Marine Viruses Exploit Their Host’s Two-Component Regulatory System in Response to Resource Limitation

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Marine viruses exploit their host's two-component regulatory system in response to resource limitation

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Summary

Phosphorus (P) availability, which often limits productivity in marine ecosystems, shapes the P-acquisition gene content of the marine cyanobacteria \textit{Prochlorococcus} \textsuperscript{[1–4]} and its viruses (cyanophages) \textsuperscript{[5, 6]}. As in other bacteria, in \textit{Prochlorococcus} these genes are regulated by the PhoR/PhoB two-component regulatory system that is used to sense and respond to P availability and is typical of signal transduction systems found in diverse organisms \textsuperscript{[7]}. Replication of cyanophage genomes requires a significant amount of P, and therefore these phages could gain a fitness advantage by influencing host P acquisition in P-limited environments. Here we show that the transcription of a phage-encoded high-affinity phosphate-binding protein gene (\textit{pstS}) and alkaline phosphatase gene (\textit{phoA})—both of which have host orthologs—is elevated when the phages are infecting host cells that are P starved, relative to P-replete control cells. We further show that the phage versions of these genes are regulated by the host’s PhoR/PhoB system. This not only extends this fundamental signaling mechanism to viruses but is also the first example of regulation of lytic phage genes by nutrient limitation in the host. As such, it reveals an important new dimension of the intimate coevolution of phage, host, and environment in the world’s oceans.
Results and Discussion

Unicellular cyanobacteria Prochlorococcus and Synechococcus are the dominant photosynthetic organisms in the oceans and they contribute significantly to global primary production [8]. The environmental availability of phosphorus (P), often a limiting nutrient in marine ecosystems, exerts strong selective pressure on Prochlorococcus genomes, which is manifested in the suite of P-acquisition genes they contain [1–4]. This selective pressure is also visible in cyanophages (viruses that infect cyanobacteria): 9 of the 16 sequenced T4-like cyanophages isolated on Prochlorococcus and Synechococcus [9] contain pstS, encoding a periplasmic high-affinity phosphate-binding protein, and 2 contain phoA, an alkaline phosphatase gene. We have suggested previously that these genes of host origin play a role in the acquisition of phosphorus required for phage DNA replication [6]. Consistent with this hypothesis, the frequency of occurrence of pstS gene in phages in the wild was shown to be higher in oceanic sites with lower phosphate [5], as is true of their Prochlorococcus hosts [4, 10].

Both pstS and phoA are upregulated in Prochlorococcus [2] and Synechococcus [11] during P starvation, a signal transduction response regulated by the bacterial phosphate sensing two-component system. We postulated that the expression of the phage versions of these P-acquisition genes (hereafter referred to as “phage pstS” and “phage phoA”) might be regulated by P availability to the hosts—possibly through the PhoR/PhoB two-component regulatory system in the host cells. To address this question, we used a cultured Prochlorococcus strain (NATL2A) and the T4-like cyanophage P-SSM2, which encodes pstS, to measure expression of selected phage and host genes, under P-depleted and P-replete conditions (Figure 1). Transcript levels of the host pstS gene increased within 8 hr in the P-limited host cells (Figure 1B) and rose steadily thereafter, signaling the onset of P starvation, which was manifested as a decrease in the culture growth rate (Figure 1A). Reintroduction of phosphate at 46 hr (arrow, Figure 1B) resulted in a rapid decline of pstS transcripts as the cells were released from P starvation. Similar patterns have been observed in other strains of Prochlorococcus under P starvation [2].

We used this system to examine the effect of host P starvation on infection kinetics and pstS expression level in infecting phage. The cultures were infected 47 hr after resuspension in P-depleted media (shaded interval in Figure 1B) when P starvation was well established in the host cells, as indicated by upregulation of pstS (Figure 1B). Both host and phage pstS transcript levels were then measured over time. Host pstS transcript levels in infected P-starved cells stayed higher than those in the P-replete cultures (Figure S1A available online), as was observed in uninfected hosts (Figure 1B). Transcript levels of phage pstS increased in P-starved hosts relative to the P-replete control (Figures 1C and S1B), whereas transcripts of genes g61 and g20—early and late T4-like phage structural genes [12–14], respectively—did not (Figures 1C and S1B), suggesting differential regulation of pstS and phage structural genes. Addition of phosphate 4 hr after infection (arrow, Figure 1C) resulted in a reduction of both host (similar to Figure 1B) and phage pstS transcripts within 4 hr (Figure 1C), suggesting that both phage and host pstS genes could be regulated by the same mechanism.

phoH encodes an ATP binding protein with unknown function [15] and is considered a phosphate (pho) regulon gene because it is upregulated by P starvation in E. coli [16, 17]. Its expression is not upregulated during P starvation in marine cyanobacteria [2], however, suggesting that it may not play the same role as in E. coli [18]. Nonetheless, because of its prevalence in T4-like cyanophages [9] and association with the pho regulon in E. coli, we examined its expression in our experiments. We found that the expression of phoH in the phage
purified bp sequences 70.8% identical to the predicted and found a putative host PhoB protein to a 2C). This cell, are the phage genes phage genes via the host pho regulon would be through binding of host PhoB to the promoters of their phages [9]. Thus we hypothesized that the likely mechanism for the regulation of these PCC6803 [19]. They have also been predicted in separated by flexible 3 bp linkers [16], have been experimentally determined in sequences, which contain conserved tandem 8 bp repeats sequences in their promoters, which bind the transcriptional activator PhoB [16].

PhoR/PhoB system (Figure 2D). As before, and as phage/host increased relative to controls in P

starved and agreeing with our results for Prochlorococcus phages (Figure S1F). The phoR mutant (doubling time 1.43 ± 0.04 days) grew slower than the WT (1.39 ± 0.04 days) in P-replete conditions, which could explain why phage production was lower in the phoR mutant than in the WT host (Figures 2A and 2B). Most importantly, phage pstS and phoA transcripts increased relative to controls in P-starved WT host cells (Figure 2C), as in our Prochlorococcus phage/host system (Figure 1C), but they did not in the mutant lacking the PhoR/PhoB regulatory system (Figure 2D). As before, and as expected, transcript levels of early and late phage structural genes g61 and g20, respectively, were not affected by P starvation of the WT or mutant cells (Figures 2C and 2D). The significance of the expression patterns of genes g172 and hsp20 in this experiment will become clear below.

We first established that upon the onset of P starvation in host cells, pstS was not upregulated in the phoR mutant, although it was in the WT cells—simply confirming the results of Tetu et al. [11] (data not shown). Once pstS was upregulated in the P-starved WT host, we infected P-starved and control cultures of both the mutant and WT strains with phage and measured phage production and the expression levels of a suite of phage genes over the course of infection (Figure 2). Phage production in P-starved cells was reduced relative to the P-replete controls for both the WT and phoR mutant (Figures 2A and 2B), confirming that the mutant was indeed P starved and agreeing with our results for Prochlorococcus phages (Figure S1F). The phoR mutant (doubling time 1.43 ± 0.04 days) grew slower than the WT (1.39 ± 0.04 days) in P-replete conditions, which could explain why phage production was lower in the phoR mutant than in the WT host (Figures 2A and 2B). Most importantly, phage pstS and phoA transcripts increased relative to controls in P-starved WT host cells (Figure 2C), as in our Prochlorococcus phage/host system (Figure 1C), but they did not in the mutant lacking the PhoR/PhoB regulatory system (Figure 2D). As before, and as expected, transcript levels of early and late phage structural genes g61 and g20, respectively, were not affected by P starvation of the WT or mutant cells (Figures 2C and 2D). The significance of the expression patterns of genes g172 and hsp20 in this experiment will become clear below.

The evidence presented thus far is compelling that the transcription of the phage pstS (and phoA) is regulated by the host PhoR/PhoB two-component system. What is the mechanism? Host genes regulated by the PhoR/PhoB system (pho regulon genes) have well-conserved pho box sequences in their promoters, which bind the transcriptional activator PhoB [16], pho box sequences, which contain conserved tandem 8 bp repeats—important for PhoB binding [21]—separated by flexible 3 bp linkers [16], have been experimentally determined in Synechocystis sp. PCC6803 [19]. They have also been predicted in Prochlorococcus, Synechococcus [22], and their phages [9]. Thus we hypothesized that the likely mechanism for the regulation of these phage genes via the host pho regulon would be through binding of host PhoB to the promoters of the phage genes—activating their transcription.

In cyanophage S-SM1, pstS is upstream of phoA, with which it overlaps. Just upstream of pstS are g172 and hsp20 (Figure 3A). When the phage infects a P-starved WT Synechococcus host cell, g172, pstS, and phoA are upregulated in the phage (Figure 2C), whereas hsp20 is not (Figure 2C). This suggests that g172, pstS, and phoA are transcribed from the same promoter—probably located between g172 and hsp20 (black bar, Figure 3A)—which is activated by binding of the host PhoB protein to a pho box. To explore this further, we searched for (by manual alignment) and found a putative pho box sequence in this region (Figures 3B and S2A) with three tandem 8 bp sequences 70.8% identical to the predicted host Synechococcus WH8102 pho box [22]. We purified recombinant Synechococcus WH8102 PhoB protein (see Experimental Procedures) and
PCR amplified a ~200 bp DNA fragment upstream of g172 (Figure 3A) containing this putative pho box and used it in binding assays. A gel mobility shift assay showed that the recombinant *Synechococcus* WH8102 PhoB protein binds to this DNA fragment (Figure 3C) and that this binding reaction is sequence specific (Figure S2B), suggesting that this putative pho box is functional. Phage gene g172, which is not found in any host genomes, is between the putative pho box and the phage pstS gene (Figure 3A). The putative pho box sequence in front of g172 could be formed or gained after g172 and pstS got inserted in the phage genome. Or, this putative pho box could be gained together with pstS from the host genome and g172 was inserted between them during gene recombination events, which could destroy the pho box. In both scenarios, there must be strong selective pressure to maintain this putative pho box in front of pstS in the phage, even though it need not be in that location in the host genome. Thus, cyanophages not only carry pstS and phoA genes, but the expression of these genes is tied into the host PhoR/PhoB phosphatesensing two-component regulatory system, which regulates the P-starvation response of the host. Although we can’t completely exclude the possibility of indirect regulation of these phage genes by the PhoR/PhoB system, the results of our binding assay strongly favors direct regulation.

Regulation of phage pstS and phoA genes by P availability to the host also suggests that these phage genes play a role in the acquisition of phosphorus for phage DNA replication during infection. For *Prochlorococcus* (Figure S1F) and *Synechococcus* (Figures 2A and 2B) infecting phages with the pstS gene, we found that phage production was reduced by P starvation (Figures S1F, 2A, and 2B), but the timing of the infective cycle was not changed (Figures S1C–S1F). In a related cyanophage/host system, in which the phage does not carry the pstS gene, the lytic cycle is lengthened during infection of P-starved host cells [23], consistent with our hypothesis that phage pstS expression may augment the P supply to the host cell to facilitate an expedient lytic cycle. Phage-encoded phoA may facilitate access to organic P pools, which again would give phage a selective advantage in phosphate-limited environments. Cyanophage S-SM1 gene g172 may play a role in this process as indicated by the fact that its expression is also induced by P starvation, although its function is still unknown. Because of the lack of genetic tools, we couldn’t knock out these cyanophage genes to see how they affect the lytic cycle, although the selective advantage for cyanophages to have pstS gene is supported by the fact that it is enriched in phage genome fragments at oceanic sites with lower phosphate concentrations [5].

Two-component regulatory systems have been found in the three kingdoms of life [7] enabling cells to better acclimate to changing environmental conditions. Although nutrient limitation has been shown to affect the lysis-versus-lysogenization decision of coliphage λ through the host ppGpp level [24, 25], to our knowledge this is the first example in which a lytic virus exploits a host two-component system to be responsive to “environmental conditions” within the host cell, which in turn is responding to nutrient limitation in the external milieu. This extends the selection pressures on the host cell to its infecting viruses, making ever more intimate the coevolution of viruses, hosts, and their environment.
Figure 1. Host and Phage Transcript Levels during Infection of *Prochlorococcus* NATL2A by Cyanophage P-SSM2 Under P-depleted and P-replete Conditions

(A and B) Uninfected host cells. P starvation was achieved by resuspending log-phase cells in P-depleted (-P) media at t=0 hr, and letting the culture grow.

(A) Cell number as a function of time in P-replete control cultures (open circles) compared to cells re-suspended in media with no amended P at t=0 hr (-P, closed circles).

(B) Host *pstS* (YP_291636.1) transcript levels in uninfected cells during P starvation relative to P-replete controls. The arrow indicates the reintroduction of phosphate at 46 hr to verify the P-starvation condition.

(C) Phage gene expression in infected cells. Aliquots from the cultures in (A) were infected with phage 47 hr after the onset of P starvation and infection was then monitored for 12 hr (shaded interval shown in (B)). Transcript levels of phage genes *pstS*, *phoH*, *g61* (DNA primase) and *g20* (portal protein,) were measured during infection of P-depleted host cells, and normalized to their levels when infecting P-replete cells. The only transcript levels that differed from the controls was that of *pstS*, thus all others are flat lines near 1 on the y-axis. The arrow indicates reintroduction of phosphate 4 hr after infection and dashed lines indicate transcript levels after phosphate reintroduction. Error bars indicate the SD of two biological replicates and are smaller than the data point when not apparent. The vertical dotted line indicates the beginning of phage burst, as determined in Figure S1F.
Figure 2. Infection and Gene Expression Patterns in Cyanophage S-SM1 Infecting a Wild-Type and Mutant Strain of *Synechococcus* WH8102 under P-depleted and P-replete Conditions.

(A and B) Phage genome copies released while infecting P-replete (control), and P-depleted (-P) WT (A) and mutant (B) strains. The WT and mutant strains were transferred to P-replete (control) and P-depleted (-P) media, and once P limitation was established in the -P cultures, as evidenced by upregulation of *pstS* in the WT strain (not shown) they were infected by phage at t= 0 hr. The vertical dotted line indicates the beginning of phage burst.

(C and D) Transcript levels of phage genes in the P-depleted host infections relative to P-replete controls: *pstS, phoA, g172, hsp20, g61, g20*. Error bars indicate the SD of two biological replicates.
Figure 3. Binding of Recombinant Synechococcus WH8102 PhoB to the Upstream Region of pstS in Phage S-SM1.

(A) Genomic organization of the S-SM1 pstS region, showing hsp20, g172, pstS and phoA. A black bar indicates the ~200 bp DNA fragment containing the putative pho box that was used in the binding assays shown in (C).

(B) Alignment of the experimentally determined pho box sequence of Synechocystis sp. PCC6803 (S6803) [19] and the putative host and phage pho box sequences. The 8 bp conserved tandem repeats are underlined, which are important for PhoB binding. Bold letters indicate conserved sequences.

(C) DNA fragment containing the putative pho box was labeled by a fluorophore and incubated with increasing concentrations of purified recombinant Synechococcus WH8102 PhoB protein. Free DNA and the shifted PhoB-DNA complex were separated in native polyacrylamide gels.
Experimental Procedures

Strains and growth conditions

Axenic Prochlorococcus NATL2A was grown in 0.2 µm filtered Sargasso seawater based Pro99 medium [26] amended with 10 mM HEPES (pH 7.5) and 12 mM sodium bicarbonate. Axenic Synechococcus WH8102 was grown in SN medium [27] made with seawater from Woods Hole, MA. Kanamycin (25 µg ml⁻¹) was used to maintain the phoR mutant [11]. Cultures were maintained at 21°C under constant cool white light (30 µmol quanta m⁻² s⁻¹).

Prior to infection, the NATL2A culture was harvested by centrifugation (15,000 x g for 10 min), washed twice in either PO₄-replete (Pro99 with 50 µM PO₄) or -depleted (Pro99 without PO₄) medium and resuspended in the same medium. WH8102 and the phoR mutant cultures were transferred 1:25 to PO₄-replete or -depleted SN medium.

Cyanophage P-SSM2 lysate was concentrated by centrifugation (5,000 x g) with Amicon Ultra-15 30K Centrifugal Filter Units (Millipore), washed twice in filtered Sargasso seawater and resuspended in the same medium. Cyanophage S-SSM1 lysate was concentrated the same way and resuspended in filtered seawater from Woods Hole. Infection was carried on with a multiplicity of infection of 3. Total cell concentration was determined by flow cytometry (Influx, Cytopeia-BD), and phage concentration was determined by the most probable number assay [28].

Quantification of phage and host genomic DNA during infection

Phage and host genomic DNA was quantified using a quantitative PCR method described previously [29]. Briefly, infected Prochlorococcus cells were filtered through polycarbonate filters (0.2 µm pore-size) to separate extracellular phage (filtrate), from host cells containing both host and intracellular phage genomic DNA. The latter were recovered from the filters. The qPCR primers used to measure DNA in the filter and filtrate fractions are listed in Table S1.

RNA extraction and transcript analysis

Samples were collected by centrifugation at 15,000 x g for 15 min at 4°C, and cell pellet was flash frozen in liquid nitrogen and stored at -80°C. Total RNA was then extracted using the Ambion mirVana RNA isolation kit and DNA was removed using the Turbo DNA-free kit (Ambion). Total RNA was reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad).

RNA copies were quantified using a QuantiTect SYBR Green PCR Kit (QIAGEN) with 0.5 µM forward and reverse primers (Supplemental Table 1) on a LightCycler 480 Real-Time PCR System (Roche Diagnostics). The qPCR primers were designed to only amplify the gene of interest. The specificity of phage (or host) pstS gene primers was confirmed by showing that they don’t amplify the host (or phage) pstS genes (data not shown). The amplification reaction consisted of an initial activation step of 15 min at 95°C, then 50 cycles of denaturation (95 °C, 15 s), annealing (56 °C, 30 s) and extension (72 °C, 30 s), followed by 5 min at 72 °C. Relative transcript abundance was determined by the ΔΔCT method [30]. The host rnpB gene was used as an internal control for our gene expression study, since transcription of this gene is stable in various conditions, including phage infection [29] (Figure S3).

Protein expression and purification

Primers 8102phoBNcoI (5′-AAAAAAACCATGGCTATGCCCCGCTGTG-3′) and 8102phoBHindIII (5′-TTTTTTAAGCTTTACCGAACCCTAGCC-3′) were used to amplify the Synechococcus WH8102 phoB gene. PCR was carried out with 0.02U/µl KOD HiFi DNA polymerase (Novagen) in 120 mM Tris-HCl (pH 8.0), 10 mM KCl, 6 mM (NH₄)₂SO₄,
0.1% Triton X-100, 0.001% BSA, 1 mM MgCl₂, 0.2 mM dNTPs and 0.4 µM of each primer. PCR cycling conditions consisted of a hot start at 94 °C for 5 min, followed by 25 cycles (98 °C for 15 sec, 50 °C for 2 sec and 74 °C for 20 sec), followed by incubation at 74 °C for 7 min. This gene was then cloned into pET30a plasmid (Novagen) with a His-tag on the 5' end and transformed into E. coli BL21(DE3) competent cells. Single colony was grown at 37 °C overnight in LB medium containing 50 µg/ml Kanamycin, diluted 1:100 with the same medium and grown at 37 °C until OD₆₀₀=0.5. Protein expression was induced by adding IPTG to a final concentration of 0.1 mM and cells were grown at 18 °C for 24 hours. Cells were harvested by centrifugation at 6,000 g for 20 minutes. The cell pellet was suspended with binding buffer (20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, pH 7.4) and disrupted by sonication. The crude lysate was centrifuged at 10,000 g for 20 min and the supernatant was loaded on a HisTrap™ FF crude column (GE Healthcare). Protein purification was performed according to the manufacturer’s instructions. Protein was eluted from the column with elution buffer (20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4). Imidazole was removed from the protein solution by centrifugation (5,000 x g, 15 min) with Amicon Ultra-15 10K Centrifugal Filter Units (Millipore), washed twice with 20 mM NaH₂PO₄ (pH 7.4) and resuspended in the same buffer. Although PhoB needs to be phosphorylated by PhoR to become active, the DNA binding ability of purified recombinant PhoB from E. coli is comparable to that of phosphorylated PhoB [20].

**Electrophoretic mobility shift assay**

Primers SSM1F187bp (5’-ATGGAATGCTGACAATTAATTACAA-3’) and SSM1IR10_6FAM (5’-6FAM-CTTTTTTTATTTGTATGTGTGAA-3’) were used to amplify a 200 bp fragment upstream of phage S-SM1 g172. The reverse primer SSM1R10_6FAM was 5’ labeled with the fluorophore 6FAM to enable in gel detection using a fluorescence scanner. The same primers without labeling were used to amplify the non-labeled specific competitor DNA fragment. Nonspecific competitor DNA fragment was amplified with primers SSM1F357bp (5’-TAAAGACGAAATCGAAGTGAACC-3’) and SSM1F187bp_RC (5’-TTGTAAATTACAGCATTCCAT-3’). PCR products were purified using QIAquick PCR Purification Kit (Qiagen). For binding assays without competitor, 3 nM labeled DNA fragment was incubated on ice for 30 min with different amounts of PhoB protein in 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 5 mM MgCl₂, 2.5 % glycerol, 5 µg/ml poly-dIdC, 2µg/ml BSA and 1 mM DTT in a 20 µl reaction. For assay with competitors, 3 nM labeled DNA fragment and 1 µM PhoB protein were incubated with different amounts of non-labeled specific competitor or nonspecific competitor. The free DNA and protein-bound complexes were separated on 5% native polyacrylamide gel with 1X TBE buffer (89 mM Tris, 2 mM EDTA, 89 mM Boric acid, pH 8.3).

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References


Inventory of Supplemental Information

Figure S1. Transcript levels and infection kinetics of Prochlorococcus NATL2A and cyanophage P-SSM2 during the P-starvation experiment shown in Figure 1C.

Figure S2. Competition assays to verify the binding of PhoB to phage pstS promoter as shown in Figure 3C.

Figure S3. rnpB transcript levels of Prochlorococcus NATL2A during infection by cyanophage P-SSM2 as mentioned in Experimental Procedures.

Supplemental Table 1. Quantitative PCR primers described in Experimental Procedures.
Supplemental Figures

Figure S1. Transcript levels and infection kinetics of *Prochlorococcus* NATL2A and cyanophage P-SSM2 during the P-starvation experiment shown in Figure 1C. The host was infected with cyanophage P-SSM2 at 47 h after the cells were resuspended in P-replete (control) or P-depleted (-P) media. Host (A) and phage (B) transcript levels were normalized to that of the host *rnpB* gene (see Experimental Procedures). Please note that the y axis of this figure is different from that of Figures 1B and 1C. In the latter we show the transcript levels in cells during P starvation relative to P-replete controls. <d.l. indicates phage transcript levels below detection limit at time 0 h. (C) Host gDNA degradation as a function of time after the onset of infection, measured as the disappearance of the host *rnpB* gene. (D) Host cell numbers over the same interval. (E, F) Phage DNA replication and release measured as the number of intra- and extra-cellular phage *pstS* gene copies (each representing a phage genome). When not visible they are smaller than the data points. Dotted line indicates the beginning of phage burst, and is reproduced on Figure 1C. Error bars indicate the s.d. from two biological replicates.
**Figure S2.** Competition assays to verify that the binding of PhoB to *pstS* promoter is sequence specific. (A) DNA sequence between cyanophage S-SM1 genes *hsp20* and *g172*. The numbers are the genomic positions. The red TAA is the stop codon of *hsp20* and the green ATG is the start codon of *g172*. The putative *pho* box sequence is shown by blue letters. (B) DNA fragment (with black underline in A) containing the putative *pho* box was labeled by a fluorophore. 3 nM labeled DNA fragment and 1 µM PhoB protein were incubated with different amounts of non-labeled specific competitor or nonspecific competitor. The same DNA fragment without labeling was used as specific competitor (lanes 3-6) and a ~200 bp DNA fragment in phage S-SM1 *hsp20* gene (with orange underline in A) was used as non-specific competitor (lanes 7-10). The first lane shows the pattern of free DNA (no PhoB protein was added in the reaction). Free DNA (F) and the shifted protein-DNA complex (S) were separated in native polyacrylamide gels. The protein-DNA complex almost disappeared in lane 6 and remained unchanged in lane 10, confirming that the binding of PhoB to phage *pstS* promoter is sequence specific.
Figure S3. *rnpB* transcript levels of *Prochlorococcus* NATL2A during infection by cyanophage P-SSM2. The host *rnpB* transcript copy number was quantified by qPCR and normalized to the total RNA (ng) used in the reverse transcription reaction. Error bars indicate the s.d. from two biological replicates.
**Supplemental Table 1. Quantitative PCR primers**

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