Diurnal Infection Patterns and Impact of Microcystis Cyanophages in a Japanese Pond

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Viruses play important roles in regulating the abundance, clonal diversity, and composition of their host populations. To assess their impact on the host populations, it is essential to understand the dynamics of virus infections in the natural environment. Cyanophages often carry host-like genes, including photosynthesis genes, which maintain host photosynthesis. This implies a diurnal pattern of cyanophage infection depending on photosynthesis. Here we investigated the infection pattern of Microcystis cyanophage by following the abundances of the Ma-LMM01-type phage tail sheath gene g9I and its transcript in a natural population. The relative g9I mRNA abundance within host cells showed a peak during the daylight hours and was lowest around midnight. The phage g9I DNA copy numbers in host cell fractions, which are predicted to indicate phage replication, increased in the afternoon, followed by an increase in the free-phage fractions. In all fractions, at least 1 of 71 g9I genotypes was observed (in tested host cell, free-phage, and RNA fractions), indicating that the replication cycle of the cyanophage (i.e., injection, transcription, replication, and release of progeny phages) was occurring. Thus, Microcystis cyanophage infection occurs in a diel cycle, which may depend on the light cycle. Additionally, our data show that the abundance of mature cyanophage produced within host cells was 1 to 2 orders of magnitude greater than that of released phages, suggesting that phage production may be higher than previously reported.

Viruses play important roles in regulating the abundance, clonal diversity, and composition of cyanobacterial populations, and thus, they have potential impact on the biogeochemical cycles through the process of virus-mediated cell lysis (29, 37). To assess their impacts on the host, the frequency of infected host cells is often estimated from viral production, which is calculated from measurements of viral abundance (10, 12, 44, 46). However, previous studies show that viral abundance varies from hour to hour and from day to day (45). Therefore, it is essential to understand viral infection dynamics in order to determine the impact of the virus on host populations.

Marine cyanophages infecting members of the cyanobacterial genera Prochlorococcus and Synechococcus, which contribute significantly to the primary production of the open ocean, often carry host-like genes involved in photosynthesis, the pentose phosphate pathway, and carbon metabolism (25, 33–35). Recent studies show that these genes direct carbon flux from the Calvin cycle to the pentose phosphate pathway, suggesting that the phage augments the production of energy (ATP) and reducing power (NADPH) to fuel phage deoxynucleoside triphosphate (dNTP) biosynthesis (1, 41).

Ma-LMM01 is a lytic myovirus infecting a strain of Microcystis aeruginosa that frequently forms noxious cyanobacterial blooms in eutrophic freshwater environments (50). The majority of predicted genes in its genome have no detectable homologues in the present databases, and thus, Ma-LMM01 was assigned as a member of a new lineage of the Myoviridae family (5, 49). In contrast to the marine cyanophages possessing photosynthetic genes, Ma-LMM01 possesses a homologue of the nblA gene that plays a central role in the degradation of phycobilisomes. M. aeruginosa has a gas vacuole conferring buoyancy, allowing it to float near surface waters, where it is exposed to high light intensity, which may rapidly lead to photoinhibition upon phage infection. Recently, a second example of cyanophage-encoded nblA was found in a phage infecting the freshwater cyanobacterium Planktothrix agardhii, which has a gas vacuole (9). The phage nblA gene is predicted to function by maintaining host photosynthesis (49). This led to the hypothesis that cyanophage infection may have a diurnal pattern dependent on photosynthesis (36). Further, the latent period of Ma-LMM01 lasts from 6 to 12 h (50), suggesting that the length of the lytic cycle fits into the hours of daylight. However, little is known about the cyanophage infection cycle associated with the light cycle in natural populations.

Synechococcus cyanophage abundance was previously determined by using the plaque assay on solid medium and the most-probable-number (MPN) method using Synechococcus isolates (38). However, it is thought that culture-based methods often underestimate abundances, since the quantifiable cyanophage is limited to the cyanophages that infect only laboratory isolates of Synechococcus (24). One approach to avoiding this problem is to use a PCR-based method (28, 32). Similarly, we have never detected Microcystis cyanophage using culture-dependent methods with several host strains (40). Therefore, to determine whether cyanophage infections have diurnal patterns associated with the light cycle, we monitored the abundances of an M. aeruginosa-infectious cyanophage gene and its transcripts using real-time PCR over 24 h in a Japanese freshwater pond.
products from environmental samples.

MATERIALS AND METHODS

Study site and sampling. Diehl changes of Microcystis aeruginosa and its cyanophages were investigated at Hirosawanokai Pond (35°02′N, 135°41′E), Japan, a small (surface area, 14 ha), shallow (mean depth, 1.5 m) reservoir in the form of a farm pond. Hirosawanokai Pond receives high nutrient input in relation to its volume due to its use for raising carp agriculturally. This results in eutrophication and cyanobacterial blooms from early summer to autumn every year (48). Samples of surface water were taken from a boat every 3 h over a period of 24 h on 15 to 16 September and on 21 to 22 October 2009 at a fixed point in the pond. Two liters of pond water were stored in brown bottles and were transported to the laboratory within 1 h. For DNA extraction, water samples were separated into a free-phase and a host cell fraction. For preparation of the free-phase fractions, 10 ml of the pond water was filtered using a 0.2-μm-pore-size polycarbonate filter. The suspension of recovery of phage particles with this procedure was 65.1% by use of a lysate of Ma-LMM01-infected M. aeruginosa NIES298 cells. The 0.2-μm filtrates were ultracentrifuged at 111,000 × g for 1.5 h at 4°C (40). The pellet was resuspended in 200 μl deionized water and was stored at −80°C. For the host cell fractions, 100 ml of the pond water was sonicated gently and was harvested using centrifugation at 1,680 × g for 10 min (51). The pellet was stored at −20°C until DNA analysis. For the transcriptional analysis of phage mRNA, 20 to 100 ml of the sample was collected on a 3-μm-pore-size polytetrafluoroethylene (PTFE) membrane filter (an RNA fraction) and was resuspended in 1 ml of Stop solution (Tris-EDTA (TE)-saturated phenol-ethanol (5:1:1) according to the work of Yoshida et al. (48). The suspension was stored at −20°C. We extracted DNA and RNA within 3 months.

A seasonal study of variations in M. aeruginosa and its cyanophages was performed at the same sampling site as that used in the diehl study once per month from 21 April to 17 November 2009. Seasonal samples were treated the same as samples for the diehl study described above.

DNA extraction. DNA was extracted from host cell fractions using the xanthogenate method as described previously (51). DNA was extracted from free viral fractions as described previously (40). To avoid contamination with dissolved DNA, the filtrate was treated with DNase I at 37°C for 1 h before DNA extraction. Purified DNAs were suspended in 30 μl deionized water. The amount and purity of the extracted DNA were determined using optical density comparison at 260 nm and 280 nm. Each DNA extract was used as the template for real-time PCR to quantify the abundances of total M. aeruginosa and its infectious cyanophages.

RNA extraction, purification, and cDNA synthesis. Total RNA was extracted from 1 ml of each stored cell suspension as described previously (48). The purified RNA was suspended in 30 μl of dimethyl dicarbonate (DMDC)-treated water. The amount and purity of the extracted RNA were determined using optical density comparison at 260 nm and 280 nm. After digestion with DNase I, 1 μg of purified RNA was reverse transcribed using random primers with the SuperScript III first-strand synthesis system (Invitrogen) according to the manufacturer’s instructions. Each cDNA was used as the template for real-time reverse transcription-PCR (RT-PCR) to quantify cyanophage mRNA.

Real-time PCR and real-time RT-PCR amplification. To quantify the abundances of total M. aeruginosa and its infectious cyanophages, a real-time PCR assay was performed using primers based on sequences of the phycocyanin intergenic spacer (PC-IGS) gene and the Ma-LMM01 tail sheath gene g91, respectively, as described previously (48). The primer pairs used, 188F-254R (16) and SheathRTF-SheathRTR (40), are shown in Table S1 in the supplemental material. The copy numbers of the g91 DNA gene were studied in two fractions: the host cell fraction and the free-phase fraction. To detect related cyanophage mRNA, we performed real-time RT-PCR with the primer sets SheathRTFH-SheathRTR and rnpRTF-rnpRTR, targeting the cyanophage g91 gene and the RNA P RNA gene (rnpB) of M. aeruginosa, respectively (see Table S1). To normalize the raw expression levels of the phage g91 mRNA, the relative abundance of g91 was compared to that of the rnpB gene transcripts of the host M. aeruginosa. A minimum of three replicates were used to quantify numbers. Real-time PCR and real-time RT-PCR were performed with 1 μl of each extracted DNA using SYBR Premix Ex Taq. Individual real-time PCR was performed for each primer set according to the following cycle parameters: for M. aeruginosa (PC-IGS), denaturation at 95°C for 15 s, annealing at 60°C for 15 s, and extension at 72°C for 30 s; for its infectious cyanophages (g91), denaturation at 95°C for 15 s, annealing at 58°C for 15 s, and extension at 78°C for 30 s; for the RNase P RNA gene (rnpB), denaturation at 95°C for 15 s, annealing at 58°C for 15 s, and extension at 78°C for 30 s.

Real-time PCR products of g91 genes were cloned into the pGEM-T Easy vector (Promega) and were then transformed into Escherichia coli IN455F competent cells (Invitrogen) according to the manufacturer’s instructions. At least 20 positive clones (white colonies) from each clone library were randomly selected and were then sequenced at the Dragon Genomics Center, TaKaRa Bio, Inc. (Osaka, Japan).

Primer design for g91 clonal analysis using TAIL-PCR. A new degenerate primer set (g91F and g91R) was designed on the basis of sequences obtained using a combination of real-time PCR products with TAIL-PCR products from environmental samples.

First, we determined the downstream flanking sequences of g91 real-time PCR products using TAIL-PCR with three specific primers (SheathTPF1 to -3) designed on the basis of the sequence of Ma-LMM01 g91 and eight arbitrary (AD1 to AD8) primer sets (Fig. 1; see also Table S1 in the supplemental material) (19, 20). The reaction conditions for the
TAIL-PCR were those described previously (15). The sheathTPF2 and AD2, -4, -5, and -7 primer sets had amplified products (1- to 1.5-kbp fragments) from all of the three fractions in environmental samples from Hirosawanoike Pond. The PCR products with the sheathTPF2-AD2 primer set were purified using a Wizard Miniprep purification kit (Promega, Madison, WI), cloned into the pTAC-1 or pTAC-2 vector (BioDynamics), and then transformed into E. coli DH5α competent cells. Ten positive clones (white colonies) from each clone library were randomly selected and were sequenced using a model 3130 genetic analyzer (Applied Biosystems, Foster City, CA) with a BigDye Terminator (version 3.1) cycle sequencing kit according to the manufacturer’s instructions (Applied Biosystems, Foster City, CA). The reverse primer g91R was designed manually by comparing the variable regions of sequence alignment of SheathTPF2-AD2-amplified products from the three fractions. In addition, we designed the forward primer g91F against variable regions of g91 real-time products from the three fractions (Fig. 1; see also Table S1).

PCR amplification with primer set g91F–g91R was performed in a total volume of 25 μl containing 10× Ex Taq buffer, 200 μM dNTP mixture, 0.5 μM each primer, 1.25 U TaKaRa Ex Taq polymerase, and 1 μl of each DNA template. The reaction conditions were as follows: initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 1.5 min, with a final extension at 72°C for 10 min. The PCR products were purified, cloned, and sequenced as described above. Maximum-parsimony network analysis was performed using the statistical parsimony program TCS, version 1.21 (7).

Nucleotide sequence accession numbers. The nucleotide sequences determined in this study have been deposited in the DDBJ/EMBL/GenBank database. The accession numbers are as follows: AB690464 to AB690490 for clones of g91 arrays from the host cell fraction, AB690491 to AB690520 for g91 arrays from the RNA fraction, and AB690521 to AB690550 for g91 arrays from the free-phage fraction.

RESULTS

Population dynamics of *M. aeruginosa*. We performed 24-h sampling on 15 to 16 September and 20 to 21 October 2009. Here we refer to these samplings as the first sampling and the second sampling, respectively. In the first sampling, the PC-IGS gene copy numbers of *M. aeruginosa* were nearly constant at approximately 10^6 copies ml^-1 from 0900 to 0000 h, increased at 0300 h, and then declined to the same level (4.9×10^6 copies ml^-1) as in the beginning sample (Fig. 2). The 0300-h peak resulted from an accumulation of *M. aeruginosa* in the surface water caused by a well-known diel vertical migration by *M. aeruginosa* (30, 43). The PC-IGS gene copy numbers in the second sampling also showed a pattern similar to that in the first (Fig. 2).

Diel infection dynamics of *Microcystis* cyanophage. We monitored the abundance of g91 DNA copy numbers in the free-phage fraction as well as in the host cell fraction. A putative site-specific recombinase gene was found in the Ma-LMM01 genome (49). However, this gene has been reannotated as a variant of the IS607 family members not related to lysogeny (15). Further, no amplicon is observed from the 29 cyanobacterial strains isolated by using g91-targeted PCR (40). This suggests that the Ma-LMM01-type phage is a virulent phage and that the g91 gene in the host cell fraction is not replicated with the host genome as a prophage but is newly replicated using host machinery immediately after infection. The gene copy numbers in the host cell fraction may be predicted as a marker to evaluate phage replication. We also monitored the diurnal pattern of cyanophage gene expression. In the first sampling, the phage g91 DNA copy numbers in the free-phage fraction were 1.3×10^5 copies ml^-1 at 0900 h, the initial sampling time, and then showed a peak at 3.9×10^5 copies ml^-1 from 1500 to 1800 h (Fig. 3A). The phage g91 DNA copy numbers in the host cell fraction were much higher than those in the free-phage fraction (Fig. 3A and B). The phage copy numbers in the host cell fraction were 1.5×10^6 copies ml^-1 at 0900 h, subsequently increased to 1.3×10^7 copies ml^-1 at 1500 h, and then decreased to 5.2×10^6 copies ml^-1 at 2100 h (Fig. 3B). The relative abundance of the cyanophage g91 mRNA was 0.0026 at 0900 h; then it showed a peak (0.0051) at 1200 h and its lowest relative abundance (0.0007) at 0300 h in the first sampling (Fig. 3C). In the second sampling, the phage g91 DNA copy numbers in the free-phage fraction showed a first peak (2.2×10^5 copies ml^-1) at 2100 h, decreased to 1.3×10^6 copies ml^-1 at 0000 h, and then showed a second peak at 1.5×10^6 copies ml^-1 from 0300 to 0600 h (Fig. 3D). Phage g91 DNA copy numbers in the host cell fraction were 2.6×10^6 copies ml^-1 at 0900 h and subsequently had a first peak at 1.2×10^6 copies ml^-1 from 1200 to 1500 h (Fig. 3E) and then a second peak at 1.1×10^6 copies ml^-1 at 0300 h (Fig. 3E). The g91 transcripts in the second sampling showed the same trend as the dynamics observed in the first sampling (Fig. 3F), suggesting that the first peak in g91 DNA copy numbers in both fractions was derived from phage production and that the 0300-h peak in phage g91 DNA copy numbers in both fractions at the second sampling was associated with accumulation of *M. aeruginosa* at the water surface during the night rather than with phage proliferation in host cells, since the transcripts showed the lowest value at 0300 h.

Relationship between g91 genotypes in all of the three fractions. Next, we accessed genetic relationships among g91 sequences in three fractions (host DNA, viral DNA, and host
transcripts). If the phage inject their DNA into cells of a host population, replicate their DNA within the cells, and release their progenies into the environment, we would obtain sequences identical to the phage sequence from all the fractions. Twenty-seven, 30, and 30 clones were sequenced from the host cell, free-phage, and RNA fractions of the first sampling, respectively. When searched against the NCBI nonredundant protein sequence database using BLAST, all the sequences showed significant similarities only to the corresponding region of Ma-LMM01 (data not shown). The 87 sequences were assigned as 71 genotypes (G1 to G71) clustered at 100% nucleotide sequence identity.

To determine the relationships between the 71 different phage g91 genotypes and Ma-LMM01, we conducted a maximum-paralogy network analysis. This network showed that the genotypes were largely divided into three sequence groups, the g91-1, g91-2, and g91-3 groups, consisting of 62, 7, and 2 genotypes, respectively, and that these groups were genetically distinct from Ma-LMM01 (Fig. 4). When the sequences of representatives from the three groups (G1 for the g91-1 group, G25 for the g91-2 group, and G57 for the g91-3 group) were compared, the numbers (percentages) of nucleotide differences between pairs were 15 (1.3%) for G1 versus G25, 60 (5.4%) for G1 versus G57, and 55 (4.9%) for G25 versus G57. Genotype G1, the predominant group g91-1 genotype, was found in all of the three fractions. Twenty-nine, 15, and 13 genotypes of group g91-1 differed from the G1 genotype by 1, 2, and 3 nucleotides, respectively. Except for one genotype, sequences of the g91-2 group were found only in the RNA fraction.

Seasonal dynamics of M. aeruginosa and cyanophage in the host cell fraction and in the free-phage fraction. The copy numbers of the M. aeruginosa PC-IGS gene ranged from $6.1 \times 10^4$ to $3.9 \times 10^7$ copies ml$^{-1}$ from April to November 2009 (Fig. 5). The cyanophage g91 DNA copy numbers in the free-phage fraction ranged from below the detection limit to $8.2 \times 10^2$ copies ml$^{-1}$, and those in the host cell fraction were between $2.5 \times 10^1$ and $1.6 \times 10^6$ copies ml$^{-1}$ during this sampling period. Throughout this year, the cyanophage g91 DNA copy numbers in the host cell fraction were 3 to 104 times higher than those in the free-phage fraction. The phage abundance fluctuates with the host abundance (Fig. 5). This trend was also found in the relationship between the free phages and their hosts in previous studies (38, 48). This suggests that a portion of the host population is always infected with the phages, albeit with diversity in the hosts and the phages (38).

**DISCUSSION**

**Infection cycle of Microcystis cyanophage.** Recent reports show that cyanophage production depends on host photosynthesis, occurs with a diurnal pattern in laboratory experiments where production of the cyanophage is suppressed completely or reduced by darkness or photosynthetic inhibitors (2,18, 21), and is correlated with the amount of light that is shown in a diurnal pattern under natural light (13) using laboratory conditions. One field survey concerning the infection patterns of natural populations demonstrated that Synechococcus cyanophage numbers increase at midnight (8). Therefore, this is the first report describing the diurnal...
infection patterns of cyanophage depending on the light cycle by determining the dynamics of phage gene replication and transcription in a natural population.

Previously, our culture-based data demonstrated that the relative abundance of the $g91$ gene showed a rapid increase at 1 h postinfection, reaching a maximum ($10^{-1}$) at 6 h postinfection (48). Thus, we suggested that the relative abundance of the $g91$ gene may be a potential marker for environmental monitoring of cyanophage infection. In the field survey reported here, the relative $g91$ mRNA abundance showed a peak during daylight and the lowest value around midnight (Fig. 3C and F); thus, the expression clearly showed a diurnal pattern. Subsequently, the phage $g91$ DNA copy numbers in the host cell fraction increased at 6 (1200 h) to 9 (1500 h) h after dawn (Fig. 3B and E), a finding compatible with the latent period of the cyanophage Ma-LMM01 (6 to 12 h under laboratory conditions) (50); this was followed by an increase in $g91$ DNA copy numbers in the free-phage fraction (Fig. 3A and D). One genotype (G1) of $g91$ sequences was observed in all three fractions. These patterns suggest that the phage genes were transcribed at the beginning of the host’s photosynthesis, at dawn; then, after 6 to 9 h, mature phages were formed and were released from host cells. This suggests that *Microcystis* cyanophage proliferation may be dependent on host photosynthetic performance associated with the light cycle and that cyanophage infection occurs in a diel cycle.

Previously, we demonstrated that Ma-LMM01-type cyanophage dynamics may affect shifts of composition of *M. aeruginosa* populations (e.g., microcystin-producing and non-micro-
cystin-producing populations) during bloom succession (47). The diurnal nature of cyanophage infection implies that this shift is produced by the accumulation of small changes throughout the “day-to-day infections.” As pointed out by Winter et al. (46), our data also show that phage production and cell lysis are not accounted for by a steady-state assumption; therefore, estimates of the numbers of infected cells, calculated from cyanophage abundances, which are necessary to assess the impact of cyanophage on their host population, depend on the sampling time.

Of 62 genotypes belonging to the g91-1 group, 17 genotypes had silent mutations relative to the G1 type sequence (Fig. 4). A previous study showed that the divergence in the phage sequences is derived from point mutations, where the majority (>90%) were silent mutations (27). In contrast, our results show that most mutations (45/62) result in amino acid changes. This may imply that structural plasticity in G91 function is not limited by amino acid changes.

Bacteria have evolved phage defense mechanisms, e.g., the restriction-modification (RM) system, the clustered regularly interspaced short palindromic repeat (CRISPR)-CRISPR-associated (Cas) system, and the abortive infection (Abi) system (17). A recent report shows that the *Microcystis* genome (strain NIES-843) contains a large number of these defense genes \( n = 492 \) among the 1,055 bacterial and archaeal genomes (22), although the presence of these genes in natural populations of *M. aeruginosa* is not addressed. Therefore, it may be likely that the cyanophage genetic variation observed in this study reflects the continuous arms race between the phage and host populations. Further, eight of the nine sequences of group g91-2 were obtained only in the RNA fraction (Fig. 4). One possible explanation is that DNA replication of cyanophages included in the g91-2 group may be prevented by one of numerous defense genes, including the Abi systems newly identified in *M. aeruginosa* (22), similarly to the abiK genes of lactococcal Abi systems (6).

**Impact of *Microcystis* cyanophages on *M. aeruginosa***. The phage g91 DNA copy numbers in the free-phage fraction were 2 to 3 orders of magnitude lower than those in the host cell fraction at the same sampling time. We observed this trend throughout the year (Fig. 5). Although the packaging efficiency of lytic cyanophage Ma-LMM01 remains unknown, as much as 10 to 100% of the input DNA was packaged under carefully optimized reaction conditions in a study of T4 phage DNA packaging (3, 14, 31). Given that the packaging ratio of Ma-LMM01 is 10%, the abundance of mature cyanophage calculated from the gene abundance in the host cell fraction was still 1 to 2 orders of magnitude higher than that of free phages. Therefore, the level of phage production estimated from mature phage abundance may be higher than that reported previously based on free-phage abundance. We speculate on explanations for this large discrepancy, as follows. (i) Most progeny phages may be trapped by host lysates or colonies, and small portions may have diffused gradually into the pond water. (ii) Progeny phages may attach to the next hosts for infection or to nonspecific particles immediately after diffusing into the pond water. Several authors suggested that adsorption onto transparent exopolymers is the primary mechanism responsible for the removal of viral production in eutrophic reservoirs (4, 26). (iii) Progeny phages released into the pond water may be rapidly degraded by UV radiation (10). (iv) Infected cells may be preyed upon by protozoa (e.g., heterotrophic nanoflagellates), resulting in the release of decreased numbers of cyanophage into the pond (23, 39).

Our real-time PCR-based method may detect small portions of the *Microcystis* cyanophages (i.e., close relatives of Ma-LMM01-type phage) because of the specificity of the PCR method. Indeed, previous studies imply that there are diverse *Microcystis* cyanophages in the natural environment (11, 42). To quantitatively evaluate the impacts of *Microcystis* cyanophage on their host cells, further studies of unknown *Microcystis* cyanophage and the multiple host-phage interactions will be necessary.

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