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Genomic Sequence and Evolution of Marine Cyanophage P60: a New Insight on Lytic and Lysogenic Phages†

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The genome of cyanophage P60, a lytic virus which infects marine *Synechococcus* WH7803, was completely sequenced. The P60 genome contained 47,872 bp with 80 potential open reading frames that were mostly similar to the genes found in lytic phages like T7, phi-YeO3-12, and SIO1. The DNA replication system, consisting of primase-helicase and DNA polymerase, appeared to be more conserved in podoviruses than in siphoviruses and myoviruses, suggesting that DNA replication genes could be the critical elements for lytic phages. Strikingly high sequence similarities in the regions coding for nucleotide metabolism were found between cyanophage P60 and marine unicellular cyanobacteria.

The discovery of highly abundant viral particles in natural waters (2, 22) reinitiated research on the ecological impacts of viral infection and lysis on marine microbes. It is now known that viruses in aquatic environments are an active and important component of the microbial loop in terms of their potential roles in regulating microbial mortality, production, community structure, and biogeochemial cycling (3, 12, 22, 29, 38). It is believed that marine viruses could mediate genetic exchange between microbes through gene transduction, thereby effecting the genetic diversity of microbial communities (12, 15, 20, 38).

Cyanophages are the viruses that infect cyanobacteria. Cyanobacteria of the genus *Synechococcus* are important primary producers and account for a substantial portion of carbon fixation in the world's oceans (17). Cyanophages that infect specific strains of marine *Synechococcus* spp. typically range from 10^3 to 10^5 ml^{-1} in nearshore and offshore waters, and sometimes they can reach concentrations in excess of 10^6 ml^{-1} (26–28, 33). All known cyanophages isolated belong to three bacteriophage families: *Myoviridae*, *Siphoviridae*, and *Podoviridae* (18, 28, 33, 35). Cyanophages are morphologically and genetically diverse (18, 35, 39) and can potentially be used to infer the phage-host interaction in natural viral communities.

Although bacteriophage dominate marine viral communities, research on marine phage genomes is still in its infancy. Currently, only one marine phage genome has been sequenced (24) while more than 100 nonmarine phage or prophage genomes were sequenced. The study on the genome of roseophage SIO1, a lytic phage of the heterotrophic marine bacterium *Roseobacter* suggested that marine and nonmarine phages are genetically related but their basic life histories may be significantly different (24). The rapidly increasing genomic data on bacteriophage have led to some new findings on phage evolution. It has been proposed that double-stranded DNA phage and prophage are mosaics that arose by horizontal gene transfer of genetic material from a global phage pool (14). A later study based on the analysis of six prophage suggested the existence of two modes of genetic evolution, depending on the phage infectivity (8). Chopin et al. (8) proposed that lysogenic phage follows the mode suggested by Hendrix et al. (14) while lytic phage would not exchange DNA outside its group.

In order to better understand the biological properties of marine and nonmarine phages and explore potential functional linkage between cyanophage and cyanobacteria, the whole genome of cyanophage P60, which infects marine *Synechococcus* spp., was sequenced.

Cyanophage P60. Cyanophage P60, which lyses marine *Synechococcus* WH7803, was isolated from one of Georgia's coastal rivers, the Satilla River. Water samples were collected near the mouth of the Satilla River, where the salinity is about 30‰, on 12 July 1988. The liquid serial dilution method was used to isolate and purify cyanophage P60, and the purified P60 was examined by transmission electron microscopy (18). P60 is a podovirus (Fig. 1) with high lytic activity. Among more than 100 cyanophages isolated in our laboratory, P60 was able to lyse the exponentially growing WH7803 culture within 10 h, while it took 1 to 7 days for other cyanophages (myoviruses and siphoviruses) to lyse the host culture. The cross infectivity of P60 was tested against 10 other marine *Synechococcus* strains, including three phycoerythrin-containing strains (WH7805, WH8103, and WH8108) and seven phycocyanin-containing strains (WH8101, WH8007, WH5701, CCMP1628, CCMP1629, CCMP1630, and CCMP1632), and only *Synechococcus* WH7805 was sensitive to P60. Compared to other cyanophages, the P60 genome is more susceptible to complete digestion with restriction endonucleases (18). The genome of P60 could be digested by restriction enzymes such as *Acc*I, *Bam*HI, and *Eco*RV. The P60 genome was insensitive to RNase but completely degraded by DNase I treatment. The buoyant density of P60 is about 1.48 g cm^{-3}. The infectivity of P60 was not significantly reduced by pretreatment with 2% chloroform. DNA of P60 was extracted by using a method described elsewhere (18).

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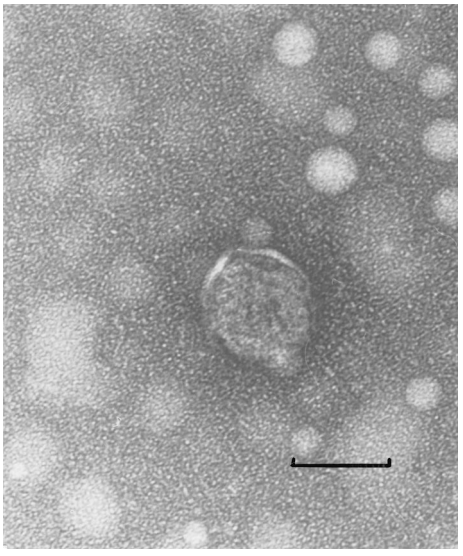

FIG. 1. Electron micrograph of marine cyanophage 60. Bar, 50 nm.

Cloning and sequencing. The P60 phage DNA was digested with restriction endonuclease *Bam*HI (Promega, Madison, Wis.), and the fragments were cloned into pUC18 plasmid with T4 DNA ligase of pGEM-T Easy vector System II (Promega). Sequencing was carried out by using ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction on a 310 Genetic Analyzer (PE Applied Biosystems, Inc., Foster City, Calif.). Inserts from the cloned libraries were sequenced with the M13 forward and/or reverse primers (Promega). The primer walking technique was used for sequencing the remaining fragments and linking different fragments.

Analysis of sequence data. Genomic sequences were assembled into contigs with the Sequencher software (Gene Code Corp., Ann Arbor, Mich.). Open reading frames (ORFs) were identified by using ORF finder at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Sequences of ORFs were used to search for homologues by using BLASTP with default parameters (1) against the Swissprot protein database (http://www.ncbi.nlm.nih.gov/blast). Homologues of the ORFs were also searched against *Synechococcus* and *Prochlorococcus* genomic sequences by using TBLASTN at the DOE Joint Genome Institute (http://spider.jgi-psf.org/JGI_microbial/html/index.html). The sequence analyses, including database searching, gene comparison, and multiple-sequence alignment, were also performed with the Wisconsin Package, version 9.0 (Genetics Computer Group, Madison, Wis.) (10). Phylogenetic analyses based on some conserved genes were conducted with PHYLIP (J. Felsenstein, PHYLIP: phylogeny inference package, version 3.5, University of Washington, Seattle).

Nucleotide sequence accession number. The complete nucleotide sequence of cyanophage P60 has been deposited in GenBank under the single accession no. AF338467.

The genome size of P60 was 47,872 bp. Eighty ORFs were identified on the P60 genome, and 19 of them could be assigned putative functions (Fig. 2). All of these ORFs were preceded by 5′ start codons (ATG) and predicated on the basis of this information plus a plausible Shine-Dalgarno prior to the start codon. In general, the ORFs of P60 were most similar to those found in bacteriophages T3, T7, phi-YeO3-12, and SIO1 (Table 1). However, ORFs 26, 27, 53, and 67 of P60 shared strikingly high similarity (>50% amino acid sequence identity) with sequences found in marine *Synechococcus* and *Prochlorococcus* strains (Table 1). These ORFs corresponded to the

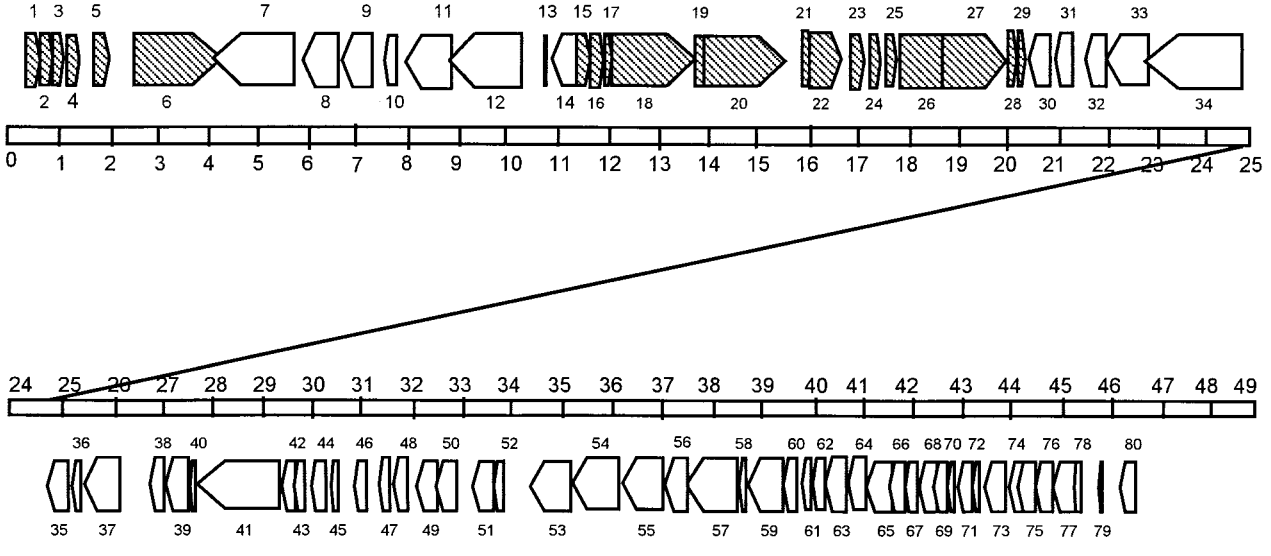

FIG. 2. Genomic arrangement of cyanophage P60. Nineteen ORFs were assigned putative functions, and they were RNA polymerase (ORF 6), endonuclease (ORFs 16 and 17), primase-helicase (ORF 18), DNA *pol* (ORF 20), exonuclease (ORF 22), ribonucleotide triphosphate reductase (ORFs 26 and 27), tail tubular protein (ORFs 34, 35, and 36), capsid protein (ORF 37), methionine codon (ORF 39), head-to-tail joining protein (ORF 41), proline-rich protein (ORF 42), thymidylate synthase (ORF 53), packaging protein (ORFs 54 and 55), and collagen alpha I chain precursor (ORF 57). The length of the arrow is proportional to the length of the predicated ORF.

TABLE 1. General features of the main putative ORFs of cyanophage P60

ORF	Start-stop (position)	% G+C content	Size (amino acids)	Function or similarity	No. identical/ total (%)	No. similar/ total (%)	GenBank accession no.
6	2589–4313	53.39	574	RNA polymerase of coliphage T3; other significant hits included bacteriophage K11, coliphage T7, and *Enterobacter* phage SP6	162/604 (26)	249/604 (40)	P07659
9	6554–7192	58.22	212	Possible hit to virulence determinants, EspB of *E. coli*	50/185 (27)	72/185 (38)	X96953.1
10	7553–7900	58.38	115	Pneumococcal surface protein A of *Streptococcus pneumoniae*	31/76 (40)	41/76 (53)	AF255908.1
16	11678–11878	47.88	66	Endodeoxyribonuclease I of coliophage T7; other significant hits included roseophage SIO1 and bacteriophage phi-YeO3-12 and T3	30/63 (47)	43/63 (67)	NC_001604.1
17	11865–12029	47.88	54	Endodeoxyribonuclease of bacteriophage phi-YeO3-12; also significant hits for coliphage T3 and T7	24/53 (45)	32/53 (60)	NC_001271.1
18	12138–12029	52.63	531	RP primase-helicase of roseophage SIO1; other significant hits included bacteriophage phi-YeO3-12 and coliphage T3 and T7	204/519 (39)	306/519 (58)	NC_002519.1
19	13720–13983	49.62	87	Possible hit to thioredoxin C3 of *Corynebacterium nephridii* and thioredoxin M of *Synechocystis* sp.	19/62 (30)	33/62 (52)	P52228
20	13967–15730	52.27	587	DNA polymerase of bacteriophage phi-YeO3-12, other significant hits included coliphage T3 and T7 and roseophage SIO1	235/716 (32)	351/716 (48)	NC_001271.1
22	16106–16837	49.31	243	Exonuclease of coliophage T3; other significant hits included coliphage T7 and bacteriophage phi-YeO3-12	83/271 (30)	129/271 (46)	P20321
26	17789–19009	56.02	406	Ribonucleotide triphosphate reductase of marine *Prochlorococcus* MIT9313 (contig 477) and marine *Synechococcus* WH8102	138/265 (52)	181/265 (68)	—[a,b]
27	18676–20016	54.81	446	Ribonucleotide triphosphate reductase of marine *Synechococcus* WH8102 (contig 51) and *Prochlorococcus* MIT9313 (contig 477)	273/441 (61)	318/441 (71)	—[a,b]
30	20409–20900	52.44	163	Possible hit to repressor of bacteriophage phi-C31	25/81 (30)	42/81 (50)	NC_001978.1
34	22697–24739	51.79	680	Tail tubular protein of coliphage T7	157/591 (27)	165/591 (45)	NC_001604.1
35	24739–25116	49.47	125	Tail tubular protein of bacteriophage phi-YeO3-12	29/86 (33)	49/86 (56)	NC_001271.1
36	25143–25346	46.08	67	Tail protein of coliphage T7	19/64 (29)	35/64 (54)	NC_001604.1
37	25465–26130	55.86	221	Minor capsid protein of coliphage T7	46/211 (21)	82/211 (38)	NC_001604.1
39	27038–27460	58.39	140	G protein coupled receptor, Saimiriine herpesvirus 2	42/109 (38)	61/109 (55)	NC_001350.1
40	27466–27705	57.08	79	Phosphatidylinositol 2-kinase of *Saccharomyces cerevisiae*	20/62 (32)	28/62 (44)	NC_001144.1
41	27650–29317	55.58	555	Head-tail connector protein of coliphage T7	195/560 (34)	293/560 (51)	NC_001604.1
53	34408–35115	47.32	235	Contig 478 of *Prochlorococcus* MIT9313; other significant hits to homospermidine synthase of *Paramecium bursaria* Chlorella virus 1 and *Synechocystis* sp. (strain PCC 6803)	128/206 (62)	160/206 (77)	—[a]
54	35244–36170	50.59	308	DNA packaging protein of bacteriophage phi-YeO3-12	111/263 (42)	160/263 (60)	NC_001271.1
55	36203–36979	53.15	258	DNA packaging protein of coliphage T3	114/218 (52)	155/218 (70)	P10310
67	41883–42161	49.82	92	Conserved hypothetical protein of *Deinococcus radiodurans*; other significant hits included *Synechocystis* sp., bacteriophage phi-C31, contig 478 of *Prochlorococcus* MIT9313, and contig 53 of *Synechococcus* WH8102	34/68 (50)	47/68 (69)	B75278
72	43211–43399	49.74	62	Possible hit to lytic enzyme of *Pseudomonas aeruginosa*	19/48 (39)	28/48 (57)	AB030826.1
73	43441–43887	55.03	148	Gp10 of *Mycobacterium* phage D29	25/55 (45)	33/55 (59)	NC_001900.1
77	44854–45234	52.76	126	Gp7 of roseophage SIO1	50/80 (62)	62/80 (77)	NC_002519.1
80	46212–46526	46.98	104	Tail fiber protein of bacteriophage phi-YeO3-12	13/34 (38)	24/34 (70)	NC_001271.1

[a] The sequence was retrieved from the website http://spider.jgipsf.org/JGI_microbial/bin/psf_blast,/psf/grenada/auspex1/draft008/Microbial/prochlorococcusB/edit_dir.001127/blast_dir.

[b] The sequence was retrieved from the website http://spider.jgi-psf.org/JGI_microbial/bin/psf_blast,/psf/inagua/draft010/microbe/projects/2351364/blast_dir.

genes that code for ribonucleoside triphosphate reductase A and B, thymidylate synthase, and an unidentified protein, respectively.

The genomic arrangements of four different podoviruses (P60, T7, phi-YeO3-12, and SIO1) were compared (Fig. 3). The genes responsible for DNA replication (e.g., primase-helicase and DNA polymerase [DNA *pol*]) appeared to be conserved among all these phages. In terms of gene organization, cyanophage P60 was more similar to phages T7 and phi-YeO3-12 than to roseophage SIO1. However, the genes involved in ribonucleotide triphosphate reduction were only conserved between marine cyanophage P60 and marine roseophage SIO1 (Fig. 3). Classes I, II, and III were defined by Dunn and Studier in 1983 (11). Briefly, the class I genes are transcribed by host RNA polymerase and include functions to overcome host restriction; the class II genes are next to be expressed and are responsible for phage DNA replication and metabolism; the class III genes are the last to be expressed and mainly include the structural genes for maturation and packaging of phage DNA.

The phylogenetic relationship constructed based on the DNA *pol* from 13 different strains of podoviruses demonstrated that the two marine podoviruses (cyanophage P60 and roseophage SIO1) were not necessarily more related than nonmarine podoviruses (Fig. 4). In fact, cyanophage P60 was more closely related to coliphages T3, T7, and phi-YeO3-12 than to roseophage SIO1 (Fig. 4). Phages that infect the same or closely related hosts appeared to be more closely related. For example, coliphages T3, T7, and phi-YeO3-12 were closely related and the *Bacillus* phages B103, M2, PZA, phi29, and GA-1 were also clustered together (Fig. 4). According to the DNA *pol* phylogeny, podoviruses can be divided into two major clusters, A and B. Interestingly, the genome sizes of podoviruses in cluster A were nearly twice as large as those in cluster B. The genome sizes of these podoviruses are compared in Table 2.

Although phages are a group of highly diverse viruses, morphology, genome size, and host relatedness could provide important clues to their evolution and taxonomy. Our data suggested that T7-like podoviruses with a genome size of about 40

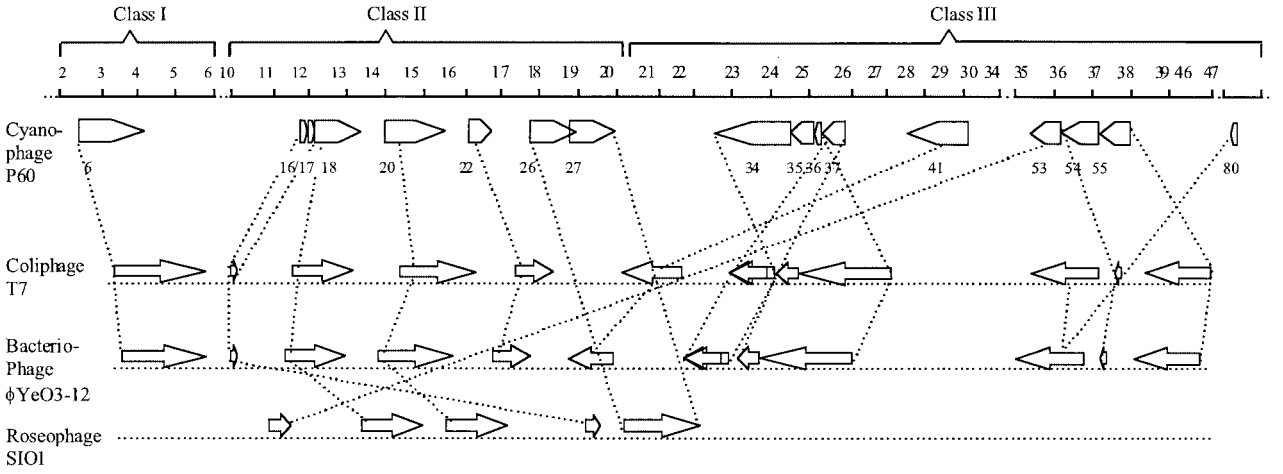

FIG. 3. Comparison of the genomic arrangements of four bacteriophages (*Podoviridae*): cyanophage P60, coliphages T7 and phi-YeO3-12, and roseophage SIO1.

kb share very similar genomic arrangements. Phylogenetic analysis based on conserved DNA *pol* further proved the kinship of T7-like phages. The phi29-like podoviruses (phages B103, BS32, GA-1, M2, Nf, phi15, phi29, and PZA) have very similar morphologies and genome sizes (~20 kb) and were shown to evolve from a common ancestor based on the genomic comparison (21). In general, viruses that infect the same host or closely related hosts have similar morphologies and are evolutionarily close. For example, the capsid assembly gene was conserved in cyanomyoviruses that infect marine *Synechococcus* (13) and several head and tail genes were found to be conserved among T4-type phage that infects enterobacteria (30). The large double-stranded DNA viruses that infect eukaryotic microalgae were also shown to be closely related on the basis of the viral DNA *pol* gene (7). Evolution of phage is likely more dependent on the host rather than the environment. The two marine phages SIO1 and P60 were not the most closely related strains in terms of genomic structure. In terms of genomic arrangement, P60 is more similar to T7 and phi-YeO3-12 than to SIO1. The RNA polymerase, an essential component of T3 and T7 life cycles, was not identified in the SIO1 genome (24).

Genomic sequences of phage provide many new insights on the biology and ecology of phage. Currently, the genomes from 109 bacteriophages have been sequenced and deposited in GenBank. Genomic sequences from *Myoviridae* (10 strains), *Podoviridae* (10 strains), *Siphoviridae* (42 strains), *Inoviridae* (16 strains), *Leviviridae* (8 strains), *Microviridae* (10 strains), and several viruses that infect archaeal bacteria are available to the public on the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov:80/PMGifs/Genomes/phg.html). According to the phage genomic database, 2 of 10 myovirus genomes and 3 of 42 siphovirus genomes are lytic phages. Lysogenic phage is the dominant form among the known myovirus and siphovirus genomes. Interestingly, the gene that codes for the integrase was found in all lysogenic

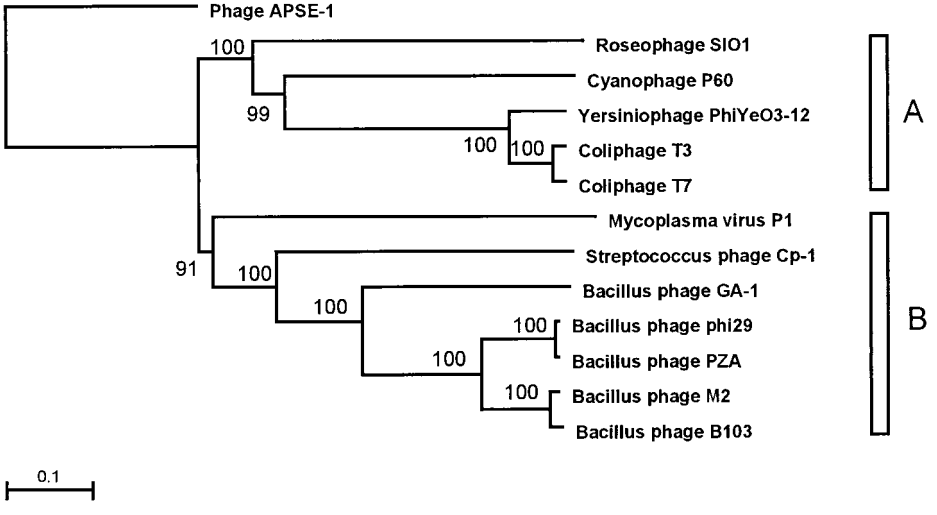

FIG. 4. Neighbor-joining tree constructed based on the aligned DNA *pol* amino acid sequences of 13 podoviruses. The alignment of whole sequences was used to construct the phylogenetic trees. Phage APSE-1 was used as an outgroup. Clusters are indicated on the right. The scale bar represents 0.1 fixed mutation per amino acid position. Bootstrap, 1,000.

TABLE 2. Genome size comparison of 11 lytic podoviruses[a]

Phage strain	Host	Genome size (bp)	Cluster[b]	GenBank accession no.
P60	*Synechococcus* sp.	47,872	A	AF_338467
SIO1	*Roseobacter* sp.	39,898	A	NC_002519
phi-YeO3-12	*Yersinia enterocolitica*	39,600	A	NC_001271
T7	*E. coli*	39,989	A	NC_001604
T3	*E. coli*	38,208	A	NC_003298
GA-1	*Bacillus* sp.	21,129	B	NC_002649
PZA	*Bacillus* sp.	19,366	B	NC_001423
B103	*Bacillus subtilis*	18,630	B	X99260
Cp-1	*Streptococcus* sp.	19,343	B	NC_001825
P1	*Mycoplasma*	11,660	B	NC_002515
APSE-1	*Acyrthosiphon pisum*	36,542	Outgroup	NC_000935

[a] Temperate phage P22 is not included.
[b] Clustering is based on DNA *pol* gene phylogeny (Fig. 4).

siphovirus and myovirus genomes, suggesting that these phages were able to integrate their genomes into the host genome and become lysogenized. A majority of bacteriophages isolated from marine environments were myoviruses and siphoviruses (18, 28, 33, 34). More than 40% of the bacterial isolates contained inducible prophage, and the percentage of lysogenic bacteria was higher in oligotrophic environments than in coastal or estuarine environments (16). It will be interesting to estimate what proportion of marine phages contains the integrase gene. Eight of nine podoviral genomes (except for phage P22) contained the DNA *pol* gene, and some other genes (e.g., primase and helicase) were associated with DNA replication. These phages have been known to be lytic phages. Although morphologically P22 is a member of *Podoviridae*, many studies have suggested that it is a member of the lambdoid family (31). Phage lambda (*Siphoviridae*) is a typical lysogenic phage. Most of the lysogenic phage genomes in *Siphoviridae* and *Myoviridae* did not contain the DNA replication genes (i.e., primase and DNA *pol*).

The evolution of lytic podovirus could be less influenced by the genetic exchange between phage and host. A conserved DNA *pol* gene contained in the viral genome could be an important inherited feature for lytic bacteriophages. The replication of lysogenic phage would be more host dependent than that of lytic phage. The genetic diversity of lysogenic phage should be much higher than that of lytic phage due to higher frequencies of lateral genetic exchange between lysogenic phage and the host. Numerous cases of horizontal gene transfer were observed among lambda-like phages upon genomic comparison (4). According to the recent study of *Lactococcus* prophages, genomic similarity of lysogenic phages is much lower than that of lytic phages (8). Chopin et al. (8) further suggested that the frequencies of horizontal genetic exchange are lower among lytic phage than lysogenic phage. Our study suggested that the evolution of DNA replication machinery of lytic podoviruses is more independent of the host than is that of lysogenic phage. This is consistent with the view that acute viruses tend not to show phylogenetic congruence with their hosts (32).

Genetic exchanges between cyanophage P60 and marine cyanobacteria occurred at the sites that code for ribonucleoside triphosphate reductase A and B, thymidylate synthase, and an unidentified protein. It is not clear why P60 maintains extensive similarity with marine cyanobacteria for genes involved in nucleic acid metabolism. Such homologues were also found between phage T4 and *Escherichia coli*. Marine roseophage SIO1 also contains these proteins that exhibit higher similarity to bacteria than to other phages (24). Ribonucleotide reductases are the key enzymes that convert ribonucleosides to deoxynucleotides, which are the immediate precursors of DNA (23). With ribonucleotide reductase and thymidylate synthase, the rate of DNA synthesis of T4 could be increased 10-fold compared to the system without these enzymes (19). Although host DNA degraded by phage-encoded ribonucleases can be incorporated into the DNA of the progeny phages, it is believed that the great bulk of the deoxynucleoside triphosphates for T4 DNA synthesis comes from de novo synthesis catalyzed by phage-encoded proteins like ribonucleotide reductase and thymidylate synthase (19). Perhaps acquisition of these DNA metabolism genes generated the rapidly growing lytic phages like P60, SIO1, and T4. Again, these genes were not common in lysogenic phages. Another example of genetic exchange between lytic phage and host is the phage-encoded PhoH, a host-borne protein typically induced under conditions of phosphate starvation. This gene was found in the lytic phage SIOI that infects marine *Roseobacter* sp. (24) but not in marine lytic phage P60.

Although no single universal genetic marker was found for all of the double-stranded DNA phages, the phylogenetic diversity of viruses in natural environments could be explored within defined groups on the basis of their infection mechanism, morphology, genome size, and host linkage. The DNA *pol* gene has been proven to be a suitable genetic marker for examining the evolutionary relationship between algal viruses and other large double-stranded DNA viruses (5–7). Our study here suggested that the DNA *pol* gene could also be used as a marker molecule to study the phylogenetic relationship or diversity of podoviruses. Recently, a partial DNA *pol* gene sequence was obtained from cyanophage ϕ12, another podovirus which infects marine *Synechococcus* WH8017 (33). The nucleotide sequence (GenBank accession no. AY063486) of partial DNA *pol* from cyanophage ϕ12 was 97.5% identical to that of cyanophage P60, suggesting that specific PCR primers could be designed at least for the podoviruses of marine *Synechococcus* spp.

Although podoviruses are not the dominant form among the phage isolates, they could represent a unique group of viruses that are important in terms of controlling bacterial mortality in natural environments due to their superinfectivity and high host specificity. Podoviruses that infect marine *Synechococcus* spp. were found to be more host specific than myoviruses, the dominant form of cyanophage isolates (18, 28, 33). However, the DNA *pol* gene did not appear to be conserved among myophages and siphophages. In our laboratory, more than six sets of degenerate primers have been designed based on the conserved regions of known DNA *pol* (families A and B, respectively) and used to amplify the gene target from 35 cyanophage isolates. In most cases, nonspecific products were amplified and sequence data did not match the DNA *pol*. In a recent study, the primers based on the T4 sequences also failed to amplify the DNA *pol* gene from the T4-type phages (30).

In order to investigate genetic diversity of myophages or siphophages in natural environments, one should probably consider viral structural genes as probes. For example, the

capsid assembly gene (g20) of myoviruses infecting marine *Synechococcus* spp. were found to be conserved (13) and the specific primers based on the g20 genes have been used to compare the genetic diversity of this group of cyanophages in natural environments (36, 37). Moreover, several major tail genes (i.e., genes 18 and 19) and the capsid gene (gene 23) were found to be conserved within T4-type phages and suitable for phylogenetic analysis (30). It was also found that the sequences of the head assembly proteins were conserved between several phiC31-like siphoviruses (prophages) that were isolated from evolutionarily diverse hosts (25). These prophages were proposed to share a common head assembly mechanism (25). Furthermore, the lysogeny-related genes were found to be conserved among lysogenic siphoviruses from an evolutionarily related branch of low-GC-content gram-positive bacteria (9).

There is no doubt that viral communities in aquatic environments are much more complex than what we have seen from their morphologies. The extent of viral diversity in marine environments is still largely unknown. More phage genomes from aquatic environments should be explored in order to better understand the evolutionary history and biological and ecological functions of marine viruses.

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