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Report

Efficient Phage-Mediated Pigment Biosynthesis in Oceanic Cyanobacteria

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Summary

Although the oceanic cyanobacterium *Prochlorococcus* harvests light with a chlorophyll antenna [1–3] rather than with the phycobilisomes that are typical of cyanobacteria, some strains express genes that are remnants of the ancestral *Synechococcus* phycobilisomes [4]. Similarly, some *Prochlorococcus* cyanophages, which often harbor photosynthesis-related genes [5], also carry homologs of phycobilisome pigment biosynthesis genes [6, 7]. Here, we investigate four such genes in two cyanophages that both infect abundant *Prochlorococcus* strains [8]: homologs of heme oxygenase (*ho1*), 15,16-dihydrobiliverdin:ferredoxin oxidoreductase (*pebA*), ferredoxin (*petF*) in the myovirus P-SSM2, and a phycocyanobilin:ferredoxin oxidoreductase (*pcyA*) homolog in the myovirus P-SSM4. We demonstrate that the phage homologs mimic the respective host activities, with the exception of the divergent phage PebA homolog. In this case, the phage PebA single-handedly catalyzes a reaction for which uninfected host cells require two consecutive enzymes, PebA and PebB. We thus renamed the phage enzyme phycoerythrobilin synthase (PebS). This gene, and other pigment biosynthesis genes encoded by P-SSM2 (*petF* and *ho1*), are transcribed during infection, suggesting that they can improve phage fitness. Analyses of global ocean metagenomes show that PcyA and Ho1 occur in both cyanobacteria and their phages, whereas the novel PebS-encoding gene is exclusive to phages.

Results and Discussion

Although they do not have the typical cyanobacterial phycobilisome antennae [4], *Prochlorococcus* cells carry both the cellular machinery for the biosynthesis of the phycobiliprotein pigments phycocyanobilin (PCB) and phycoerythrobilin (PEB) (Figure 1, Table 1) and one of the three phycobilisome structural proteins [4]. Several lines of evidence suggest that these are playing some functional role [9–12]. The occurrence of phycobilisome-related genes in some marine cyanophage genomes (Table 1) is further evidence in support of a role for these genes in cell fitness. Although these types of genes are not found in cyanophage podoviruses [13–15], cyanophage myovirus genomes contain between one and three phycobilisome-related genes [14, 16, 17]. The two *Prochlorococcus* myovirus genomes available include some combination of the putative bilin reductase genes *pebA* and *pcyA*, heme oxygenase (*ho1*), and ferredoxin (*petF*). *Synechococcus* myoviruses, on the other hand, carry *cpeT* (a putative phycobiliprotein lyase) alone. The phage protein PetF is similar to a plant-type [2Fe-2S] ferredoxin [18], suggesting that it might serve as an electron donor for Ho1 and PebA (renamed PebS) in myovirus P-SSM2.

pebA from the Cyanophage P-SSM2 Encodes a Phycoerythrobilin Synthase

To better understand the role of these genes in cyanophages, we first investigated whether the phage *pebA*-encoded homolog, PebA_P-SSM2, whose sequence is highly divergent from cyanobacterial PebA, encodes a functional ferredoxin-dependent bilin reductase (FDBR). We found that recombinant PebA_P-SSM2 (Figure S1 available online) was highly active in vitro with biliverdin IXα (BV) as a substrate. However, instead of the expected two-electron-reduced product 15,16-dihydrobiliverdin (15,16-DHBV) (Figure 1), the PebA_P-SSM2-catalyzed reaction yielded the four-electron-reduced chromophore PEB (Figure 2 and Figure S2). Thus, the phage enzyme directly converts BV to PEB, whereas the host cells require the sequential action of the two enzymes PebA and PebB [7]. Because of this new FDBR activity, we renamed PebA_P-SSM2 as PebS, phycoerythrobilin synthase, by analogy with phytochromobilin synthase [6, 19]. PebS is only the second FDBR, after PcyA [20], to perform a formal four-electron reduction. The PebS-mediated reduction proceeds faster with PetF_P-SSM2 than with standard assay ferredoxin as a redox partner (data not shown), likely because of more efficient electron transfer among the phage proteins.

Phycoerythrobilin Synthase Converts BV via the Semireduced Intermediate 15,16-DHBV

By slowing down the in vitro reaction, we were able to observe the transient accumulation of the semireduced reaction intermediate 15,16-DHBV (Figure 2), as well as the appearance of the reaction product PEB. Hence, the sequence of reductions performed by the phage PebS is identical to that in the consecutive action of PebA and PebB in cyanobacterial and algal cells [21, 22]. Specifically, PebS and PebA alike catalyze the reduction of BV at the 15,16 double bond; PebS holds onto this 15,16-DHBV intermediate, whereas PebA passes it to PebB [22]. PebS and PebB then catalyze a reduction of the A-ring vinyl moiety of 15,16-DHBV, a formal 2,3 reduction most likely followed by isomerization to *3Z*-PEB.

PebS accepts the intermediate 15,16-DHBV as a substrate and completes its reduction to PEB, indicating that substrate recognition is not inhibited by a reduced 15,16 double bond. Nevertheless, the overall turnover rate of 15,16-DHBV to PEB in vitro was lower than that of the proper substrate BV (data not shown), possibly because of the instability of free 15,16-DHBV or because of an association rate constant (k_{on}) that is lower for free 15,16-DHBV's alternate conformation. We therefore propose that the intermediate is never released from the

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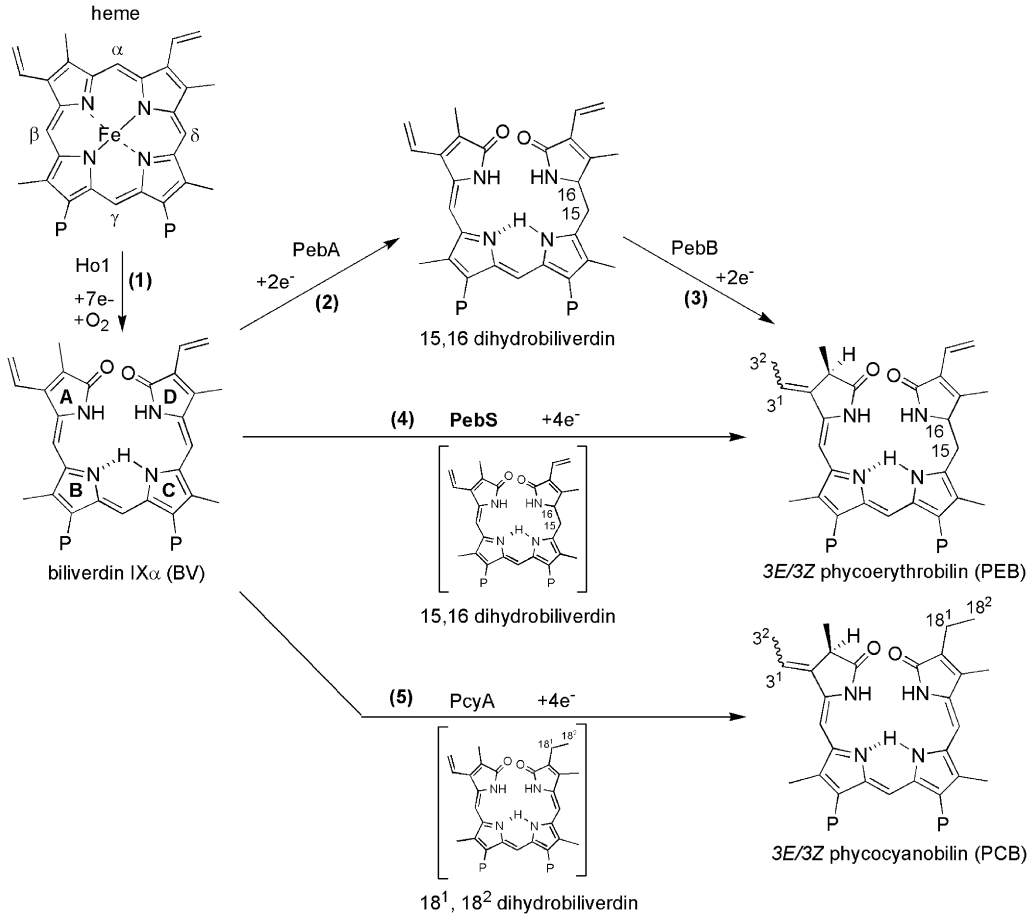

Figure 1. Biosynthesis of Open-Chain Tetrapyrroles in Cyanophages and Their Hosts

The first open-chain product biliverdin IXα (BV) is derived from heme by a heme oxygenase-catalyzed reaction (1). This open-chain product is the substrate for various enzymes of the FDBR family. Two sequential two-electron reductions catalyzed by PebA (2) and PebB (3) yield phycoerythrobilin (PEB). Phycoerythrobilin synthase (PebS) catalyzes a unique four-electron reduction of BV IXα to PEB (4). In a similar reaction, BV is reduced to phycocyanobilin (PCB) by the action of PcyA (5). All FDBRs obtain the required number of electrons from the redox cofactor ferredoxin. Enzymes catalyzing reactions 2 and 3 are found in cyanobacterial host cells. Enzymes catalyzing reactions 1 and 5 are found in both cyanobacterial host cells and cyanophages. The PebS enzyme (4) is found solely in cyanophage populations. The following abbreviations are used: propionate side chain (P), heme oxygenase (Ho1), PCB:ferredoxin oxidoreductase (PcyA), 15,16-DHBV:ferredoxin oxidoreductase (PebA), PEB:ferredoxin oxidoreductase (PebB), and PEB synthase (PebS).

enzyme but is directly converted to PEB. Similarly, cyanobacterial PebA and PebB have been postulated to associate transiently to perform metabolic channeling, the direct enzyme-to-enzyme transfer of the unstable intermediate 15,16-DHBV [22].

Reconstruction of Cyanophage Bilin Biosynthetic Pathways in *E. coli*

The functionality of the full-phage-encoded bilin biosynthetic pathway was further tested by reconstruction of the pathway with several expression vectors in *E. coli*, a useful system because *E. coli* can synthesize heme but cannot catabolize it. Extracted pigment from an *E. coli* culture expressing pTD*ho1* was indistinguishable from that of a BV IXα control (Figure S2), demonstrating that *ho1*_P-SSM2 encodes an active heme oxygenase that regiospecifically cleaves the heme macrocycle at the α-meso carbon.

Full reconstruction of PEB and PCB biosynthesis in *E. coli* was achieved with two more vectors, pTD*ho1pebS* and pTD*ho1pcyA*. Again, high-pressure liquid chromatography (HPLC) elution profiles of extracted expression culture pigments confirmed the expected products: The *pebS* construct yielded *3E*- and *3Z*-isomers of PEB, and the *pcyA* construct yielded *3E*- and *3Z*-isomers of PCB (Figure S2). Thus, expressed *ho1*_P-SSM2 and *pebS* can transform endogenous *E. coli* heme to PEB, and expressed *ho1*_P-SSM2 and *pcyA*_P-SSM4 can transform endogenous heme to PCB, in each case using only endogenous electron donors.

Finally, we wondered whether bilins produced by the cyanophage-encoded enzymes could be incorporated in phytochromes with the expected spectroscopic signatures. Here, the bilin biosynthesis vectors were used in coexpression experiments with bacterial BV-binding and cyanobacterial PCB- and PEB-binding apo-phytochromes, as previously described [23, 24]. Coexpression of pTD*ho1* with pASK_*bphP*, encoding the bacterial phytochrome of *Pseudomonas aeruginosa*, yielded a functional holophytochrome; red- and far-red-light difference spectroscopy revealed a typical phytochrome signature [25] (Figure S3). A similar result was obtained by coexpression of pTD*ho1pcyA* with the PCB-binding cyanobacterial phytochrome Cph1 of *Synechocystis* sp. PCC6803 (Figure S3). Notably, coexpression of pTD*ho1pebS* with *cph1* yields a highly fluorescent Cph1-PEB adduct (phytofluor)

Table 1. The Presence or Absence of Bilin Biosynthesis Genes in the Genomes of Fully Sequenced Cultured Marine Cyanobacteria and Cyanophages

Taxon	Strain	*pebS*	*pebA*	*pebB*	*pcyA*	*ho1*	*petF*	*cpeT*
Prochlorococcus podovirus	P-SSP7	–	–	–	–	–	–	–
Synechococcus podoviruses	Syn5	–	–	–	–	–	–	–
Synechococcus podoviruses	P60	–	–	–	–	–	–	–
Prochlorococcus myoviruses	P-SSM2	+	–	–	–	+	+	–
Prochlorococcus myoviruses	P-SSM4	–	–	–	+	–	–	–
Synechococcus myoviruses	S-PM2	–	–	–	–	–	–	+
Synechococcus myoviruses	Syn9	–	–	–	–	–	–	+
HL *Prochlorococcus*	6 strains[a]	–	+	+	+	+	+	–
LL *Prochlorococcus*	6 strains[b]	–	+	+	+	+	+	+
Synechococcus	6 strains[c]	–	+	+	+	+	+	+

+ indicates that the gene is present in the genome, and – indicates that the gene has not been identified in the genome. Each member of three cyanobacterial host cell groups (high-light [HL] adapted *Prochlorococcus,* low-light [LL] adapted *Prochlorococcus* and *Synechococcus*) contained the same bilin biosynthesis genes under investigation here; these data are grouped together under the appropriate taxon heading. Host genomes used here are strains as available as of October 15, 2007 at Microbes Online (http://www.microbesonline.org/).

[a] HL *Prochlorococcus* strains: MED4, MIT9215, MIT9301, MIT9312, MIT9515, and AS9601.

[b] LL *Prochlorococcus* strains: NATL1A, NATL2A, SS120, MIT9211, MIT9303, and MIT 9313.

[c] *Synechococcus* strains: CC9311, CC9605, CC9902, WH8102, JA-2-3B'a(2-13), and JA-3-3Ab.

[26], a useful biotechnology tool (Figure S4). Taken together, these experimental findings demonstrate that cyanophage enzymes are sufficient to efficiently produce the predicted bilin metabolites in a heterologous background.

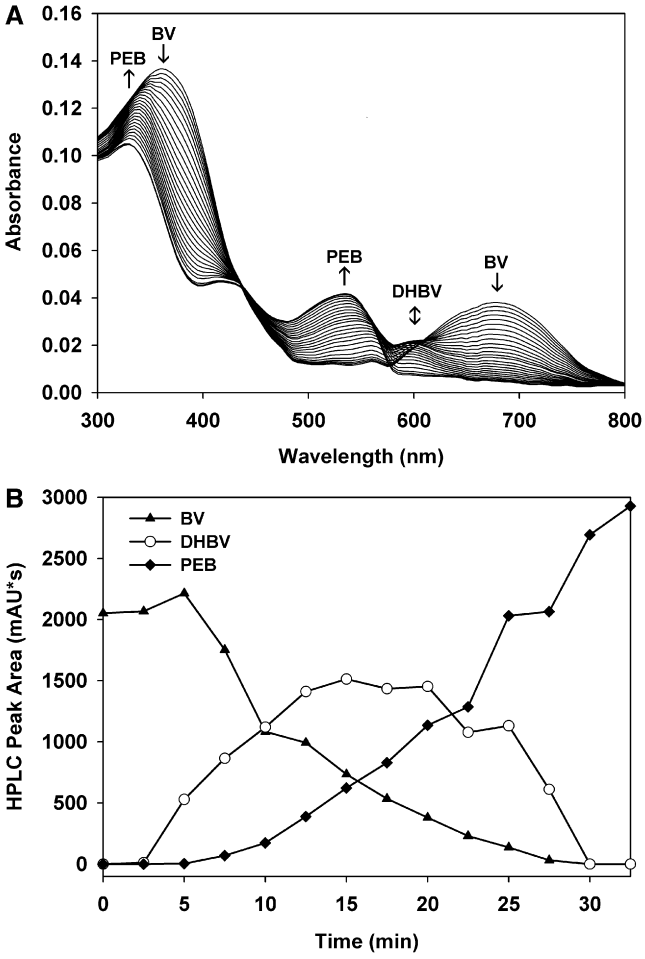

Figure 2. Time-Resolved Electron-Transfer Activity of PebS

(A) In vitro enzymatic conversion of 5 μM biliverdin IXα (BV) to phycoerythrobilin (PEB) was monitored by absorbance spectroscopy at 75 s intervals. Substrate-specific absorbance at 380 and 690 nm decreases, whereas the product-specific absorbance at 330 and 540 nm increases. Absorbance changes around 600 nm correspond to the appearance and disappearance of a partially reduced intermediate.

(B) Samples of the reaction were collected at 150 s intervals and subjected to HPLC analyses. HPLC peaks were identified by comparison to known standards. The integrated HPLC peak areas were plotted against sampling time. Triangles indicate BV IXα, circles indicate 15,16-dihydrobiliverdin (15,16-DHBV), and squares indicate *3E*- and *3Z*-PEB. The occurrence of 15,16-DHBV fits the approximately 600 nm absorbance change observed in (A), identifying 15,16-DHBV as the semireduced intermediate in this reaction. Detection wavelengths were 650 nm for BV IXα and 560 nm for 15,16-DHBV and PEB. Values for PEB correspond to the sum of both *3E*- and *3Z*-isomers.

Cyanophage-Encoded Bilin Biosynthesis Genes Are Expressed during Infection

Having shown that the *pebS*, *petF*, and *ho1* genes carried by the phage P-SSM2 encode proteins functional in vitro, we tested whether these three genes are expressed during infection of the low light (LL) *Prochlorococcus* strain NATL1A by P-SSM2. We used quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) to detect expression of *pebS*, *petF*_P-SSM2, and *ho1*_P-SSM2 at the messenger RNA (mRNA) level in infected and control cultures. In all infected cultures, all three phage transcripts were unambiguously detected as mRNA by 1 hr (Figure 3) after infection began ($p < 0.000003$ for each biological sample, binomial probability distribution; see the Supplemental Data). The early induction of these genes is consistent with the timing of phage gene induction of the core photosynthetic reaction center gene, *psbA*, in a *Prochlorococcus* podovirus phage-host system [27]. In light of the functionality of the recombinant proteins, the fact that all three genes in P-SSM2's PEB biosynthesis pathway are expressed during infection suggests that, rather than being excess genomic baggage en route to degradation, these genes likely play a functional role in this phage-host interaction.

Bilin Biosynthesis Genes in Host and Phage Genomes from Oceanic Communities

In the seven complete cultured cyanophage genomes, the *pebS* gene occurs only once, in P-SSM2 [13–17]. Furthermore, it is absent from the 18 *Prochlorococcus* and *Synechococcus* host genomes sequenced to date (S.C.B., M.B.S., and S.W.C., unpublished data, with data from [28] and unpublished

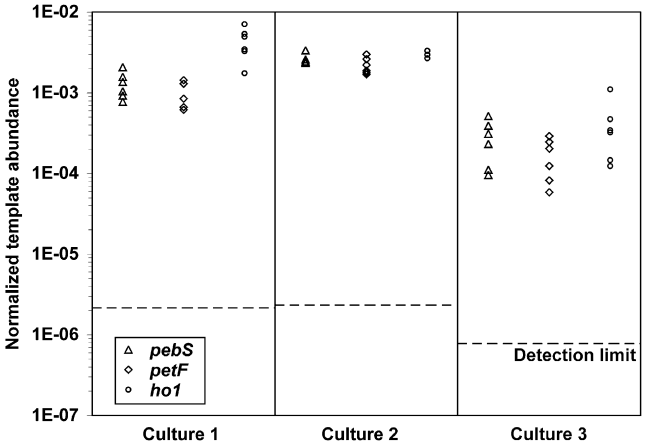

Figure 3. Bilin Biosynthesis Gene Expression during Infection of *Prochlorococcus* NATL1A by P-SSM2

Quantitative reverse transcriptase-polymerase chain reaction was used for the measurement of levels of cyanophage-encoded *pebS* (triangles), *petF* (diamonds), and *ho1* (squares) mRNA relative to host-encoded *rnpB* mRNA 1 hr after infection of triplicate NATL1A cultures. Dotted lines indicate the theoretical detection limit (i.e., the starting abundance of template that would allow a reaction to reach the threshold fluorescence during the final cycle). At t = 1 hr, all P-SSM2 genes investigated were expressed as mRNA in all infected cultures, whereas the phage genes were undetectable in both the uninfected cultures (biological negative control; data not shown) and 17 of 18 replicates of *pebS* primed reactions with reverse transcriptase omitted (technical negative control; the 18th technical replicate, with template abundance 5.3 e-4, is taken to be an outlier as described in the Supplemental Data).

genomes available at http://www.microbesonline.org/). Conversely, *pebA* (like its pathway partner *pebB*) is found in all of these host genomes and none of the cyanophages (Table 1), suggesting that the canonical *pebA* and *pebB* pathway plays an essential role in *Prochlorococcus* and *Synechococcus* but that this pathway may be absent in their phages. To see whether the exclusive association of *pebS* with phage genomes and *pebA* with host genomes persists in the wild, we examined the Global Ocean Survey (GOS) metagenomics dataset [29–31], which consists of DNA fragments extracted from 0.1–0.8 μm diameter particles in surface waters of the Atlantic and Pacific Oceans and from specialized aquatic environments (e.g., lakes, hypersaline ponds, estuaries).

After curation of low-stringency recruits and length normalization (see the Supplemental Data and Table 2), PebA and PebS protein-sequence queries recruited 164 and 137 DNA fragments, respectively, from the GOS database (Table 2). The recruited read and the reverse sequence read (paired end) from each piece of cloned DNA were then used as BLAST queries for the comparison of the taxonomic assignments of the two queries' top hits. The taxonomic affiliation of the recruited and paired-end reads' best hits were strikingly similar (Figure 4), allowing us to classify each environmental sequence as originating from *Synechococcus* PebA (31 sequences), high light (HL)-adapted *Prochlorococcus* PebA (133 sequences), or phage PebS (137 sequences). We then examined the phylogenetic clustering of these PebA and PebS homologs (Figure 4). All of the recruited reads inferred by BLAST to be *Prochlorococcus* or *Synechococcus* clustered together with PebA from HL *Prochlorococcus* and *Synechococcus* isolates, respectively. Likewise, recruited reads already inferred by BLAST to be cyanophage clustered together with PebS from P-SSM2. Thus, it appears that environmental PebA-like sequences are found only in cyanobacterial host cells, whereas PebS-like sequences are found only in phages. Notably, the 137 "cyanophage" metagenomic PebS sequences originated from 24 sampling sites (Table 2, Figure 4) in varied environments, suggesting that this "viral" PebS bilin biosynthesis strategy is not geographically restricted.

We next examined the environmental distribution of other bilin pathway proteins, PcyA, Ho1, and PebB (PetF was not examined; see the Supplemental Data). We identified 159 PcyA, 264 Ho1, and 159 PebB homologs (Table 2). The origins of these PcyA and Ho1 sequences, as inferred from BLAST and clustering (Figures S5 and S6), are split between cyanobacteria and phage, with 46 PcyA and 91 Ho1 sequences of cyanophage origin. In contrast, BLAST analyses of the recruited and paired-end reads argue that the recruited PebB DNA fragments likely originated solely from cyanobacteria (Table 2;

Table 2. Occurrence of Phage and Host Bilin Biosynthesis Gene Homologs in the Global Ocean Survey Metagenomic Dataset

		Bit Score > 100, Any Size	Bit Score > 100 *and* Over 140 Amino Acids in Size				
Query Protein	Query Size (Amino Acids)	Total Recruited Sequence Reads	Total Recruited Sequence Reads	Number[a] of Putative Pro Sequences	Number[a] of Putative Syn Sequences	Number[a] of Putative Phage Sequences	Number of GOS Sites with Phage Sequences
PebA	234	166 (236)	115 (164)	93 (133)	22 (31)	0 (0)	0
PebB[b]	257	167 (216)	123 (159)	108 (140)	15 (19)	0 (0)	0
PebS	234	127 (181)	96 (137)	0 (0)	0 (0)	96 (137)	24
PcyA	237	124 (174)	113 (159)	58 (82)	22 (31)	33 (46)	15
Ho1	242	232 (319)	192[c] (264)	94 (130)	28 (39)	66 (91)	18

The "total" columns represent the sequence reads with bit score similarities greater than 100 to the query sequence, of any size or restricted to over 140 amino acids length as indicated. To allow for cross-query comparisons, we normalized the number of recruits to query size as follows: The normalized number of recruited reads is equal to the number of recruited reads divided by the protein query size times an average protein size of 333 amino acids; these data are presented in parentheses. These tabulated data represent the summary of inferences made with a two-tiered approach for the inference of the origin of each environmental DNA fragment: phylogenetic clustering and paired-end analysis (see the Supplemental Data, Figure 4, Figures S5 and S6, and Table S1).

[a] Taxon assignments were determined by BLAST similarity of the recruited and paired-end sequences, as well as phylogenetic clustering of the recruited sequence read (see the Supplemental Data, Figure 4, and Figures S5 and S6).

[b] No tree is presented for PebB because there are no phage sequences available to make phylogenetic clustering inferences meaningful—tabulated data are presented in Table S1.

[c] For Ho1, of the 192 recruited sequence reads, the original organism for four DNA fragments could not be confidently inferred with either phylogenetic clustering or paired-end analysis.

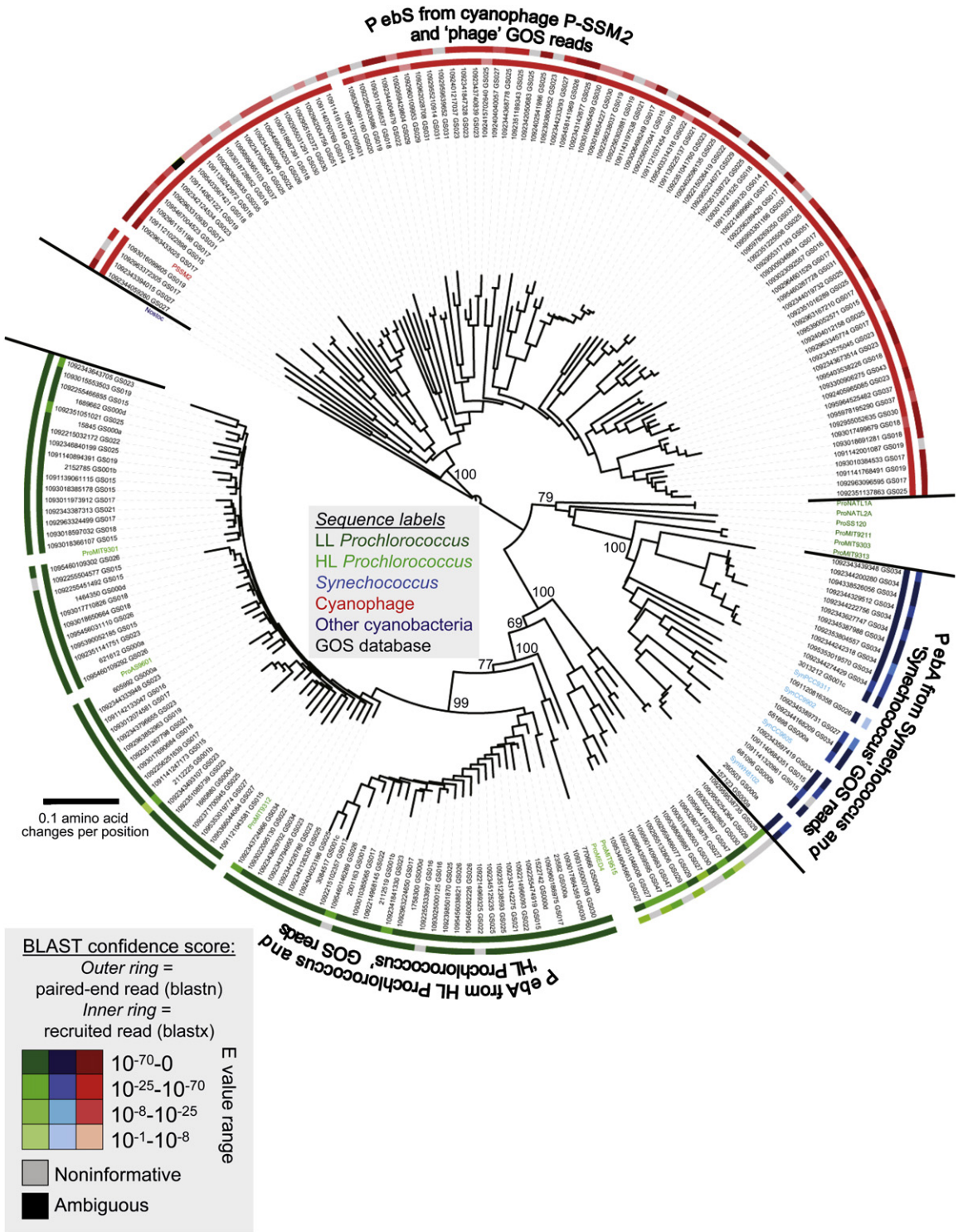

Figure 4. Analysis of PebA and PebS Proteins from Cultured Cyanobacteria and Cyanophages and of DNA Fragments from Wild Populations

Neighbor-joining distance tree constructed from the PebA and PebS sequences from cultured cyanobacteria and cyanophages (colored), respectively, as well as sequences recruited from the Global Ocean Survey (GOS) database (black) [29–31]. Statistical (neighbor-joining bootstraps) support is displayed only at critical nodes of the tree that help delineate cyanophage, *Prochlorococcus*, and *Synechococcus* sequence lineages. For environmental sequences, the read number is displayed, as well as the GOS site number (GS###) from which the DNA was originally obtained. The BLAST-based taxonomic assignments (see the Supplemental Data) are presented for each recruited and paired-end sequence from the wild DNA fragments as two color rings along the outside of the tree. The colors represent organismal identifications made from the taxon label of the top BLAST alignment of the recruited or paired-end reads against the GenBank nucleotide (NT) database; the intensity of the color reflects the confidence score of the BLAST hit. BLAST results were considered noninformative if there were no hits better than e-value 1e-1 from either cyanobacteria or cyanophages or, in the case of the paired-end reads, if the blast alignment was only to the query gene because the recruited read was already counted for that gene. BLAST results were considered ambiguous if the

Table S1). These findings suggest that cyanophages exploiting PEB synthesis may exclusively utilize the cyanophage PebS pathway rather than the "cyanobacterial" PebA and PebB pathway.

Phylogenetic analyses shed light on the evolutionary history of these genes. The monophyletic clusters observed for "phage" copies of these genes suggested that PebA and PebS and Ho1 were obtained from marine cyanobacteria only once, whereas PcyA phage sequences group into two clusters (Figure 4, Figures S5 and S6) and may have been obtained twice. In comparison, the genes encoding the core photosystem II reaction center proteins, PsbA and PsbD, are thought to have been obtained repeatedly (four and two times, respectively) by cyanophages from their cyanobacterial hosts [32].

Finally, although we recognize that the phage signal in these cell-fraction metagenomic data are predominantly intracellular phages unlikely to represent the entire free-phage community, we estimated the fraction of T4-like phages in the metagenome that contain phage bilin biosynthesis genes. By using normalized recruit frequencies that account for variable gene sizes, we observed 137 phage PebS, 46 phage PcyA, and 91 phage Ho1 DNA fragments (Table 2), as compared to 1018 DNA fragments (Document S2) recruited for the T4-like portal protein-encoding gene (gene 20), which is universal among T4-like phages, one of the most common phage types observed in marine metagenomes to date [33, 34]. Thus, as much as 13%, 5%, and 9% of the T4-like phages captured in these samples contained PebS, PcyA, and Ho1, respectively, suggesting that these biosynthetic pathways are an important component of wild T4-like phage populations. Notably, one *pcyA* sequence resides on a 12.7 kb phage genomic fragment (JCVI_SCAF_1096626959277) that also includes a *pebS* homolog; thus, some phage genomes may be capable of both PEB and PCB biosynthetic pathways.

Conclusions

We have demonstrated that the cyanophage protein PebS has acquired a novel activity, combining the functions of two separate enzymes in the host cell, PebA and PebB. Further, all evidence suggests that cyanophages that maintain bilin biosynthesis activity have replaced the canonical PebA and PebB pathway found in host cells with the single PebS enzyme. We hypothesize that the maintenance of two individual genes might allow for tighter regulation (e.g., feedback inhibition) in the cell or a metabolic branchpoint upstream of an alternative, beneficial function for 15,16-DHBV. Conversely, phage fitness may be influenced more by short-term efficiency rather than long-term flexibility; a one-enzyme system would allow a cyanophage to channel all PebS-bound BV toward a single metabolic fate. Further, phage genomes are likely under tighter size selection than microbial genomes because of headful packaging; again, the single enzyme would prove advantageous because PebS requires less than half of the genetic material as the PebA and PebB system.

More broadly, the auxiliary metabolic genes in oceanic viral genomes presumably reveal the evolutionary swapping of metabolic components critical to phage and host reproduction. The importance of these components to cyanophages is not always clear. In the case of the bilin biosynthesis genes studied here, their role is not even well understood in the host cells from which the phage genes were derived. Nonetheless, we find that cyanophage-encoded bilin biosynthesis genes are functional, that they are expressed during infection, and that they are represented in wild populations of phage. Moreover, PebS has a novel activity and a sequence found only in cyanophages. Together, these data contribute to our growing understanding of cyanophages as a laboratory for metabolic innovation.

Experimental Procedures

Standard methods for manipulation of nucleic acids and purification of recombinant proteins were used throughout. Full experimental procedures and associated references are in the Supplemental Data available online.

Supplemental Data

Experimental Procedures, six figures, one dataset, and one table are available at http://www.current-biology.com/cgi/content/full/18/6/442/DC1/.

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top hits included both cyanobacteria and cyanophage. Sequences from *Prochlorococcus* MIT9303 and MIT9313 often cluster with homologs from *Synechococcus* [28], as observed here. Together these analyses identify the environmental PebA and PebS sequences as having originated either from cyanobacteria or cyanophages, respectively.

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