

Cloning, expression of the major capsid protein gene from marine algae *Emiliania huxleyi* virus and the possible use in detection of virus infection

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Abstract

Here we described the cloning, bioinformatic characterization and expression in Escherichia coli of the major capsid protein (MCP) from marine unicellular algae Emiliania huxleyi virus EhV-99B1 isolate. The purified recombinant MCP was used to develop a polyclonal antibody for testing viral infection. The full length openreading frame (ORF) of MCP encodes a protein of 496 amino acids with a calculated molecular mass of 55 kDa and Ip 6.34. Hydropathy analysis of MCP showed that there were 6 largely hydrophobic domains, which may be important for the interaction with the envelope protein. The conserved region of EhV strains MCP had high similarity in amino acid sequence and secondary structure which allow us to develop a specific biomarker for EhVs infection detection. The full length ORF was subcloned into expression vector pGEX-4T-3 for overexpression in E. coli as glutathione-Stransferase-L1 (GST-L1) fusion protein and the soluble recombinant protein was used to generate polyclonal antibodies in mice. The obtained antisera reacted in Western immunoblots with the same protein both in purified EhV-99B1 virions and infected host cells sample. These shows that the antiserum against recombinant EhV-MCP offers the potential to develop immunofluorescence techniques for the detection of EhVs infected cells.

Introduction

Viruses are found to be able to infect and lyse a wide range of primary producers and have the potential significance in aquatic ecology as mortality agents for phytoplankton.^{1,2} Through cellular lysis, viruses indirectly affect the fluxes of energy, nutrients, and organic matter and lateral and horizontal gene transfer, especially during algal bloom events when biomass is high.^{3,4} Viral-mediated mortality of phytoplankton can occur at significantly high rates in the field. Although the importance of viruses is presently recognized, it is apparent that many aspects of viral-mediated mortality of phytoplankton are still poorly understood.⁵ Estimates for total cell lysis rates in natural phytoplankton vary widely in time and space.⁶⁻ ⁸ Reports of viral lysis rates of phytoplankton in the field are limited because of the restricted number of suitable methods available for measuring viral-induced mortality.

There have been several approaches developed for estimating the magnitude of viralmediated mortality rates of phytoplankton. To calculate cell lysis from the total number of viruses produced and burst size per cell.9,10 Critical assumptions of this approach are that burst size is known, and that the virus of interest can be discriminated from other viruses in the samples. Another, the impact of viral infection on phytoplankton mortality can be estimated by using decay rates of viral particles in which burst size must be known.⁵ An existing dilution technique estimating microzooplankton grazing of phytoplankton was modified to partition the mortality.¹¹ This method has the advantage that the natural samples do not need any pre-treatment, but the duration of the latent period and the concentration of algal cells of interest are critical factors with this technique.⁵ Another method to estimate viral production involves addition of fluorescently labeled viral tracers and this approach has the disadvantage that the fluorescent dve is lightsensitive and incubations in the light are not recommended.¹² The approach based on the frequency of infected cells seems the most straightforward and has the important advantage that culturing or incubation of samples is unnecessary and the fraction of viral-infected cells is calculated by adjusting for the proportion of the lytic cycle during which viral particles can be observed. This method is very useful but time-consuming and transmission electron microscopy is required; the number of samples analyzed is therefore limited.

The infection between virus and cells start up by virus major capsid protein (MCP) and cell membrane conjugation.The difference of virus infection in specificity and susceptibility is due to the viral capsid and virus infection can be diagnosed by detection of antibody to the capsid protein. Monospecific antibodies produced against viral capsid is available for the unique antigenic determinants by immunofluorescent and is a powerful tool for detecting virus infection.¹³ Anti-capsid antibodies raised against recombinant capsid antigen have been used to detect viral infection in immunofluorescence assay.¹⁴⁻¹⁶

Emiliania huxleyi (Haptophyceae) (Lohmann) Hay & Mohler has worldwide distribution and form massive blooms in both oceanic and coastal waters.¹⁷ It belongs to the Coccolithophores, a class of unicellular phytoplankton. The main mechanism for these boom-and-bust cycles was thought to be infecCorrespondence: Huinong Cai, Bioengineering College of Jimei University, Xiame 361021, China.

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Key word: *Emiliania huxleyi* virus, major capsid protein, bioinformatic characterization, antiserum reaction.

Acknowledgments: the authors would like to thank Prof. Gunnar Bratbak (Department of Biology, University of Bergen) for providing the *Emiliania huxleyi* and *E. huxleyi* virus strains.

Contributions: ZLZ, YQC, did experiments; XHL, data analyzing; JWL, manuscript writing; HNC, manuscript reviewing and references search.

Conflict of interests: the authors declare no potential conflict of interests.

Funding: this work was supported by Chinese Public science and technology research funds projects of ocean (No. 201305027), Fujian Province Nature Science Foundation, China (No. 2010J01261) and the Foundation for Innovative Research Team of Jimei University, China (No. 2010A007).

Received for publication: 5 February 2013. Revision received: 23 April 2013. Accepted for publication: 15 May 2013.

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tion and lysis by E. huxleyi-specific viruses (EhV).¹⁸ Virus induced mortality has been estimated to account for 25-100% of the total net mortality during blooms of E. huxleyi in large outdoor mesocosms.^{9,10} Numerous large dsDNA viruses specific to E. huxleyi have been isolated,^{9,19-21} and phylogenetic analysis of the DNA polymerase genes of these viruses suggests that EhV belongs to a new genus within the family of Phycodnaviridae-Coccolitho*virus*.^{19, 22,23} It is reported that EhV-86 enters its host via either an endocytotic or an envelope fusion mechanism in which an intact nucleoprotein core encapsulated by MCP, which is different from that employed by other algal NCLDVs (nucleocytoplasmic large DNA viruses).²⁴ These literatures indicate that MCP could be expected to be as a marker for the detection of EhVs infection host cells.

In this study, we aimed to clone and express recombinant EhV-99B1 MCP, and then to develop the antisera raised against purified recombinant MCP. Given the antisera can react



specifically with purified EhV-99B1 virons, it will offer the potential to develop immunofluorescence techniques for the detection of EhVs infected cells in field. In addition, the purified recombinant EhV MCP also can be used for further research in its characteristics, functions, viral susceptibility and virion assembly.

Materials and Methods

Materials

The restriction endonucleases (*EcoR* I and *Not* I) and T4 DNA ligase were purchased from Takara. *E. coli* BL21, pUCM-T and the expression vector pGEX-4T-3 were purchased from Invitrogen. KOD DNA polymerase was purchased from TOYOBO. *E. coli* strain Top10, stored in our laboratory, was used for the transformation and propagation of recombinant plasmids. *E. coli* strain BL21 with glutathione S-transferases (GST) was used as host to express recombinant proteins.

The algal specie and its virus used in this study were *E. huxleyi* (EhBOF92) and *E. huxleyi* virus (EhV- 99B1),²⁵ which were from the culture collection at the Department of Biology, University of Bergen, Norway.

Viral purification and DNA isolation

To produce purified virus, the virus was amplified in 1.5L culture grown in f/2 medium (Guillard, 1975). Exponentially growing culture was infected with filtered EhV-99B1. The amount of virus added gave an initial virus-tohost ratio of ca 1.5. The debris in the lysates was removed by centrifugation at 10,000 ×g in a Beckman JA-14 centrifuge at 4°C. The supernatant was passed sequentially through 0.45 and 0.2 µm filters for removing large cellular debris. The virus filtrates were concentrated by tangential flow ultrafiltration with a 50kMW size cut-off unit (Prep/Scale TFF-1, PTOK50, Millipore). The viral concentrates were added with polyethlene glycol (PEG 8000; 100 g L^{-1} final conc.) and incubated overnight at 4°C. The viral particles were precipitated by centrifugation at 15,000 ×g in a Beckman JA-25.50 rotor for 1 h at 4°C. The pellets were resuspended with 3 mL SM buffer (10 mM NaCl, 50 mM Tris, 10 mM MgSO₄ and 0.1% gelatin, pH 7.5) and incubated overnight at 4°C. The viral suspensions were added with CsCl to a final concentration of 0.5 g \times mL⁻¹ and ultracentrifuged at 200,000 × g using a T-8100 rotor (CS150GXL Micro Ultracentrifuge) for 24 h at 4°C. The CsCl purified EhV suspensions were stored at 4°C until further uses. The viral DNA was isolated from EhV99B1 by using CTAB method.

Cloning and sequencing of major capsid protein gene

MCP gene of EhV99B1 was amplified by PCR using one pair specific primers designed according to the ORF of MCP of genomic sequences of EhV99B1 (GenBankTM accession number: FN429076.1). The forward primer was F5'-GAC<u>GAATTC</u>CTCTGGATTTGGTGGTGG-3' (*EcoR* I). The reverse primer was 5'-ACC<u>GCG-GCC</u>GCTCAGTTCGAGTATAATA-3' (*Not* I).

Virus DNA (100 ng) was added to 25 μ L of a PCR reaction mixture which contained KOD DNA polymerase, assay buffer (50 mM Tris-HCl pH 9.0, 1 mM EDTA, 1 mM DTT, 0.001% Tween 20, 0.001% NP-40, 50% glycerol), 1.5 mM MgSO4, 0.2 mM each deoxyri-bonucleoside triphosphate, 10 pmol of each primer, and 0.5 U of KOD DNA polymerase. PCR was conducted with a Tgradient 96 cycler (Biometri) (94°C for 1 min, 30 cycles at 94°C for 15 sec, 55°C for 30 sec, and 68°C for 2 min). The PCR product was purified with agarose gel DNA purification kit Ver.2.0 (TaKaRa) and then was cloned into the pUCM-T Vector System (Invitrogen) for sequence.

Construction and expression of recombinant EhV99B1-MCP

To obtain a sufficient amount of soluble recombinant protein, MCP was overproduced in E. coli BL21 using the pGEX-4T-3 vector. The recombinant plasmid was designated pGEX-MCP. E. coli BL21 was transformed with the pGEX-MCP construct, and a single positive colony was inoculated in 1 liter of LB medium plus ampicillin and grown at 37°C. Then fusion protein was induced by the addition of isopropyl-1-thio-b-D-galactopyranoside (Sigma) to a final concentration of 1.0 mM, and growth was continued for another 5 h. In order to obtain more soluble recombinant protein, the recombinant strain, harboring the plasmid pGEX-MCP. was induced with different concentrations of IPTG (0.1 mM, 0.3 mM and 0.6 mM) at different temperatures (20, 25 and 30°C) for different induction durations (5 h, 8 h and 10 h) respectively. In order to yield enough expression supernatant for purification of recombinant proteins, a single bacterial colony carrying pGEX-MCP was used to inoculate an overnight preculture in LB medium with 100 mg L⁻¹ ampicillin at 37°C. This preculture was used to inoculate 1 L of fresh LB medium with 100 mg L⁻¹ ampicillin. Approximately 4 hours later, the A_{600} of the bacterial broth reached 0.5. The flasks were then transferred to 20°C for 30 min, and 0.3 mM IPTG was added for another 8 h induction.

Analysis and purification of expressed major capsid protein

The harvested cell pellet was resuspended in

50 mL of 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 5 mM EDTA and lysed through sonication, centrifuged, and separated into soluble and insoluble fractions. Both the fractions were analyzed by 10% or 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

The cells were lysed by sonication in buffer A (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% triton X-100) containing 1% β-Mercaptoethanol and 1 mM PMSF. The soluble GST-MCP fusion protein was purified by hydrophobic interaction chromatography. The total lysate was treated with DNase I (200 U \times mL⁻¹) for 45 min at 25°C and then centrifuged at 12,000 \times g for 30 min at 4°C, the supernatant containing recombinant protein was applied at $1 \text{ mL} \times \text{min}^{-1}$ to an Phenyl-Sepharose CL 4B column (GE Amersham bioscience) (18×100 mm). The protein fractions were eluted at 1 mL \times min⁻¹ with a linear gradient of $100\% \sim 0\%$ buffer A and 0 ~ 100% buffer B (20 mM Tris-HCl pH 8.0, 5 mM EDTA, 1% triton X-100) within 60 min. The purified fusion protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Antiserum production

To raise the specific antibody against MCP, the purified GST-MCP fusion protein was used as antigen to immunize five mice four times by intradermal injection. For the first injection, antigen (50 µg) was mixed with an equal volume of Freund's complete adjuvant (Sigma). After two weeks, the following three injections were conducted using 50 µg antigen mixed with an equal volume of Freund's incomplete adjuvant once every a week. Four days after the last injection, mice were exsanguinated and the antisera were collected. The titres of the antisera were approximately 1:20000 as determined by ELISA using horseradish peroxidase conjugated goat anti-mouse IgG (Invitrogen mmunodetection, Zymed Laboratories). For a negative control, antigen was replaced with 1×PBS.

Western blots

The ability of the immune antiserum to recognize native viral protein was examined using Western blot analysis. Virus particles were concentrated and purified as described above. The concentrated virus was centrifuged by CsCl equilibrium gradient and ultracentrifugation. The pellets were suspended in SDS sample buffer and heated to 100°C for 10 min and then were subjected to 10% SDS-PAGE. In addition, exponentially growing cultures were infected with concentrated EhV lysate. Control culture with no virus additions was run at the same conditions. 25 h after inoculation, in the second lytic cycle when the majority of the cells were infected, 1L of the cultures were harvested by centrifugation at 2800 ×g for 8 min. The crude



protein extracts were prepared by sonicating cells in 50 mM Tris-HCl extraction buffer (pH 7.4). The extracts were centrifuged at $13500 \times g$ for 1h at 4oC to remove cell debris and protein was resolved by 10% SDS-PAGE.

The protein were then electrophoretically transferred onto a nitrocellulose membrane (Amersham) by electric current (Bio-Rad-Mini-Protein and Mini Trans-Blot) at a constant current of 0.5 mA/cm² of membrane surface for 1.5 h. The membrane was blocked with 2% BSA in TBST (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween-20) at 4°C overnight, followed by incubation with polyclonal mouse anti-MCP serum diluted 1:5000 in TBST with 1% BSA for 1 h at room temperature. Subsequently, goat anti-mouse IgG conjugated with horseradish peroxidaseconjugated (Invitrogen mmunodetection, Zymed Laboratories) (1:200 dilution) was used as the secondary antibody and detection was performed with a substrate solution (NBT/BCIP, Promega). Negative controls were run by loading the same amount of total protein on the gel, and the blot underwent the same immunodetection procedures in the absence of the MCP-antiserum.

Bioinformatics analysis of EhV99B1-MCP gene

After sequencing, homology searches with DNA sequences were carried using the BLAST algorithm provided by the Internet Service of the National Centre for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/ Blast.cgi). The secondary structure of the N-terminal conserved domains of EhVs MCP predicted by using the program ESPrip (http://espript. ibcp.fr/ESPript/ESPript/index.php). Hydropathy analysis and transmembrane helices and sheets prediction of EhV99B1 MCP were carried out through the web-based tool MPEx (Jaysinghe *et al.* 2006; *data not cited*) using the Wimley-White octanol scale. The prediction of conformation was performed using SWISS MODEL method (http://swissmodel. expasy.org/).

Results

Gene clone and bioinformatics analysis of EhV99B1-MCP

Sequencing resulted in amplification of the EhV99B1-MCP ORF with the size of 1491 bp. There is about $93\% \sim 100\%$ identity in the conserved domain of nucleotide and 100% deduced amino acid sequence with other EhV isolates. Multiple sequence alignment of MCP from EhV isolates showed that the secondary structure of the N-terminal domain is well preserved across all EhV isolates including one α -helix (α 1) and five β -pleated sheets ($\beta 1 \sim \beta 5$) and without an apparent organelle targeting (Figure 1). ANTHEPROT and structural analysis revealed that MCP ORF contained 6 distinct hydrophobic regions from N -to C-terminal residues 43H-60Q; 74D-94A; 232L-288V; 344N-366K; 402P-431S; 470L-493N concomitance 6 predicted cytoplasmic transmembrane segments. The N-terminal first hydrophobic domain of the capsid protein (aa 43H-60Q) may be important for the interaction with the envelope protein. SWISS-

MODEL was applied to predict the conformation of MCP, and the result showed that the peptide was composed of 1 α - helix, 15 β -Sheet, and 10 loops connected by small peptides, and moved together to each other to

Expression and purification of recombinant EhV-MCP

form a spherical molecule (Figure 2).

E. coli strain BL21 was used as host for the expression of recombinant EhV-MCP (Figure 3), but GST tag showed poor soluble protein



Figure 2. The three-dimensional structures of EhV99B1 Msjor Capsid Protein (MCP) predicted by the SWISS-MODEL (http://swissmodel.expasy.org/). The range between ASP-23 and GLN-42 is predicted to be transmembrane segments. Red: α helices; yellow: β -sheets; green: loops.





Figure 3. Agarose gel electrophoresis of plasmid PCR and the digest product of the pGEX-MCP recombinant plasmid. M_1 : DNA marker (λ -Hind III digest marker); line1: pGEX-MCP recombinant plasmid; line 2: pGEX-MCP/*EcoR* I+ *Not* I ; M_2 : Gene RuLerTM 100 bp DNA ladder marker; line 3: PCR products



production with the majority of the production found in the insoluble fraction. In order to obtain more soluble recombinant protein, the recombinant strain was induced with different concentrations of IPTG at different temperatures for different induction durations as described previously (data not shown). By optimizing the expression conditions, the production of soluble GST-MCP fusion protein increased to 15% of total cellular proteins and about 26% of the products appeared in the cellular supernatant fraction (Figure 4), when the recombinant strain was induced with 0.3 mM IPTG at 20°C for 8 h.

In our construct, a GST tag was fused to the N terminus of EhV-MCP in order to increase the soluble yields and facilitate the purification of the desired protein by affinity chromatography. Unfortunately, poor results were obtained with GST-MCP fusion protein in affinity chromatography specific for GST tag (data not shown). The soluble GST-MCP fusion fraction was purified by using a Phenyl-Sepharose CL-4B hydrophobic column and the purified fusion protein migrated on SDS-PAGE with an apparent molecular mass of 76 kDa (Figure 4).

Antiserum to EhV-99B1 MCP react with MCP in Western blots

EhV recombinant MCP was purify and raise mouse antiserum as described in Materials and Methods. Western blot analysis showed that mouse anti-MCP antiserum reacted specifically with the same protein in purified EhV-99B1 virions as well as the infected host cells sample (Figure 5). The 54,000-molecularweight protein (54K protein), a major band corresponding to the expected capsid protein (54 kDa), was immunodetected in both samples, confirming the authenticity of the recombinant protein.

Discussion and Conclusions

It is known that the difference of virus infection in specificity and susceptibility is due to the viral capsid and viral infection can be diagnosed by detection of antibody to the capsid proteins. The infection between virus and cells starts-up by virus MCP and cell membrane conjugation. In this study, we obtained purified recombinant MCP in E. coli. ORF of EhV-99B1-MCP encode a predicted protein with a molecular mass of 55 kDa. A BLAST P search indicated that there is about $93\% \sim 100\%$ identity in the conserved domain of nucleotide and 100% deduced amino acid sequence with other EhV isolates, which suggests that EhV MCP might have been conserved throughout this family. Based upon the sequence homology and the high conserved secondary structural domain among EhV isolates, it could be expected that the antibody generate from MCP could serve as a specific marker for the detection of EhVs infected cells. In addition, it has been shown that EhV-86 is an enveloped virus and it enters host cell via either an endocytotic or an envelope fusion mechanism, with an intact nucleoprotein core encapsulated by its capsid.²⁴ Therefore, to detect the infection of EhVs by the antibody to the capsid proteins is practicable theoretically.

From N-to C-terminal residues of EhV99B1-MCP were largely hydrophobic, which may be important for the interaction with the envelope protein. Enveloped-virus fusion protein, generate a hydrophobic conformer capable of inserting into and disrupting cell membranes. These hydrophobic domains are necessary for infectivity and are probably as specific fusion peptide sequence for envelop fusion reactions,²⁶ and contribute to form stably capsid structure by the noncovalent interactions and allow efficient assembly of the particle in the cytosol or nucleus of host cells.^{26,27} Enveloped viruses employ specific proteins to fuse with and thereby introduce their genetic material into host cells, it is likely that cellular fusion reaction are also protein-mediated.²⁸ The acquisition of hydrophobic properties may be a general prerequisitc to fusion.

In order to express soluble heterologous proteins in E. coli, two approaches have been used. One approach was to co-express molecular chaperones which aid in protein folding. Another approach was the use of gene fusions. In this study, GST was chosen as carrier fused to the N-terminus of EhV-MCP. Unfortunately, GST tag showed poor soluble protein production with the majority of the production found in the insoluble fraction. By optimizing the concentration of ammonium sulfate in loading buffer, the GST-MCP fusion protein was purified by Phenyl-Sepharose CL 4B column at 1 M ammonium sulfate in loading buffer. The EhV-MCP expressed by the E. coli system was in soluble form with a molecular weight of Mr 55 kDa, which is similar with the size reported.19

The use of the antiserum raised from purified recombinant EhV MCP in western blot analysis has shown that the polyclonal serum recognized the same protein both in purified VLP samples and EhV-99B1 infected host cells, suggesting that the reactivity in this test may be a useful diagnostic response. This observation also implied that the EhV MCP is a major epitope in other assay systems, such as immunofluorescence or immunoprecipitation. Although it is known that the difference of virus infection in specificity and susceptibility is due to the viral capsid and viral infection can be diagnosed by detection of antibody to the capsid proteins, no report is done about its use in the detection of marine algal virus. This is the first report to show that the MCP protein

alone is able to detect EhV virus particles when expressed in *E. coli*, which highlights its potential as biomarker for viral infection in the oceans and will be invaluable tools to help understand some of these complex interactions. Based on these observations, it is anticipated that the antisera described here will prove to be valuable tools for specific detection



Figure 4. Purification of EhV 99B1 Major Capsid Protein (MCP) after recombinant expression in E. coli. Shown is an SDS-PAGE analysis of GST-MCP fusion proteins by Coomassie blue-stained polyacrylamide gel. After induction with IPTG, the cells were lysed and the soluble GST-MCP fusion fraction was purified by using a Phenyl-Sepharose CL- 4B hydrophobic column. Lane M, Mr markers, sizes of which are indicated to the left. lanes 1-2, whole cell lysates uninduced and induced for GST-MCP expression; lines 3-4, cell extract supernatant and cell extract sediment fraction. lane 5, the purified fusion protein migrated on SDS-PAGE with an apparent molecular mass of 76 kDa.



Figure 5. The obtained antiserum reacted in Western immunoblots with the same protein both in purified EhV-99B1 virions (A) and host cells sample at 25 h post EhV infection (B). Lane M, Mr markers, sizes of which are indicated to the left. Line 1, purified EhV-99B1 virions; line 2, negative control: same treatment as in lane 1 but without Major Capsid Protein (MCP) antiserum. Lines 3-4, host cells sample at 25 h post EhV infection and uninfected cells.



of EhV infection cells and also be used for the study of EhV assemblage. The successful development of MCP specific mouse antiserum encourages us to develop monoclonal antibodies specific for EhV using the recombinant MCP which will offer the potential to develop immunofluorescence techniques for the detection of EhVs infected cells in field.

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