A unicellular algal virus, *Emiliania huxleyi* virus 86, exploits an animal-like infection strategy

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*Emiliania huxleyi* virus 86 (EhV-86) belongs to the family *Phycodnaviridae*, a group of viruses that infect a wide range of freshwater and marine eukaryotic algae. *Phycodnaviridae* is one of the five families that belong to a large and phylogenetically diverse group of viruses known as nucleocytoplasmic large dsDNA viruses (NCLDVs). To date, our understanding of algal NCLDV entry is based on the entry mechanisms of members of the genera *Chlorovirus* and *Phaeovirus*, both of which consist of non-enveloped viruses that ‘inject’ their genome into their host via a viral inner-membrane host plasma membrane fusion mechanism, leaving an extracellular viral capsid. Using a combination of confocal and electron microscopy, this study demonstrated for the first time that EhV-86 differs from its algal virus counterparts in two fundamental areas. Firstly, its capsid is enveloped by a lipid membrane, and secondly, EhV-86 enters its host via either an endocytotic or an envelope fusion mechanism in which an intact nucleoprotein core still encapsulated by its capsid is seen in the host cytoplasm. Real-time fluorescence microscopy showed that viral internalization and virion breakdown took place within the host on a timescale of seconds. At around 4.5 h post-infection, virus progeny were released via a budding mechanism during which EhV-86 virions became enveloped with host plasma membrane. EhV-86 therefore appears to have an infection mechanism different from that employed by other algal NCLDVs, with entry and exit strategies showing a greater analogy to animal-like NCLDVs.

INTRODUCTION

Viruses are the most abundant biological entity in the world’s oceans, constituting the greatest genetic diversity and playing a major role in biogeochemical cycles (Fuhrman, 1999; Suttle, 2005). *Emiliania huxleyi* virus strain 86 (EhV-86), from the genus *Coccolithovirus* within the family *Phycodnaviridae* of algal viruses (Schroeder et al., 2002), plays an important role in phytoplankton bloom control of its host *E. huxleyi* (Bratbak et al., 1996; Jacquet et al., 2002; Wilson et al., 2002). *E. huxleyi* is a marine calcifying unicellular phytoplankton, which can form vast oceanic blooms at temperate latitudes and is a key player in global biogeochemical cycles (Paasche, 2001). Its production of intricately formed calcium carbonate coccoliths and its role in CO2 cycling and dimethyl sulphide production make *E. huxleyi* an important species with respect to marine primary productivity, sediment formation and climate (Charlson et al., 1987; Westbroek et al., 1993).

EhV-86, a lytic virus, is icosahedral in shape and approximately 170–190 nm in diameter (Wilson et al., 2002), and is the largest algal virus fully sequenced to date. EhV-86 has a genome size of 407 339 bp containing 472 coding sequences (GenBank accession no. AJ890364; Wilson et al., 2005). EhV-86 belongs to a group of viruses...
known as nucleocyttoplasmic large dsDNA viruses (NCLDVs) that replicate completely or partly in the cytoplasm of eukaryotic cells (Iyer et al., 2001). NCLDVs are a diverse group of viruses composed of five families, *Phycodnaviridae*, *Poxviridae*, *Asfarviridae*, *Iridoviridae* and *Mimiviridae* (Allen et al., 2006b), that are united by sharing at least five conserved synapomorphic genes (Iyer et al., 2001; Raoult et al., 2004). Diversity is seen at all levels within the NCLDV group and within its families.

NCLDVs have evolved highly diverse and complex infection strategies. Two areas that show high levels of variation are viral entry and exit. Variations are seen not just between the five NCLDV families but also within families and individual viral species. Vaccinia virus (VACV), family *Poxviridae* is a classic example of the complexity seen in NCLDV entry and exit. VACV internal processing and exit from its host are intrinsically linked to its subsequent entry into its sequential host. This is highlighted in the unique non-fusogenic outer-membrane dissolution mechanism of the viral particles containing a double membrane envelope ([Law et al., 2006](#)). This results in loss of the outer envelope, allowing the inner envelope to fuse with the host plasma membrane, releasing an intact virion core into the host cytoplasm ([Armstrong et al., 1973](#); [Brown et al., 2006](#); [Chang & Metz, 1976](#); [Doms et al., 1990](#); [Law et al., 2006](#)).

Entry via host vacuoles is seen in the families *Iridoviridae*, *Asfarviridae* and recently *Mimiviridae*. *Acanthamoeba polyphaga* mimivirus (APMV, family *Mimiviridae*) entry into amoeba is most likely due to a phagocytic process. Upon vacuole internalization, APMV genetic material is released into the host cytoplasm by the opening of its ‘stargate’ portal and fusion of the inner viral and vacuole membranes ([Suzan-Monti et al., 2007](#)). Both enveloped frog virus 3 (FV-3, family *Iridoviridae*) and African swine fever virus (ASFV, family *Asfarviridae*) enter via adsorptive endocytosis and exit by viral budding ([Braunwald et al., 1985](#); [Valdeira & Geraldes, 1985](#)). FV-3 is also found in a non-enveloped form when released from lysed cells that either injects its DNA by a possible fusion of the internal viral lipid membrane (a lipid membrane that underlies the capsid) with the host plasma membrane or enters via endocytosis ([Braunwald et al., 1985](#)). Fusion of an internal membrane with either host plasma membrane or host vacuoles is seen throughout the NCLDVs.

Almost all that is known about algal virus entry is based on chloroviruses and phaeoviruses. Members of these genera are icosahedral, non-enveloped viruses with an internal lipid-containing bilayer membrane underlying an outer capsid shell ([Wolf et al., 1998](#); [Van et al., 2000](#)). Fusion of this inner membrane with the host plasma membrane results in the ‘injection’ of viral DNA and associated proteins into the host cytoplasm ([Van Etten et al., 2002](#)). In the case of chloroviruses, viral DNA injection is preceded by viral attachment to and digestion of the carbohydrate-based host cell wall with the resultant virus capsid left on the host cell surface ([Meints et al., 1984](#)). In contrast, phaeoviruses infect their host’s gametes or spores, which lack a cell wall ([Maier et al., 2002](#)), leaving remnants of their capsid on the cell surface ([Maier & Muller, 1998](#)). *E. huxleyi* lacks a cell wall but its lipid plasma membrane is surrounded by calcium carbonate coccoliths that form the coccosphere ([Paasche, 2001](#)). These differences in host cell-surface environment are likely to play key roles in the entry and exit strategies exploited by the members of the *Phycodnaviridae*. In this study, we describe for the first time the entry and exit stages of a coccolithovirus. Using confocal and electron microscopy, we have shown that EhV-86 is an enveloped virus that, in contrast to other algal viruses, employs a distinctly animal-like infection strategy.

### METHODS

#### Host and virus

All host work was carried out using *E. huxleyi* strain CCMP 1516. A stock culture of calcifying *E. huxleyi* in f/2-Si medium ([Guillard, 1975](#)) and incubated at 15 °C in a constant temperature room with a 16:8 h light–dark illumination cycle. Cell counts were made with an improved Neubauer haemocytometer.

Virus particles were obtained by adding 5 ml EhV-86 stock to 500 ml exponentially growing host *E. huxleyi* in f/2-Si. After clearance of the host culture (6 days), the lysate was filtered through a 0.8 μm nitrocellulose filter (to remove cell debris), followed by a 0.2 μm nitrocellulose filter ([Schroeder et al., 2002](#)). Virus concentration was calculated by analytical flow cytometry using SYBR Green I as described by [Jacquet et al. (2002)](#) and/or epifluorescence microscopy using 4’,6-diamidino-2-phenylindole, dilactate (DAPI dilactate; Sigma) based on a method developed by [Suttle (1993)](#). Virus viability was determined by plaque assay analysis ([Schroeder et al., 2002](#)).

#### Virus staining

Viral DNA and lipid were stained with DAPI and N-(3-triethylammoniumpropyl)-4-[4-(dibutylamino)styryl] pyridinium dibromide (FM 1-43; Invitrogen), respectively. One millilitre of the lysate was filtered viral lysate was incubated with 5 μl DAPI (final concentration 5 μg ml⁻¹) for 30 min at 4 °C in the dark. FM 1-43 was added to the viral particles at a final concentration of 10 μM, mixed briefly and incubated at 4 °C in the dark for 10 min. The stained viral particles were captured onto 0.02 μm Anodisc 25 mm filters ([Suttle, 1993](#)) and mounted in FM 1-43 (10 μM in f/2-Si (0.02 μm filtered) and topped with a coverslip. The samples were viewed with a ×60 oil objective using a Nikon Eclipse E1000 microscope with a Radiance 2100 confocal laser scanning system with excitation wavelengths of 405 nm (DAPI) and 488 nm (FM1-43).

#### Solvent/detergent treatment

Solvent/detergent (SD) treatment was based on methods described by [Roberts (2008)](#). A 1 : 1 (v/v) stock solution of Triton X-100 and tributyl phosphate (TBP) was diluted to one millilitre of Triton X-100 and tributyl phosphate (TBP) was diluted to 1 : 1 for 30 min at 4 °C in the dark. FM 1-43 was added to the viral particles at a final concentration of 10 μM, mixed briefly and incubated at 4 °C in the dark for 10 min. The stained viral particles were captured onto 0.02 μm Anodisc 25 mm filters ([Suttle, 1993](#)) and mounted in FM 1-43 (10 μM in f/2-Si (0.02 μm filtered) and topped with a coverslip. The samples were viewed with a ×60 oil objective using a Nikon Eclipse E1000 microscope with a Radiance 2100 confocal laser scanning system with excitation wavelengths of 405 nm (DAPI) and 488 nm (FM1-43).
E. huxleyi was decalcified by gently spinning down (4000 g, 4 min) 1.5 ml of cells obtained from a settled culture as described above. Gentle centrifugation avoided pelleting the cells but resulted in them settling on the side of the microfuge tube, allowing careful removal of the supernatant. The supernatant was replaced with an equal volume of 25 mM ethylene glycol tetraacetic acid (EGTA) in calcium-free artificial seawater (EGTA-ASW) and the cells were mixed gently with a transfer pipette (Taylor & Brownlee, 2003). After 15 min incubation, the cells were gently spun out, allowing the replacement of EGTA-ASW with seawater (EGTA-ASW) and the cells were mixed gently with a transfer pipette. After 15 min of incubation, the cells were gently spun out, allowing the replacement of EGTA-ASW with ASW. Cells were checked visually for decalcification and the production of new coccoliths (results not shown).

The viability of viruses stained with DAPI and FM-143 was tested by infecting 10 ml E. huxleyi (-1 x 10^6 cells ml^-1) with 100 µl virus (-1 x 10^7 particles ml^-1) stained with the dye concentrations described above. Unstained virus and non-virus (100 µl sterile f/2-Si with the above dye concentrations) controls were set up. All experiments were conducted in triplicate. The cultures were analysed for lysis at 6 days post-infection (p.i.).

Transmission electron microscopy (TEM). TEM was used to view both concentrated virus lysate and virus-infected E. huxleyi cells. Virus concentrates (as described above) were negatively stained with uranyl acetate (Schroeder et al., 2002), whilst virus-infected E. huxleyi cells were prepared for TEM by (a) glutaraldehyde fixation of a plaque assay at 3 and 36 h p.i. or (b) glutaraldehyde fixation of virus-infected host cells at 30 min p.i. followed by centrifugation. For (a), duplicate plaque assays with an m.o.i. of 0.1 and a negative (no virus) control were prepared as described by Schroeder et al. (2002). Briefly, 50 ml exponentially growing E. huxleyi cultures were concentrated by centrifugation at 4330 g for 5 min at 4 °C. The cells were resuspended in 900 µl f/2-Si before the addition of 100 µl diluted viral lysate to give a final m.o.i. of 0.1. The solution was incubated for 2 h at 15 °C with constant illumination, prior to being mixed with 3 ml molten 0.4 % (w/v) electrophoresis-grade agarose in f/2-Si (40 °C). This solution was overlaid onto pre-poured 1.5 % (w/v) agarose bottom layers in 100 mm Petri dishes. Once cooled, the plates were incubated at 4 °C. For (b), a 50 ml culture of E. huxleyi at the end of its exponential growth phase (-2 x 10^6 cells ml^-1) was centrifuged at 4330 g for 5 min at 4 °C. The pellet was resuspended in 900 µl f/2-Si and incubated with 100 µl of a 1:10 dilution of viral lysate for 30 min before fixation with 2.5 % (v/v) glutaraldehyde. This was then pelleted as above and processed for TEM.

The top layer of the virus-infected plaque assay was removed in fragments. Both plaque assay and pelleted virus-infected E. huxleyi were washed in PBS base buffer (PBS + 0.42 M NaCl) prior to staining with 2 % osmium tetroxide for 1 h at room temperature. The staining solution was removed and the sample dehydrated in an increasing ethanol series and then in absolute ethanol overnight. Samples were infiltrated with increasing concentrations of Spurr resin (TAAB Laboratories) in ethanol at 35 °C, followed by overnight embedding in 100 % resin at 70 °C. Ultrathin sections (~70 nm) were cut with a Micro-Star diamond knife and placed onto carbon-stabilized Formvar-coated copper grids. The grids were stained with 2 % uranyl acetate solution, followed by 4 % lead citrate solution. All imaging was conducted on a JEOL 1200 EX II transmission electron microscope at 120 kV at varying magnifications from ×15000 to ×150000.

### RESULTS

#### EhV-86 virion structure

Electron micrographs of freshly collected EhV-86 lysates revealed a distinct ~20 nm coat surrounding a nuclear-dense central core (Fig. 1a). We previously reported that this coat constituted the virus capsid (Schroeder et al., 2002). However, an aged EhV-86 lysate (stored at 4 °C for 90 days) revealed a distinct membrane detaching from the capsid-encapsulated nucleoprotein core (CENPC, Fig. 1b), resulting in an enlarged virion diameter of ~250 nm. Fig. 1(c) gives a diagrammatic view of fresh and aged virus, highlighting the fact that the lipid envelope pulls away from the CENPC in the aged virus. To examine the nature of this membrane further, freshly purified virus was stained with the fluorescent lipophilic styryl dye FM 1-43. FM 1-43 is water soluble and concentrates into the outer layer of surface membranes, where it emits a strong fluorescent signal (Nishikawa & Sasaki, 1996). Fig. 1(d) shows that the strong fluorescence of FM 1-43 (orange) consistently corresponded with the fluorescence from nucleic acid-bound DAPI (green). The co-localization of FM 1-43 and DAPI indicated the presence of a lipid membrane. Some lipid membranes were present in the absence of a DAPI signal, indicating the presence of viral particles without a nucleic acid core.

To investigate virion structure and stability further, EhV-86 particles were exposed to varying levels of the anionic detergent Triton X-100 and the organic solvent TBP (Fig. 2). At a low concentration of Triton X-100 (≤0.01 %, v/v), the virus remained intact and was similar in appearance to the control (Fig. 2a, b). At 0.1 % Triton X-100, fluorescence corresponding to individual viral lipid (orange) was present, but all green fluorescence had disappeared, indicating the loss of nucleic acid material (Fig. 2c). At higher concentrations (≥0.5 %), lipid staining was of a less defined viral size, with lipid aggregations present on the filter membrane (Fig. 2d–f). Aggregations contained spots with greater fluorescence intensity that corresponded to the size range of EhV-86. Taken together, the above observations by TEM and confocal microscopy strongly indicate...
that the virus particles are surrounded by an external lipid membrane.

**EhV-86 infection strategy**

To investigate EhV-86 entry and the early stages of infection (<5 h p.i.), real-time confocal microscopy was used. TEM was also used to view samples at 30 min, ~3 h and 36 h p.i. Following DAPI treatment, *E. huxleyi* showed autofluorescence that corresponded with the chloroplast (data not shown) and that faded rapidly under laser exposure. Chloroplast and genomic DNA DAPI fluorescence was generally low, allowing viral particles to be easily distinguishable (Fig. 3). Inoculation of cultures with

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**Fig. 1.** Electron micrographs of fresh (a) and aged (b) EhV-86. Note the increased size of aged particles. The diameters of the aged particles shown were 265 nm (upper) and 241 nm (middle). (c) Diagrammatic views of fresh and aged virus, highlighting that, in the aged virus, the CENPC remains integral but the surrounding lipid membrane (LM) pulls away from the CENPC. (d) Confocal image of virus particles stained with DAPI (green) and FM 1-43 (orange) filtered onto 0.02 μm Anodisc filters. The zoomed area is four times the original image size. Regions of overlap between orange FM 1-43 and green DAPI fluorescence are shown in yellow. Circles highlight viral particles with no green fluorescence. Bars, 170 nm (a); 200 nm (b); 5 μm (d).

**Fig. 2.** SD treatment of EhV-86 virus with anionic detergent Triton X-100 and organic solvent TBP at varying concentrations: (a) no solvent/detergent; (b) 0.01 % (v/v); (c) 0.1 %; (d) 0.5 %; (e) 1 %; (f) 2 %. All images are a merge of DAPI fluorescence (green) and lipid-bound FM-143 (orange). Bars, 5 μm.
Fig. 3. (a, b) EhV-86 particles bound to *E. huxleyi* plasma membrane of different cells. Arrows indicate bound virus. (c, d) Sequential confocal microscope images of EhV-86 bound to *E. huxleyi* (c) and the same cell imaged 2 min later with no EhV-86 present (d). (e–h) Different *E. huxleyi* cells with internalized EhV-86 viral particles. Images were from initial scans of cells. Arrows indicate internalized virus. The main images are a merge of the insets showing nucleic acid fluorescence (green, DAPI) and lipid membrane fluorescence (orange, FM 1-43). All confocal images are of decalcified *E. huxleyi* mixed with EhV-86. (i, j) TEM analysis of a healthy uninfected *E. huxleyi* cell (i) and *E. huxleyi* with internalized EhV-86 (j) at 30 min p.i. Bars, 5 μm (a–h); 1 μm (i, j); 200 nm (j, inset).
stained virus showed that EhV-86 staining with DAPI and FM 1-43 had no adverse effects on host cell lysis and the dyes had no visible adverse effect on host cell growth (data not shown). FM 1-43 fluorescence of the viral membrane was not visible in these images against the high host-membrane FM 1-43 fluorescence.

Images captured during the early stages of viral infection (<40 min p.i.) of decalcified *E. huxleyi* showed attachment of EhV-86 to the host plasma membrane (Fig. 3a, b). Upon identification of an attached viral particle, images were taken approximately every minute for 10 min to limit laser damage to the host. Attached virus particles were generally inactive, remaining attached to the cell surface for the full 10 min, suggesting that attachment to the host plasma membrane was irreversible and did not sequentially result in viral DNA internalization. Fig. 3(c, d) illustrates the disappearance of a viral particle associated with the plasma membrane of a decalcified cell within 2 min of being identified. Sequential Z-sections confirmed that this disappearance was not due to a shift in focus. Several images were obtained of viral-like DAPI-stained particles, apparent as bright punctate fluorescence within the host cytosol at <40 min p.i. (Fig. 3e–h). The section thickness of these images (1.10–1.21 μm) confirmed that the signal, shown in green, was from an internal source and not from externally bound EhV-86. No viral particles were ever observed attached to the host membrane of calcified cells at <40 min p.i.

TEM images taken at 30 min p.i. also indicated that EhV-86 entered the host cell intact with its nucleoprotein core encapsulated by the major capsid protein (MCP) (Fig. 3j). TEM images at 3 h p.i. showed the start of formation of new progeny viruses (Fig. 4). Fig. 4(a) shows an incomplete viral particle devoid of a dense DNA core, suggesting the early stages of viral DNA packaging. In all images of newly formed viral particles, there was no clear lipid envelope surrounding the nucleoprotein core. By TEM, we observed a viral budding mechanism (Fig. 4c, d) whereby EhV-86 acquired a lipid membrane from its host. Real-time sequential imaging showed viral budding at 4 h 35 min p.i. (Fig. 4e). Individual viral particles appeared on the cell surface of a calcified cell over a period of several minutes (Fig. 4f–j), with some particles observed to detach from the plasma membrane into the surrounding medium. Viral particles only became DAPI stained once exposed on the host surface.

EhV-86 binding to the calcified coccosphere appeared to be a reversible process. Fig. 5 shows sequential images (approx. one image s⁻¹) of a calcified *E. huxleyi* cell and an attached viral particle detaching from the host coccosphere at 1 h p.i. At approximately 2 h p.i., *E. huxleyi* exposed to EhV-86 started exhibiting plasma membrane patchiness (membrane blebbing). The projected Z-series shown in Fig. 6(a, b) highlight the intense lipid spots appearing on the cell surface. Membrane blebbing was also seen in TEM images taken at 3 h p.i. (Fig. 6c, d).

### DISCUSSION

In this study, we have shown for the first time that: (i) EhV-86 is an enveloped virus, unlike previously characterized members of the *Phycodnaviridae*, based on FM 1-43 virion labelling, TEM images of aged and fresh viral particles, and effects of SD treatment and membrane coating of the virus particle during budding; (ii) EhV-86 binds irreversibly to the host plasma membrane as seen using confocal microscopy; (iii) the CENPC enters the host intact, where it rapidly disassembles, based on both the confocal and sectioned electron microscopy images; (iv) *E. huxleyi* exposure to virus leads to membrane blebbing due to increased production of a dense lipid that is excreted on the host cell surface (shown by FM 1-43 confocal microscopy images); and (v) virus particles exit the host via a budding mechanism whereby EhV-86 gains an outer membrane as seen from the thin sections using electron microscopy. A simplified life cycle of EhV-86 based on the data presented in this study and of the literature discussed is shown in Fig. 7.

Previously characterized algal viruses have been shown to be non-enveloped, ‘injecting’ their DNA across their host plasma membrane via the fusion of an internal lipid membrane (Van Etten et al., 2002). The results presented here identify EhV-86 as exhibiting a unique lifestyle among members of the *Phycodnaviridae* that is more analogous to animal-like NCLDV. We hypothesize that the viral envelope plays an intrinsic role in viral entry, as seen with all other enveloped viruses. It is known that virus age and storage conditions (such as temperature and light exposure) affect virus viability and lipid envelope integrity (data not shown). SD treatment disrupted virion stability, resulting in nucleic acid leaking of virus particles leaving intact envelopes at low SD concentrations but membrane disintegration at higher concentrations. SD treatment is a standard technique used to deactivate enveloped viruses in blood plasma by disruption of virion structure (Horowitz et al., 1992; Roberts, 2008). Morphological changes of viruses after treatment with organic molecules known to disrupt membranes have shown nucleoprotein core leaking, leaving ‘empty’ virus structures (Thormar et al., 1987; Vollenbroich et al., 1997). *E. huxleyi* is known to produce large quantities of diverse lipids (Rieley et al., 1998) with increased lipid production during viral exposure, consistent with the demonstration here of increased lipid body extrusion in virus-infected cells.

Currently, all characterized viruses belonging to the family *Phycodnaviridae* have been shown to contain an internal lipid membrane. The internal membrane plays an essential role in the release of the viral nucleoprotein core (Maier et al., 2002; Meints et al., 1984; Wolf et al., 1998). The presence of an internal lipid membrane has yet to be investigated in EhV-86. Further higher resolution TEM and cryoelectron microscopy to provide subnanometer resolution (Huiskonen & Butcher, 2007) are required for undisputable evidence for the presence or absence of an
Fig. 4. (a, b) Internal viral particles at 3 h p.i. (c, d) EhV-86 being released into the extracellular space via a budding mechanism at 36 h p.i. Viral particles gain an outer lipid host-derived membrane. Arrows indicate acquisition of host plasma membrane. (e–j) Real-time sequential images of virus budding from an *E. huxleyi* cell. All images are of the same cell. Arrows indicate individual viral particles. Calcified *E. huxleyi* was mixed with EhV-86 (~100 virus particles per cell) and imaged from 30 min p.i. The images are a merge of green fluorescence (DAPI) and orange lipid fluorescence (FM 1-43). Bars, 1 μm (a, b); 200 nm (a, b, insets); 100 nm (c, d, including insets); 5 μm (e–j).
internal membrane. None the less, EhV-86 encodes an A32-type ATPase that has been shown to be expressed within 33 h of infection (Wilson et al., 2005). A32-type ATPases are putative DNA packaging proteins that, in addition to containing regular Walker A and B motifs, carry a conserved A32-specific motif (Iyer et al., 2006; Strömsten et al., 2005). It has been proposed that these form multiprotein DNA pumps that transport viral DNA

**Fig. 5.** Live sequential images (a–f) of bound virus detaching from the calcite coccosphere (not seen) of *E. huxleyi*. Calcified *E. huxleyi* was mixed with EhV-86 (~100 virus particles per cell) and imaged from 30 min p.i. The images are a merge of green fluorescence (DAPI) and orange lipid fluorescence (FM 1-43). Bars, 5 μm.

**Fig. 6.** (a, b) *E. huxleyi* exhibiting membrane blebbing. Projected Z-series (*n*=67, 0.15 μm section thickness) of decalcified *E. huxleyi* stained with FM 1-43 and exposed to EhV-86. (c, d) TEM images of calcified *E. huxleyi* exposed to EhV-86. Times p.i. are shown. Dense spots are due to host-produced lipids. Bars, 5 μm (a, b); 1 μm (c, d).
into the pro-capsid, with the A32 motif acting as a membrane anchor that is encapsulated into the final virion (Strömsten et al., 2005). This motif has been identified in all sequenced NCLDVs that have an internal membrane (Iyer et al., 2006; Strömsten et al., 2005).

The lack of viral particles attached to the plasma membrane of calcified cells suggests that the coccosphere provides a line of virus defence. Anecdotal microscopy data indicate that, although virus binding to the coccosphere occurs, it is reversible. These observations are supported by other evidence showing that viral infection is orders of magnitude higher during the G2 + M stage of the life cycle (unpublished results). During this cell division phase, the coccosphere is incomplete, thus increasing exposure of the plasma membrane to viral particles. Virus infection can still occur with an intact coccosphere, possibly due to gaps between coccoliths or incomplete coccospheces, which are seen naturally in *E. huxleyi* (Paasche, 2001).

In this study, we showed that EhV-86 attachment to the host plasma membrane was irreversible but did not always result in internalization. Membrane-bound viral particles appeared to be tightly associated with the membrane. Most (but not all) of the non-internalized plasma membrane-bound virus particles were attached to a region of plasma membrane in close proximity to the chloroplast (see Fig. 3). This may explain why internalization of these viral particles did not occur. Another possible explanation why viral particles were not always internalized is that a type of superinfection exclusion mechanism may be exhibited by infected *E. huxleyi*. Possible expression of early viral genes may alter the host cell (possibly cell-surface receptors), preventing further viral internalization. Strong evidence for this strategy has been identified recently in VACV (Turner & Moyer, 2008). It should also be noted that *E. huxleyi* has at least two more membranes underlying the plasma membrane constituting the peripheral endoplasmic reticulum (Billard & Inouye, 2004), possibly presenting an additional barrier for EhV-86 to cross.

Our observations indicated that viral binding to the host membrane and internalization is a rapid process occurring potentially within 2 min of binding. Internalization may occur via endocytosis (Fig. 7, step 1a) followed by envelope fusion with the vacuole membrane (analogous to ASFV; Fig. 7, step 2) or direct fusion (Fig. 7, step 1b) of the viral envelope with the host membrane (analogous to VACV intracellular mature virus). Whichever mechanism is employed, the CENPC enters intact. To the best of our knowledge, every enveloped virus fuses its membrane with a host-cell membrane, releasing its capsid-encapsulated genome into the cytoplasm (Earp et al., 2005). This process can be direct viral envelope fusion with the host plasma membrane, such as with VACV (Armstrong et al., 1973), or fusion with a host vacuole membrane, such as with ASFV (Valdeira et al., 1998). For NCLDVs that contain an internal and external (envelope) membrane, loss or fusion of the external membrane would be necessary for sequential fusion of the inner membrane with a host membrane. Therefore, for an enveloped virus with an internal lipid membrane to inject its core through the host plasma membrane, a non-fusogenic loss of its envelope would have to occur. Although non-fusogenic loss of the outer membrane of VACV extracellular enveloped virus has been described (Law et al., 2006), internalization still takes place by fusion of the outer membrane with the host plasma membrane. Hence, in accordance with all previously identified enveloped NCLDV entry mechanisms, EhV-86 is internalized with an intact CENPC.

Once the virus has entered the cell, the nucleoprotein core rapidly disassembles, releasing its DNA into the host cytoplasm or directly into the nucleus (an order of seconds; Fig. 7, step 3). This is implied by the fast disappearance of internal viruses when seen with real-time microscopy (see Fig. 3). Due to the large volume of the cell occupied by the nucleus, the distance required to travel by internalized viral particles would be minimal (~1 μm). Transcriptomic
studies have shown that viral RNA polymerase genes are not transcribed until at least 2 h p.i. (Allen et al., 2006a), suggesting that the EhV-86 genome may target the nucleus where host RNA polymerases recognize early viral promoter sequences (Allen et al., 2006a).

Using TEM and real-time confocal microscopy, we observed virus production (Fig. 7, steps 4–5) and release via budding (Fig. 7, step 6) occurring from 3 h and 4.5 h p.i., respectively. EhV-86 microarray studies have shown that the MCP component of virions is expressed from 2 h p.i. (Allen et al., 2006a), making virus release at this time feasible. It should be noted that many of the other proteins known to make up the virion were not expressed by 4 h p.i. However, this could reflect undetectable expression levels due to only a small proportion of cells releasing mature virions at this relatively early stage of infection. Although chloroviruses are released solely by a lytic event, assembled viruses are present by 3–4 h p.i. (Meints et al., 1986). Both ASFV and FV-3 are released by a budding mechanism similar to that proposed by us for E. huxleyi, with the virus gaining an envelope from its host (Chinchar, 2002; Dixon et al., 2004). As mentioned previously, both ASFV and FV-3 in their enveloped form enter by endocytosis. EhV-86 budding explains the large virus titres observed and explains how the low m.o.i. of 0.001 rapidly lyases an E. huxleyi culture (Wilson et al., 2002).

EhV-86 infection and release mechanisms are novel for phycodnaviruses, with viral entry, internal processes and exit having greater similarities to other family members of the NCLDV group than to members of its own family. EhV-86 is not only genetically distinct from other members of the Phycodnaviridae (Wilson et al., 2005) but is also morphologically distinct. The increasing evidence that E. huxleyi has greater similarities to animal-like viruses than phycodnaviruses adds further weight towards reclassification into its own subfamily of coccolithoviruses.

ACKNOWLEDGEMENTS

We thank Roy Moate and Peter Bond from the Plymouth Electron Microscope Centre for their assistance. C.B. and D.C.S. are funded by NERC and through the NERC core strategic research programme Oceans2025 (R8-H12-52). A.V. was supported by the Carnegie Corporation of New York.

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