Cyanobacteria are photosynthetic organisms responsible for ~25% of organic carbon fixation on the Earth. These bacteria began to convert solar energy and carbon dioxide into bioenergy and oxygen more than two billion years ago. Cyanobacteria, which infect these bacteria, have an important role in regulating the marine ecosystem by controlling cyanobacteria community organization and mediating lateral gene transfer. Here we visualize the maturation process of subcellular components, including thylakoid membranes, carboxysomes and polyribosomes, as well as phages, inside the congested cytosol of the infected cell. By correlating the structural features and relative abundance of viral progeny within cells at different stages of infection, we identify distinct Syn5 assembly intermediates. Our results indicate that the procapsid releases scaffolding proteins and expands its volume at an early stage of genome packaging. Later in the assembly process, we detected full particles with a tail either with or without an additional horn. The morphogenetic pathway we describe here is highly conserved and was probably established long before that of double-stranded DNA viruses infecting more complex organisms.

Cyanobacteria, and presumably their cyanophages, predate the emergence of enteric bacteria and mammalian viruses by ~2.7 billion years. Cyanophages containing double-stranded (ds)DNA infect a wide range of photosynthetic cyanobacteria. A key question in the assembly of dsDNA viruses is the coordination between protein shell assembly and genome packaging. Here we use a relatively new electron cryo-microscopy approach to follow the maturation process of wild-type cyanophage Syn5 as it occurs inside its host, *Synechococcus* sp. WH8109.

We preserved the native structure of the phage-infected cells by plunge-freezing and maintaining them at liquid nitrogen temperature during imaging. The frozen, hydrated cells were imaged in an electron microscope equipped with a Zernike phase plate, a thin carbon film with a central hole, placed in the back focal plane of the objective lens. This imaging modality yields dramatic enhancement of image contrast over conventional cryoET and thus facilitates the direct identification of subcellular components, including thylakoid membranes, carboxysomes and polyribosomes, as well as phages, inside the congested cytosol of the infected cell. By correlating the structural features and relative abundance of viral progeny within cells at different stages of infection, we identify distinct Syn5 assembly intermediates. Our results indicate that the procapsid releases scaffolding proteins and expands its volume at an early stage of genome packaging. Later in the assembly process, we detected full particles with a tail either with or without an additional horn. The morphogenetic pathway we describe here is highly conserved and was probably established long before that of double-stranded DNA viruses infecting more complex organisms.

W18109 cells were imaged before infection and 65–70 min after infection. Even at this late infection time, some cells seemed to be newly infected. We reconstructed 58 Zernike phase contrast (ZPC) tomograms of WH8109 cells (Figs 1a and 2, Supplementary Videos 1–4 and Methods). The cells range from 0.7 to 1.0 μm in diameter. Although the cell envelope and thylakoid membrane (Fig. 1a, b) are roughly concentric, the thylakoid membrane does not fully enclose the inner compartment of the cell, nor does it seem to directly interact with the cell membrane. This differs from the organization seen in other cyanobacteria. Cyanobacteria also contain carboxysomes, polyhedral compartments encapsulating enzymes for carbon fixation. Each WH8109 cell has, on average, four or five carboxysomes, with diameters ranging from 920 to 1,160 Å (Fig. 1c). Ribosomes are abundant and widespread, forming numerous intracellular patches that contain polyribosomes (Fig. 1d).

Cyanophage Syn5 that infects WH8109 cells is a short-tailed icosahedral phage with a unique horn appendage at the vertex opposite to the tail. Initial segmentation of our tomograms...
of infected cells identified Syn5 particles on the cell surface, floating in the extracellular medium, and Syn5 progeny inside the cell. Multiple full and empty phage particles are seen attached to the cell surface. Injection of viral DNA occurs at multiple sites on the bacterial envelope and does not seem to be a coordinated process. Figure 1e shows a tubular density extending from the phage tail through the periplasm to the cytoplasm (Supplementary Video 4), similar to observations in other phage-infected bacteria\textsuperscript{14,15}. As infection progresses, increasing numbers of Syn5 phage progeny are observed inside the cells. Late in infection, the cell membrane deforms and ruptures, releasing the phage progeny (Fig. 2).

We extracted 470 subvolumes of intracellular Syn5-like particles and classified them into three morphological types on the basis of their size, shape and internal density. The particles were then subjected to volume average, there is a density extending inward at one location of the shell, which we interpret to be the Syn5 portal. However, we could not locate theicosahedral symmetry axes in these particles because of their spherical shape, and thus cannot definitively assign the inward pointing density to an icosahedral vertex. This putative portal is connected to an inner spherical density (∼220 Å in diameter) that resembles the scaffolding proteins seen in procapsids of other dsDNA phages\textsuperscript{18,20}. Given these characteristics, we conclude that this particle type represents Syn5 procapsids\textsuperscript{18}.

The third particle type is characterized by an angular shape (Fig. 3d) and has not previously been reported in \textit{in vitro} structural studies. Fifty-three subvolumes comprise this group. Thirty-seven are quantitatively confirmed to have icosahedral symmetry (Methods). Model-free alignment of the 37 particles produced an average with an icosahedral shell and a ∼200 Å internally protruding density at one of its 12 vertices. The size of the average (∼660 Å diameter, 40 Å shell thickness) matches that of mature Syn5 (refs 13, 18). The internally protruding density could correspond to the full-length portal protein complex, possibly with additional proteins and/or DNA. Although the raw individual particles display internal density of variable contrast, shape, size and distribution, their average appears empty (except for the portal) because the locations of these densities with respect to the capsid and the portal are not uniform and are thus averaged out. In closely examining this subvolume average, we noticed that a flat protruding density (∼40 Å in length) outside the capsid shell is present at the portal vertex. This density cannot correspond to the tail hub, which is assembled after the DNA is fully encapsulated. Although our resolution is insufficient to positively identify the density as being the terminase, this density occurs at the position and stage of assembly expected for the terminase\textsuperscript{21}.

This third particle type may correspond to either an abortive particle or a functional intermediate between the procapsid and DNA-containing capsid types. We carried out the following analysis to resolve this ambiguity. Phage attachment to the outer cell membrane and DNA injection into the host cells are not synchronized under our experimental conditions. Therefore, multiple stages of infection and phage assembly are present in different cells at 65 min after phage infection. To investigate the order in which the above phage types occur before maturation, we used the number of DNA-containing particles (type 1) per cell as a proxy for the progression of productive infection (Fig. 3f)—the greater the number of DNA-containing capsids inside a cell, the farther the infection has proceeded. As infection progresses, the total number of phage progeny observed increases from 2 to 84 per cell. In cells at early infection stages, procapsids and expanded capsids are seen before DNA-containing capsids appear (Fig. 3f, inset). This indicates that procapsids and expanded progeny are assembled before the DNA-containing capsids. With progression of infection, the numbers of procapsid and
expansion of the first ten ranks. The numbers of the phage progeny in each cell were plotted against the cell tomogram ranking. The numbers of DNA-containing capsids. The numbers of the phage progeny with progression of infection. Forty-seven tomograms of intact cells were ranked by the number of DNA-containing capsids.

Figure 3 | Phage progeny average maps reveal diverse assembly intermediates during phage assembly. a–e. Phage progeny classified as DNA-containing capsid (a–c), expanded capsid (d) and procapsid (e). The left three panels show 54 Å slabs containing representative particles. Yellow arrows, tails; red arrows, horns. Right panels show the averaged maps, with the number of subvolumes (n) and resolutions (d). f. Number of the three types of phage progeny with progression of infection. Forty-seven tomograms of intact cells were ranked by the number of DNA-containing capsids. The numbers of the phage progeny in each cell were plotted against the cell tomogram ranking. The inset shows an expansion of the first ten ranks.

expanded capsids increase only slightly. This slow rate of increase suggests that these species are short-lived, and progeny exit these states at almost the same rate as they enter them throughout the infection process. The lack of accumulation of procapsid and expanded capsids is consistent with previous biochemical experiments, and supports the notion that the expanded particles are assembly intermediates after the capsid shell has expanded and acquired icosahedral angularity.

It was not previously known whether shell expansion and DNA encapsulation occur sequentially or simultaneously. Our identification of the expanded intermediates (Fig. 3d) reveals that, in Syn5 and probably some other phages, the conformational changes of the capsid, the expansion of the shell, and acquisition of angularity, are completed before the full length of viral DNA is packaged (Fig. 4). Those expanded capsids without icosahedral symmetry (16 out of 53, and not included in the average shown in Fig. 3d) may be in the midst of transformation from procapsid to the expanded capsid upon DNA entry.

The intracellular assembly of many dsDNA phages and viruses—including adenoviruses and herpesvirus—proceeds through assembly of a precursor procapsid shell, containing scaffolding proteins and a cyclic portal complex defining a unique vertex. Our results show that Syn5 shares the same procapsid-forming pathway as enteric bacteriophages and eukaryotic viruses (Fig. 4). Because cyanobacteria precede enteric bacteria in evolution, it is reasonable to propose that enteric bacteriophages might have inherited this assembly pathway from cyanophages.

We examined the impact of phage infection on cell physiology by evaluating subcellular components throughout infection. The number of carboxysomes, which often reflects the cellular metabolism level, remained invariant (Extended Data Fig. 3). This observation suggests that phage production does not profoundly perturb the host cell’s metabolism until lysis.

Our study demonstrates the first application of ZPC cryoET to examine cellular processes without labelling or sectioning. Post-tomographic analyses allowed us to mine the rich trove of spatial and temporal information conveyed by the complex biological process of phage infection and maturation in situ. The value of our imaging approach lies in its ability to study the ancient process of phage assembly in its natural intracellular environment at nanometre resolution, offering the potential to characterize cyanobacterial strains modified for a wide range of applications, including bioenergy development.

METHODS SUMMARY

Synechococcus sp. strain WH8109 cells were grown in artificial sea water. Cells at exponential phase were infected with Syn5 phage at a multiplicity of infection (m.o.i.) of 5. At 65–75 min after infection, the cells were collected for cryo-specimen preparation. Tilt series of frozen, hydrated cells were collected in a JEM2200FS electron microscope (JEOL) operated at 200 kV and specially equipped with a π/2 thin carbon film Zernike phase plate. Low-dose tilt series were manually collected. IMOD was used to align tilt series and reconstruct tomograms.

Subvolumes of Syn5 progeny phages were extracted from tomograms of infected cells and visually classified into three types based on size, shape and intra-capsid density. For each type, a symmetry-searching algorithm was used to determine whether the particles possessed icosahedral symmetry. If symmetry was confirmed, one of the 12 vertices and a twofold axis of each particle were aligned along the z and y axes, respectively. Expanded and DNA-containing capsids that had detectable icosahedral symmetry were then subjected to further template-free classification and alignment to bring the portal vertex into proper register across particles. The contrast of the subvolumes extracted from our ZPC tomograms was high enough to classify them both visually and quantitatively. For procapsids, the symmetry-searching program failed to identify icosahedral symmetry, so they were aligned using hierarchical ascendant classification to obtain their final average resolution. The resolution of each of the particle averages was estimated by computing the 0.143 Fourier shell correlation criterion from two independent subsets of averages of each type and subtype particle subvolumes.
Segmentation and annotation of the cell tomograms were done in Avizo (Visualization Sciences Group, FEI).

General linear modelling was performed to correlate carboxysome number with progression of infection using PROC GLM.

Online Content Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Author Contributions W.D., D.R. and C.F. prepared the samples and conducted the infection experiments under the advice of C.H.-P. and J.P. W.D. collected the image data and reconstructed the tomograms; C.F. and H.A.K. established the Zernike phase plate imaging conditions in the microscope; K.N. provided the phase plates for imaging and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to W.D. (wch@bcm.edu).
METHODS

Host growth and infection. The Syn5 host Synchococcus sp. strain WH8109 cells were grown in artificial sea water (ASW) with continuous aeration in gas dispersion bottles. To make the ASW medium, nine salts (428 mM NaCl, 9.8 mM MgCl₂·6H₂O, 6.7 mM KCl, 17.8 mM NaNO₃, 14.2 mM MgSO₄, 3.4 mM CaCl₂·2H₂O, 0.22 mM K₂HPO₄·3H₂O, 5.9 mM NaHCO₃, 9.1 mM Tris) were dissolved in MilliQ water, then pH was adjusted to 8.0 with HCl. After autoclaving and cooling down to room temperature, a trace solution was added to the following final concentrations: 0.77 mM ZnSO₄·7H₂O, 7.0 µM MnCl₂·4H₂O, 0.14 µM CoCl₂·6H₂O, 30 µM Na₂MoO₄·2H₂O, 30 µM ferric citrate. Also, a supplementary salt solution was added to a final concentration of 100 µM Na₂CO₃ and 15 µM Na₂EDTA·2H₂O.

To carry out the infection experiments, cells at exponential phase were infected with Syn5 phage at a multiplicity of infection (m.o.i.) of 5. The cells then were kept in a controlled water bath at 28 °C under continuous cool white fluorescent light at an intensity of 1,200 lx, and a shaking speed of 110 r.p.m. At 65–75 min after infection, cells were centrifuged at 8,500 x g for 5 min. The cell pellet was gently re-suspended in fresh ASW medium and concentrated 100-fold. Tomographic tilt series acquisition and reconstruction. For cryoET, Syn5-infected WH8109 cell sample was first mixed with 100 Å gold fiducial markers to facilitate alignment in data processing. An aliquot of 3.5 µl sample was applied to 2.0/1.0 µm Quantifoil holey grids (Quantifoil) and plunge frozen using a Vitrobot (FEI). The frozen, hydrated samples were transferred to a Gatan cryo-holder (Gatan, Inc.) and kept at −170 °C throughout the imaging session in a JEOL2200FS electron microscope (JEOL). This electron microscope has an in-column energy filter and the slit was set to 15 eV. In addition, the microscope was equipped with an airlock system to allow insertion of ∼2/3 Zernike phase plate made of a thin carbon film with a 0.7 µm central hole2,6,8. The illumination setting used was: spot size 1; condenser aperture = 70 µm; objective aperture = 60 µm.

Tilt series of frozen, hydrated samples were collected manually with an electron energy of 200 kV under low-dose conditions on a Gatan 4k × 4k CCD camera (Gatan, Inc.) at ×25,000 microscope magnification. The sampling of the data was calibrated to be 4.52 Å per pixel. Typically, a tilt series ranged from −60° to 60° in 3° step increment. The accumulated exposure for each tilt series was 40–50 electrons Å⁻². We set the defocus to near zero for imaging a 0° tilt specimen as judged by the computed fast Fourier transform (FFT) of a region next to the specimen of interest, and then used pre-calibrated defocus change for subsequent tilts within a tilt series. We could not afford to use computer-aided focusing for every tilt, because we wanted to minimize the damage to the phase plate from electron exposure. However, we recorded a moderate dose image during our tilt series every 5–10 images to monitor the defocus setting, and to determine the charging status of the phase plate. In general, the defocus values were consistent within 1 µm. The manner and extent of change observed in the FFT varies depending on the initial quality of the phase plate and the cumulative electron exposure to the phase plate. In certain cases, a ring similar to a contrast transfer function (CTF) zero appears at low spatial frequency, as previously shown7. In other cases, an FFT of the image can display different patterns indicative of charging. Once a phase plate was found to suffer from charging, we replaced it with a new one. Of note, there were many times that we had to stop image acquisition in the middle of a tilt series because there was no good phase plate replacement available.

IMOD29 was used to align tilt series and reconstruct tomograms. Because the images of tilted specimens were taken with low dose, it was impossible to detect the CTF rings. However, with resolution limited to ~50–70 Å, we can tolerate defocus as high as 5 µm without a need for CTF phase flipping. Our targeted defocus values were substantially less than this, so CTF correction was not necessary.

Subvolume alignment and averaging. Subvolumes of progeny phages, found inside infected WH8109 cells, were extracted from 22 good cell tomograms. Initial classification of the procapsid, the expanded capsid and the DNA-containing capsid was done by visual inspection based on the size and shape of the particles. The classified particles were then subjected to symmetry search27,28 (e2symsearch.py from EMAN2, described below), alignment, classification and averaging.

For DNA-containing capsids (type 1 particles) we used the symmetry-searching algorithm to align particles to the icosahedral symmetry axes. Then the particles were subject to a model-free all-vs-all alignment scheme (incorporating hierarch-}

ical ascendant classification16,17,29), constrained to search only the 12 vertices to obtain an initial model, in which the special vertex in each particle (containing the portal) was registered to the positive z axis. The high contrast of the ZPC cryoET subvolumes allowed us to further partition the particles manually into three subtypes: with neither tail nor horn, with tail only, and with tail and horn on opposite vertices.

The expanded capsid particles (type 3) have an apparent angular shape. We used the symmetry-searching algorithm to determine whether the particles had icosahedral symmetry. Those with identified icosahedral symmetry (37 out of 53) were subjected to the alignment scheme described above to bring the portal vertex in each particle into proper register27. The final average shows few missing wedge artefacts. In addition, the strong icosahedral symmetry of the capsid shell validates our symmetry-free alignment and averaging procedure.

The procapsid particles (type 2 subvolume) are smooth and pseudo-spherical, without apparent icosahedral symmetry as determined by the symmetry search algorithm. We thus used the model-free alignment scheme searching the entire rotational space to obtain an initial model from a subset of particles. We iteratively refined the alignment of the entire data set using this initial model until no further improvement was observed. The final average lacked detectable icosahedral symmetry.

Symmetry searching algorithm. To align the Syn5 particles to their symmetry axis, we developed a new algorithm: first the algorithm takes a particle in its current orientation and applies icosahedral symmetry to it. If the orientation of the raw particle is aligned near the conventional icosahedral symmetry axes as defined in EMAN2, the symmeterized particle’s icosahedral features will be enhanced. If the particle is not so aligned, the symmetrization will smear out the particle into a ball. Next the algorithm computes the normalized cross-correlation between the symmetrized and unsymmetrized particles16,17,29, accounting for the missing wedge. A GSL multidimensional simplex minimizer (http://www.gnu.org/software/gsl/) varies the three Euler angles and three translation coordinates of the raw particle, applies icosahedral symmetry to the particle in this new orientation, and computes a new cross-correlation score. The GSL minimizer continues to generate new particle orientations by going downhill until the (negative of the) cross-correlation decreases by less than 0.01.

The simplex minimizer does not guarantee a global minimum. We thus repeated the above process ‘n’ times, each time applying a random rotation to the same particle as generally used in a Monte Carlo optimization algorithm. In this process, we will find the global minimum of the cross-correlation if ‘n’ is sufficiently big. To speed up the search, we centred the particles before performing the cross-correlation computation. In our experience n = 10 is sufficient to determine if the particle has icosahedral symmetry. The judgment of whether the particle has icosahedral symmetry is based not only on the score but also a visual inspection, that is, the symmetrized particle should have distinct vertices and the correct capsid surface features.

Resolution estimates of the subvolume averages. There is not yet a widely accepted and rigorous standard for resolution estimates of subvolume averages; however, the gold-standard Fourier shell correlation (FSC) methodology30,31 used in single particle analysis can be adapted to produce good estimates. Each of the subvolumes for the five phage assembly intermediates were split into two subsets, to compute two independent averages for each, then the FSC was computed between the two averages. The resolution for the combined average of each phage assembly intermediate is then measured using an FSC threshold of 0.143 (refs 30, 32).

Cut-on frequency correction. The effect on low-resolution contrast is primarily due to the low-pass frequency imposed by the central hole in the Zernike phase plate. The Fourier coefficients of these images below 1/300 Å⁻¹ would be lost and between 1/300–1/20 Å⁻¹ would be highly enhanced. We undertook the following steps to re-scale the Fourier components of the map. We computed a 1D structure factor derived from a published single particle reconstruction of Syn52 from conventional cryo-electron microscopy (cryo-EM). This 1D structure factor is simply the rotationally averaged power spectrum of the particle structure. It is applied to our subvolume averages such that their 1D structure factors match this ‘known’ curve. As a rotationally symmetric linear filter, this does not impose any new features on the structure. We used the EMAN2 procdm command to process the averaged subvolumes with the options of ‘apix=9.04 setd=sym5-structure-factor lp=40’. This corrects the amplitudes, and then applies a 40 Å low-pass filter to suppress high-resolution noise. Note that no correction was attempted for subvolume averages of procapsid and expanded capsid because an appropriate structure factor was not available.

Visualization and segmentation. Visualization of the cell tomograms and averaged maps was done using Chimera32. Segmentation and annotation of the cell tomograms were done using Avizo (Visualization Sciences Group, FEI).

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Extended Data Figure 1 | ZPC improves contrast of cryoET images and reveals detailed structural features of Syn5-infected cells. a, A conventional EM image of a Syn5-infected WH8109 cell. b, A ZPC image of the same cell as shown in a under the same imaging conditions.
Extended Data Figure 2 | ZPC-cryoEM single-particle images of biochemically purified mature Syn5 phage. The particles are shown with the tail pointing down and the wavy horn pointing up. The tail fibres appear to have variable conformations.
Extended Data Figure 3 | General linear modelling of cellular carboxysome number with progression of infection. The number of carboxysomes remains roughly constant as infection progresses, indicating that their variation does not correlate with progression of infection. The solid line indicates linear regression; the dashed lines indicate the 95% confidence limits.