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Identification of a Diagnostic Marker To Detect Freshwater Cyanophages of Filamentous Cyanobacteria†

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Cyanophages are viruses that infect the cyanobacteria, globally important photosynthetic microorganisms. Cyanophages are considered significant components of microbial communities, playing major roles in influencing host community diversity and primary productivity, terminating cyanobacterial water blooms, and influencing biogeochemical cycles. Cyanophages are ubiquitous in both marine and freshwater systems; however, the majority of molecular research has been biased toward the study of marine cyanophages. In this study, a diagnostic probe was developed to detect freshwater cyanophages in natural waters. Oligonucleotide PCR-based primers were designed to specifically amplify the major capsid protein gene from previously characterized freshwater cyanomyoviruses that are infectious to the filamentous, nitrogen-fixing cyanobacterial genera *Anabaena* and *Nostoc*. The primers were also successful in yielding PCR products from mixed virus communities concentrated from water samples collected from freshwater lakes in the United Kingdom. The probes are thought to provide a useful tool for the investigation of cyanophage diversity in freshwater environments.

The first report of a cyanophage infecting a freshwater cyanobacterium was provided by Safferman and Morris (31), who isolated the podovirus LPP-1, infectious to the filamentous cyanobacterial genera *Lyngbya*, *Plectonema*, and *Phormidium*. Since this initial isolation, strains of the LPP-1 group have been found in numerous habitats, including lakes, fishponds, and sewage settling ponds (2, 27). Freshwater cyanophages that exhibit the myovirus and siphovirus morphology have also been isolated, including AS-1, a myovirus infectious to the unicellular hosts *Anacystis* and *Synechococcus* (32), and S-1, a siphovirus infectious to *Synechococcus* (1). The vast majority of studies on freshwater cyanophages were conducted during the 1960s and 1970s; consequently, limited knowledge of their abundance and diversity exists. A few more recent studies have provided estimations of freshwater cyanophage abundance, including that of Manage et al. (21), who estimated concentrations of freshwater cyanophage infectious to *Microcystis aeruginosa* at 10^4 PFU/ml in a pond in Japan. Tucker and Pollard (38) also recently reported the successful isolation of a lytic cyanophage infectious to *Microcystis* from a lake in Australia. This cyanophage, Ma-LBP, was reportedly found at densities as high as 5.56×10^4 ml^{-1} and plays an important role in *Microcystis* population dynamics.

There are very few molecular studies of freshwater cyanophages, with the vast majority of research focused on their marine counterparts (35). Cyanophages are considered to be key components of aquatic microbial communities, influencing host populations (12, 20), biogeochemical cycling (13), and primary production (36). The study of freshwater cyanophages is therefore imperative to enable a fuller understanding of the functioning of aquatic microbial communities. PCR-based probes have been successfully used to study marine cyanophage communities following the development of primers that amplify the capsid assembly gene, g20 (6, 45). PCR products generated using g20-specific primers have subsequently been used either as tools for studies of the presence or absence of marine cyanophages or in studies to determine cyanophage diversity by combining PCR with sequencing (45), terminal restriction fragment length polymorphism (39), or denaturing gradient gel electrophoresis (DGGE) (43).

Bacteriophage taxonomy is largely based on their morphology (5); however, genotypic analysis of similar morphotypes often identifies sequence homologues between bacteriophages that infect genetically distant hosts. There are numerous examples of this, particularly as more sequence information is becoming available in a range of databases. Perhaps the best-studied bacteriophages are the T4-like myoviruses, for which phylogenetic analysis using a range of different genes, such as those encoding the capsid assembly protein, tail sheath protein, and major capsid protein (MCP), has revealed a grouping of bacteriophages that infect a range of genetically distinct hosts (8, 23, 37). This trend has also been observed in genes of the podoviruses, with high sequence similarity of the DNA polymerase genes between cyanophage P60, coliphage T7, and also roseophage SIO1 (3). Evidence also exists that geographically separated bacteriophages show sequence homology. *Sulfolobus*-infecting viruses collected from hot springs in Iceland and the United States are being characterized and genomically sequenced. Eighteen open reading frames, present in all of the

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isolates, that were highly similar across U.S. and Icelandic bacteriophage isolates were identified (41).

The recent surge in the sequencing of phage genomes has highlighted numerous homologous genes in different phages. An example is the *Pseudomonas*-infecting phage gh-1, which was found to exhibit 31 genes similar to those of T7, with at least 50% amino acid identity (16). A recent study by Hambly et al. (8) investigated the g20 sequences of numerous T4-like myoviruses, including the marine cyanomyovirus S-PM2. They concluded that marine and nonmarine phages, including T4 and RB69, share homologous regions of the g20 gene with S-PM2, suggesting that these bacteriophages, infectious to phylogenetically distant hosts, are actually related. As S-PM2, a phage infectious to cyanobacteria, was shown to be similar to those that infect *Escherichia coli*, it seemed highly probable that a freshwater cyanophage and a marine cyanophage of the same family would also contain these homologous regions, as they share hosts from a common taxonomic group.

Due to their conserved nature, structural genes, such as the capsid assembly gene and the major capsid protein gene, are often used as diagnostic markers in molecular virus ecology studies (4, 6, 40). The major capsid protein is typically the predominant protein in virus particles and, as a result, has been the subject of numerous studies (40). Indeed, there are many sequences of the major capsid protein available on GenBank, serving as a good base for comparative genetics. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is commonly used as a tool to characterize viral proteins, with the most abundant protein commonly assigned the function of the major capsid protein (18).

The aim of this study was to develop oligonucleotide primers that could specifically amplify regions of DNA from freshwater cyanophages. The success and viability of the designed primers were evaluated by testing them on previously characterized cyanophages, AN-15, A-1 (L), and N-1 (1, 15, 17). All three of these freshwater cyanophages are virulent myoviruses that are infectious to the filamentous cyanobacterial genera *Anabaena* and *Nostoc*.

MATERIALS AND METHODS

Strains and media. Cyanophages AN-15, N-1, and A-1 (L) were obtained from the American Type Culture Collection (ATCC 29106-B1, 2737347, and 27893-B16, respectively). The host strain, *Anabaena* sp. strain PCC 7120, was obtained from the Pasteur Culture Collection. Liquid cultures of *Anabaena* strain PCC 7120 were grown in 100-ml batch cultures in BG11 medium in 250-ml conical flasks under constant illumination (24 μmol photon m^{-2} s^{-1}) at 30°C, with shaking at 110 rpm. Cyanophage lysate was prepared by growing *Anabaena* strain PCC 7120 in BG11 to mid-exponential phase (optical density at 700 nm, 0.3 to 0.5) and infecting it with the cyanophage at a multiplicity of infection between 0.3 and 3.0 PFU per cell. The cultures were left for 3 to 7 days until they had cleared to transparency, indicating complete lysis. Cellular debris was removed by centrifugation for 5 min at 6,000 rpm in a Megafuge (Hereus) rotor no. 3360. The supernatant was carefully poured off and was treated with DNase and RNase (both at 20 μg μl^{-1}) at room temperature for 1 h to degrade any extracellular nucleic acid. The supernatant was centrifuged for a further 10 min at 4,500 rpm in the Megafuge (Hereus) 3360 rotor and was retained and stored at 4°C.

Cyanophage concentration and DNA extraction. Cyanophages were recovered from 50-ml volumes of lysate by polyethylene glycol (PEG) precipitation (44); 58.4 g $liter^{-1}$ solid NaCl and 10% (wt/vol) PEG 6000 were dissolved in the cyanophage lysate and left for at least 2 h at 4°C. Cyanophage particles were pelleted by centrifugation at 6,000 rpm for 30 min in the Megafuge (Hereus) 3360 rotor and subsequently resuspended in 1 ml cyanophage buffer (5 mM $MgCl_2$, 5 mM $CaCl_2$, 10 mM NaCl, 10 mM HEPES, pH 7.0). Any remaining PEG was removed by dialysis overnight against cyanophage buffer. DNA was extracted from the dialyzed cyanophage concentrate using one phenol extraction followed by at least one round of 1:1 (vol/vol) phenol-chloroform extraction. The final aqueous layer was retained, and the proteins were extracted by adding 0.5× volume 7.5 M ammonium acetate. The suspension was incubated at room temperature for 30 min before centrifugation at 14,000 rpm for 15 min in the MSE HiSpin 21 microcentrifuge using the 4311S-112 rotor. The supernatant was retained, and the DNA was retrieved by ethanol precipitation overnight at 4°C. The DNA pellet was washed in 200 μl 70% ethanol and was subsequently allowed to air dry before resuspension in up to 50 μl TE (10 mM Tris-Cl, 1 mM EDTA).

Environmental-sample collection. Environmental water samples were collected from three freshwater lakes—Priest Pot, a small eutrophic pond in the Lake District, United Kingdom (OS SD355975); Esthwaite Water, an oligotrophic lake in the Lake District, United Kingdom (OS SD365965); and Burrator Reservoir, an oligotrophic lake in Dartmoor, Devon, United Kingdom (OS SX555685). Twenty-liter water samples were collected from the surface waters of the lakes and were returned to the laboratory within 1 h of sample collection. The samples were prefiltered through coarse gauze to remove any debris and then through a 0.45-μm Suporcap filter (PALL Gellman). The fraction that passed through the 0.45-μm filter was concentrated to approximately 20 ml by tangential-flow filtration using a 50-kDa filter Viva Flow 200 system (Sartorius). One-milliliter aliquots of the resulting concentrate were snap frozen in liquid nitrogen and stored at −80°C before DNA extraction using the cetyltrimethylammonium bromide (CTAB) method detailed below (7).

DNA extraction from environmental samples. Concentrated water samples were subjected to DNA extraction using the CTAB method (7); 500 μl lysis buffer (0.5% SDS, 20 μg ml^{-1} proteinase K) was added to 1-ml volumes of concentrated water samples, and the samples were incubated at 55°C for 1 h. Eighty microliters of 5 M NaCl and 150 μl of CTAB buffer (2% [wt/vol] CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-Cl), prewarmed to 60°C, were added to each sample, which was incubated at 60°C for 15 min. Proteins were removed by extraction in an equal volume of chloroform-isoamyl alcohol (24:1). DNA precipitation was carried out in 0.6× volume isopropanol at room temperature for 1 h. The DNA was recovered by centrifugation for 30 min at 14,000 rpm in the MSE HiSpin 21 microcentrifuge using the 4311S-112 rotor. The supernatant was removed and discarded, and the pellet was washed in 200 μl 70% (vol/vol) ethanol. The supernatant was again discarded, and the DNA pellet was air dried and resuspended in 30 μl TE (10 mM Tris-Cl; 1 mM EDTA).

Southern hybridization. Following gel documentation, gels were briefly washed in 0.25 M hydrochloric acid and then rinsed in water. The Southern hybridization apparatus was assembled, and the gels were subjected to blotting overnight using 0.4 M NaOH-1 M NaCl as the blotting buffer and Nylon Hybond N^+ membrane (Amersham) as the transfer membrane. Hybridization and detection was carried out using the DIG High Prime DNA Labeling Detection Starter Kit 1 (Roche Molecular Biochemicals) following the manufacturer's instructions. Probes were prepared via the PCR amplification of regions of marine cyanophage DNA and were purified by agarose gel electrophoresis and labeled using the kit.

Purification of the major capsid protein. Protein separation was carried out on an SDS-PAGE Mighty-Small II gel rig from Hoefer Scientific Equipment. PEG-precipitated and dialyzed cyanophages were denatured at 95°C for 5 min and were then added to an equal volume of sample buffer (0.125 M Tris-Cl, pH 6.8, 4% [wt/vol] SDS, 20% [wt/vol] glycerol, 10% [wt/vol] 2-mercaptoethanol). Samples were loaded onto a polyacrylamide gel comprised of a 4% (vol/vol) stacking gel (4% acrylamide, 0.1% SDS, 0.05% ammonium persulfate, 0.005% *N,N,N′,N″*-tetramethylethylenediamine in 4× stacking buffer [0.5 M Tris-Cl, pH 6.8]) layered on top of a 10% (vol/vol) separating gel (10% acrylamide, 0.1% SDS, 0.05% ammonium persulfate, 0.005% *N,N,N′,N″*-tetramethylethylenediamine in 4× resolving buffer [1.5 M Tris-Cl; pH 8.8]). Samples were electrophoresed through the 4× stacking gel for approximately 30 min at 15 mA and then for approximately 1 h at 30 mA through the 10% (vol/vol) separating gel in SDS-PAGE running buffer (0.025 M Tris-Cl, pH 8.3, 0.192 M glycine, 0.1% [wt/vol] SDS). Sigma SDS-PAGE-6H protein markers were also run on the gel to provide size estimates. The gels were subsequently rinsed for 30 min in water and were then stained with GelCode (Pierce) protein stain following the manufacturer's instructions.

SDS-PAGE gels to be blotted were not stained but were removed from the tank and soaked in CAPS (3-[cyclohexylamino]-1-propanesulfonic acid) buffer (10 mM CAPS, pH 11, 10% [vol/vol] high-performance liquid chromatography [HPLC] grade methanol) for 1 h to remove excess Tris, glycine, and SDS. A gel-size piece of polyvinylidene difluoride (Bio-Rad) membrane was briefly rinsed in 100% HPLC grade methanol prior to use and was then equilibrated in 10 mM CAPS buffer. The gel was then subjected to semidry electroblotting at 50 mA for 90 min. The membrane was subsequently thoroughly rinsed in water to

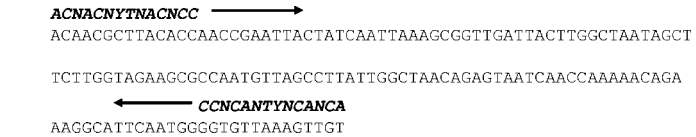

FIG. 1. Partial sequence of the putative AN-15 major capsid protein generated from the PAN15F1 primer. The boldface indicates where the forward primer has annealed, as expected, and also how the degeneracy of the forward primer has enabled it to anneal downstream and also act as the reverse primer.

remove any salts and was dried thoroughly. To verify protein transfer, the membrane was stained for a few seconds in 0.005% (wt/vol) sulforhodamine B stain (0.005% [wt/vol] sulforhodamine B, 30% [vol/vol] HPLC grade methanol, 70% [vol/vol] water containing 0.2% [vol/vol] HPLC grade acetic acid). The membrane was dried thoroughly, and the bands were excised and N-terminally sequenced at the University of Leeds protein-sequencing facility.

PCR and oligonucleotide primers. The oligonucleotide primers used to amplify the major capsid protein gene of the freshwater cyanophages were AN15 MCPF5 (5′-GTT CCT GGC ACA CCT GAA GCG T-3′) and AN15 MCPR5 (5′-CTT ACC ATC GCT TGT GTC GGC ATC-3′). PCRs were carried out in a PTC-100 thermal cycler (MJ Research) in 50-μl volumes containing 1× *Taq* polymerase buffer, 1.5 mM $MgCl_2$, 0.025 mM (each) deoxynucleoside triphosphate, 20 pmol forward primer, 20 pmol reverse primer, 0.2 units *Taq* polymerase (Promega), and approximately 50 ng template DNA. PCR commenced with an initial denaturation step at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 56°C for 1 min, extension at 72°C for 1 min, and then a final extension step at 72°C for 5 min. Ten microliters of PCR product was verified by agarose gel electrophoresis on a 1.2% (wt/vol) agarose gel in 1× TBE (89 mM Tris-Cl, 89 mM boric acid, 2 mM EDTA, pH 8.0) and was subsequently stained with ethidium bromide and viewed with a UV transilluminator.

PCR sequencing. PCR products were purified using the QIAex II gel extraction kit (QIAGEN) and were cloned into the TA cloning vector (Invitrogen). Positive clones were screened for inserts using M13 primers according to the manufacturer's instructions. Any PCR product generated was subjected to sequencing using the ABI Big-dye version 3.1 sequencing kit. Sequencing reactions were carried out at 1/16 dilution using approximately 70 ng template DNA, and the products were incubated in a PTC-100 thermal cycler (MJ Research) at 96°C for 1 min, followed by 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 min. Reaction mixtures were stored for a maximum of 48 h at 4°C. Dye terminators were removed from the sequencing reactions using Genetix dye removal cleanup plates, following the manufacturer's protocol. Sequencing reactions were run on the ABI Prism 3100 Genetic analyzer using a 50-cm array.

Sequence data was automatically collated and analyzed using the ABI sequencing analysis software and was subsequently manually verified. Similarities between cyanophage sequences and published sequences were determined using BLAST (Basic Local Alignment Search Tool) within the National Center for Biotechnology Information (NCBI) database (http://www.//ncbi.nlm.nih.gov/BLAST). Searches were undertaken at the nucleotide level (BLASTn) to identify similar sequences, as well as identifying any potential contamination. The tBLASTx protein database translated the nucleotide sequence and was used to examine sequences at the protein level to determine any similarity. Significantly similar sequences and the associated BLAST "scores" were recorded.

RESULTS

Identifying homology between freshwater and marine cyanophages. Existing primer sets, CPS1/2 (6), CPS1/8 (45), and g20fD2/CPS2 (9), designed for marine cyanophages, were tested on the freshwater cyanomyoviruses AN-15, A-1 (L), and N-1 in order to establish whether a gene homologous to g20 in marine cyanophages was present in freshwater cyanophages. The primer sets tested were unsuccessful in amplifying a product from the freshwater cyanophages, even when the PCR stringency had been significantly reduced (data not shown). Consequently, a series of Southern hybridizations were conducted to investigate further potential homologies between

FIG. 2. Full nucleotide sequence for the putative MCP of AN-15. The N-terminal sequence match (amplified using the PAN15F1 primer set) is underlined. The positions of the primers MCP5F5 (forward) and MCPR5 (reverse) are underlined and boldface (forward primer position, 564 to 586; reverse primer position, 894 to 918). The primers amplified a 350-bp region of the MCP gene.

freshwater and marine cyanophages. The Southern hybridizations were performed using g20-generated PCR products (6) amplified from the marine cyanophage S-PM2 as a probe; however, no hybridization of the probes could be identified in any of the blots with the AN-15 DNA or any of the other freshwater samples containing virus concentrates, even when the stringency was reduced (data not shown).

Isolation of the AN-15 MCP and development of specific PCR primers. SDS-PAGE of AN-15 purified cyanophage particles revealed a dominant protein at approximately 37 kDa that was assumed to be the major capsid protein (see Fig. S1 in the supplemental material). N-terminal sequencing of the Western transferred band was successful in generating seven amino acid residues for the putative major capsid protein; alanine-leucine-threonine-threonine-leucine-threonine-proline. A degenerate primer, PAN15F1 (5′-ACN ACN YTN ACN CC-3′), was designed based on this amino acid sequence. The reverse primer sequence was derived from an alignment of 20 major capsid protein bacteriophage sequences obtained from GenBank (data not shown). An amplified PCR product of approximately 900 bp was predicted from this primer pair; however, a product of approximately 150 bp was amplified. PCR containing the forward primer only, and also sequence analysis of the fragment, revealed that PAN15F1 had also annealed as a reverse primer downstream (Fig. 1).

The full major capsid protein gene sequence was eventually obtained from random shotgun cloning and sequencing of the AN-15 cyanophage genome. The 150-bp fragment (Fig. 1) was matched to the N-terminal region of an open reading frame deduced from a contiguous sequence found by AN-15 random sequencing (Fig. 2). The sequence match indicated that this open reading frame was the major capsid protein gene, and it was used to design a new pair of primers, designated MCPF5 and MCPR5 (Fig. 2). The full putative major capsid protein

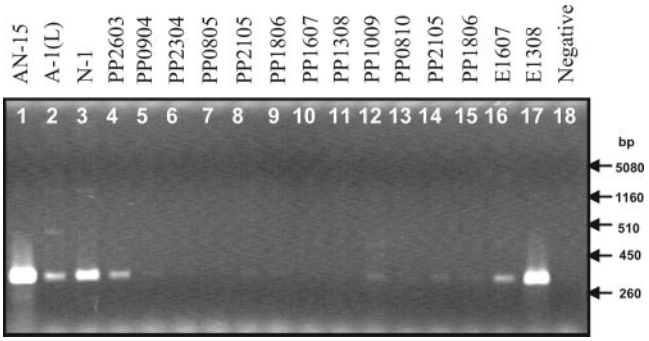

FIG. 3. Analysis of the major capsid protein gene by PCR, using the MCPF5/R5 primer pair. Twenty microliters of the PCR products was run on a 1.2% agarose gel, which was subsequently stained with ethidium bromide. Cyanophage strains: AN-15 (lane 1), A-1 (L) (lane 2), and N-1 (lane 3). Virus concentrates collected from freshwater lakes, with the date of collection (day/month/year) and DNA extraction method (p, phenol; c, CTAB): Priest Pot 260302 (p) (lane 4), Priest Pot 090402 (p) (lane 5), Priest Pot 230402 (p) (lane 6), Priest Pot 080502 (p) (lane 7), Priest Pot 210502 (p) (lane 8), Priest Pot 180602 (p) (lane 9), Priest Pot 160702 (p) (lane 10), Priest Pot 130802 (p) (lane 11), Priest Pot 100904 (p) (lane 12), Priest Pot 081002 (p) (lane 13), Priest Pot 210502 (c) (lane 14), Priest Pot 180602 (c) (lane 15), Esthwaite 160702 (p) (lane 16), Esthwaite 130802 (p) (lane 17), and negative control (lane 18). The positions of the λ *Pst* markers are labeled on the right-hand side of the gel.

gene sequence was compared to database major capsid protein sequences using BLAST; however, no strong homology to known sequences was identified, at either the protein or nucleotide level.

The PCR primer pair MCPF5/R5 was successful in yielding PCR products of approximately 350 bp from cyanophage strains AN-15, N-1, and A-1(L), and also from virus concentrates collected from Priest Pot, Esthwaite Water, and Burrator Reservoir (Fig. 3). In negative control reactions, no PCR products or nonspecific PCR products were detected in the viruses S-PM2 (marine cyanophage), T4, λ, *Chlorella* virus PBCV1, *Micromonas pusilla* virus, and *Emiliania huxeyli* virus strain 86 (data not shown). PCR products generated from virus concentrates in environmental samples were cloned and sequenced; a total of 26 sequences, which were almost identical at the amino acid level, were successfully generated (data not shown). However, there were differences at the nucleotide level (Table 1; see Fig. S2 in the supplemental material). Eleven different genotypes (labeled A to K) were identified from the cyanophage isolates and also from the virus samples collected from the freshwater lakes. Genotypes A and F were the most frequently encountered in the virus concentrates collected. Genotype A, however, was detected only in samples collected from the eutrophic pond, Priest Pot. Genotype F was detected in samples collected from Priest Pot, Burrator Reservoir, and Esthwaite Water, as well as being identical to the genotypes of AN-15 and A-1 (L). Genotypes B, C, D, E, H, G, and J were all detected only once in the PCR products that generated sequences. All of the genotypes except genotype K had greater than 97% identity to genotype A.

DISCUSSION

A major result of this study is the discovery that the freshwater cyanomyoviruses infecting filamentous cyanobacteria that were investigated are genetically very different from

TABLE 1. Sample locations, sampling dates, and GenBank accession numbers for the sequences generated in this study

Sequence reference no.	Sample location	Date collected (day/mo/yr)	GenBank accession no.	Genotype reference	% Identity to genotype A
263-2	Priest Pot	26/03/02	DQ314216	A	100
94-24	Priest Pot	09/04/02	DQ314217	F	98.944
263-5	Priest Pot	26/03/02	DQ314218	A	100
94-26	Priest Pot	09/04/02	DQ314219	C	99.648
263-6	Priest Pot	26/03/02	DQ314220	D	99.648
263-10	Priest Pot	26/03/02	DQ314221	B	99.648
215-52	Priest Pot	21/05/02	DQ314222	J	99.296
215-53	Priest Pot	21/05/02	DQ314223	E	99.296
215-54	Priest Pot	21/05/02	DQ314224	A	100
E167-83	Esthwaite Water	16/07/02	DQ314225	F	98.944
E167-84	Esthwaite Water	16/07/02	DQ314226	G	98.592
E167-87	Esthwaite Water	16/07/02	DQ314227	I	97.887
B75-66	Burrator reservoir	07/05/03	DQ314228	F	98.944
B75-69	Burrator reservoir	07/05/03	DQ314229	F	98.944
E138-103	Esthwaite Water	13/08/02	DQ314230	F	98.944
E138-104	Esthwaite Water	13/08/02	DQ314231	F	98.944
E138-105	Esthwaite Water	13/08/02	DQ314232	F	98.944
E138-106	Esthwaite Water	13/08/02	DQ314233	F	98.944
263-8	Priest Pot	26/03/02	DQ314234	A	100
94-21	Priest Pot	09/04/02	DQ314235	F	98.944
94-25	Priest Pot	09/04/02	DQ314236	I	97.887
E138-101	Esthwaite Water	13/08/02	DQ314237	H	98.592
E138-102	Esthwaite Water	13/08/02	DQ314238	F	98.944
AN-15	Michigan	NA[a]	DQ314239	F	98.944
A-1 (L)	Leningrad, Russia	NA	DQ314240	F	98.944
N-1	Wisconsin	NA	DQ314241	K	77.622

[a] NA, not available.

marine cyanomyoviruses infecting unicellular cyanobacteria. A g20 homologue, originally developed as a diagnostic marker for marine cyanophages (6), could not be identified in the freshwater cyanophages AN-15, A-1 (L), and N-1, suggesting that these cyanophages possess a capsid assembly protein gene very different from that identified in the T4-like coliphages and marine cyanophages. The result of the Southern hybridization using the S-PM2 genome as a probe suggested there were no strong homologous sequences within the AN-15 genome. The full sequence obtained for the putative major capsid protein also appeared to be unique, with no significant similarity being detected in other sequences available in the databases. It appears, however, that the major capsid protein gene is conserved within the freshwater cyanomyovirus group (specifically, those infecting filamentous cyanobacteria), with the three cyanophages used in this study showing similar-size major capsid proteins and also similar sequences (see Fig. S2 in the supplemental material).

One explanation for the lack of sequence homology between the marine cyanophage S-PM2 and the freshwater cyanophage AN-15 is the diverse hosts that they infect. S-PM2 infects the unicellular phycoerythin-containing *Synechococcus*, whereas AN-15 infects the multicellular, freshwater, heterocystous, phycocyanin-containing *Anabaena* and *Nostoc* (29). Cyanobacterial phylogenetics based on morphological differences, as well as 16S rRNA gene sequences, consistently group *Synechococcus* species and *Anabaena* species in separate clades, indicating that the two genera are derived from separate evolutionary lineages (14, 19, 25). It is thus plausible that the cyanophages infectious to these different groups are also derived from separate lineages, thereby accounting for the major differences between them. This result was not expected, particularly since recent studies have detected homologous sequences between phage infecting evolutionarily distant hosts (8, 10, 26, 30), including sequence homologies between the gene encoding the capsid assembly protein in the enterobacteriophage T4 and the marine cyanophage S-PM2 (8).

It is evident from transmission electron microscopy that, although the freshwater cyanomyovirses AN-15, A-1 (L), and N-1 share similar *Myoviridae* characteristics with S-PM2, they vary slightly in capsid morphology (9, 15). S-PM2 possesses a more elongated head (9) than AN-15, A-1 (L), and N-1, which all possess more isometric capsids (15). In addition to the morphological differences, the freshwater cyanophages studied here also exhibit smaller capsids, approximately 60 nm in diameter, whereas S-PM2 has a larger capsid diameter of 85 nm (9), which may account for the MCP differences. Potentially, when more sequences become available, freshwater major capsid protein gene sequences may show similarities to those of cyanophages with more isometric heads. It is also feasible that the variations in the major capsid protein gene sequences are a product of the requirement of some cyanophages for certain cations. Evidence has highlighted the requirement for calcium ions in AN-15 (24), and it is thought that the ions play a functional role in maintaining capsid structure. A-1 (L) and N-1 have also been described as being dependent on magnesium ions, and in their absence, capsid degradation and tail separation occur (28). Differences in genome size and type of nucleic acid are also important factors to consider with respect to conserved genes, with the freshwater cyanomyoviruses having genomes of approximately 65 kb (15), whereas the marine cyanomyoviruses possess genomes of approximately 195 kb (9).

The results of the g20 Southern hybridizations and PCRs (data not shown) strongly suggest that a g20 homologue is absent in the freshwater cyanophages considered in this study. Recent studies carried out by Dorigo et al. (4) and Short and Suttle (34), however, utilized this gene to investigate cyanophage community diversity in freshwater lakes. In both studies, g20 PCR products obtained using the CPS1/8 primer set were subjected to sequence analysis and showed homology to marine cyanophage g20 sequences. These studies concluded that marine and freshwater cyanophages exhibit strong sequence homology and potentially share a common ancestor. It is important to acknowledge, however, that neither of these studies used the g20 primers on any freshwater cyanophage isolate controls, such as those employed in our study.

The CPS1/8 primers were tested in this study to try to amplify PCR products from the cyanophage isolates AN-15, A-1 (L), and N-1; however, the result was always negative over a range of amplification conditions (data not shown). As with all PCR-based diagnostic probes, specificity is never certain unless all the potential templates have been isolated, particularly within extremely diverse virus communities. It is therefore possible that in the aforementioned studies the CPS1/8 primers did not amplify freshwater cyanophages and actually amplified other bacteriophages within the lake that possess similar marine g20 sequences. As numerous studies have revealed, evolutionarily distant and geographically isolated bacteriophages show sequence homologies between particular genes, making it plausible that CPS1/8 primers amplified other bacteriophages with sequences homologous to the marine cyanophage g20 sequences (30, 42).

The most straightforward conclusion from the results of this study is that freshwater cyanophages that infect filamentous cyanobacteria are heterologous to marine cyanophages infecting unicellular strains. It could be hypothesized that g20 homologues from marine cyanomyoviruses (with unicellular hosts) would most likely be found in freshwater cyanophages that infect unicellular cyanobacteria, such as species of *Synechococcus* and *Anacystis*. It this therefore possible that the sequences amplified by Dorigo et al. (4) and Short and Suttle (34) were indeed from cyanophages but were actually derived from cyanophages with unicellular cyanobacterial hosts. This is difficult to verify until the appropriate cyanophage-host systems are isolated. In addition, all of the marine g20 work conducted to date has been undertaken on cyanophages infectious to unicellular hosts, such as *Synechococcus* and *Prochlorococcus*, with none having analyzed cyanophages that infect filamentous cyanobacteria. It is therefore hypothesized that there may also be differences between these cyanophage groups in marine systems. Further work in testing this new primer set on cyanophages infecting marine filamentous cyanobacteria could help resolve this issue.

The MCP primers designed in this study were able to amplify PCR products from a range of freshwater samples. It was found that some samples did not yield a PCR product and some samples produced much stronger, more intense bands. It is possible that PCR efficiency relates to cyanophage abundance, with the stronger PCR products indicating higher cya-

nophage abundance. The sample that yielded the strongest PCR product was that collected from Esthwaite Water on 13 August 2002, where a filamentous cyanobacterial bloom was dispersing (Stephen Maberley, personal communication) (Fig. 3, lane 17). As discussed above, it is widely thought that cyanophages play an important role in cyanobacterial-bloom dynamics, with strong evidence suggesting cyanophages are responsible for the collapse of the blooms (20, 22). The strong PCR product generated from this sample implies that a large number of cyanophages were present in the water at this time, probably infecting filamentous cyanobacteria present in the bloom and inducing its decline. Ten different genotypes were detected in freshwater samples collected from Priest Pot, Esthwaite Water, and Burrator Reservoir (Table 1; see Fig. S2 in the supplemental material). Particular genotypes, F, G, and H, were found only in Esthwaite Water samples, suggesting that the hosts of these cyanophages are adapted to waters more oligotrophic than the nutrient-rich waters of Priest Pot. The reverse can also be said for genotype A, which was detected only in samples collected from Priest Pot. Genotypes B, C, and E were detected only in samples collected from Priest Pot at a specific time of year and were not detected in any of the other samples, suggesting that they were present only during that time of year and thus indicating a dynamic viral community.

Our data indicate that the MCP gene has been conserved through the *Cyanomyoviridae* group, infecting freshwater filamentous cyanobacteria; however, it is variable enough at the nucleotide level for diversity analysis and is proposed as a genetic marker to differentiate between different strains of this group of cyanophages. Major capsid protein probes have proven to be successful in amplifying genes from freshwater isolates [AN-15, A-1 (L), and N-1] and from virus concentrates in freshwater environments. Consequently, the major capsid protein probes are ideal diagnostic markers for presence/absence-based studies of freshwater cyanophages and for the assessment of their diversity. In addition, these primers could be developed further for use in denaturing gradient gel electrophoresis or terminal restriction fragment length polymorphism applications to provide information on the cyanophage community over different time scales, and also spatially. A longer study could be undertaken incorporating the study of the cyanobacterial population and thus providing further information on host- virus interactions.

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