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Resistance to Co-Occurring Phages Enables Marine *Synechococcus* Communities To Coexist with Cyanophages Abundant in Seawater†

JOHN B. WATERBURY* AND FREDERICA W. VALOIS

Department of Biology, Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543

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Recent reports documenting very high viral abundances in seawater have led to increased interest in the role of viruses in aquatic environments and a resurgence of the hypothesis that viruses are significant agents of bacterial mortality. *Synechococcus* spp., small unicellular cyanobacteria that are important primary producers at the base of the marine food web, were used to assess this hypothesis. We isolated a diverse group of *Synechococcus* phages that at times reach titers of between 10^3 and 10^4 cyanophages per ml in both inshore and offshore waters. However, despite their diversity and abundance, we present evidence in support of the hypothesis that lytic phages have a negligible effect in regulating the densities of marine *Synechococcus* populations. Our results indicate that these bacterial communities are dominated by cells resistant to their co-occurring phages and that these viruses are maintained by scavenging on the relatively rare sensitive cells in these communities.

The presence of phages in seawater has been known for some time, but they have received little attention, and knowledge of their ecology is quite limited (16). Recent observations with transmission electron microscopy (TEM) of seawater samples concentrated by either filtration or centrifugation have shown that viruses can be present at high concentrations ranging from 10^5 to 10^8 viral particles ml^{-1} of seawater (2, 3, 5, 6, 8, 9, 18–20, 26, 27). These observations have led to a resurgence of the hypothesis that viruses are significant agents of bacterial mortality and have generated considerable interest within the oceanographic community concerning the potential impact of viruses on trophodynamics at the base of the marine food web.

To assess this hypothesis, we have isolated a diverse group of lytic cyanophages that infect marine *Synechococcus* spp. These unicellular cyanobacteria were chosen for this study because they are abundant in the surface waters of the world's oceans, are capable of rapid growth, and are responsible for 5 to 25% of the total oceanic primary productivity (31). In addition, they can be counted in nature specifically and accurately and there exists a large collection of axenic strains suitable as host clones for phage isolation (31).

The taxonomy of unicellular cyanobacteria is still in flux. The terminology used here is described in *Bergey's Manual of Systematic Bacteriology* (29). *Synechococcus* spp. are a suprageneric group composed at present of six clusters that are equivalent to genera. Strains belonging to two of these clusters (marine cluster A and marine cluster B) have been used in this study as host clones for the isolation of marine cyanophages. Members of marine cluster A can be isolated from both open ocean and coastal waters, have phycoerythrin as their primary light-harvesting pigment, and have elevated salt requirements for growth that reflect the chemistry of seawater. Members of marine cluster B have been isolated only from coastal waters and do not possess phycoerythrin, but instead use phycocyanin as their primary light-harvesting pigment, and do not have elevated salt requirements for growth but are halotolerant.

Titers of marine *Synechococcus* phages are shown to vary seasonally, and at times they reach population densities between 10^3 and 10^4 cyanophages per ml in both inshore and offshore waters. However, despite their diversity and abundance, we present compelling evidence that cyanophages are not significant agents of *Synechococcus* mortality and have a negligible effect in regulating the densities of marine *Synechococcus* populations. Instead, our results indicate that *Synechococcus* communities are dominated by cells resistant to their co-occurring phages and that these cyanophages are maintained by infecting the relatively rare sensitive cells in these communities. Our findings agree with studies that have examined the dynamics of virulent coliphages and *Escherichia coli* in chemostats and with mathematical models (7, 11–13) and show that field populations of *Synechococcus* spp. and their phages follow patterns predicted from these laboratory studies.

MATERIALS AND METHODS

Cultures. The 20 cyanobacteria used in this study came from two culture collections. Strains preceded by WH came from the Woods Hole Collection of Cyanobacteria (Biology Department, Woods Hole Oceanographic Institution), and strains preceded by PCC came from the Pasteur Collection of Cyanobacteria (Unité de Physiologie Microbienne, Institut Pasteur, Paris, France). Isolation data and strain properties are presented for the Woods Hole strains in reference 31 and the Pasteur strains in reference 22.

Stock cultures of cyanobacteria. Medium SN (31, 32) and medium BG11 (21) were used for the growth of marine and freshwater cyanobacteria, respectively. Cultures were incubated at 23°C in constant light (20 to 30 microeinsteins m^{-2} s^{-1}) with Vitalux fluorescent lamps (Luxor Light Products, Lindhurst, N.J.) (32).

* Corresponding author.

† Contribution number 8241 of the Woods Hole Oceanographic Institution.

Isolation of *Synechococcus* spp. *Synechococcus* enrichment and isolation were carried out in medium SNAX with procedures described in references 31 and 32. *Synechococcus* enrichments to document resistance to co-occurring phages were established by diluting a filtrate (pore size, 1.0 μm) of Woods Hole seawater in 10-fold increments (i.e., 1 to 10, 1 to 100, and 1 to 1,000 dilutions).

***Synechococcus* cell counts.** *Synechococcus* cells were counted by epifluorescence microscopy with techniques described in reference 31.

Electron microscopy. Phage ultrastructure was determined from preparations negatively stained with 1.0% aqueous uranyl acetate on Formvar-coated grids with a Philips 300 transmission electron microscope. Material for thin sections was fixed and prepared for TEM as described previously (30).

Phage isolation. *Synechococcus* phages were isolated and enumerated with liquid dilution cultures rather than from plaques on solid media because lawn formation was erratic for many strains belonging to *Synechococcus* marine cluster A. Either fresh seawater or seawater stored at 4°C with a few drops of chloroform was used to isolate cyanophages. Seawater samples were centrifuged at 6,000 rpm in a Sorvall HS-4 swinging bucket rotor for 12 min at 5°C to remove microorganisms. Ten-fold dilutions of the supernatant were made into successive 0.1-ml drops of a concentrated cell suspension of an appropriate host clone of a *Synechococcus* sp. contained in 24-well tissue culture plates (Falcon, no. 3047). Exponentially growing host clones were concentrated by centrifugation to a final concentration of approximately 10^8 cells per ml. The concentrated cell suspension containing the supernatant dilutions was incubated for 1 h at room temperature to allow for phage adsorption before being diluted with 1.5 ml of growth medium SN. The tissue culture plates were incubated in constant illumination (20 to 30 microeinsteins m^{-2} s^{-1}) at room temperature and were monitored for cell growth or lysis. Lysed cultures were examined by TEM to verify the presence of phages, which were then purified by a combination of successive serial dilutions in liquid media and from plaques on agar plates. Lawns of strains belonging to *Synechococcus* marine cluster A were produced by spreading a heavy cell suspension of exponentially growing cells on plates of medium SN (31) supplemented with 2.0 mM sodium sulfite and 20.0 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (Sigma Chemical Co., St. Louis, Mo.) at pH 8.0 (both were added aseptically just prior to the pouring of plates).

Phage titers. Phage titers were determined by the most-probable-number technique (10). The protocol was the same as described above, except that 10 multiples of each of four 10-fold dilutions were prepared. The dilutions were monitored for cell lysis weekly for 3 weeks.

Host range and specificity. Purified phage isolates were screened for host range and specificity in liquid cultures in tissue culture wells prepared and incubated as described for phage isolation.

Theoretical calculations of phage-mediated *Synechococcus* mortality. The percentage of *Synechococcus* populations that might be infected per day was calculated from theoretical rates of phage adsorption and measured concentrations of *Synechococcus* cells and phages.

Calculations were made with a theoretical value of adsorption for coliphage T4, a phage similar in structure and belonging to the same viral family as the majority of *Synechococcus* phage isolates (25). The time necessary for a phage to contact a host cell was calculated with the equation $\ln (P - 1) - \ln P = kNt$, where N is the number of *Synechococcus* cells, P is the phage titer, t is the time (minutes) for a phage to contact the host cell, k is the adsorption rate constant ($4\pi RCf = 0.196 \times 10^{-8}$ cm^3 min^{-1} for marine *Synechococcus* cells with the diffusion constant [C] for phage T4 [2.4×10^{-6} cm^2 min^{-1}], a *Synechococcus* volume of 1.0 μm^3 giving a spherical radius [R] of 0.65 μm, and with f, the fraction of collisions that results in adsorption, = 1 [i.e., all collisions result in adsorption]). The percentage of *Synechococcus* cells infected per day was calculated with the equation $(1{,}400/tN) \times 100$. In making these calculations, we assumed that adsorption led to cell lysis and that all cells in the natural population of *Synechococcus* cells were sensitive to phage infection, when in reality the majority of the population was later shown to be resistant to co-occurring phages. These calculations consequently overestimate the percentage of *Synechococcus* populations infected by phages.

RESULTS AND DISCUSSION

Using liquid dilution cultures, we have isolated a large collection of *Synechococcus* phages from both inshore and open ocean waters. Examination by TEM indicates that our 75 cyanophage isolates include representatives from each of the three families of tailed phages (4, 14) (Fig. 1), with the vast majority being contractile tailed phages of the family *Myoviridae*. Solely on the basis of ultrastructural characteristics, it is evident that these *Synechococcus* phage isolates are a diverse group that should represent a number of cyanophage species.

Even more striking than the morphological diversity of the *Synechococcus* phages were their very high titers. *Synechococcus* phage titers, determined by the most-probable-number technique, seasonally reached concentrations of between 10^3 and 10^4 phage per ml in both coastal and open ocean waters. This is in marked contrast to freshwater systems, in which only six cyanophage isolates have been found that infect unicellular cyanobacteria (23) and in which natural titers, although not specifically measured, are presumably low because the isolation of these phages occurred very rarely during an extensive survey (22a) or required the enrichment of at least a liter of sample (24a).

An example of the morphological diversity and range of *Synechococcus* phage titers within a single seawater sample is shown in Fig. 2. The sample collected in the Gulf Stream contained 1.1×10^4 *Synechococcus* cells per ml. By using *Synechococcus* strains from marine clusters A and B, we were able to demonstrate that this Gulf Stream sample contained eight morphologically distinguishable *Synechococcus* phages with as many as five types seen by TEM in the primary enrichment of a single host strain (i.e., WH8012). The titers of *Synechococcus* phages for host strains in marine cluster A ranged from 3.5×10^1 (host strain WH8108) to 7.0×10^3 (host strain WH8017) phage per ml and 1.3×10^1 phage per ml for the one host strain in marine cluster B.

The considerable differences seen in the phage titers for specific host strains in the Gulf Stream sample illustrated in Fig. 2 led us to screen *Synechococcus* phages to determine their host range and specificity (Fig. 3). Some phages will infect as many as 10 of 13 strains tested within marine cluster A (phage 9), whereas others (phages 5 and 7) will infect only the host strain used for isolation. However, phage 20, isolated on a host from marine cluster A, not only will infect

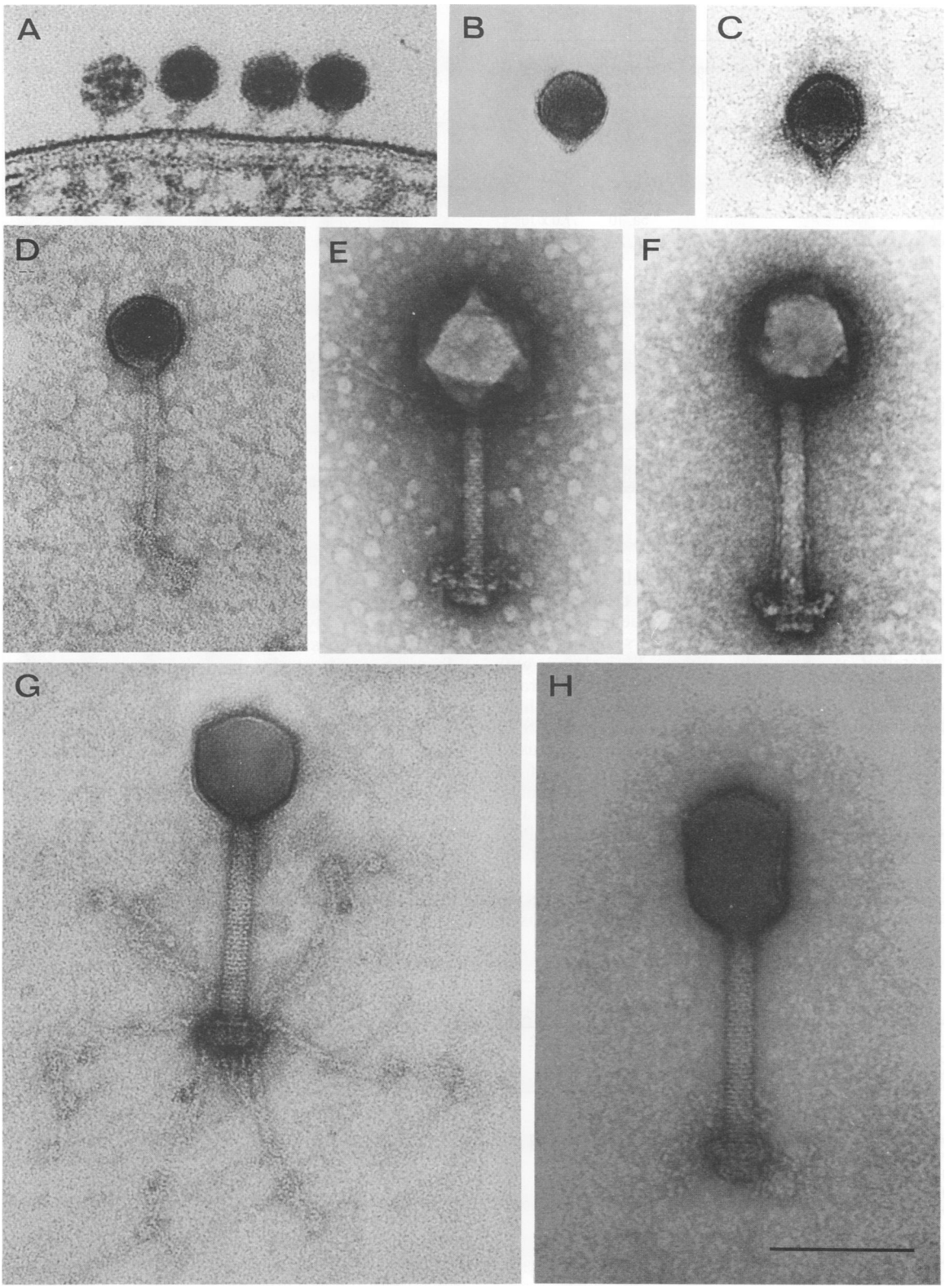

FIG. 1. Transmission electron micrographs of representative *Synechococcus* phages. (A) A thin section showing phage 12 attached to the cell surface of *Synechococcus* strain WH8017. (B to H) Negatively stained phage preparations. (B) Phage 12 isolated from the Gulf Stream (37°N, 73°40′W) in December 1990 with strain WH8018. (C) Phage 5 isolated from the Sargasso Sea (34°N, 60°W) in July 1990 with strain WH8109. (D) Phage 4 isolated from Woods Hole Harbor in August 1990 with strain WH5701. (E) Phage 14 isolated from the Gulf Stream (37°N, 73°40′W) in December 1990 with strain WH8103. (F) Phage 19 isolated from the Sargasso Sea (34°N, 60°W) in July 1990 with strain WH8109. (G) Phage 1 isolated from Woods Hole Harbor in August 1990 with strain WH8101. (H) Phage 10 isolated from the Gulf Stream (37°N, 73°40′W) in December 1990 with strain WH8017. (A to C) Viral family *Podoviridae*. (D) Viral family *Styloviridae*. (E to H) Viral family *Myoviridae*. Bar, 100 nm.

Host *Synechococcus* (Culture No.)	*Synechococcus* Taxonomic Group	Type Phage Isolated	Type Phage Observed in Enrichment	Phage Titers / ml
WH 8012	Marine A	7	1, 2, 4, 7, 9	4.3×10^3
WH 8103	Marine A	7	7	6.2×10^2
WH 8108	Marine A	7	7	3.5×10^1
WH 8018	Marine A	5, 7	5, 7	2.5×10^3
WH 8109	Marine A	5, 7	5, 7	1.3×10^2
WH 8017	Marine A	5a, 10	5a, 10	7.0×10^3
WH 7803	Marine A	1	1, 2	6.2×10^2
WH 8101	Marine B	1	1, 2	1.3×10^1

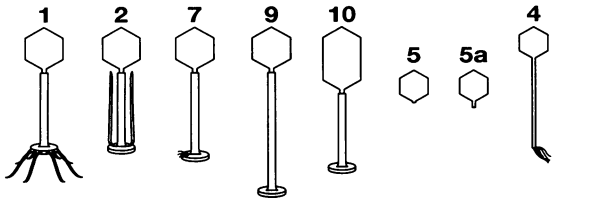

FIG. 2. Morphological diversity and range of *Synechococcus* phage titers in a surface seawater sample collected in the Gulf Stream (36°58'N, 73°42'W) on 2 December 1990.

TABLE 1. Percentage of *Synechococcus* populations that might be infected per day calculated from theoretical rates of phage adsorption (25) and measured concentrations of *Synechococcus* cells and phages[a]

Sample characteristics	Cells/ml	Phage titer/ml	% infected/ day
Woods Hole Harbor annual cycle, 1991			
5 June	5.0×10^4	1.9×10^1	0.005
24 July	3.8×10^5	1.14×10^4	3.2
14 August	8.0×10^3	3.3×10^3	0.9
10 September	8.6×10^4	5.6×10^2	0.16
7 October	7.1×10^4	9.2×10^2	0.26
Slope Water (38°N, 70°W), 10 September 1990	1.5×10^4	2.1×10^3	0.6
Gulf Stream (37°N, 73°W), 2 December 1990	1.1×10^4	4.3×10^3	1.2
		7.0×10^3 (WH8017)	2.0

[a] Sampling dates in Woods Hole Harbor are identified as points 1 to 5 in Fig. 4. Titers were measured with host WH8012, except for one sample from the Gulf Stream in which host WH8017 was used (Fig. 2).

other strains of marine cluster A but will also infect strain WH8101 from marine cluster B, which is consistent with phylogenetic analyses using 16S rRNA sequences that have shown that *Synechococcus* marine clusters A and B are closely related (7a). Similarly, phage 1, isolated with WH8101 from marine cluster B, will infect 5 of 13 strains tested that belong to marine cluster A. None of the phage clones tested would infect *Synechococcus* strains WH5701 and WH8007, which are currently placed in marine cluster B, nor would they infect freshwater strain PCC6307 of the *Cyanobium* cluster, which is on the same phylogenetic lineage as marine clusters A and B. More distantly related members of the *Synechococcus* group (i.e., PCC 6301, PCC 6716, and PCC 7335) (29) were not infected by any of the phages tested.

Preliminary characterization indicates that our isolates are virulent phages. The broad host range within marine cluster A may be due to the variable possession of host resistance, immunity to specific phages, or restriction and modification systems. We have observed that resistant *Synechococcus* clones frequently grow up and can be isolated from cultures after phage lysis. However, attempts to induce lysogeny in *Synechococcus* strains by using temperature shock, light shifts, UV and X radiation, and mitomycin have not been successful.

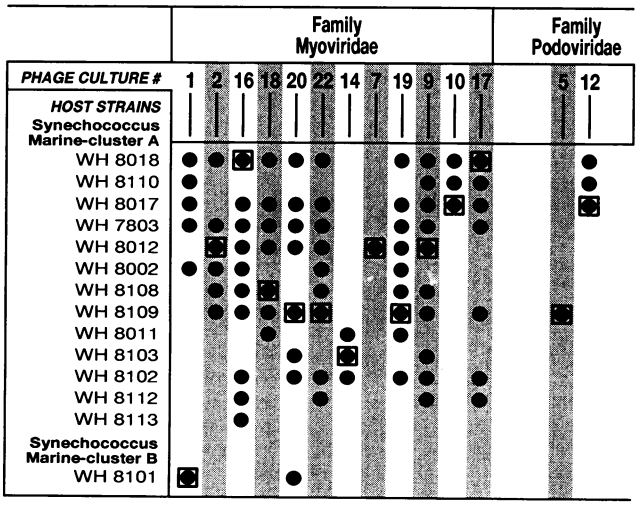

FIG. 3. Host ranges of representative *Synechococcus* phages. □, host used for phage isolation.

To assess the effects of cyanophages on *Synechococcus* mortality, we determined the percentage of natural populations that might be lysed by viral infection according to theoretical rates of phage adsorption and measured abundances of *Synechococcus* spp. and their phages. Using *Synechococcus* concentrations and phage titers measured on five dates in Woods Hole waters during the summer of 1991, we calculated that the percentage of the population that might be lysed by phages ranged from 0.005%/day at the end of the spring bloom to 3.2%/day during a *Synechococcus* peak in July (Table 1 and Fig. 4). Offshore samples from Slope Water, south of Cape Cod, and from the Gulf Stream yielded similar results of 0.6%/day and 1.2 to 2.0%/day, respectively (Table 1). These calculations indicate that cyanophages would not be major contributors to *Synechococcus* mortality in either inshore or offshore oceanic waters.

Using another approach, Proctor and Fuhrman (18, 19) calculated that viral infection might account for as much as 30% of *Synechococcus* mortality in seawater. They observed by TEM that 0.8 to 2.8% of *Synechococcus* cells in natural marine populations contained assembled phages. Their value for *Synechococcus* mortality was derived with data for *Cytophaga marinoflava* in which assembled phages were visible by electron microscopy during the latter 10% of the latent period (28). Had they used published values for cyanobacteria in which assembled phages were visible during 50% of the latent period (17), their calculations would be in closer agreement with ours. We have also monitored the lytic cycle of one of our *Synechococcus* phages (no. 12) by electron microscopy and have shown that assembled phages were visible during 60% of the latent period.

To assess the role of phages on *Synechococcus* natural population dynamics, we compared results on the annual cycle of *Synechococcus* spp. and their phages in Woods

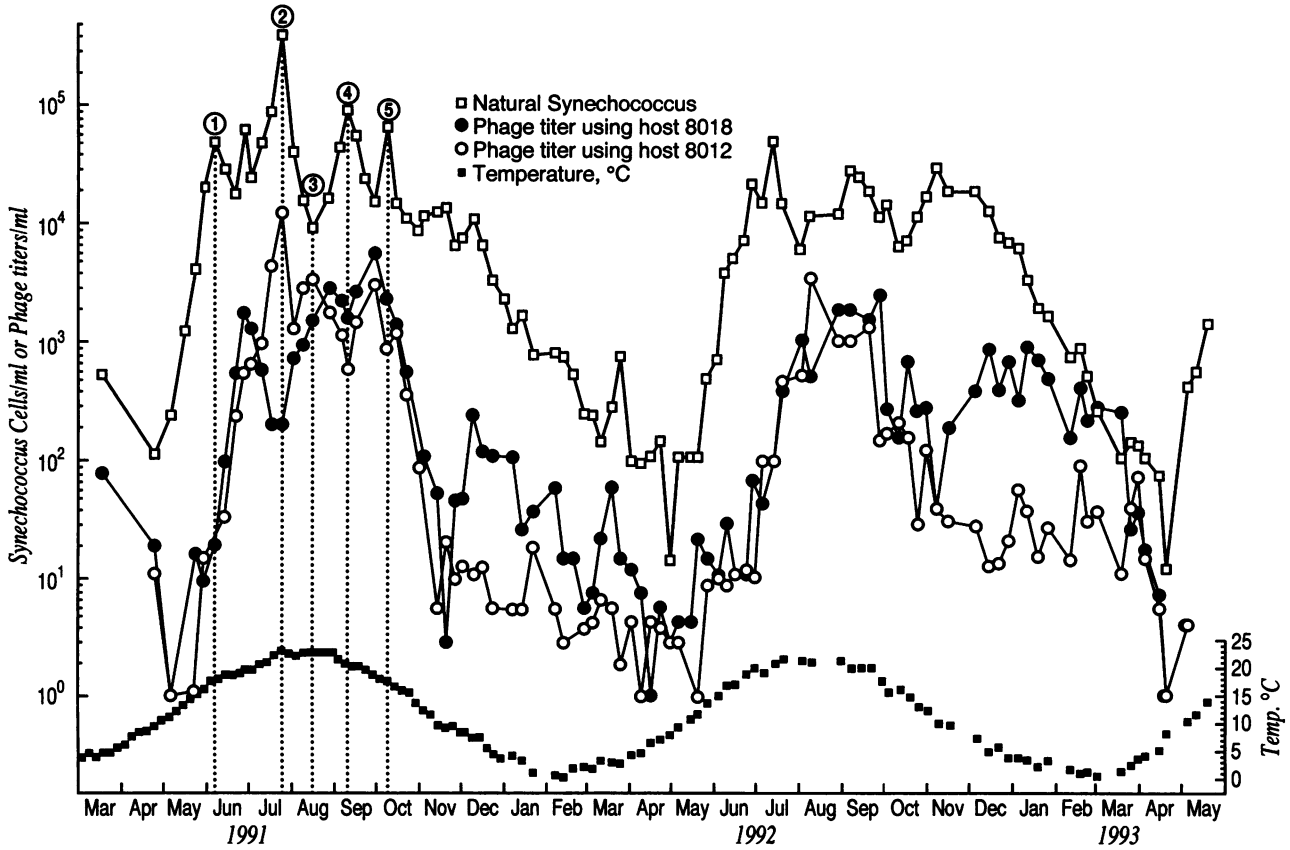

FIG. 4. Annual cycle of *Synechococcus* spp. and *Synechococcus* phages in Woods Hole Harbor. Cell counts were determined by epifluorescence microscopy. Phage titers were determined by the most-probable-number technique in tissue culture wells with the host strains WH8018, isolated from Woods Hole in 1980, and WH8012, isolated from the Sargasso Sea in 1980. These strains were chosen because of their sensitivity to a broad spectrum of *Synechococcus* phages (Fig. 3). Points 1 to 5 refer to dates in Table 1.

Hole waters with the results of laboratory experiments conducted by Levin, Lenski, and their colleagues describing the dynamics of interactions between *E. coli* and virulent coliphages (7, 11–13). The annual cycle of *Synechococcus* abundance is driven by water temperature and is very repeatable from year to year (31) (Fig. 4). We noted that during 15 years of monitoring their annual cycle, *Synechococcus* spp. have never been observed to deviate dramatically from their temperature-driven pattern in a way that might suggest the occurrence of a massive lytic phage infection. The annual cycle of the *Synechococcus* phage titers was monitored with two host strains (WH8018 and WH8012). These titers (Fig. 4) begin to increase about a month after the onset of the *Synechococcus* spring bloom. During the summer months, the phage titers oscillate between 10^3 and 10^4 phage per ml and are about 10-fold less abundant than their host population. During the winter months, the phage titers fell below 100 phage per ml, and on five occasions since March 1991 they were undetectable with one host strain but were never undetectable with both host strains at the same time (Fig. 4).

Laboratory studies by Wiggins and Alexander (33) with a variety of bacteria and phages have indicated that there is a minimum host density threshold of approximately 10^4 cells per ml required for phage replication. Results from the annual cycles of *Synechococcus* spp. and their phages in Woods Hole waters indicate that this threshold is not universal. Apparently in this aquatic environment, there is sufficient time for phage adsorption to occur at host and phage densities well below a theoretical threshold of 10^4 host cells per ml (Fig. 4). Lower thresholds in the systems examined by Wiggins and Alexander (33) may have been masked by the rapid growth rates of the chemoheterotrophic bacteria they studied, whereas growth rates of *Synechococcus* spp. during the spring bloom are slow, averaging a doubling every 4.3 days (31).

One of the principal findings from resource-limited chemostat studies with *E. coli* and virulent coliphages is that the *E. coli* population rapidly acquires resistance to its co-occurring phages (7, 11–13). To test for *Synechococcus* resistance to co-occurring cyanophages, clones of *Synechococcus* cells and *Synechococcus* phages were isolated from a single water sample collected in Woods Hole Harbor on 17 September 1991. After enrichment, 10 clonal *Synechococcus* isolates were established from single colonies picked from agar plates. From the same original sample, seven clones of *Synechococcus* phages were established with liquid enrichments in tissue culture wells and were purified by successive serial dilution. The 10 clonal isolates of *Synechococcus* cells were then challenged by the seven phage isolates. Results from this experiment (Table 2) indicated that the natural population of *Synechococcus* spp. sampled during September 1991 followed the pattern predicted from laboratory experiments with *E. coli*. The *Synechococcus* clones iso-

TABLE 2. Resistance of *Synechococcus* clones to co-occurring phages isolated from Woods Hole Harbor on 17 September 1991

Strain[a]	Isolation dilution	Cyanophage resistance or sensitivity[b]						
		WH8018				WH8012		
		A	B	C	D	A	B	C
1-01	10^{-1}	+	−	−	−	−	−	−
1-02	10^{-1}	+	−	−	+	−	−	−
1-04	10^{-1}	+	−	−	−	−	−	−
4-01	10^{-2}	+	+	+	+	+	+	+
4-02	10^{-2}	−	+	+	+	+	+	+
4-03	10^{-2}	+	+	+	+	+	+	+
2-01	10^{-3}	−	−	±	−	+	+	+
2-02	10^{-3}	+	+	−	+	+	+	+
3-01	10^{-3}	+	+	+	+	+	+	+
3-07	10^{-3}	+	±	±	+	+	+	+

[a] The cells were small cocci (0.7 by 1.6 μm in diameter).

[b] All seven phage isolates were identified by TEM as members of the family *Myoviridae*. Symbols: +, resistant, with cell growth equal to that of controls; ±, resistant, with cell growth not as good as that of controls; −, sensitive, with cells lysed.

lated from the 100- and 1,000-fold dilutions were resistant to most of the phage isolates, indicating that a majority of the *Synechococcus* population at the time of sampling was resistant to its co-occurring phages.

Population stability in resource-limited chemostat experiments requires that a small population of sensitive cells persists, becomes infected, and releases phages. This results in a phage population that is typically about an order of magnitude less abundant than its host. It is believed that sensitive cells persist as a minority population because they possess a subtle ecological advantage over their resistant counterparts (7, 12). Predominantly sensitive *Synechococcus* clones were isolated only from enrichments of the lowest dilution (10^{-1}) (Table 2). This indicated that they represented a minor component of the natural population and that, when present, they were capable of outcompeting their resistant counterparts during the enrichment process. Finally, *Synechococcus* phage titers in both inshore and offshore waters were consistently lower than their host cell densities. This agrees with similar observations made in chemostat studies dominated by bacterial strains resistant to co-occurring phages (7, 11–13) and with observations for natural populations (1, 24).

The acquisition and maintenance of resistance by *Synechococcus* spp. to their co-occurring phages permit these cyanobacteria to coexist stably with a diverse group of cyanophages that can be present at high titers in seawater. As a consequence of this stable coexistence, *Synechococcus* cyanophages may have little effect on the overall population densities of marine *Synechococcus* cells but should be important in determining and enhancing the clonal composition of these populations (7, 11, 12, 15). An indication that clonal shifts and clonal diversity may occur within *Synechococcus* populations can be seen in Fig. 4 at points 2 and 4 and in the diversity of *Synechococcus* clones that we have isolated from both inshore and open ocean waters (31).

It has been suggested that the isolation of phages is best accomplished with bacterial strains isolated from the same sample that contains the natural phage population (15, 16). The observation that bacteria are resistant to their co-occurring phages suggests that this procedure will lead both to underestimates of phage abundances and to the isolation of a restricted group of phages. In our experience, the use of *Synechococcus* strains cultured in the absence of phage pressure for several years yields the best results. This coincides with the observation that phage-resistant *Synechococcus* clones begin to revert to phage sensitivity after approximately 2 years of culture in the absence of phages.

The results presented here show that predictions made from laboratory experiments describing the interactions of bacteria and virulent phages (7, 11–13) were applicable to natural populations of marine *Synechococcus* spp. and their phages and should in the future be shown to apply to a wide variety of phage-host systems in aquatic environments. Conversely, recent suggestions that phages might be responsible for a large fraction of procaryotic mortality in seawater seem unlikely. Earlier in this century, a similar situation occurred in medical microbiology in which viruses were advanced as agents to treat diseases caused by bacteria. In each case, virus therapy failed, mainly because of the ability of the pathogenic bacteria to defend themselves against viral attack by rapidly acquiring resistance.

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