need to live, and certain cyanobacteria, including species common to the Pacific Northwest, can release toxins that are dangerous to animals and humans. These impacts should stress the importance of understanding algal bloom dynamics and their evolution over a bloom season. As more viral genomes are analyzed and catalogued, a greater understanding of their evolution and of their host's evolution will surely follow.

The genetic comparison between this freshwater phage and its marine relatives is also valuable. Analyses based on gene sequence and gene content indicates a close phylogenetic relationship to the marine cyanomyophages infecting *Synechococcus* and *Prochlorococcus* (Dreher et al., 2011). Such relatedness suggests that freshwater and marine phages can draw on a common gene pool; 29% of its total ORFs contain gene homology to at least one of 17 closely related myophages that lytically infect marine *Synechococcus* and *Prochlorococcus* (Dreher et al., 2011). This data supports the argument that related phages can be found in marine and freshwater environments.

The data from this study could also contribute to further research pertaining to

controlling Pacific Northwest harmful cyanobacterial blooms, defining the cyanobacteria-cyanophage relationship over the course of a bloom season, or phage as an influential factor in bloom growth and decline. Although this phage has not been shown to infect toxic cyanobacteria, the information gathered about this phage is valuable in the freshwater phage community where few genomes have been sequenced.

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