

ORIGINAL ARTICLE

Structure and function of a cyanophage-encoded peptide deformylase

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Bacteriophages encode auxiliary metabolic genes that support more efficient phage replication. For example, cyanophages carry several genes to maintain host photosynthesis throughout infection, shuttling the energy and reducing power generated away from carbon fixation and into anabolic pathways. Photodamage to the D1/D2 proteins at the core of photosystem II necessitates their continual replacement. Synthesis of functional proteins in bacteria requires co-translational removal of the N-terminal formyl group by a peptide deformylase (PDF). Analysis of marine metagenomes to identify phage-encoded homologs of known metabolic genes found that marine phages carry PDF genes, suggesting that their expression during infection might benefit phage replication. We identified a PDF homolog in the genome of *Synechococcus* cyanophage S-SSM7. Sequence analysis confirmed that it possesses the three absolutely conserved motifs that form the active site in PDF metalloproteases. Phylogenetic analysis placed it within the Type 1B subclass, most closely related to the *Arabidopsis* chloroplast PDF, but lacking the C-terminal α -helix characteristic of that group. PDF proteins from this phage and from *Synechococcus elongatus* were expressed and characterized. The phage PDF is the more active enzyme and deformylates the N-terminal tetrapeptides from D1 proteins more efficiently than those from ribosomal proteins. Solution of the X-ray/crystal structures of those two PDFs to 1.95 Å resolution revealed active sites identical to that of the Type 1B *Arabidopsis* chloroplast PDF. Taken together, these findings show that many cyanophages encode a PDF with a D1 substrate preference that adds to the repertoire of genes used by phages to maintain photosynthetic activities.

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Introduction

Bacteriophages (phages) frequently carry metabolic genes acquired from host genomes. Not being essential for replication *per se*, these are termed *auxiliary metabolic genes* (AMGs) (Breitbart *et al.*, 2007). The list of AMGs identified in phage genomes and thought to benefit the phage during lytic replication now includes those functioning in photosynthesis (Mann *et al.*, 2003; Sharon *et al.*, 2009; Sharon *et al.*, 2011), the pentose phosphate pathway (Thompson *et al.*, 2011), phosphate acquisition (Goldsmith *et al.*, 2011; Zeng and Chisholm, 2012), nucleotide metabolism (Rohwer *et al.*, 2000; Mann *et al.*, 2005; Sullivan *et al.*, 2005; Dinsdale

et al., 2008) and cytoskeletal construction (Kraemer *et al.*, 2012), among others.

Marine cyanophages carry a rich array of AMGs, including genes encoding components of photosystem II (PSII). PSII is subject to photodamage, particularly the D1 and D2 proteins that compose the functional heterodimer at its core and that are responsible for the binding of pigments and cofactors necessary for primary photochemistry (Millard *et al.*, 2004). To maintain sufficient active D1/D2, the proteins are protected by high-light-inducible proteins that assist by dissipating excess light energy, and are also regularly recycled and replaced (Millard *et al.*, 2004).

During a cyanophage infection, continuing photosynthesis is required to provide both energy (ATP) and reducing power (NADPH) for phage replication (Mann *et al.*, 2003; Lindell *et al.*, 2005). Numerous cyanophages encode D1 proteins plus at least one other component of PSII (Lindell *et al.*, 2005). During infection of *Prochlorococcus* MED4 by the T7-like cyanophage P-SSP7, phage D1 protein is

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expressed and partially compensates for the decline in host D1 synthesis (Lindell *et al.*, 2005). Other factors likely contribute as well to the maintenance of photosynthetic activity during infection, for example, phage-encoded high-light inducible proteins. On the other hand, following infection of *Synechococcus* sp. WH7803 by S-PM2 phage, host D1 (*psbA*) mRNA initially increases 18-fold and remains slightly elevated even late in infection, while a high level of phage D1 transcripts was sustained throughout (Clokie *et al.*, 2006). Clearly D1 synthesis, either from phage or host genes, is a priority during cyanophage infection.

During protein translation in bacteria, the first amino-acid residue inserted is *N*-formylmethionine. The formyl moiety is then co-translationally removed from at least 98% of bacterial proteins by a peptide deformylase (PDF) (personal communication; Sharon *et al.*, 2011; reviewed in Meinnel and Giglione, 2008). Frequently the *N*-terminal methionine is also removed by methionine aminopeptidase. Both modifications take place immediately as the nascent polypeptide chain emerges from the portal of the ribosome exit tunnel, before protein folding blocks enzyme access to the *N*-terminus. In *E. coli*, deletion of the PDF gene is lethal (Mazel *et al.*, 1994).

Inhibition of PDFs by actinonin, a naturally occurring antimicrobial, inhibits bacterial growth (Chen *et al.*, 2000), and actinonin treatment *in vivo* also leads to decline of photosynthetic function of the plastids in plants and green algae. In the unicellular alga *Chlamydomonas*, actinonin destabilizes D2, shunting it to a degradative pathway, thus interfering with the assembly of PSII (Giglione *et al.*, 2003). In vascular plants, where the translation rate of D1 is 50–100 times greater than those of other PSII proteins (Kyle *et al.*, 1984), the effect of actinonin on D1 synthesis and assembly is most pronounced (Hou *et al.*, 2004). Therefore, the chloroplast PDF is essential for maintenance of PSII. If this *N*-terminal methionine excision system is overwhelmed, as might be expected by the high level of protein synthesis during phage replication, improperly processed and non-functional proteins would result. Cyanobacteria, important aquatic primary producers, share high functional similarity to chloroplasts (Giovannoni *et al.*, 1988), but it is not known if their PDF shares functional or structural similarity to the PDF of chloroplasts.

PDFs are a subclass of the metalloprotease superfamily of enzymes known as the ‘clan MA and MB’ metalloproteases. Proteins from this family share a common structure containing a three-stranded β strand facing a catalytic metal and a HEXXH motif-containing α helix (Giglione *et al.*, 2004). Although PDFs display variability in amino-acid sequence and overall length, they share three absolutely conserved and unique motifs that together form the entire active site: the G-motif (G Φ G Φ AAXQ where Φ is any hydrophobic amino acid and X is any amino acid), H-motif (QHEXDHLXG) and C-motif (EGCXS)

(Giglione *et al.*, 2004). PDFs are classified into three types based on sequence homologies and structural distinctions. Type 1 PDFs (PDF1) are found in bacteria and bacterial-derived organelles of eukaryotes (plastids, mitochondria and apicoplasts), while Type 2 PDFs are restricted to the Gram-positive Bacteria. These PDFs all possess the three conserved motifs and all act on the same substrates (*N*-formyl methionine polypeptides), although some variation in substrate specificity has been found in that the high-turnover D1 protein from PSII is a preferred substrate for the PDF in plant plastids (Dirk *et al.*, 2008). These active PDFs differ in the secondary structure of their C-terminal domain; it is an α helix in Type 1 proteins and a supplementary β strand in Type 2 proteins. Within the PDF1, the C-terminal α -helix displays variable length and is not required for catalytic activity (Meinnel *et al.*, 1996).

The less-studied Type 3 PDFs, found in Archaea and in the mitochondria of trypanosomatids, differ in their ‘conserved’ motifs. Archaea do not use *N*-formylmethionine for translation initiation; the formylated substrate for the archaeal PDFs is currently unknown (Bouzaidi-Tiali *et al.*, 2007).

Evidence that phages encode AMGs has often come from the identification of metabolic homologs in sequenced phage genomes. Recently a novel method was used to search marine metagenomes for virally-encoded microbial metabolic genes (Sharon *et al.*, 2011). By recognizing phage scaffolds in these marine metagenomes and then identifying metabolic genes within them, this approach is not limited to sequenced genomes. The most abundant metabolic genes found by this survey were PDFs, thereby predicting that PDFs are a significant part of the metabolic repertoire carried by marine phages.

Bringing all these threads together, we hypothesized that encoding and expressing a PDF could be one of the strategies used by cyanophages to manipulate host metabolism during infection. Here, we report identification of a PDF gene in the genome of a marine cyanophage (S-SSM7) and solution of its crystal structure. The PDF encoded by cyanophage S-SSM7 belongs to the Type 1B PDF subgroup, which is widely distributed among marine phage. We also determined the enzymatic activity and structure of the previously annotated PDF protein from *Synechococcus elongatus* PCC 6301. Comparison of these enzymes demonstrates that the S-SSM7 enzyme is highly active with a substrate preference for the high-turnover PSII protein D1, consistent with our hypothesis that phage encode PDFs to manipulate host metabolism and help sustain photosynthesis during infection.

Materials and methods

Target selection

The gene for the *Synechococcus* phage PDF, YP_004324347, was identified in the genome of

Synechococcus phage S-SSM7 during a bioinformatic survey looking for unknown phage proteins that are abundant in environmental metagenomes but under-represented in the PhAnToMe database phage genome database (<http://www.phantome.org/>). Protein sequences identified in phage genomes using Glimmer V3.02 were compared against all publicly available metagenomes (from <http://edwards.sdsu.edu/cgi-bin/mymgdb/show.cgi>) using tBLASTn (*E* value cutoff 10^{-5}). ORFs were annotated using the PhAnToMe database with BLASTp (*E* value cutoff 10^{-5}). The encoded protein is referred to throughout the text as the phage PDF.

The gene for the cyanobacterial PDF, *YP_170923*, in the genome sequence of *S. elongatus* PCC 6301, was identified based on gene annotations in the NCBI Reference Sequence database (<http://www.ncbi.nlm.nih.gov/RefSeq/>). (The original host strain for S-SSM7, *Synechococcus* WH8109, has not been sequenced.) The encoded protein is referred to throughout the text as the bacterial PDF.

Gene design

Starting from the amino-acid sequences for these two PDF proteins, gene sequences were designed for expression in *E. coli* using Gene Composer (Emerald BioSystems, Bainbridge Island, WA, USA) (Lorimer *et al.*, 2009; Lorimer *et al.*, 2011). Back-translation of the amino-acid sequences employed a Universal Codon Usage Table designed to accommodate expression in *E. coli* with a minimum usage threshold of 2%. Restriction enzyme recognition sequences for *Bam*HI and *Hind*III were excluded from the sequence to facilitate cloning. The engineered gene sequences were synthesized by DNA2.0 (Menlo Park, CA). The genes were sub-cloned into an expression vector containing an isopropyl- β -thiogalactopyranoside-inducible (IPTG-inducible) T7 promoter using the PIPE cloning method (Klock *et al.*, 2008; Raymond *et al.*, 2009; Raymond *et al.*, 2011). The vector provides an N-terminal hexahistidine-Smt tag; the Smt tag is specifically and efficiently removed by UlpI protease (Mossessova and Lima, 2000).

Gene expression and protein purification

E. coli BL21(DE3) cells expressing the engineered PDF gene from *Synechococcus* phage S-SSM7 or *S. elongatus* were cultured at 37 °C to an A600 of ~ 0.6 in TB medium (Teknova, Hollister, CA, USA). Cultures were induced with 1 mM IPTG and incubated overnight at 25 °C. The bacterial cells were harvested by centrifugation at 4 °C and the resulting cell paste was stored at -80 °C. For protein purification, the cells were thawed in 25 mM Tris-HCl (pH 8.0), 200 mM NaCl, 50 mM arginine, 10 mM imidazole, 0.02% CHAPS detergent, 0.5% glycerol, 1 mM Tris(2-carboxyethyl)phosphine (TCEP), 100 mg lysozyme, 250 U μ l $^{-1}$ Benzonase (Novagen, Madison, WI, USA) and one complete EDTA-free Protease

Inhibitor Cocktail tablet (Roche, Indianapolis, IN, USA), and then lysed by sonication using a Misonix S-4000 sonicator (Qsonica, Newtown, CT, USA) at 70% power (67–69 W), 2 s on/1 s off, 3 min total. Immediately after sonication, the crude lysate was clarified by centrifugation at 18 000 *g* for 35 min at 4 °C. The tagged proteins were initially purified using the protein maker (Smith *et al.*, 2011). Briefly, lysates were applied to a 5-ml HisTrap FF nickel-chelate column (GE Healthcare, Waukesha, WI, USA) in 25 mM Tris-HCl (pH 8.0), 200 mM NaCl, 50 mM arginine, 10 mM imidazole, 0.25% glycerol and 1 mM TCEP. The column was washed with three column volumes of the same buffer, then eluted in three steps: a 5-ml elution with 30 mM, a 5-ml elution with 206 mM and two 5 ml elutions at 500 mM imidazole. Elution fractions containing partially purified PDF proteins were identified based on molecular weight by SDS polyacrylamide gel electrophoresis (SDS-PAGE). The fraction containing the target protein was treated with 50 ml of 1 mg ml $^{-1}$ of 6 \times His-tagged ubiquitin-like protease 1 overnight to cleave the Smt-PDF fusion and remove the His tag. The resulting protein was dialyzed against 1 l of 25 mM Tris-HCl (pH 8.0), 200 mM NaCl, 50 mM arginine, 10 mM imidazole, 0.25% glycerol and 1 mM TCEP, and then run over a second nickel-chelate column as described above. The flow-through, wash, and elution fractions were analyzed by SDS-PAGE and the fraction containing PDF protein was concentrated using an Amicon 10 kDa MWCO concentrator (Millipore, Billerica, MA, USA) to ~ 5 ml and further purified by size exclusion chromatography on a Sephacryl S-100 10/300 GL column (GE Healthcare) in 25 mM Tris-HCl (pH 8.0), 200 mM NaCl, 1.0% glycerol and 1 mM TCEP. Peak fractions (3.0 ml) were concentrated to ~ 10 mg ml $^{-1}$ and flash frozen in liquid nitrogen in 100 μ l aliquots. Selenomethionine-labeled proteins were produced in *E. coli* BL21(DE3) cells grown in M9 minimal medium supplemented with 5 mg ml $^{-1}$ thiamine, 60 mg l $^{-1}$ selenomethionine and other metabolites to inhibit methionine biosynthesis (Doublé, 1997), followed by purification as described above.

Enzyme assays

PDF enzyme activity was assayed using a coupled PDF/FDH (formate dehydrogenase) assay. The formate released by PDF from the fMAS substrate is oxidized by FDH with the concomitant reduction of NAD $^{+}$ to NADH (Lazennec and Meinel, 1997). NADH was measured by absorbance at 340 nm using a SpectraMax M2 96-well microplate reader (Molecular Devices, Sunnyvale, CA, USA). The substrate was 1 mM fMAS. Initial rates of reaction (v_{app}) were calculated from the initial linear portion of plots of NADH produced versus time. K_m and V_{max} were extrapolated from plots of v_{app} versus substrate concentration (GraphPad Prism, La Jolla, CA, USA). k_{cat} was calculated by dividing V_{max} by enzyme

concentration. The inhibitor data were obtained using 0.2 nM enzyme.

N-terminal tetrapeptides

The N-terminal tetrapeptide sequences encoded by annotated ribosomal protein genes in *S. elongatus* PCC 6301 (Genbank: AP008231.1), *Synechococcus* sp. CC931 (Genbank: NC_008319.1), *Synechococcus* sp. CC9605 (Genbank: NC_007516.1) and *Synechococcus* sp. CC9902 (Genbank: NC_007513.1) were tabulated to yield a total 259 proteins (64, 65, 66 and 64 from each genome). The most frequently occurring tetrapeptides consisted of one with six occurrences (fMAKK) and 17 with four occurrences (including fMSRV and fMARI). The peptides fMAKK, fMSRV and fMARI were used as substrates in the PDF specificity assays, compared against the encoded N-terminal tetrapeptides of four representative D1 proteins: the D1 of *Synechococcus* phage S-SSM7 (Genbank: GU071098.1) and three bacterial D1 proteins from *S. elongatus* PCC 6301 (Table 1).

Phylogenetic analyses

For phylogenetic analysis, in addition to the phage and bacterial PDFs, 49 representative annotated PDF genes were selected from the sequenced genomes in The SEED database (<http://theseed.uchicago.edu/FIG/>) (Overbeek *et al.*, 2005). A multiple sequence alignment was generated at the amino-acid level using ClustalW (Chenna *et al.*, 2003) and the tree was drawn using FigTree v1.31 (<http://tree.bio.ed.ac.uk/software/figtree/>).

Table 1 Tetrapeptide substrates for PDF activity assays

Protein	Tetra-peptide	Source	Number of occurrences
<i>D1</i>			
Genbank:			
GI:310003945	fMLIS	Cyanophage S-SSM7	
GI:56686061	fMTSI	<i>Synechococcus elongatus</i> PCC 6301	
GI:56685134, GI:56685615	fMTTA	<i>S. elongatus</i> PCC 6301	
<i>Ribosomal proteins</i>			
GI:56750900	fMAKK	<i>S. elongatus</i> PCC 6301	6
GI:56751081		<i>S. elongatus</i> PCC 6301	
GI:78213979		<i>S. sp.</i> CC9605	
GI:78212121		<i>S. sp.</i> CC9605	
GI:78185723		<i>S. sp.</i> CC9902	
GI:78185359		<i>S. sp.</i> CC9902	
GI:56751466	fMSRV	<i>S. elongatus</i> PCC 6301	4
GI:113953218		<i>S. sp.</i> CC9311	
GI:78212935		<i>S. sp.</i> CC9605	
GI:78184654		<i>S. sp.</i> CC9902	
GI:56751895	fMARI	<i>S. elongatus</i> PCC 6301	4
GI:113953764		<i>S. sp.</i> CC9311	
GI:78211907		<i>S. sp.</i> CC9605	
GI:78185543		<i>S. sp.</i> CC9902	

Abbreviations: fM, formylmethionine; PDF, peptide deformylase.

Global PDF distribution

The 70 phage-encoded PDFs reported by Sharon *et al.* (Sharon *et al.*, 2011) were compared against all 12 672 581 sequencing reads in the Global Ocean Survey (GOS) metagenomes (Yooseph *et al.*, 2007) using BLASTX (Altschul *et al.*, 1990) (parameters: $-e\ 1e-5 -F\ F -r\ 1 -q\ -1 -v\ 5 -b\ 5$). GOS sequences with at least 30% amino-acid identity and 50% similarity to any of the 70 PDFs were tallied as phage-encoded PDF homologs. The normalized relative abundance was calculated for each sampled location as the number of phage-encoded PDF homologs divided by the total number of sequencing reads in that data set. The map was generated using ArcGIS version 10.1. The mapped chlorophyll concentrations are the September, 2012, values from the NASA Earth Observations site (<http://neo.sci.gsfc.nasa.gov>).

Crystallization

Crystallization trials of the phage PDF (native and Se-Met) were carried out using sitting drop vapor diffusion at 16 °C in a drop of a 1:1 mixture of protein (10.17 mg ml⁻¹) and reservoir solution. Crystals grew from 0.1 M HEPES, pH 7.5; 70% (v/v) (+/-)-2-methyl-2,4-pentanediol. For co-crystallization with the inhibitor, actinonin, the native PDF protein was incubated at 4 °C overnight with a five-fold molar excess of actinonin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) before crystallization. Crystals were frozen in liquid nitrogen in a solution of 30% (w/v) ethylene glycol for cryoprotection.

Crystallization of the native bacterial PDF was achieved as described above except that the crystals grew in 0.15 M KBr, 30% PEG-MME 2000.

Crystallography: phage protein

For the apo Se-Met phage protein data set (PDB 3UWA), X-ray diffraction data were collected at the Advanced Photon Source, beamline 19ID (Argonne, IL, USA) at a wavelength of 0.9791750 Å. The structure was solved by Se-SAD (Supplementary Tables S2 and S3). Crystals display a symmetry of $P2_12_12_1$ with cell dimensions $a = 47.5$ Å, $b = 58.3$ Å, $c = 62.4$ Å. For the actinonin-bound native phage protein data set (PDB 3UWB), the data were obtained using a Rigaku SuperBright FR-E + rotating-anode X-ray generator with Osmic VariMax HF optics and a Saturn 944 + CCD detector. Data were collected to 1.7 Å resolution and the structure was solved by molecular replacement using the phage structure, PDB 3UWA, as the search model (Supplementary Tables S2 and S3). In both cases, a single crystal was used for each complete data set.

Crystallography: bacterial protein

A data set for the apo native bacterial crystal (PDB 4DR8) was collected at the Stanford Synchrotron Radiation Lightsource, beamline 7-1. Data were

collected to 1.55 Å resolution and the structure was solved by molecular replacement using PDB 1LRY as the search model (Supplementary Tables S4 and S5). PDB 1LRY, the PDF from *Pseudomonas aeruginosa*, was the PDF with a solved structure that had the highest similarity to the *Synechococcus* protein. Crystals display P1 symmetry with cell dimensions of $a = 43.40$ Å, $b = 65.36$ Å, $c = 69.03$ Å. To generate the actinonin-bound bacterial protein, the apo crystals were soaked overnight with actinonin (1 mM in crystallization mother liquor). The actinonin-bound data set was collected in-house as described for the phage data sets. Data were collected to 1.9 Å resolution and the structure (PDB 4DR9) was solved by molecular replacement using the native structure (PDB 4DR8) as the search model (Supplementary Tables S4 and S5).

Results

Global distribution of phage PDF homologs

The initial report of abundant phage-encoded PDFs in marine environments (Sharon *et al.*, 2011) did not address their biological function or the question of their global distribution. Therefore, we first searched all available GOS metagenomes for homologs of those phage-encoded PDFs using BLASTX. Mapping of the relative abundance of these homologs at all the GOS-sampling locations makes their widespread distribution apparent (Figure 1, Supplementary Table S1), thus demonstrating their importance in the environment. Additional studies will be required to correlate their pattern of distribution with ecological factors such as host distribution and nutrient levels; however, we have

extensively characterized the structure and function of a phage-encoded PDF.

Synechococcus phage PDF activity

Polypeptide deformylation activity of the PDF protein from *Synechococcus* phage S-SSM7 (referred to throughout as the 'phage PDF') was compared with the activity of the PDF from *S. elongatus* PCC 6301 (referred to throughout as the 'bacterial PDF') using a real-time PDF/FDH enzyme-coupled assay (see Methods). The assay was optimized to measure initial rates accurately and produced z' values > 0.8 (data not shown). This assay was first used to evaluate the sensitivity of the phage PDF to inhibition by actinonin, a naturally occurring antibiotic demonstrated to be a potent inhibitor of bacterial PDFs (Chen *et al.*, 2000). Results in Figure 2a show that actinonin is a potent inhibitor of phage and bacterial PDFs with an apparent IC_{50} value of 10 nM against both enzymes, suggesting that both enzymes have similar catalytic active sites.

The deformylation activity of the phage PDF was assayed using formylated N-terminal tetrapeptides derived from D1 proteins from cyanophage S-SSM7 and *S. elongatus* PCC 6301 as well as the most frequently occurring N-terminal tetrapeptides of ribosomal proteins from four *Synechococcus* genomes (Table 1 and Methods). The phage PDF has significantly higher specific activity than the bacterial enzyme on the substrates used above (average k_{cat} values of 615 versus 192 s^{-1} , Table 2). Likewise, the phage PDF is significantly more efficient than the bacterial PDF at deformylating the D1-derived tetrapeptides (k_{cat}/K_m for the phage PDF being 14.7,

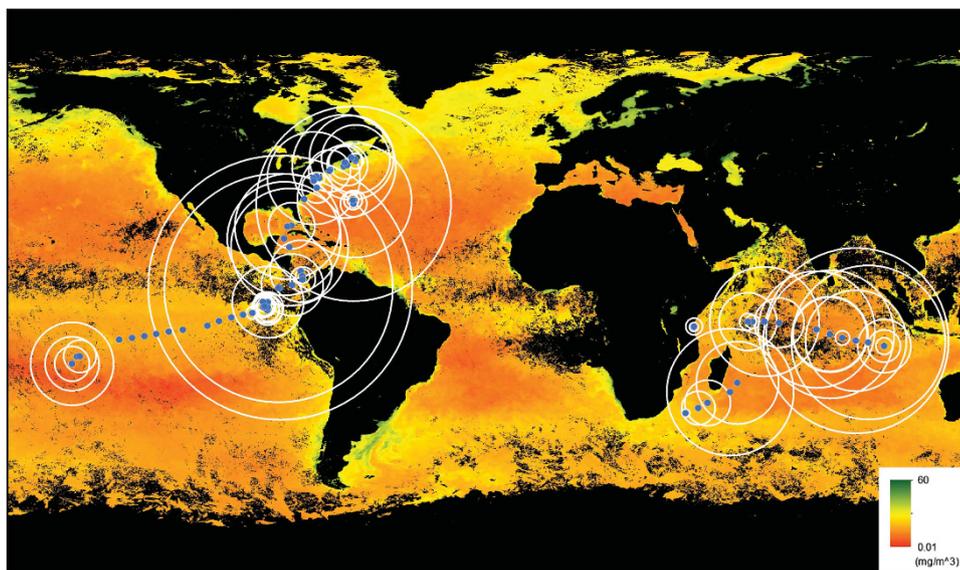


Figure 1 Global distribution of phage-encoded PDFs. GOS-sampling locations (blue dots) and relative abundances of phage-encoded PDFs (white circles) in the GOS metagenomes are plotted on a global map of marine chlorophyll concentrations. See Supplementary Table S1 for the data plotted.

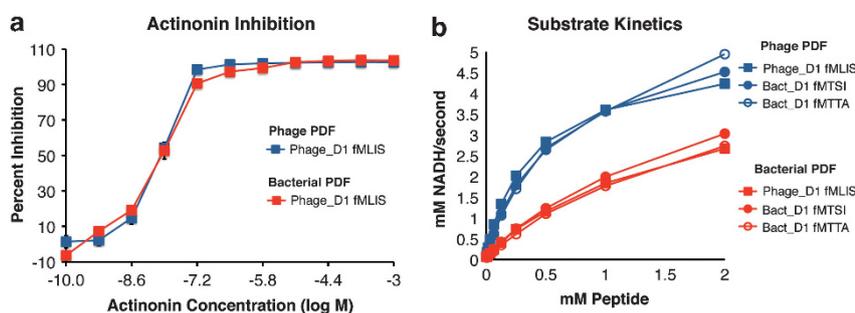


Figure 2 (a) Inhibition of the *Synechococcus* phage S-SSM7 PDF (blue) and the PDF from *Synechococcus elongatus* PCC 6301 (red) by actinonin. Enzyme: 0.2 nM PDF. Substrate: 1.5 mM fMLIS, the N-terminal tetrapeptide of the D1 protein encoded by the phage. (b) Kinetic assays comparing the activity of the *Synechococcus* phage S-SSM7 PDF (blue) with that from *S. elongatus* PCC 6301 (red) on three different N-terminal tetrapeptide substrates derived from phage or bacterial D1 proteins (see Table 1). Red square = phage D1, fMLIS; solid red circle = bacterial D1, fMTSI; open red circle = bacterial D1, fMTTA.

Table 2 Activity of phage and bacterial PDFs on N-terminal tetrapeptides derived from D1 proteins and cyanobacterial ribosomal proteins. (See Table 1 for substrate details.)

Enzyme	Substrate								
	Phage D1: fMLIS			Bacterial D1: fMTSI			Bacterial D1: fMTTA		
	K_m (nM)	k_{cat} (s^{-1})	k_{cat}/K_m	K_m (nM)	k_{cat} (s^{-1})	k_{cat}/K_m	K_m (nM)	k_{cat} (s^{-1})	k_{cat}/K_m
Phage	0.31	683	2204	0.48	767	1597	0.52	800	1538
Bacterial	1	150	150	1.2	183	153	1.5	183	122
Phage/Bact	0.3	4.6	14.7	0.4	4.2	10.5	0.3	4.4	12.6

	Ribosomal proteins: fMAKK			Ribosomal proteins: fMARI			Ribosomal proteins: fMSRV		
	K_m (nM)	k_{cat} (s^{-1})	k_{cat}/K_m	K_m (nM)	k_{cat} (s^{-1})	k_{cat}/K_m	K_m (nM)	k_{cat} (s^{-1})	k_{cat}/K_m
Phage	1.3	583	449	0.2	87	433	0.6	767	1278
Bacterial	1.9	167	88	0.8	250	313	1.1	217	197
Phage/Bact	0.7	3.5	5.1	0.3	0.3	1.4	0.5	3.5	6.5

10.5 and 12.6 times that of the bacterial PDF; Table 2). Comparison of the substrate kinetics of these two enzymes on tetrapeptides derived from phage and bacterial D1 proteins shows the consistently greater activity of the phage PDF (Figure 2b). The differences in efficiency were less when three different tetrapeptides from ribosomal proteins served as the substrate (the phage efficiency here being only 5.1, 1.4 and 6.5 times that for the bacterial enzyme). Taken together, these results clearly show that the phage PDF is a catalytically active deformylase with overall properties similar to the bacterial PDF.

Phage PDF specificity

The two substrate classes (D1 and ribosomal proteins) were chosen to determine if either PDF displayed any selectivity for deformylating D1 proteins. Results in Table 2 show that the specificity constants (k_{cat}/K_m) (Eisenthal *et al.*, 2007) for the phage PDF are significantly higher for the

D1-derived peptides compared with the ribosomal-derived peptides (1780 versus 720). In contrast, when comparing the two substrates against the bacterial PDF, the opposite trend was observed, although the difference was not nearly as significant (141 versus 199). These results show that the phage PDF is much more efficient at deformylating the D1 substrates than the bacterial PDF.

Phylogenetic analysis of cyanophage S-SSM7 PDF

Members of the PDF family, while displaying variability in amino-acid sequence, share three essential conserved motifs (the G, H and C motifs) that together build the entire active site (Giglione *et al.*, 2004). Alignment of the predicted phage PDF sequence with PDFs of known structure from eukaryote organelles (mitochondria and chloroplasts of *Arabidopsis thaliana*, and the non-photosynthetic plastid-derived apicoplast of *Plasmodium falciparum*) and a cyanobacterium (*S. elongatus* PCC 6301) demonstrates the conservation of these motifs

from the kinetic data, the structures of all three enzymes are also identical when bound to actinonin (data not shown), thus providing strong structural evidence that the phage protein is a functional PDF enzyme. The C-terminal α -helix that is characteristic of Type 1B PDF proteins is absent from the phage protein (Supplementary Figure S1). The amino-acid residues that compose the active site, which are conserved and are predicted to interact with a zinc ion, are observed in identical conformations in these three enzymes (Figure 4c). Comparison of the amino-acid residues positioned at the entry to the active site in these three proteins also shows compelling similarity (Figure 4d), with the notable exception that the tyrosine in both the bacterial and chloroplast proteins is replaced by an asparagine in the phage PDF.

The catalytic zinc has been shown to coordinate the PDF inhibitor actinonin (Guilloteau *et al.*, 2002) and is responsible for the high affinity binding of this universal PDF inhibitor. The structure of the phage PDF bound to actinonin was determined (Figures 5a and b) shows the interactions between bound actinonin and active site residues of the phage PDF.

Discussion

The growing list of AMGs identified in phage genomes includes PDFs. The deformylation of the N-terminal formylmethionine catalyzed by PDFs is an essential step in the co-translational processing of nascent polypeptides in all bacteria and bacterial-derived organelles (Giglione *et al.*, 2004). Deformylation, and often excision of the N-terminal methionine, is necessary for further post-translational processing and proper folding. Only two representatives of this enzyme family had been previously recognized in sequenced phage genomes, those being in two phages of *Vibrio parahaemolyticus* (Seguritan *et al.*, 2003). However, virus-affiliated PDF genes were recently found to be abundant in marine metagenomes (Sharon *et al.*, 2011). All of these sightings relied solely on homology; enzymatic

activity had not been demonstrated for any phage-encoded PDF.

We report here the identification of a gene encoding a predicted PDF in the sequenced genome of a cyanophage (S-SSM7) isolated from a marine cyanobacterium (*Synechococcus* WH8109). For comparison, the predicted bacterial PDF from the genome of a similar host, the freshwater cyanobacterium *S. elongatus* PCC 6301, was also characterized. Both protein sequences display the three absolutely conserved motifs (G, H and C) known to form the PDF active site (Giglione *et al.*, 2004), thus suggesting that both might be catalytically active (Figure 3a).

The argument can be made that having a PDF gene on board may be more necessary for a cyanophage than for phages infecting heterotrophs. A common theme of lytic infection by any phage is the shutting down of transcription and translation of host proteins, with the concomitant redirection of the host machinery to phage replication. In the case of cyanophages, this is complicated by the need to maintain photosynthesis to provide the energy (ATP) and reducing power (NADPH) required for efficient phage replication (Lindell *et al.*, 2005). Maintenance of photosynthesis, in turn, requires ongoing synthesis of functional proteins, the synthesis of D1 being particularly critical because its high rate of photo damage requires continual replacement. Numerous cyanophages encode PSII proteins (D1 and at least one other) (Lindell *et al.*, 2005). In *Prochlorococcus* and *Synechococcus*, synthesis of phage-encoded D1 supplements production of the host protein (Lindell *et al.*, 2005; Clokie *et al.*, 2006). Although it is likely that cyanophages use additional tactics (for example, high-light-inducible proteins) to maintain a functional PSII, synthesis and co-translational processing of D1 is a priority.

PDFs remove the N-terminal formyl group from at least 98% of all bacterial proteins (reviewed in Meinnel and Giglione, 2008). Actinonin, an antimicrobial produced by *Streptomyces roseopallidus* (Giglione *et al.*, 2004), is a universal PDF inhibitor. When PDF activity in plant chloroplasts is inhibited by actinonin *in vivo*, photosynthetic function declines and an albino phenotype is typically evident (Giglione *et al.*, 2003; Hou *et al.*, 2004), demonstrating an essential role for PDFs in maintaining photosynthesis. In vascular plants, actinonin reduces D1 synthesis and PSII assembly, leading to reduced PSII function (Hou *et al.*, 2004). Notably, the PDF1B in *Arabidopsis* chloroplasts has a higher catalytic efficiency when deformylating D1 compared with other proteins (Dirk *et al.*, 2002).

The predicted PDF encoded by phage S-SSM7 was expressed and was found to have a high level of deformylase activity and sensitivity to actinonin, a universal PDF inhibitor (Figure 1a). Its closest homolog, the type IB PDF from *A. thaliana* chloroplasts (Figure 2b), is more efficient at deformylating D1 than other proteins (Dirk *et al.*, 2002).

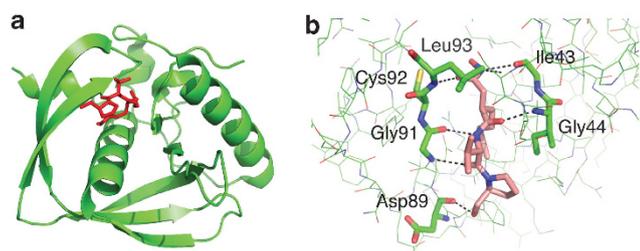


Figure 5 (a) Crystal structure of *Synechococcus* phage S-SSM7 PDF binding the inhibitor actinonin. (b) Interaction of actinonin with the active site residues of the phage PDF showing polar interactions. Residues from the C- and G-motifs are shown as green sticks, actinonin as orange sticks.

Likewise, catalytic efficiency (k_{cat}/K_m) of the phage PDF is higher on the D1-derived tetrapeptide substrates (Figure 2b). In contrast, the bacterial PDF from *S. elongatus* PCC 6301 does not show this preference (Table 2). That the differences in efficiency were less when tetrapeptides from ribosomal proteins served as the substrate suggests that the greater efficiency of the phage PDF on the D1-derived tetrapeptides cannot be attributed solely to the higher activity (larger k_{cat}) of the phage enzyme. The factors responsible for this difference were not apparent in the actinonin-bound enzyme structures and their elucidation would presumably require obtaining structures of the PDF enzymes bound to substrate molecules. Attempts were made to co-crystallize the enzymes with the substrates, and to soak existing crystals with peptide substrates, but substrate bound structures could not be obtained (data not shown).

These observations suggest that this cyanophage-encoded PDF assists in the maintenance of an active PSII during infection. Moreover, all 70 of the PDFs identified in marine metagenomes are from cyanophage genomes, thus indicating that carrying a PDF gene is a tactic employed by many marine cyanophage (personal communication; Sharon *et al.*, 2011). These phage-encoded PDF genes are also widely distributed in the world's oceans (Figure 1). Combined, these factors indicate that cyanophage PDFs have a significant role in the global interplay between cyanophage and their hosts. Comparison of the solved structures of both the phage and bacterial PDFs to the Type 1B PDF from *A. thaliana* chloroplasts demonstrated striking similarity of the protein folds, position of the catalytic zinc, and the amino-acid residues forming the cavity of the active site (Figure 4). The phage and chloroplast enzymes also show identical conformation while binding the inhibitor actinonin (data not shown). Phylogenetic analysis locates both the phage and the bacterial PDFs within the Type 1B group (Figure 3). As noted above, the phage PDF and its closest homolog, the *A. thaliana* chloroplast PDF—but not the bacterial enzyme from *S. elongatus*—demonstrate preferential specificity for D1-derived tetrapeptide substrates, reflecting the importance of efficient deformylation of this protein. The source of this substrate preference, despite the close similarity of their active sites, has been postulated to be due to the tyrosine moiety at position 178 located at the entry to the active site where it might sterically hinder the entrance of non-D1-like N-terminal polypeptides (Dirk *et al.*, 2008). A tyrosine occupies the same location in the bacterial PDF, but in the phage protein this tyrosine is replaced by an asparagine (N99) pointing in the opposite direction (Figure 4d).

The *Synechococcus* phage S-SSM7 lacks the C-terminal α -helical domain that is a defining character of Type 1B PDFs. There is considerable variability in the length and secondary structure of

this domain in PDF1 (Giglione *et al.*, 2009), and the domain is not required for catalytic activity (Meinzel *et al.*, 1996). Nevertheless, it is striking that the domain is lacking in all three PDFs identified in sequenced phage genomes to date (that is, *Synechococcus* phage S-SSM7, and the vibriophages VP16C and VP16T). It is also lacking from at least 52 of the 70 marine viral PDF sequences identified in the marine metagenomes (Sharon *et al.*, 2011; Béjà, personal communication). It was recently proposed, based on studies of the Type 1 PDF of *E. coli*, that Type 1 PDF proteins interact with the ribosome via their helical C-terminal domain (Bingel-Erlenmeyer *et al.*, 2008; Giglione *et al.*, 2009; Kramer *et al.*, 2009). According to this model, the C-terminal domain assists in positioning the enzyme in close proximity to the translation ribosome exit tunnel, thereby providing efficient co-translational processing. In addition to supporting biochemical and structural evidence, experiments *in vivo* under PDF-limiting conditions found that truncation of the C-terminal helix reduced the bacterial growth rate (Bingel-Erlenmeyer *et al.*, 2008).

However, the PDF of *Synechococcus* phage S-SSM7, without a C-terminal domain, has higher catalytic activity *in vitro* than the bacterial PDF from *S. elongatus* that possesses the domain (Table 2, Figure 2). It is not known whether the truncation might have contributed to the higher activity of the phage enzyme. Microbially-derived genes within phage genomes tend to be shorter than their microbial counterparts (Daubin and Ochman, 2004), suggesting that perhaps these phages, for genomic economy, have eliminated a domain that is not essential during infection. Alternatively, it is possible that the truncation enables the phage PDF to compete more effectively with the host enzyme for access to the ribosome.

Encoding AMGs gives phage the potential to modulate host metabolism and energy flow to favor their own replication. Countering environmental stressors and maintaining essential host systems appear to be the priorities. Increasingly, we are aware that the well-studied example of the photosystem genes (for example, *psbA* that encodes D1) carried by cyanophages is but one of many sophisticated mechanisms used to ensure their efficient replication. Another example: carbon metabolism genes carried by several cyanophages, including the Calvin cycle inhibitor protein CP12, serve to shuttle energy (ATP) and reducing power (NADPH) away from carbon fixation and into anabolic pathways (for example, dNTP synthesis), thereby facilitating phage replication (Lindell *et al.*, 2005; Sharon *et al.*, 2009; Thompson *et al.*, 2011). The cyanophage-encoded PDF characterized here adds a novel strategy contributing to the maintenance of photosynthetic processes during infection and redirection of their products toward phage replication. The enzymatic and structural studies reported here also demonstrate that the structure, efficiency and

specificity of acquired AMGs can be subsequently fine-tuned to better serve the interests of the phage. Further work is needed to monitor expression of both phage and host PDF during cyanophage infection and to measure the contribution of the phage PDF to co-translational protein processing. Also remaining to be elucidated is how the function and efficiency of phage PDFs are influenced by the absence of a C-terminal alpha helix.

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References

- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. (1990). Basic local alignment search tool. *J Mol Biol* **215**: 403–410.
- Bingel-Erlenmeyer R, Kohler R, Kramer G, Arzu Sandikci S, Maier T, Schaffitzel C *et al.* (2008). A peptide deformylase–ribosome complex reveals mechanism of nascent chain processing. *Nature* **452**: 108–111.
- Bouzaidi-Tiali N, Giglione C, Bulliard Y, Pusnik M, Meinnel T, Schneider A. (2007). Type 3 peptide deformylases are required for oxidative phosphorylation in *Trypanosoma brucei*. *Mol Microbiol* **65**: 1218–1228.
- Breitbart M, Thompson LR, Suttle CA, Sullivan MB. (2007). Exploring the vast diversity of marine viruses. *Oceanography* **20**: 135–139.
- Chen DZ, Patel DV, Hackbarth CJ, Wang W, Dreyer G, Young DC *et al.* (2000). Actinonin, a naturally occurring antibacterial agent, is a potent deformylase inhibitor. *Biochemistry* **39**: 1256–1262.
- Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, Higgins DG *et al.* (2003). Multiple sequence alignment with the clustal series of programs. *Nucleic Acids Res* **31**: 3497–3500.
- Clokier MRJ, Shan J, Bailey S, Jia Y, Krisch HM, West S *et al.* (2006). Transcription of a ‘photosynthetic’ T4-type phage during infection of a marine cyanobacterium. *Environ Microbiol* **8**: 827–835.
- Daubin V, Ochman H. (2004). Bacterial genomes as new gene homes: the genealogy of ORFans in *E. coli*. *Genome Res* **14**: 1036–1042.
- Dinsdale EA, Edwards RA, Hall D, Angly F, Breitbart M, Brulc JM *et al.* (2008). Functional metagenomic profiling of nine biomes. *Nature* **452**: 629–632.
- Dirk L, Williams MA, Houtz RL. (2002). Specificity of chloroplast-localized peptide deformylases as determined with peptide analogs of chloroplast-translated proteins. *Arch Biochem Biophys* **406**: 135–141.
- Dirk LM, Schmidt JJ, Cai Y, Barnes JC, Hanger KM, Nayak NR *et al.* (2008). Insights into the substrate specificity of plant peptide deformylase, an essential enzyme with potential for the development of novel biotechnology applications in agriculture. *Biochem J* **413**: 417–427.
- Doublé S. (1997). [29] Preparation of selenomethionyl proteins for phase determination. *Methods Enzymol* **276**: 523–530.
- Eisenthal R, Danson MJ, Hough DW. (2007). Catalytic efficiency and k_{cat}/K_M : a useful comparator? *Trends Biotechnol* **25**: 247–249.
- Giglione C, Boularot A, Meinnel T. (2004). Protein N-terminal methionine excision. *Cell Mol Life Sci* **61**: 1455–1474.
- Giglione C, Fieulaine S, Meinnel T. (2009). Cotranslational processing mechanisms: towards a dynamic 3D model. *Trends Biochem Sci* **34**: 417–426.
- Giglione C, Vallon O, Meinnel T. (2003). Control of protein life-span by N-terminal methionine excision. *EMBO J* **22**: 13–23.
- Giovannoni SJ, Turner S, Olsen GJ, Barns S, Lane DJ, Pace NR. (1988). Evolutionary relationships among cyanobacteria and green chloroplasts. *J Bacteriol* **170**: 3584–3592.
- Goldsmith DB, Crosti G, Dwivedi B, McDaniel LD, Varsani A, Suttle CA *et al.* (2011). Development of *phoH* as a novel signature gene for assessing marine phage diversity. *Appl Environ Microbiol* **77**: 7730–7739.
- Guilloteau JP, Mathieu M, Giglione C, Blanc V, Dupuy A, Chevrier M *et al.* (2002). The crystal structures of four peptide deformylases bound to the antibiotic actinonin reveal two distinct types: a platform for the structure-based design of antibacterial agents. *J Mol Biol* **320**: 951–962.
- Hou CX, Dirk LMA, Williams MA. (2004). Inhibition of peptide deformylase in *Nicotiana tabacum* leads to decreased D1 protein accumulation, ultimately resulting in a reduction of photosystem II complexes. *Am J Bot* **91**: 1304–1311.
- Klock HE, Koesema EJ, Knuth MW, Lesley SA. (2008). Combining the polymerase incomplete primer extension method for cloning and mutagenesis with micro-screening to accelerate structural genomics efforts. *Proteins* **71**: 982–994.
- Kraemer JA, Erb ML, Waddling CA, Montabana EA, Zehr EA, Wang H *et al.* (2012). A Phage tubulin assembles dynamic filaments by an atypical mechanism to center viral DNA within the host cell. *Cell* **149**: 1488–1499.
- Kramer G, Boehringer D, Ban N, Bukau B. (2009). The ribosome as a platform for co-translational processing, folding and targeting of newly synthesized proteins. *Nat Struct Mol Biol* **16**: 589–597.
- Kyle D, Ohad I, Arntzen C. (1984). Membrane protein damage and repair: selective loss of a quinone-protein function in chloroplast membranes. *Proc Natl Acad Sci USA* **81**: 4070.
- Lazennec C, Meinnel T. (1997). Formate dehydrogenase-coupled spectrophotometric assay of peptide deformylase. *Anal Biochem* **244**: 180–182.
- Lindell D, Jaffe JD, Johnson ZI, Church GM, Chisholm SW. (2005). Photosynthesis genes in marine viruses yield proteins during host infection. *Nature* **438**: 86–89.
- Lorimer D, Raymond A, Mixon M, Burgin A, Staker B, Stewart L. (2011). Gene composer in a structural genomics environment. *Acta Crystallogr Sect F Struct Biol Cryst Commun* **67**: 985–991.

- Lorimer D, Raymond A, Walchli J, Mixon M, Barrow A, Wallace E *et al.* (2009). Gene composer: database software for protein construct design, codon engineering, and gene synthesis. *BMC Biotechnol* **9**: 36–58.
- Mann NH, Clokie MRJ, Millard A, Cook A, Wilson WH, Wheatley PJ *et al.* (2005). The genome of S-PM2, a ‘photosynthetic’ T4-type bacteriophage that infects marine *Synechococcus* strains. *J Bacteriol* **187**: 3188–3200.
- Mann NH, Cook A, Millard A, Bailey S, Clokie M. (2003). Marine ecosystems: bacterial photosynthesis genes in a virus. *Nature* **424**: 741–741.
- Mazel D, Pochet S, Marliere P. (1994). Genetic characterization of polypeptide deformylase, a distinctive enzyme of eubacterial translation. *EMBO J* **13**: 914.
- Meinzel T, Giglione C. (2008). Tools for analyzing and predicting N-terminal protein modifications. *Proteomics* **8**: 626–649.
- Meinzel T, Lazennec C, Dardel F, Schmitter JM, Blanquet S. (1996). The C-terminal domain of peptide deformylase is disordered and dispensable for activity. *FEBS Lett* **385**: 91–95.
- Millard A, Clokie MR, Shub DA, Mann NH. (2004). Genetic organization of the psbAD region in phages infecting marine *Synechococcus* strains. *Proc Natl Acad Sci USA* **101**: 11007–11012.
- Mossessova E, Lima CD. (2000). Ulp1-SUMO crystal structure and genetic analysis reveal conserved interactions and a regulatory element essential for cell growth in yeast. *Mol Cell* **5**: 865–876.
- Overbeek R, Begley T, Butler RM, Choudhuri JV, Chuang HY, Cohoon M *et al.* (2005). The subsystems approach to genome annotation and its use in the project to annotate 1000 genomes. *Nucleic Acids Res* **33**: 5691–5702.
- Raymond A, Haffner T, Ng N, Lorimer D, Staker B, Stewart L. (2011). Gene design, cloning and protein-expression methods for high-value targets at the Seattle structural genomics center for infectious disease. *Acta Crystallogr Sect F Struct Biol Cryst Commun* **67**: 992–997.
- Raymond A, Lovell S, Lorimer D, Walchli J, Mixon M, Wallace E *et al.* (2009). Combined protein construct and synthetic gene engineering for heterologous protein expression and crystallization using gene composer. *BMC Biotechnol* **9**: 37–52.
- Rohwer F, Segall A, Steward G, Seguritan V, Breitbart M, Wolven F *et al.* (2000). The complete genomic sequence of the marine phage roseophage SIO1 shares homology with nonmarine phages. *Limnol Oceanogr* **45**: 408–418.
- Seguritan V, Feng IW, Rohwer F, Swift M, Segall AM. (2003). Genome sequences of two closely related vibrio parahaemolyticus phages, VP16T and VP16C. *J Bacteriol* **185**: 6434–6447.
- Sharon I, Alperovitch A, Rohwer F, Haynes M, Glaser F, Atamna-Ismael N *et al.* (2009). Photosystem I gene cassettes are present in marine virus genomes. *Nature* **461**: 258–262.
- Sharon I, Battchikova N, Aro EM, Giglione C, Meinzel T, Glaser F *et al.* (2011). Comparative metagenomics of microbial traits within oceanic viral communities. *ISME J* **5**: 1178–1190.
- Smith ER, Begley DW, Anderson V, Raymond AC, Haffner TE, Robinson JI *et al.* (2011). The protein maker: an automated system for high-throughput parallel purification. *Acta Crystallogr Sect F Struct Biol Cryst Commun* **67**: 1015–1021.
- Sullivan MB, Coleman ML, Weigele P, Rohwer F, Chisholm SW. (2005). Three *Prochlorococcus* cyanophage genomes: signature features and ecological interpretations. *PLoS Biol* **3**: e144.
- Thompson LR, Zeng Q, Kelly L, Huang KH, Singer AU, Stubbe J *et al.* (2011). Phage auxiliary metabolic genes and the redirection of cyanobacterial host carbon metabolism. *Proc Natl Acad Sci USA* **108**: E757–E764.
- Yooseph S, Sutton G, Rusch DB, Halpern AL, Williamson SJ, Remington K *et al.* (2007). The sorcerer II global ocean sampling expedition: expanding the universe of protein families. *PLoS Biol* **5**: e16.
- Zeng Q, Chisholm SW. (2012). Marine viruses exploit their host’s two-component regulatory system in response to resource limitation. *Curr Biol* **22**: 124–128.



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