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Transcriptomic response during phage infection of a marine cyanobacterium under phosphorus-limited conditions

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Summary

The transcriptomic responses of bacteria to environmental stresses have been studied extensively, yet we know little about how the stressed cells respond to bacteriophage infection. Here, we conducted the first whole transcriptome sequencing (RNA-seq) study of stressed bacteria to phage infection by infecting the marine picocyanobacterium *Prochlorococcus* NATL2A with cyanomyovirus P-SSM2 under P limitation, a strong selective force in the oceans. Transcripts of the P-acquisition genes in the uninfected cells were enriched after P limitation, including the high-affinity phosphate-binding protein gene *pstS*. They were still enriched in the infected cells under P-limited conditions. In contrast, transcripts of adenosine triphosphate (ATP) synthase and ribosomal protein genes were depleted in the uninfected cells after P limitation but were enriched during phage infection of P-starved cells. Cyanophage P-SSM2 contains *pstS*, and *pstS* and its adjacent gene *g247* of unknown function were the only phage genes that were enriched under P-limited conditions. We further found that the host *pstS* transcript number per cell decreased after infection, however, it was still much higher in the P-limited infected cells than that in the nutrient-replete cells. Moreover, phage *pstS* transcript number per cell was ~20 times higher than the host copy, which may help maintain the host phosphate uptake rate during infection.

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Introduction

The unicellular cyanobacterium *Prochlorococcus* is the most abundant photosynthetic organism in the oceans and is thought to be responsible for 50% of the total primary production in many oligotrophic regions (Liu *et al.*, 1997; Partensky *et al.*, 1999). Three morphotypes of lytic viruses (cyanophages) with double-stranded deoxyribonucleic acid (DNA) genomes have been found to infect *Prochlorococcus* (Sullivan *et al.*, 2003): T4-like cyanomyoviruses, T7-like cyanopodoviruses and cyanosiphoviruses.

Cyanophage genomes contain many host-like metabolic genes (for a review, see Puxty *et al.*, 2014). These genes are involved in photosynthesis (Mann *et al.*, 2003; Lindell *et al.*, 2004; Millard *et al.*, 2004; Sharon *et al.*, 2009), photosynthetic electron transfer (Philosof *et al.*, 2011; Sharon *et al.*, 2011), pigment synthesis (Dammeyer *et al.*, 2008; Busch *et al.*, 2011), nucleotide metabolism (Chen and Lu, 2002; Pope *et al.*, 2007; Millard *et al.*, 2009; Clokie *et al.*, 2010; Huang *et al.*, 2012; Dwivedi *et al.*, 2013; Sakowski *et al.*, 2014), pentose phosphate pathway and phosphate acquisition (Mann *et al.*, 2005; Sullivan *et al.*, 2005; 2010; Weigele *et al.*, 2007; Clokie *et al.*, 2010). The complete absence of Calvin cycle genes in cyanomyovirus genomes and the presence of the Calvin cycle inhibitor gene *cp12* suggested to us (Thompson *et al.*, 2011) that cyanomyoviruses use host-like metabolic genes to augment the light reactions of photosynthesis for phage DNA replication rather than carbon fixation.

Cyanophages not only contain host-like metabolic genes but also exploit the host's signal transduction system (Zeng and Chisholm, 2012). Phosphorus (P) limits phytoplankton production in some regions of the oceans (Wu *et al.*, 2000; Thingstad *et al.*, 2005), so much so that some use non-phosphorus lipids in response to P limitation (Van Mooy *et al.*, 2009). The availability of phosphorus also exerts strong selective pressure on the gene content of microbial genomes (Martiny *et al.*, 2006; Coleman and Chisholm, 2007; Martiny *et al.*, 2009; Coleman and Chisholm, 2010). The suite of P-acquisition genes in a *Prochlorococcus* cell, for example, is related to P availability in its habitat, not phylogeny (Coleman and

Chisholm, 2010). Many of these genes are upregulated in *Prochlorococcus* during P starvation (Martiny *et al*., 2006; Reistetter *et al*., 2013), which is controlled by the PhoR/PhoB two-component signal transduction system (Makino *et al*., 1989; Tetu *et al*., 2009). The selective pressure of P availability is also visible in cyanophages. Many cyanomyoviruses carry P-acquisition genes of host origin (high-affinity phosphate-binding protein gene *pstS* and alkaline phosphatase gene *phoA*) (Sullivan *et al*., 2005; 2010), and, as in the host cells, the frequency of these genes in cyanophages in the wild is a function of P availability (Williamson *et al*., 2008; Kelly *et al*., 2013). This suggests that these genes play a role in phage DNA replication by facilitating host P-acquisition during infection (Sullivan *et al*., 2005). Moreover, we used quantitative reverse transcription polymerase chain reaction (RT-PCR) to show that phage *pstS* and *phoA* genes were upregulated when infecting P-starved host cells, and their expression was also controlled by the host's PhoR/PhoB system (Zeng and Chisholm, 2012). This is the first example in which a lytic virus exploits a host's two-component system to be responsive to environmental conditions (Zeng and Chisholm, 2012). A regulatory sequence for the expression of P-acquisition genes has been identified in front of cyanophage *pstS* and *phoA* genes (Zeng and Chisholm, 2012). Similar sequences were also found in front of other cyanophage genes (Sullivan *et al*., 2010; Kelly *et al*., 2013), indicating that there may be more cyanophage genes that are responsive to P limitation of the host cells.

While 30 genes were upregulated in *Prochlorococcus* strain MED4 under P-limited conditions (Martiny *et al*., 2006), their expression patterns during phage infection have not been examined systematically, which is helpful to understand the adaptation of cyanophages in P-limited oceans. We performed RNA sequencing (RNA-seq) analysis (Nagalakshmi *et al*., 2008) to study the transcriptomic profiles of *Prochlorococcus* strain NATL2A during infection by cyanomyovirus P-SSM2 under nutrient-replete and P-limited conditions, using the RNA samples from our previous experiment (Zeng and Chisholm, 2012). In total, 48 libraries were sequenced by Illumina HiSeq 2000, with 1–8 million non-rRNA reads per library (NCBI Short Read Archive accession no. SRP048525; see Table S1 for the library information and Table S2 for the number of mapped reads per gene). We first analysed phage transcriptome dynamics after infection under nutrient-replete and P-limited conditions to identify all the phage genes that were differentially expressed when the host cells were P-starved. We then compared the transcriptomes of uninfected and infected host cells under P-limited and nutrient-replete conditions to see whether the host's signalling pathway for phosphate sensing was altered by phage infection. Finally, we quantified the absolute transcript numbers per cell of *pstS*, one of the P-acquisition genes. Additionally, we identified three differentially expressed host antisense RNAs in response to P limitation.

Results and discussion

Infection kinetics

The host, *Prochlorococcus* NATL2A, was infected with cyanomyovirus P-SSM2 at 47 h after log phase cells were spun down and re-suspended in nutrient-replete or P-limited media (see Fig. S1 for the experimental design) when the host P-starvation response had been initiated (Zeng and Chisholm, 2012). Infections were carried out with a multiplicity of infection of 3. After infection under nutrient-replete (control) and P-limited conditions, the host genomic DNA decreased (Fig. S2A), and the intracellular phage genomic DNA increased (Fig. S2B). At 8 h after infection, progeny phages were released into the extracellular media (Fig. S2C) under both conditions, with fewer being produced under P-limited conditions.

Temporal expression of phage genes when infecting a nutrient-replete host

Similar to *Escherichia coli* phage T4 (Luke *et al*., 2002), also a myovirus, cyanomyovirus P-SSM2 has early, middle and late gene clusters according to their initial expression time when infecting *Prochlorococcus* (Fig. 1A). The early cluster contained mostly genes of unknown function (Table S3), but it is likely, from similar studies of coliphage T4 that the genes in this cluster play a role in redirecting transcription from the host towards the phage (Miller *et al*., 2003). Similar to T4 (Miller *et al*., 2003), the middle cluster contained genes involved in DNA metabolism and replication and the late cluster consisted of genes involved in phage particle formation (Table S3). Notably, host-like metabolic genes *psbA* (photosystem II subunit), *talC* (transaldolase), *cp12* (Calvin cycle inhibitor) and *pstS* were expressed as middle cluster genes (Table S3) similar to cyanopodovirus P-SSP7 (Lindell *et al*., 2007). These genes may facilitate energy harvesting and deoxynucleotide precursor production, which is necessary for cyanophage DNA replication.

Host transcriptomic response to P limitation

The overarching goal of this study was to understand how the transcriptomes of both host and phage are affected when a phage infects a P-starved host cell – a condition that may be frequently encountered in wild *Prochlorococcus* (Van Mooy *et al*., 2009; Coleman and Chisholm, 2010). As an 'infection control', we first

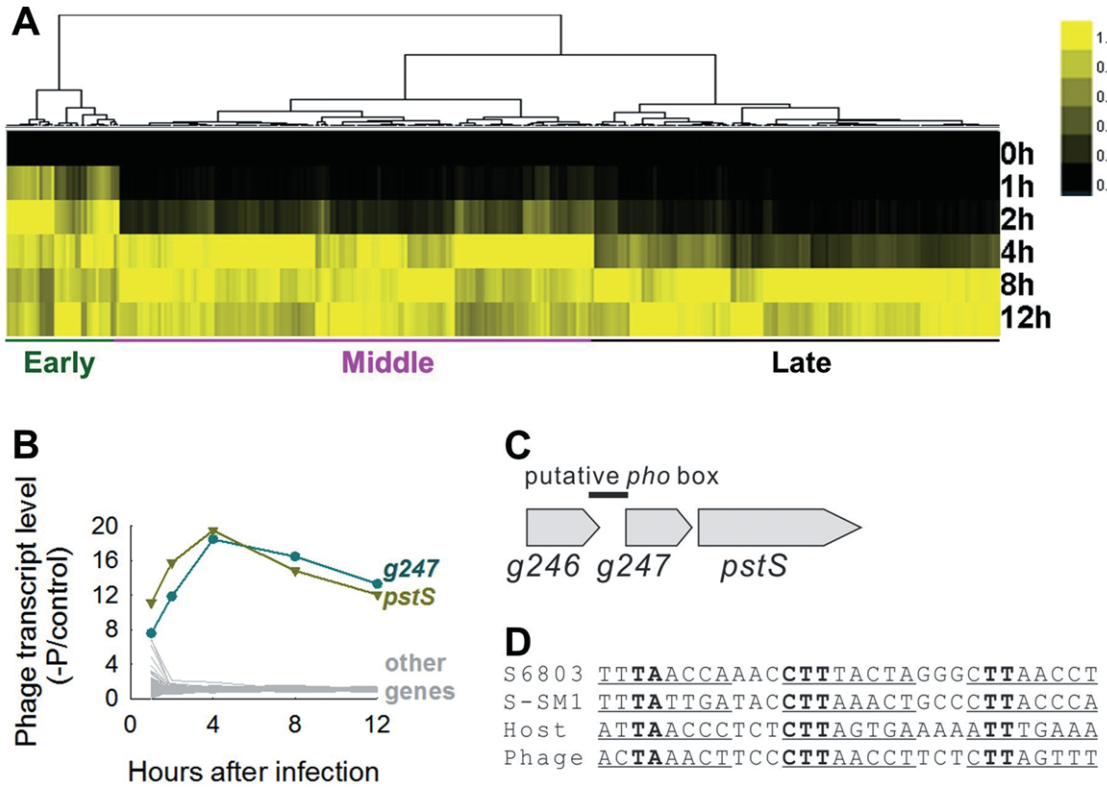

Fig. 1. Cyanophage P-SSM2 transcript levels during infection of *Prochlorococcus* NATL2A under nutrient-replete (control) and P-limited (-P) conditions.
A. Temporal expression dynamics of phage P-SSM2 genes under nutrient-replete conditions. Phage transcript level at each time point was normalized to its maximum expression level in the 12 h infection period. Yellow indicates maximum expression, and black indicates no expression. CLUSTER 3.0 was used to build the hierarchical tree (uncentered correlation with complete linkage).
B. Phage transcript levels during infection of P-limited host cells were normalized to those of nutrient-replete cells. The only transcript levels that differed significantly in the P-limited cells from the nutrient-replete controls were those of *pstS* and *g247* (with adjusted *P*-values $< 10^{-10}$ for all the time points). Data shown in A and B are the average of two biological replicates.
C. Genomic organization of the P-SSM2 *pstS* region, showing *g246*, *g247* and *pstS*. A black bar indicates a putative *pho* box in front of *g247*.
D. Alignment of the *pho* box sequences of *Synechocystis* sp. PCC6803 (S6803) (Suzuki *et al.*, 2004), cyanophage S-SM1 (Zeng and Chisholm, 2012), *Prochlorococcus* NATL2A (host) and cyanophage P-SSM2 (phage). The 8 bp conserved tandem repeats are underlined, which are important for PhoB binding (Makino *et al.*, 1996). Bold letters indicate conserved sequences.

examined the P-starvation response of the uninfected *Prochlorococcus* NATL2A cells in an experiment similar to that reported for a related *Prochlorococcus* strain MED4 (Martiny *et al.*, 2006). After P limitation, the transcripts of 19 genes were enriched (Fig. 2A, top panel), and those of 15 genes were depleted (Fig. 2A, bottom panel). As expected, when P was added back to the P-starved cultures, the relative transcript abundances changed in the opposite direction (Fig. 2A), confirming that they were tightly coupled to P limitation. Indeed, many genes with enriched transcripts (*phoB*, *phoR*, *pstB*, *pstS*, *phoA*, *phoE*, *ptrA*, *pstC* and *pstA*) are related to P acquisition (*pho* regulon genes), which have been identified in *Prochlorococcus* MED4 (Martiny *et al.*, 2006). Glyceraldehyde-3-phosphate dehydrogenase (*gap1*), *mfs* (a major facilitator superfamily transporter) and several genes of unknown function (PMN2A_1997, PMN2A_0442 and PMN2A_0438) are located adjacent to the P-acquisition genes (Fig. 2C), and their transcripts were enriched in the P-starved cultures, suggesting they may also play a role in the P-acquisition process.

Most genes with depleted transcripts in the P-starved cultures (Fig. 2A, bottom panel) belong to the ATP synthase operon (*atpA*, *atpB*, *atpE*, *atpF*, *atpG*, *atpH* and *atpI*). The proton gradient generated through the light reactions of photosynthesis is used by ATP synthase to produce ATP, and the pool size of AtpB is known to be correlated with growth rate in some *cyanobacteria* (Mackenzie *et al.*, 2005). Because *Prochlorococcus* cells grew slower under P-limited conditions (Zeng and Chisholm, 2012), this could explain this response. The ATP synthase response was only detected after prolonged (>46 h) P limitation (Fig. 2A; Table S4), which may explain why it was not observed in shorter duration experiments with *Prochlorococcus* MED4 (Martiny *et al.*, 2006). The transcripts of the leucine biosynthesis gene *leuB* and

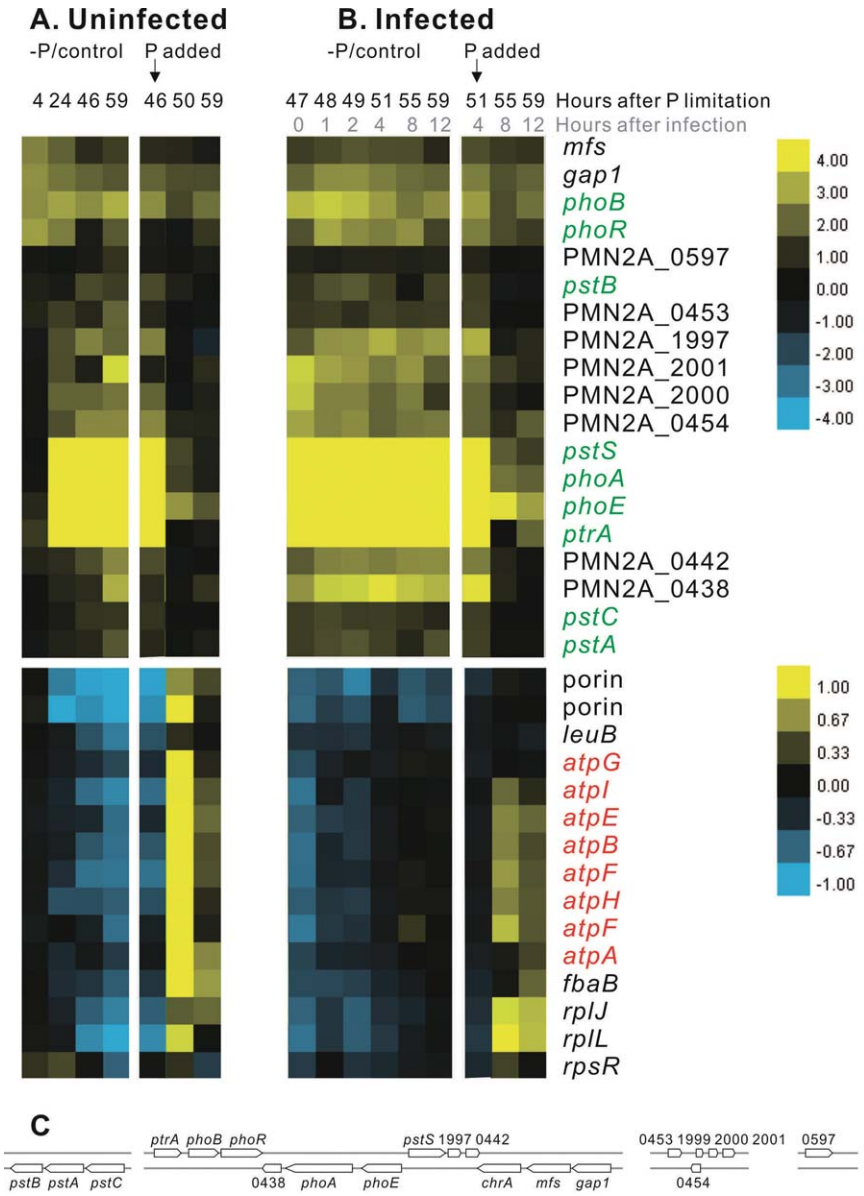

Fig. 2. Heat map showing the relative transcript abundances of *Prochlorococcus* NATL2A during infection by cyanophage P-SSM2 under nutrient-replete and P-limited conditions and after rescue from P starvation.
A. Uninfected cultures, transcript abundances of P-limited (-P) normalized to nutrient-replete (control) cultures. At 46 h of P limitation, P was added back to aliquots of the P-limited cultures to rescue the cells from P starvation. The colour bar represents log2 fold change of the transcripts (-P/control). Genes whose transcripts were relatively more abundant under P limitation (yellow tones) were shown in the upper panels, and those whose transcripts were relatively less abundant (blue tones) were shown below. The top and bottom panels have different colour bars. The P-acquisition genes were shown in green letters, and the ATP synthase operon genes were shown in red letters.
B. Infected cultures, transcript abundances of P-limited normalized to nutrient-replete cultures. After 47 h of growth, aliquots from both P-limited and nutrient-replete cultures were infected by cyanophage P-SSM2. Note that 47 h after P limitation corresponds to 0 h after phage infection, as shown above the heat map. At 51 h, P was added back to aliquots of the P-limited culture to release the infected cells from P starvation. Data shown are the average of two biological replicates. The log2 fold change, *P*-value and description of each gene are shown in Table S4.
C. The genomic positions of genes showing higher transcript abundances under P-limited conditions.

the ribosomal protein genes *rplJ*, *rplL* and *rpsR* were also depleted in the P-starved cultures (Fig. 2A, bottom panel), suggesting a reduced protein synthesis rate after P limitation. Consistent with our data, the protein abundances of several ribosomal proteins were found to be lower under P-limited conditions in the marine cyanobacterium *Synechococcus* WH8102 (Cox and Saito, 2013).

Genome-wide transcription of antisense RNAs (asRNAs) has been reported for *cyanobacteria* (Mitschke et al., 2011; Waldbauer et al., 2012; Voigt et al., 2014), including *Prochlorococcus* strains MED4 and MIT9313 (Waldbauer et al., 2012; Voigt et al., 2014). For *Prochlorococcus* NATL2A, we found that 75.6% of the total 2229 genes had antisense transcript abundances >5% of the sense transcript abundances. An asRNA responsive to P limitation has been identified in *Synechococcus* WH7803, but its function is still not clear (Gierga *et al.*, 2012). Here, we found two asRNAs (antisense to *phoR* and *chrA*) with enriched transcripts in P-limited conditions and one asRNA (antisense to PMN2A_0453) with depleted transcripts (Fig. 3A). These asRNAs are all transcribed from the genomic loci containing most of the P-acquisition genes (Fig. 2C). *phoR* (encoding a phosphate-sensing histidine kinase) and PMN2A_0438 of unknown function are on the opposite DNA strands (Fig. 2C). *phoR* asRNA seems to be from leaky transcription of PMN2A_0438, since their transcripts were both enriched in P-starved cultures (Fig. 2A, top panel). The antisense/sense transcript ratio of *phoR* was 4% at the beginning of P limitation and increased to

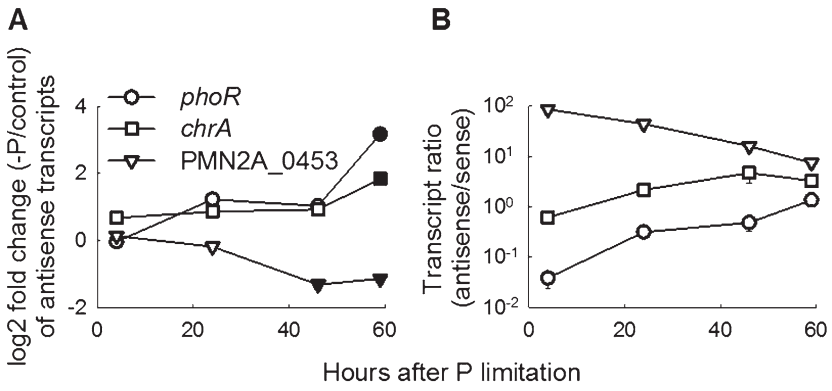

Fig. 3. Differentially expressed antisense RNAs of *Prochlorococcus* NATL2A in response to P limitation.
A. Log2 fold change (-P/control) of host antisense transcripts after P limitation. Filled symbols indicate adjusted *P*-values < 10%.
B. The ratio of antisense to sense transcripts after P limitation. Error bars show the standard deviation. Data shown are the average of two biological replicates.

137% at 59 h (Fig. 3B). If *phoR* asRNA forms a duplex with *phoR* messenger RNA (mRNA), the translation of *phoR* mRNA could be inhibited. This may serve as a self-regulatory mechanism for the PhoR/PhoB two-component system, such that less PhoR is produced under prolonged P limitation. *chrA* is a chromate transporter gene and PMN2A_0453 is of unknown function. The differentially expressed asRNAs of these two genes may have some regulatory functions under P-limited conditions. It should be noted that we identified these asRNAs solely based on our RNA-seq data. Future 5' RACE and Northern blotting experiments are needed to verify them and map their exact positions.

Phage transcriptomic response during infection of P-starved host cells

In a previous study of limited scope (Zeng and Chisholm, 2012), using quantitative RT-PCR, we observed that cyanophage P-SSM2 genes *g247* (gene cluster name PhCOG173) (Kelly *et al.*, 2013) and *pstS* were upregulated when the phage infected a P-starved host (Zeng and Chisholm, 2012). *pstS* encodes a periplasmic high-affinity phosphate-binding protein, and *g247* is upstream of it (Fig. 1C) with unknown function. There is a putative *pho* box sequence in front of *g247* (Fig. 1C and D). Under P-limited conditions, the host response regulator PhoB can bind to the conserved *pho* box sequences in the promoter regions of host and phage P-acquisition genes and activate their transcription (Wanner, 1993; Zeng and Chisholm, 2012).

Cyanophage P-SSM2 also has several predicted *pho* box sequences in front of other genes (Sullivan *et al.*, 2010; Kelly *et al.*, 2013). We wondered whether there are more phage genes that respond to P starvation of the host cells. To explore this possibility, we compared the whole-genome expression profiles of cyanophage P-SSM2 in P-limited and nutrient-replete conditions. To our surprise, in the entire phage genome of 335 annotated genes, *g247* and *pstS* were the *only* two genes differentially expressed when comparing infection of a P-limited with a nutrient-replete host (Fig. 1B). In the uninfected host cells, *pstS* is one of the most enriched genes in response to P starvation (Fig. 2A). Its presence and regulation in the phage genome suggests that *pstS* gene controls the rate-limiting step of host P acquisition. Although we do not know the function of *g247*, its association and co-expression with *pstS* highlights its importance for cyanophage replication under P-limited conditions.

The expression of host P-starvation response genes after phage infection

We identified 34 host P-starvation response genes, which were differentially expressed in response to P limitation (Fig. 2A). We wondered whether phage infection would influence their expression in different ways. To examine this, we compared *Prochlorococcus* NATL2A transcript abundances after infection by cyanomyovirus P-SSM2 under P-limited and nutrient-replete conditions (Fig. 2B). The transcripts of most host genes which were enriched in response to P limitation in the uninfected cells, including the P-acquisition genes (e.g. *pstS*, Fig. S3), were still enriched in the infected cells under P-limited conditions (Fig. 2B, top panel). In contrast, the transcripts of most host genes with depleted transcripts under P-limited conditions in the uninfected cells, including the ATP synthase (e.g. *atpH*, Fig. S3) and ribosomal protein genes, were enriched in the transcript pool in the infected cells under P-limited conditions (Fig. 2B, bottom panel). The enriched transcripts of the ATP synthase and ribosomal protein genes may allow the infected host cells to maintain ATP production and protein synthesis under P-limited conditions. This could be a stress or defense response of the infected host cells, or could be a manifestation of an infection strategy used by the phage to facilitate phage DNA and protein synthesis under P-limited conditions. Considering that progeny phages contain large amount of DNA and protein, the enriched transcripts of the ATP synthase and ribosomal

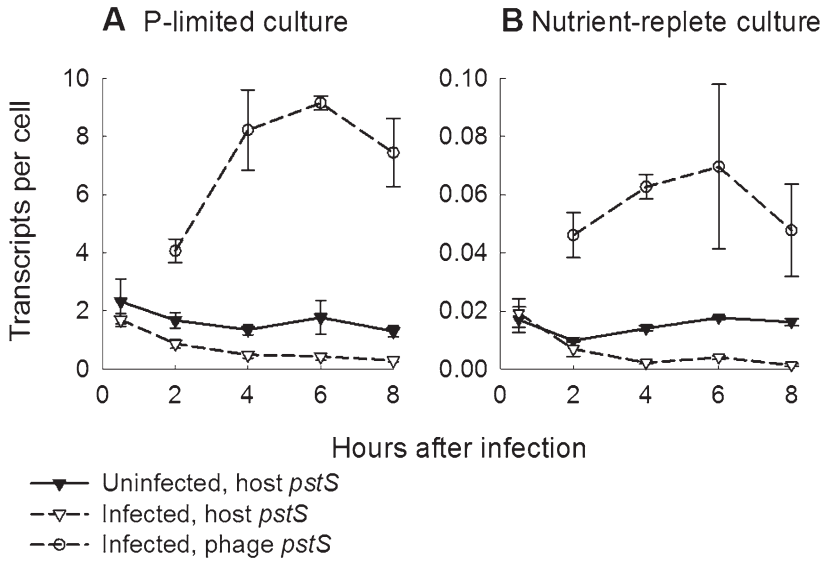

Fig. 4. The host and phage *pstS* transcript numbers per cell after *Prochlorococcus* NATL2A was infected by cyanophage P-SSM2 under P-limited (A) or nutrient-replete (B) conditions.
After growth in P-limited (A) or nutrient-replete (B) media for 47 h, *Prochlorococcus* NATL2A was infected by cyanophage P-SSM2. The host and phage *pstS* transcript numbers were calculated using the spike-in internal RNA standard. At 0.5 h after infection, the phage *pstS* transcript number was below the detection limit (<0.00001 copies per cell) and therefore was not shown in the graph. At each time point after infection, the uninfected control cultures were also sampled, and the host *pstS* transcript number was quantified. Error bars indicate the standard deviation from two biological replicates. When not visible they are smaller than the data points. Note that A and B are in different scales.

protein genes could be more beneficial to the phage than to the host.

Quantification of host and phage pstS *transcript numbers per cell*

Our RNA-seq data showed that the relative transcript abundances of the host P-acquisition genes in the infected cells were higher under P-limited conditions than those under nutrient-replete conditions (Fig. 2B). In order to know whether the transcript number per cell of these genes was affected during phage infection, we repeated the infection experiment and added a spike-in internal RNA standard to the samples before RNA extraction. Then this spike-in RNA was used to quantify the host and phage transcript numbers by quantitative RT-PCR (see *Experimental procedures*). On average, there were 2457 ± 328 copies of 16S rRNA (Fig. S4A) per *Prochlorococcus* NATL2A cell, which did not change significantly after P limitation or phage infection (Figs. S4A). Previously, the transcript abundance of the host *rnpB* gene (encoding RNA component of RNase P) was shown to be stable when *Prochlorococcus* was infected by cyanophages (Lindell *et al*., 2007; Zeng and Chisholm, 2012). In our experiments, its transcript number per cell was also stable under phage infection and P limitation (Fig. S4B).

For the P-acquisition genes, we quantified the transcript numbers of both host and phage *pstS* genes (Fig. 4). After 47 h of P limitation, there were about two host *pst*S transcripts per cell (Fig. 4A), which is about 100 times higher than that in the nutrient-replete cells (~0.02 copies per cell, Fig. 4B). Under P-limited conditions, *pstS* might be one of the highest expressed genes, considering that in *E. coli* genes with high protein expression were found to have 0.05 to 5 transcripts per cell (Taniguchi *et al*., 2010). After infection, the host *pstS* transcript number decreased under both P-limited (Fig. 4A) and nutrient-replete conditions (Fig. 4B) during the ~8 h latent period. However, in the infected cells, it was still much higher under P-limited conditions (~0.43 copies per cell at 6 h after infection) than that under nutrient-replete conditions (~0.004 copies per cell at 6 h after infection) (Fig. 4). This explains why our RNA-seq data showed that the relative transcript abundance of the host *pstS* in the infected cells were higher under P-limited conditions than that under nutrient-replete conditions (Figs 2B and S3). Although the host *pstS* transcript number per cell decreased after infection, the phage *pstS* transcript number per cell was more than 21.2 and 17.6 times higher than the host copy at 6 h after infection under P-limited and nutrient-replete conditions respectively (Fig. 4).

The expression patterns of host and phage *pstS* genes are quite similar to the inverse temporal expression of the host and phage *psbA* genes (Lindell *et al*., 2005), which encode the photosystem II D1 protein. After *Prochlorococcus* MED4 was infected by cyanopodovirus P-SSP7, the host *psbA* transcripts declined to 50–60% of the maximal level, while the phage *psbA* transcripts increased to ~50% of the total *psbA* transcripts (host plus phage) (Lindell *et al*., 2005). During infection, photosystem II efficiency of the host cells only decreased very slightly, suggesting that the phage *psbA* transcripts might supplement host photosynthesis (Lindell *et al*., 2005). The high-affinity phosphate-binding protein PstS and its associated ABC transporter (encoded by genes *pstA*, *pstB* and *pstC*) is the predominant P acquisition system in bacteria (Hsieh and Wanner, 2010), including *Prochlorococcus* (Moore *et al*., 2005). During P limitation, the *pstS* transcript abundance of *Prochlorococcus* MED4

increased, and the cellular phosphate uptake rate also increased (Krumhardt *et al*., 2013; Reistetter *et al*., 2013). With *pstS* transcripts contributed by the infecting phage, we speculate that the phosphate uptake rate of the infected host cells might not drop significantly or even increase during infection.

Broader implications

The environmental availability of phosphorus exerts a strong selective pressure on the evolution of cyanobacterial genomes (Martiny *et al*., 2006; 2009; Coleman and Chisholm, 2007; 2010). Due to a high nucleic acid to protein ratio (Jover *et al*., 2014), P availability is also a strong selective pressure for cyanophages (Williamson *et al*., 2008; Kelly *et al*., 2013), with many cyanomyoviruses carrying P-acquisition genes (*pstS* and *phoA*) of host origin (Sullivan *et al*., 2005; 2010). Using *Prochlorococcus* NATL2A and cyanomyovirus P-SSM2, we conducted the first RNA-seq study of bacteria under the combined stress of P limitation and phage infection. After infection, the transcript number per cell of host *pstS* was reduced under both P-limited and nutrient-replete conditions (Fig. 4). However, in P-limited infected cells, we found that the transcript abundances of the host P-acquisition genes were much higher than those of nutrient-replete infected (Fig. 2B) and nutrient-replete uninfected cells (Fig. 4). In P-limited oceans, with ~20 times more *pstS* transcripts from the infecting phage (Fig. 4), *Prochlorococcus* cells might continue to acquire phosphorus during infection, which could be used to build virus particles and eventually contribute to the marine dissolved organic phosphorus pool (Jover *et al*., 2014). Future studies of the phosphate uptake rates of infected *Prochlorococcus* cells will help understand how cyanophages manipulate the host phosphorus status and elemental stoichiometry to deal with environmental P limitation.

Experimental procedures

Strains and growth conditions

Axenic *Prochlorococcus* NATL2A cultures were maintained in the Pro99 medium at 21°C under constant cool white light (30 μmol quanta m^{-2} s^{-1}). The Pro99 medium (Moore *et al*., 2002) was based on 0.2 μm filtered natural seawater and amended with 10 mM HEPES (pH 7.5) and 12 mM sodium bicarbonate. Sargasso seawater was used to make the Pro99 medium for the RNA-seq experiment, and Port Shelter (Hong Kong) seawater was used for the experiment of quantification of transcript number per cell.

For the RNA-seq experiment, cyanophage P-SSM2 lysates were concentrated with Amicon Ultra-15 30K Centrifugal Filter Units (Millipore) at 5000 x g for 15 min, washed twice with filtered seawater and re-suspended in the same medium. A final concentration of 5.99 x 10^{10} phages ml^{-1} was determined by quantitative polymerase chain reaction (qPCR). Infectious phage concentration was determined by the most probable number assay (Tillett, 1987). For the fresh P-SSM2 lysates, 42.7% of the total phages were infectious. Prior to infection by cyanophages, log phase *Prochlorococcus* NATL2A cells were pelleted by centrifugation at 15 000 x g for 10 min at 21°C, washed twice in either PO_4-replete Pro99 medium (with 50 μM PO_4) or PO_4-depleted Pro99 (without added PO_4) and re-suspended in the same medium. After 47 h of growth, the cell concentration of each culture was determined by flow cytometry (Influx, Cytopeia-BD). The cell concentration of the nutrient-replete cultures was adjusted by Pro99 to 2.6 x 10^7 cells ml^{-1}, and the P-depleted cultures were adjusted by PO_4-depleted Pro99 to the same concentration. For each biological replicate, 800 ml of *Prochlorococcus* NATL2A culture was infected by cyanophage P-SSM2 with a multiplicity of infection of three.

Quantification of phage and host genomic DNA during infection

A qPCR method was used to quantify phage and host genomic DNA during infection (Lindell *et al*., 2007). Briefly, the infected *Prochlorococcus* cultures were filtered through 0.2 μm polycarbonate filters. Extracellular phage particles were collected from the filtrate. Host cells with intracellular phage genomic DNA were recovered from the filters. In order to reduce the attached phages, the filters were washed three times with sterilized seawater and once with 3 ml preservation solution (10 mM Tris, 100 mM EDTA, 0.5 M NaCl, pH 8). At each time point, it took 10 to 30 min to process all the samples. The qPCR primers were listed in Table S5.

Strand-specific RNA-seq library preparation for Illumina sequencing

At each time point, 70 ml *Prochlorococcus* culture was spun down at 15 000 x g for 15 min at 4°C, and cell pellets were flash frozen in liquid nitrogen and stored at –80°C. Total RNA was extracted using the Ambion mirVana RNA isolation kit, and residual genomic DNA was removed using the Turbo DNA-free kit (Ambion). Ribonucleic acid was then concentrated with the RNA Clean & Concentrator-5 kit (Zymo Research). Total RNA of 150 ng was fragmented to the range of 60–200 nt by magnesium catalysed hydrolysis (40 mM Tris-Acetate, pH 8.1, 100 mM KOAc, 30 mM MgOAc) for 4 min at 83°C, and purified with the RNA Clean & Concentrator-5 kit. Strand-specific RNA-seq libraries were prepared using a deoxyuridine triphosphate (dUTP) second-strand marking protocol as described elsewhere (Levin *et al*., 2010). Briefly, first-strand complementary DNA (cDNA) was synthesized from fragmented RNA with random primers (Invitrogen) and Superscript III Reverse transcriptase (Invitrogen). Second-strand cDNA was synthesized using dUTP instead of thymidine triphosphate (dTTP). Paired-end libraries for Illumina sequencing were prepared from purified double-stranded cDNA (Agencourt AMPure XP beads) as recommended by Illumina. Second strands of cDNA containing dUTP were removed with the Uracil cleavage system

(Enzymatics), and libraries were amplified with the sequencing primers. A duplex-specific nuclease (DSN)-based method (Yi *et al*., 2011), which has been successfully used in *Prochlorococcus* (Giannoukos *et al*., 2012), was used to remove the 16S and 23S rRNA molecules. This nuclease preferentially degrades the abundant and fast re-annealing cDNA molecules of 16S and 23S rRNAs, while preserves the less abundant and slow re-annealing cDNA molecules of non-rRNA transcripts. Illumina sequencing primers with barcodes were used to amplify the DSN-treated libraries. About 20 bar-coded libraries were pooled with equal proportion in one lane, and paired-end sequencing was done by Illumina HiSeq 2000 (40 nt for insert + 6 nt for barcode). One million to eight million mapped reads were recovered from each library. Because of DSN treatment, the percentage of host 16S and 23S rRNA reads in each library was reduced to 2–10% (compared with 96% in the untreated samples).

RNA-seq data analysis

Reads from each library were separated based on their bar codes and aligned to *Prochlorococcus* NATL2A and cyanophage P-SSM2 genomes using the Burrows–Wheeler Aligner (BWA). GenBank annotations (NC_007335 for NATL2A and AY939844 for P-SSM2) were used to generate Sequence Alignment/Map (SAM) alignment files using BWA. SAMtools (Li *et al*., 2009) and pysam were then used to calculate the number of reads perfectly aligning to the sense and antisense strands of open reading frames (ORFs), rRNAs, transfer RNAs and intergenic regions. For reads spanning two ORFs, they were counted once for each ORF. The host 16S and 23S rRNA reads were removed manually from the total reads before further analysis. The number of reads per gene is listed in Table S2.

Identification of differentially expressed genes

Since our RNA-seq protocol can only measure relative transcript abundances, we used the common term 'differentially expressed genes' for relative transcript abundances (the net result of transcription minus transcript degradation). Differentially expressed genes were identified using the variance analysis package DESEQ (Anders and Huber, 2010) with default parameters. Two biological replicates were conducted for each time point. For each sample, the number of host reads per gene was normalized by DESEQ based on the total number of mapped reads in that sample (phage plus host reads without 16S and 23S rRNA reads). Similarly, the number of phage reads per gene was normalized by DESEQ based on the total number of mapped reads for that sample. An adjusted *P*-value (*P*-value with a multiple-test correction done by DESEQ) < 10% (the default value by DESEQ) indicates a significant difference.

Verification of RNA-seq results

To evaluate the reliability of our RNA-seq results, we performed qPCR on three representative cyanophage genes (*g20*, *g61*, and *pstS*) during infection using the same RNA samples as used for RNA-seq. Complementary DNA copies were quantified using a QuantiTect SYBR Green PCR Kit (QIAGEN) with 0.5 μM forward and reverse primers (Table S5) as described previously (Zeng and Chisholm, 2012). The relative transcript abundances of these genes were normalized to the host *rnpB* gene (Lindell *et al*., 2007; Zeng and Chisholm, 2012). Phage gene expression patterns determined by RNA-seq correlated well with those determined by RNA-seq (Fig. S5).

Quantification of transcript number per cell

At each time point, 30 ml of *Prochlorococcus* NATL2A culture was collected by centrifugation at 20 000 x g for 5 min at 4°C. Cell pellets were re-suspended with filtered seawater. An aliquot of the re-suspended pellet was taken for cell count by flow cytometry, and the rest was frozen at −80°C. To measure the absolute transcript number per cell, we used a spike-in internal RNA standard. The spike-in RNA (1.8 kb) was *in vitro* transcribed using a HiScribe T7 High Yield RNA Synthesis Kit (NEB), and the FLuc plasmid (containing the firefly luciferase gene) provided in this kit was used as the template. The quantity of spike-in RNA was determined using a Quant-iT RiboGreen RNA kit (Life Technologies) by NanoDrop 3300 Fluorospectrometer. Before RNA extraction, the spike-in RNA was added to the cell pellet at a ratio of 18 copies per cell (~0.5% of total RNA weight, as recommended by Satinsky *et al*., 2013). Total RNA was then extracted with the ZR RNA MiniPrep kit (Zymo Research), and DNA was removed with the Turbo DNA-free kit (Ambion). Total RNA was reverse transcribed with the iScript cDNA Synthesis Kit (Bio-Rad). Complementary DNA copies were quantified using iTaq Universal SYBR Green Supermix (Bio-Rad) with 0.2 μM forward and reverse primers (Table S5) on a LightCycler 480 Instrument (Roche Diagnostics GmbH). The absolute transcript abundance was determined by normalizing to the spike-in FLuc RNA abundance (18 copies per cell). Using this method, we estimated that there were 2457 ± 328 copies of 16S rRNA per cell. Assuming that 16S rRNAs contribute to ~27% of total RNA by mass (Neidhardt, 1987), there should be ~5.9 fg of total RNA per *Prochlorococcus* cell, which is close to our experimental data (4.57 ± 1.77 fg per cell) and also agrees with marine bacteria (Moran *et al*., 2013).

Data access

The sequencing data from this study have been submitted to the National Center for Biotechnology Information Short Read Archive (http://www.ncbi.nlm.nih.gov/Traces/sra/sra.cgi) under accession no. SRP048525. The descriptions of the RNA-seq libraries and their individual accession numbers are listed in Table S1.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1. Experimental design.
Fig. S2. Infection kinetics of *Prochlorococcus* NATL2A by cyanophage P-SSM2 under nutrient-replete (control) and P-limited (-P) conditions.
Fig. S3. Plots showing *Prochlorococcus* NATL2A *pstS* and *atpH* transcript abundances during infection by cyanophage P-SSM2 under P-limited (-P) and nutrient-replete (control) conditions.
Fig. S4. Transcript numbers of *Prochlorococcus* NATL2A under different conditions.
Fig. S5. Verification of RNA-seq results by qPCR.
Table S1. Description of the RNA-seq libraries and their short read archive accession numbers.
Table S2. Mapped reads per gene. The sense and antisense reads of a gene are listed separately. Reads from both strands of an intergenic region are combined. Reads mapped to the host 16S (PMN2A_R0036) and 23S (PMN2A_R0039) rRNAs are not listed.
Table S3. Gene expression clusters of cyanophage P-SSM2 during infection of *Prochlorococcus* NATL2A under nutrient-replete conditions.
Table S4. Log2 fold change (P-limited/nutrient-replete) of *Prochlorococcus* NATL2A transcripts in the uninfected and infected cultures.
Adjusted *P*-values (padj) smaller than 10% are indicated by red numbers. Log2 fold change bigger than 1 or smaller than −1 is indicated by bold numbers. Hours are the time after the onset of P limitation.
Table S5. Quantitative PCR primers.