Proteomic analysis of the EhV-86 virion
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Abstract

Background: Emiliania huxleyi virus 86 (EhV-86) is the type species of the genus Coccolithovirus within the family Phycodnaviridae. The fully sequenced 407,339 bp genome is predicted to encode 473 protein coding sequences (CDSs) and is the largest Phycodnaviridae sequenced to date. The majority of EhV-86 CDSs exhibit no similarity to proteins in the public databases.

Results: Proteomic analysis by 1-DE and then LC-MS/MS determined that the virion of EhV-86 is composed of at least 28 proteins, 23 of which are predicted to be membrane proteins. Besides the major capsid protein, putative function can be assigned to 4 other components of the virion: two lectin proteins, a thioredoxin and a serine/threonine protein kinase.

Conclusion: This study represents the first steps toward the identification of the protein components that make up the EhV-86 virion. Aside from the major capsid protein, whose function in the virion is well known and defined, the nature of the other proteins suggest roles involved with viral budding, caspase activation, signalling, anti-oxidation, virus adsorption and host range determination.

Background

Emiliania huxleyi is the most numerically abundant coccolithophore in the world's oceans and is well known for forming vast blooms that can cover up to 10,000 km² [1,2]. Viruses have been shown to be a major cause of E. huxleyi bloom termination [3-5]. Emiliania huxleyi virus 86 (EhV-86) is the type species of the genus Coccolithovirus within the family Phycodnaviridae [3]. EhV-86 was originally isolated from a coccolithophore bloom off the coast of England in 1999 and is a large, double-stranded DNA (dsDNA) virus that infects the marine coccolithophorid E. huxleyi [4]. The fully sequenced 407,339 bp genome is predicted to encode 473 protein coding sequences (CDSs) and is the largest Phycodnaviridae sequenced to date [5]. Of the 473 predicted CDSs just 66 are annotated with functional product predictions on the basis of sequence similarity or protein domain matches. With the completion of the EhV-86 genomic DNA sequence and its annotation our research has now focused on the functional analysis of the gene products. Essential to the functional analysis is the identification of the proteins associated with the virion particle. Due to the relative simplicity of virus proteomes, 1-DE followed by LC-MS/MS was selected for...
proteomic analysis to determine the protein composition of the EhV-86 virion.

**Methods**

Viral particles were purified from the lysate of an *E. huxleyi* 1516 culture previously infected with EhV-86. Briefly, *E. huxleyi* strain 1516 was cultured in 10 litres of f/2 medium at 15°C in a Sanyo MLR-350 incubator with 16 h: 8 h light-dark illumination [6]. Exponentially growing (10 litres, 1.2 × 10⁶ cells ml⁻¹) cells were infected with 10 ml of fresh EhV-86 lysate as described previously [5]. Once clearing of the host culture was observed (5 days later), the lysate was passed through a 0.2 μm Supor membrane filter (Pall) and the filtrate concentrated by tangential flow filtration (Vivaflow200, Sartorius) to 50 ml. Virus particles were purified by CsCl gradient centrifugation (1.1 g/ml, 1.2 g/ml, 1.3 g/ml, 1.4 g/ml). CsCl-purified virus was dialysed against 30 mM Tris pH 7.4, 1 mM EDTA for 24 h and stored at 4°C. Virion proteins were then precipitated overnight at -20°C in 0.1 M ammonium acetate in methanol. Following centrifugation at 3 000 g for 10 mins, the pellet was washed in 80% 0.1 M ammonium acetate solution then again in 80% acetone. The protein pellet was desiccated to remove traces of acetone, then run on a 10% linear gradient 1-D SDS PAGE mini-gel and stained with colloidal coomassie.

The next stage was the identification of proteins within the gel. Approximately 1 mm slices were cut successively from the whole length of each track, 16 in total. The whole track was excised in this way in order to identify as many proteins as possible, and not just those which stained strongly with colloidal coomassie. Proteins within the gel pieces were first reduced, carboxyamidomethylated, and then digested to peptides using trypsin on a MassPrepStation (Waters, Manchester, UK). The resulting peptides were applied to a LC-MS/MS. For LC-MS/MS, the reverse phase liquid chromatographic separation of peptides was achieved with a PepMap C18 reverse phase, 75 μm i.d., 15-cm column (LC Packings) on a nanoAcquity LC system (Waters) attached to QTOF Premier (Waters) mass spectrometer. The MS/MS fragmentation data achieved was used to search the National Center for Biotechnology Information database using the MASCOT search engine [7]. Probability-based MASCOT scores were used to evaluate identifications. Only matches with P < 0.05 for random occurrence were considered significant. The data has been submitted to the PRIDE database under accession number 3182 [8]. Functional and structural annotation was predicted using InterProScan [9,10]. Similarity searches were performed using BLASTP against nonredundant protein sequences [11,12], and transmembrane domains were predicted using HMMTOP v2 [13].

**Results and Discussion**

An indeterminate number of faint protein gel bands were visible by SDS-PAGE in the 10 to 200 kDa range, which were dominated by two major bands at 60 kDa and 40 kDa (Figure 1). LC-MS analysis revealed that the virion of EhV-86 is composed of at least 28 proteins (Tables 1 and 2). The 60 kDa band seen by SDS-PAGE (Figure 1) is likely to correspond to the major capsid protein (predicted weight of 59.9 kDa), the 40 kDa band is likely to be a composite of the protein products from ehv067, ehv100, ehv149, and ehv175 with predicted weights of 41.9, 40.0, 40.0, 40.6 kDa, respectively (Tables 1 and 2).

Virus particles are essentially composed of structural proteins and nucleic acids. Only one protein identified in this study has a known function associated with it. The role of the major capsid protein is well defined in viral systems and it typically comprises approximately 40% of the total virion protein mass in phycodnaviruses [14]. Major capsid proteins consist of two consecutive "jelly roll" domains (antiparallel β-barrels) and are a conserved component of the capsid structures in ssRNA, dsRNA, ssDNA and dsDNA viruses [14,15].

It is surprising that of the remaining 27 proteins identified in this study, 23 are predicted to be membrane proteins (Table 3). It is possible that these proteins are associated with an internal membrane, akin to that observed in other
Phycodnaviridae [16,17]. However, electron microscopy imagery and flow cytometry data has shown that virus release occurs via budding at the host membrane (unpublished). This would suggest that virion particles may be coated in a lipid-protein membrane as they are released from infected cells. One or more of the membrane proteins identified here may be responsible for coordinating this viral budding through the formation of lipid rafts at the plasma membrane. This hypothesis is further enhanced by the previous identification of a sphingolipid biosynthesis pathway in the virus genome [5,18]. Sphingolipids are essential structural components of membranes and are found to be enriched in lipid rafts [18]. An enveloped virus release mechanism has been shown to occur in the retroviruses, paramyxoviruses, orthomyxoviruses and filoviruses [19]. Indeed, this type of mechanism has also been shown to occur with poxviruses, a virus family distantly related to the coccolithoviruses. Hitherto, phycodnaviruses were not thought to be associated with an outer membrane, but the identification of these putative membrane proteins in the EhV-86 virion and the budding release mechanism suggests this is a distinct possibility for the coccolithoviruses.

Despite extensive database interrogation, no putative function can be assigned to 23 of the 28 proteins identified in this study (Table 3). Besides the major capsid protein, putative function can be assigned to 4 proteins: two lectin proteins, a thioredoxin and a serine/threonine protein kinase. Lectin proteins are well known for their specific and reversible binding to carbohydrate moieties and may function as a binding site during virus adsorption to the host cell. The presence of a putative thioredoxin protein is intriguing since their role as antioxidants is well documented [20], particularly since EhV-86 virions have been shown to be sensitive to reactive oxygen species [21]. Indeed, elevated levels of cellular reactive oxygen species have been shown to be present in the latter stage of infection, suggesting the role of the thioredoxin protein may be to prevent damage during virion assembly [22]. The protein kinase is likely to be involved in an as yet unidentified signalling pathway.

No known components of transcriptional machinery, DNA repair or modification were detected in this study, despite being encoded on the viral genome. Several factors may account for the lack of data for these proteins including the relative abundance, size and hydrophobic-
ity of the proteins. Other related viruses from the Nucleo-
cytoplasmic Large DNA Virus (NCLDV) family such as the
mimivirus and Vaccinia virus virions have been shown to
contain 114 and 75 proteins respectively, so it is likely
that, further and more extensive characterisation will
reveal a larger number of proteins in the EhV-86 virion
[23-25].

Previous work has shown that 39 virus gene transcripts
can be detected 1 hour into infection, a further 194 two
hours post infection, and a further 71 at 4 hours post
infection [25]. Interestingly, 14 of the 28 proteins identified
here fail to be expressed even at 4 hours post infection
(see Table 1, genes expressed > 4 h post infection), sug-
iproporting that the majority of virion construction and
assembly occurs later than 4 hours post infection. This
would make sense if they are packaged into the capsid,
since their genes are probably expressed last.

Intriguingly, six proteins (EHV301, EHV325, EHV333,
EHV340, EHV356 and EHV358) have been detected in the virion whose genes are located in a 100 kbp region of
unknown origin or function [26,27]. Many of the genes in
this region (but none of these six) are associated with a
novel promoter element and are the only genes expressed
during the first hour of infection [25]. Transcriptional
work has shown that the novel promoter associated genes
are among the most highly expressed during infection,
however not one of their gene products has been identi-
fied in the virion proteome in this study [5,26]. Of partic-
ular interest is the presence of a caspase cleavage site
(YVAD) in EHV325. Host caspase activity (induced by
coccolithoviruses upon infection) has recently been pos-
tulated to play a fundamental role in the viral replication
strategy [28]. The cleavage of EHV325 by an activated host
caspase-like protease, prior to its incorporation as a
mature protein into the virion, may account for delay in
virus infection observed when caspase function is inhib-
itiated in *E. huxleyi*. Caspases are responsible for the initia-

**Table 2: Unique peptides used in the identification of proteins from the EhV-86 virion.**

| Gene   | Peptides used for identification of protein product |
|--------|--------------------------------------------------|
| eHV015 | RDIILDPNASPSDKR                                  |
| eHV034 | KCIAPDYNK, KLYLNETVSGYFR                         |
| eHV035 | KDAPLISERNGK, KDSXIDELQMMNQLDR, KEGYDQNFIVGYSVRD, KGIIGVALELLUG, KIPYVYNPLYRK, KITAPTAAALAEAAKL, KLAVFYCGCSGKT, KLATTVESDIEK, KNISSLDEKLKE, KNYDSDYFKE, KQIETTAELEPLAEDK, KQMEQOFQFEKD, KTSDTGLCCTTKV, KYVGGPITVRN, RIAQSEHEVAQSLISETKNK, RLNSLGVLSTNQILKNK, KESEADLAEEAK, RELGAETTEELDGDACK |
| eHV036 | KESADLAEEAK, RELGAETTEELDGDACK                   |
| eHV038 | KTTLDNIAEADK                                      |
| eHV055 | KDDVDAWKE, KDDVDAWKEESFVMRA, KTDFNSAVKKS         |
| eHV060 | KIDSWEPIGELAEYDSTRV                              |
| eHV067 | KELNLYLPKGTGK, KLANVEIDNKL, KLIAPARTAHR, RYMTPLDVARE |
| eHV085 | KANKDADGSHNFSGIGGGRD, KDGDHFNFSGIGGGRD KDGDHFNFSGIGGGRD PDVSAELFNNTAVR, KEQIAEAKN, KFTNGLAGGLYSN-, KIVLPGKGK, KVGATIDTIWSELLFAMELMGRA, KYNAAALPVYAAQMTPEMDFYYWTEAIFHLIKR, RASLECTYHLEAERD, RDLANTAGTQLIVQHQLQVNNIVNQTRL, RDPVSAELFNNTAVR, RLDWSVETALTQDFFGAADHANSELFVQVR, RLSETTIGR, RNVPISDDHLRA, RLEQILYVPLWFYFTKHK, RQGDLLSWMYLKI, RQGDLLSWMYLKI, RLSETTIGR, RRPTELMK, RRPTELMK, RSNLVLVAERNN, RTQPKAVWRA |
| eHV100 | KTPGAIQGPDQKD, KGTQCGNLTQCTTEKG                  |
| eHV149 | KCIPDLATICTGK, KKYDCAPGTKY, KLEPGADDNCCIVA, KLNNVSTGAKK, KVGPLGEKC, KPYDCAPGTK, RAAAAWAAATRG, RQMGASAAAGATSSAAXS |
| eHV168 | KNSALMEVMKS, KSTMGAGELEVARQ, KWTGAAAGAAAPSAADVIYKR, RGGYGPQAPDSSTGKT |
| eHV175 | RRPPNLPKVM, RYFEDIFNPRN                            |
| eHV182 | KEISDEPIDLYK, KSTCMFEADRS, KYTEDESSSPARK, RFVGDPIINNONQKGL |
| eHV189 | KVVDDLYDFRI, RYNAQOSSIRD                          |
| eHV191 | KQNLGQSOGDNLLRA                                  |
| eHV195 | RSNNQYVNOQR                                     |
| eHV200 | KSNGYDDNFPGVNNKS, KVMVATSGTTAVR, RVNVSPVPYPRN   |
| eHV250 | KSFEADAATTPYLSARS                                |
| eHV301 | RSMVPNPDINT, RSNENVNDMIARS                      |
| eHV325 | KEQPNTVSGERV                                     |
| eHV333 | KGYDVAAVQRI                                     |
| eHV340 | KAIPEGEMPGMIRA                                   |
| eHV356 | RGQTDPSONQPVVDTFR, KNPSIIGAEEK                    |
| eHV358 | KSADELNTYKE                                     |
| eHV442 | KYANGSDEVYLFNYDPKN                               |
| eHV454 | KIPTATYITRQ, RW5SGYDLEIKK, KSAVTSITLTDLEQVRV     |
| eHV461 | KTNAAELRL, KTVYVYSFKN, RLTEELRF, RVGAHGPWEIVR    |
tion and execution of programmed cell death (PCD), a process which is also thought to be affected by ceramide [28,29]. Since the virus genome contains gene homologues for an almost complete biosynthetic pathway for ceramide production, it is clear PCD may have some important role to play during infection. It is speculative to suggest that EHV325, in its newly identified role as a virion component, may be involved in this process.

There are 4 proteins identified whose genes are located at a distinct loci of the EhV-86 genome: EHV034, EHV035, EHV036 and EHV038. Although no known function has been determined for these proteins, their presence in the virion particle is intriguing since a microarray based genome wide survey has suggested that all four genes are either absent or their sequence is sufficiently divergent to cause a negative hybridisation in two Norwegian coccolithovirus strains [30]. Furthermore, ehv034 and ehv031 were found to be absent (or their sequence sufficiently divergent) in nine and seven of the twelve strains tested, respectively. The presence, absence or divergence of the products of these genes in virions may account for the variability in host range observed for different coccolithovirus strains.

Conclusion
This study represents the first steps toward the identification of the protein components that make up the EhV-86 virion. Hitherto, only one virus protein (the major capsid protein) has been confirmed as part of the virion structure. Here, we have used a simple approach to determine that the EhV-86 virion is composed of at least 28 different proteins. Aside from the major capsid protein, whose function in the virion is well known and defined, the nature of the other proteins suggest roles involved with viral budding, the caspase pathway, signalling, antioxidation, virus adsorption and host range determination.

Competing interests
The author(s) declare that they have no competing interests.

Table 3: Analysis of proteins identified in the EhV-86 virion.

| Gene Number | Top Blast Hita | Blast Scorea | TMsb | InterProScan Resultsc |
|-------------|----------------|--------------|------|-----------------------|
| ehv015      | hypothetical protein, *Trichomonas vaginalis* | 0.009 | 1 | No hits reported. |
| ehv034      | predicted protein, *Ostreococcus lucimarinus* | 0.016 | 1 | No hits reported. |
| ehv035      | similar to SCM2 protein, *Bos taurus* | 0.058 | 2 | No hits reported. |
| ehv036      | HlyD family secretion protein, *Agrobacterium tumefaciens* | 0.004 | 2 | No hits reported. |
| ehv038      | hypothetical protein, *Bos taurus* | 0.32 | 1 | No hits reported. |
| ehv055      | hypothetical protein, *Methanothermobacter thermautotrophicus* | 5e-06 | 6 | No hits reported. |
| ehv060      | No significant match | n/a | 1 | C type lectin 2 domain |
| ehv067      | Hypothetical protein, *Giardia lamblia* | 1.4 | 0 | No hits reported. |
| ehv085      | major capsid protein, *Heterosigma akashiwo virus 01* | 7e-39 | 0 | Capsid domain (iridovirus-like) |
| ehv100      | predicted protein, *Nematostella vectensis* | 5e-10 | 2 | No hits reported. |
| ehv149      | hypothetical protein, *Aedes aegypti* | 0.43 | 2 | C type lectin 1 domain |
| ehv168      | hypothetical protein, *Novosphingobium aromaticivorans* | 2.4 | 1 | No hits reported. |
| ehv175      | Putative serine/threonine protein kinase, *Populus tomentosa* | 0.66 | 0 | Protein Kinase |
| ehv182      | diaminopimelate decarboxylase, *Streptococcus pneumoniae* | 0.48 | 1 | No hits reported. |
| ehv189      | pol-like protein, *Nasonia vitripennis* | 2.0 | 0 | No hits reported. |
| ehv191      | No significant match | n/a | 1 | Proline rich extensin signature |
| ehv195      | hypothetical protein, *Salinispora arenicola* | 0.27 | 2 | No hits reported. |
| ehv200      | hypothetical protein, *Bacillus sp.* | 0.23 | 1 | No hits reported. |
| ehv250      | GCN5-related N-acetyltransferase, *Rhodospseudomonas palustris* | 9.6 | 1 | No hits reported. |
| ehv301      | NB-ARC domain containing protein, *Oryza sativa* | 0.31 | 0 | No hits reported. |
| ehv325      | envelope glycoprotein, Simian immunodeficiency virus | 1.1 | 1 | No hits reported. |
| ehv333      | CRISPR-associated protein, *Cse1 family, Pseudomonas mendocina* | 0.35 | 2 | No hits reported. |
| ehv340      | Putative fimbrial associated sortase-like protein, *Corynebacterium diphtheriae* | 0.42 | 1 | No hits reported. |
| ehv356      | No match | n/a | 1 | No hits reported. |
| ehv358      | hypothetical protein, *Paramecium tetraurelia* | 2e-58 | 1 | Thioredoxin domain |
| ehv42       | conserved hypothetical protein, *Stigmatella aurantiaca* | 0.008 | 2 | No hits reported. |
| ehv454      | hemocyanin isoform 1, *Nucula nucleus* | 2.2 | 2 | No hits reported. |
| ehv461      | Fatty acid/phospholipid synthesis protein, *Herminiimonas arsenicoxydans* | 2.6 | 1 | No hits reported. |

aBLASTP analysis [12] against nonredundant protein sequences performed on 12th December 2007. Transmembrane (TM) domains predicted by TMTOP v2.0. Functional and structure predicted by InterProScan [10].
Authors’ contributions
MIA conceived of the study, designed the study, carried out the virus purification and protein preparation, analysed the data and drafted the manuscript. JAH carried out the SDS-PAGE and LC/MS, and analysed the data. KSL helped draft the manuscript. WHW conceived of the study, participated in its design and helped to draft the manuscript. All authors read and approved the final manuscript.

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