Cloning, expression and characterization of serine palmitoyltransferase (SPT)-like gene subunit (LCB2) from marine *Emiliana huxleyi* virus (*Coccolithovirus*)

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Abstract

The authors have isolated and characterized a novel serine palmitoyltransferase (SPT)-like gene in marine *Emiliana huxleyi* virus (EhV-99B1). The open-reading frame (ORF) of EhV99B1-SPT encoded a protein of 496 amino acids with a calculated molecular mass of 96 kDa and pI 6.01. The results of sequence analysis showed that there was about 31%–45% identity in amino acid sequence with other organisms. The maximum likelihood phylogenetic tree suggested that the EhV99B1-SPT gene possibly horizontally transferred from the eukaryote. Hydrophobic profiles of deduced amino acid sequences suggested a hydrophobic, globular and membrane-associated protein with five transmembrane domains (TMDs) motifs. Several potential N-linked glycosylation sites were presented in SPT. These results suggested that EhV99B1-SPT was an integral endoplasmic reticulum membrane protein. Despite lower sequence identity, the secondary and three-dimensional structures predicted showed that the “pocket” structure element composed of 2 α -helices and 4 β -sheets was the catalytic center of this enzyme, with a typical conserved “TFTKSFG” active site in the N-terminal region and was very close to those of prokaryotic organisms. However, the N-terminal domain of EhV99B1-SPT most closely resembled the LCB2 catalysis subunit and the C-terminal domain most closely resembled the LCB1 regulatory subunit of other organisms which together formed a spherical molecule. This “chimera” was highly similar to the prokaryotic homologous SPT. For a functional identification, the EhV99B1-LCB2 subunit gene was expressed in *Escherichia coli*, which resulted in significant accumulation of new sphingolipid in *E. coli* cells.

Key words: *Emiliana huxleyi* virus, serine palmitoyltransferase (SPT), clone expression and characterization

1 Introduction

Serine palmitoyltransferase (SPT, EC 2.3.1.50) is the first and committed enzyme in *de novo* sphingolipid synthesis. SPT condenses serine and palmitoyl-CoA to form 3-ketosphinganine. SPT is present in animals, higher plants, and fungi, as well as in prokaryotes (Ikushiro et al., 2001; Nagiec et al., 1994; Weiss et al., 1997). These SPT homologs are distinct from each other in their structure, but they are conservative functionally (Salzmann et al., 2000). Prokaryotic SPTs are homodimer containing two symmetric subunits (Ikushiro et al., 2001),

whereas eukaryotic SPTs are heterodimer comprised of LCB1 and LCB2 subunits (Buede et al., 1991). SPT belongs to the small conserved enzyme family called the α -oxoamine synthases (AOS). AOS has a conserved TFTKSFGSVG motif inducing the combination of pyridoxal phosphate (PLP) with an α -amino acid to form a compound called a Schiff base (Ikushiro et al., 2001).

The coccolithophore *Emiliana huxleyi* is a globally important unicellular marine phytoplankton. It is capable of forming immense open ocean blooms and can be detected via satellite imagery due to light reflection from the calcium carbonate coccoliths (Holligan

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et al., 1983). Because of this bloom formation and its massive calcifying activity, *E. huxleyi* is considered to be one of the largest single carbonate sinks in oceanic carbonate cycling (Baumann et al., 2004; Field et al., 1998). When subjected to grazing or during viral infection, *E. huxleyi* produces dimethylsulfonio propionate (DMSP), the precursor of dimethyl sulphide (DMS), which is linked to marine cloud formation and even climate regulation (Charlson et al., 1987; Iglesias-Rodriguez et al., 2008; Stefels et al., 2007).

Algal viruses are widely distributed in nature and virus infection is one of the most important factors to induce lysis and mortality of phytoplankton (Brussaard, 2004). It is now generally accepted that the *E. huxleyi* virus (EhV) contributes to the collapse of these blooms (Bratbak et al., 1993). Virus-mediated mortality of *E. huxleyi* is thought to play an important role in ocean biogeochemistry now. *E. huxleyi* virus 86(EhV-86) was originally isolated from the demise of an *E. huxleyi* bloom off the coast of England (Wilson et al., 2002). Now, more than ten such viruses have been isolated (Bratbak et al., 1993; Wilson et al., 2002; Wilson et al., 2005). Phylogenetic analysis of the DNA polymerase genes of these viruses suggests that EhV belongs to a new genus within the family of *Phycodnaviridae-Coccolithovirus* (Allen et al., 2006; Bratbak et al., 1993; Larsen et al., 2008; Wilson et al., 2005). EhV is one of the best-studied eukaryotic phytoplankton host-virus systems (Bidle et al., 2004).

The significance of marine viruses in oceanic ecosystems has only recently been fully appreciated; not only is there an enormous and diverse viral load in the seas (the average bucket of seawater contains in excess of 10^8 viral particles per milliliter) but viruses are implicated in mediating horizontal gene transfer between species, as well as generating transient local niches through their cell-lysing effects (Suttle, 2007). Marine viruses are, therefore, important players in both the evolution and lifecycle of many aquatic organisms.

A recent study sequenced and characterized a large, double-stranded DNA (dsDNA) lytic virus (EhV-86) with a genome of 407 kbp, making this the one of largest Phycodnaviridae genome sequenced to date, in which some genes were unidentified in all known viruses but existed in its host widely (Wilson et al., 2005). These genes are predicted to encode a vast array of functional proteins involved in sugar metabolism, sphingolipid biosynthesis, reactive

oxygen species (ROS) regulation, and signaling pathway (Bidle et al., 2011; Monier et al., 2009; Wilson et al., 2005). EhV-86 especially has some unusual CDSs, including seven genes encoding sphingolipid synthesis enzymes (Monier et al., 2009). This biosynthetic pathway appears to function during lytic infection and programmed cell death (PCD) with corresponding activation of an algal metacaspase, an essential activity for EhV replication. Therefore, it changed the ecological niches of the *E. huxleyi* and turned over the *E. huxleyi* metabolic pathways for viral replication (Allen et al., 2008; Rohwer et al., 2009). The sequencing of the EhV-86 genome revealed a gene predicted to encode SPT, which has never been identified in a virus (Wilson et al., 2005). Moreover, SPT encoded by the EhV-86 genome not only contributes to regulating sphingolipid levels in *E. huxleyi* cells but also exhibits a unique substrate preference to produce novel ceramide (Jermy, 2010). Ceramide-induced PCD has been observed in *E. huxleyi* cells during virus infection (Bidle et al., 2004; Jermy, 2010). This gene was expressed in yeast and was thought to rescue yeast LCB1 and LCB2 mutants (Han et al., 2006). Undoubtedly, SPT gene is involved in the interaction between EhV strains and their hosts, as yet, their precise role remains unknown (Michaelson et al., 2010; Pagarete et al., 2009).

This study focuses on cloning and characterization of a new SPT-like gene from *E. huxleyi* virus 99B1(EhV-99B1) isolation and uses recent surge bioinformatics methods to investigate the compositional properties, structural feature, functional class and phylogenetic profile, which will contribute to our understanding of evolution and horizontal transfer between marine virus and its host. To elucidate the catalytic function of the EhV99B1-SPT protein, we describe the expression of EhV99B1-SPT subunit gene (LCB2) involved in sphingolipid biosynthesis in *E. coli* cells.

2 Materials and methods

2.1 *Bacterium strains, vectors and enzymes*

The restriction endonucleases (*Avr* II and *Not* I), T4 DNA ligase and agarose gel DNA purification kit were purchased from Takara. The clone vector pTA2-T and KOD DNA polymerase were purchased from TOYOBO. *E. coli* strain Top10, stored in our laboratory, was used for the transformation and propaga-

tion of recombinant plasmids. *E. coli* strain origami (Top10) was used as host to clone the recombinant vector.

2.2 Viral purification and DNA isolation

The algal species and their respective viruses used in this study were *Emiliania huxleyi* (EhBOF92) and *Emiliania huxleyi* virus (EhV99B1) (Castberg et al., 2002) from the culture collection at the Department of Biology, University of Bergen, Norway. To produce purified virus, the virus was amplified in 1.5 L culture grown in f/2 medium (Guillard, 1975). Exponentially growing culture was infected with filtered EhV99B1. The debris in the lysates was removed by centrifugation at 10 000 $\times$ g in a Beckman JA-14 centrifuge. The supernatant was passed sequentially through 0.45 μ m and 0.2 μ m filters for removing large cellular debris. The virus filtrates were concentrated by tangential flow ultrafiltration with a 50 kMW size cut-off unit (Prep/Scale TFF-1, PTKK50, Millipore). The viral concentrates were added with polyethylene glycol (PEG 8000; 100 g/L final concentration) and incubated overnight at 4°C. The viral particles were precipitated by centrifugation at 15 000 $\times$ g in a Beckman JA-25.50 rotor for 1 h. The pellets were resuspended with 3 ml SM buffer (10 mmol/L NaCl, 50 mmol/L Tris, 10 mmol/L 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/ml and ultracentrifuged at 200 000 $\times$ g (4°C) using a T-8100 rotor (CS150GXL Micro Ultracentrifuge) for 24 h. The CsCl purified EhV suspensions were stored at 4°C until further uses. The viral DNA was isolated from EhV99B1 by using CTAB method (Sambrook et al., 2001).

2.3 Cloning and sequencing of SPT gene

SPT gene of EhV99B1 was amplified by PCR using one pair specific primers designed according to SPT ORF of genomic sequences of EhV-86 (GenBankTM accession number: NC_007346). The forward primer F5'- AAT CCT AGG ATG TAC ACG GCC GTA TTC AT -3' and reverse primer R5'- ATT TGC GGC CGC CTA CGA CAG ATA CTC -3', that introduce an *Avr* II and a *Not* I site at the N terminus and C terminus of the coding sequence, respectively. Virus DNA (100 ng) was added to 25 μ l of a PCR reaction mixture which contained KOD DNA polymerase, assay buffer (50 mmol/L Tris-HCl; pH 9.0, 1 mmol/L EDTA, 1 mmol/L DTT, 0.001% Tween 20, 0.001%

NP-40, 5% glycerol), 1.5 mmol/L 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) (98°C for 2 min, 35 cycles at 94°C for 15 s, 50°C for 30 s, 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 pTA2-T Vector System (TOYOBO) for sequencing after poly A was added by Target cloneTM (TOYOBO).

2.4 Bioinformatics analysis of EhV99B1-SPT gene

After sequencing, homology searches with DNA sequences were carried out using the BLAST algorithm provided by the Internet Service of the National Centre for Biotechnology Information (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Maximum likelihood phylogenetic tree of SPT was predicted by using the program Mega 4.1 (<http://www.megasoftware.net>). The molecular mass and theoretical pI of EhV99B1-SPT were calculated by Expasy pI/Mw tool (http://us.expasy.org/tools/pi_tool.html). The amino acid composition of EhV99B1-SPT was accounted by SARS (http://www.isrec.isb-sib.ch/software/SAPS_form.html). Hydropathy analysis was carried out by ProtScale (<http://www.expasy.org/cgi-bin/protscale.pl>). Transmembrane helices and sheets prediction of EhV99B1-SPT were predicted by using the program TMHMM (<http://www.cbs.dtu.dk/services/TMHMM/>). The subcellular localization of EhV99B1-SPT was predicted by PTARGET (<http://www.expasy.org/cgi-bin/protscale.pl>). The catalytic center of this enzyme was predicted by Prosite (<http://au.expasy.org/prosite/>). The potential N-linked glycosylation sites were predicted by DictyOGlyc1.1 (<http://www.cbs.dtu.dk/services/DictyOGlyc/>). The secondary structure of EhV99B1-SPT were predicted by using the program ESPrip (<http://escript.ibcp.fr/ESPrpt/ESPrpt/index.php>). The prediction of conformation was performed using SWISS MODEL method (<http://swiss-model.expasy.org/>). The GenBank/NCBI accession number of EhV99B1-SPT sequence reported in this paper is JF429838.

2.5 Expression of recombinant EhV99B1-LCB2 and functional characterization

To elucidate the catalytic function of the EhV99B1-SPT protein, we described the expression

of its catalytic subunit gene (LCB2) in *E. coli* cells. To obtain EhV99B1-LCB2 gene, specific primers were derived from the recombinant EhV99B1-SPT, (forward) 5'-CGGATCCATGTACACGGCCGTATTCATA-3' and (reverse) 5'-TTGCGGCCGCATGCGTTTTCTT-TGACTCGG-3'. The fragments were subcloned and sequenced. To obtain a sufficient amount of soluble recombinant protein, LCB2 was overproduced in *E. coli* BL21 using the pGEX4T-3 vector. The insert of LCB2 cloned into the pMD19-T vector was digested with *Bam*H I and *Not* I and cloned into the *Bam*H I/*Not* I sites of the pGEX4T-3 expression vector. The recombinant plasmid was designated pGEX4T-3-LCB2. *E. coli* BL21 was transformed with the pGEX4T-3-LCB2 construct, and a single positive colony was inoculated in 1 L of LB medium plus ampicillin and grown at 37°C until A_{600} reached 0.7. Then fusion protein was induced by the addition of isopropyl-1-thio- β -D-galactopyranoside (Sigma) to a final concentration of 1.0 mmol/L, and growth was continued for another 5 h. Cells from one liter culture were harvested by centrifugation at $12\,000 \times g$ for 10 min at 4°C.

To confirm that the recombinant pGEX4T-3-LCB2 were capable of forming sphingoid bases in *E. coli* cells, total lipids were extracted by the Bligh and Dyer method (Bligh et al., 1959). To obtain total sphingoid bases, total lipids were hydrolysed with 1 ml of 1 mol/L HCl in methanol at 95°C for 2 h. The production was adjusted to alkalescence with 5 mol/L NaOH. The sphingoid bases were extracted with diethyl ether and subjected to a silica-gel TLC plate developed with chloroform: methanol: acetic acid (19:0.8:0.1, by volume) as a solvent system. The plates are largely sprayed until translucent with 1% (w/v) cupric sulfate in 8% phosphoric acid and dried for 5 min at 150°C. The sphingoid bases would form black spots and were visualized on the plate.

3 Results

3.1 Gene clone and bioinformatics characterization of EhV99B1-SPT

PCR with specific primers and EhV99B1 genome DNA as a template resulted in the amplification of the full length SPT ORF with the size of 2 613 bp. The amplification product was cloned into the pTA2-T vector and transformants verified by digest with *Avr* II/*Not* I (Fig. 1) and then sequenced.

3.1.1 Phylogenetic inference tree between the sequences of SPT gene for members of the Coccolithovirus family

A phylogenetic tree based on the SPT homologous regions of *Coccolithoviridae* revealed that EhV99B1 was about 97%–99% identity in the nucleotide sequence with other EhV isolates. EhV99B1 from surface water, Raunefjorden, Norway (RN) was high homologous to other EhV isolates same from RN (EhV-163, EhV-V1 and EhV-V2), with bootstrap value 95. Virus isolates (EhV-201, EhV-202, EhV-205, EhV-207 and EhV-208) from Western English Channel, off the coast of Plymouth, UK (PE) were different to virus isolates (EhV-84, EhV-86 and EhV-88) from RN. Virus isolates (EhV-84, EhV-86) from 15 m depth water, RN which belong to the same branching position were different to EhV-88 from 5 m depth water, RN (Fig. 2).

Fig.1. Agarose gel electrophoresis of the digest product of the pTA2-SPT recombinant plasmid. M represents λ -Hind III digest DNA Marker, Lane 1 pTA2-SPT recombinant plasmid, Lane 2 pTA2-SPT/*Not* I+*Bln* I, Lane 3 PCR product of the pTA2-SPT recombinant plasmid.

3.1.2 Primary structure analysis of EhV99B1-SPT deduced amino acid sequence

EhV99B1-SPT gene had an open reading frame of 2 613 bp, which contains a deduced amino acid sequence with 871 residues including 88 acidic amino acids (account for 10.1%), 75 alkaline amino acid (account for 8.6%), 79 hydrophobic residues-vlaine

Fig.2. Phylogenetic inference tree between the sequences of SPT gene for members of the *Coccolithovirus* family. The number of substitutions per site is indicated under the scale bar.

(account for 9.1%). Hydrophobic profiles predicted that EhV99B1-SPT contained five hydrophobic regions (scores > 1.7). The significant hydrophobic region resided in the N-terminal region of EhV99B1-SPT. Transmembrane domains (TMDs) were predicted to have five transmembrane domains (scores > 700) including three inside to outside helices and two outside to inside helices, and were highlighted by blue boxes (Figs 3 and 4). One obvious transmembrane domain resided in the EhV99B1-LCB2 between 160 and 180 residues (Fig. 3). It was a one-to-one correspondence between the TMDs and hydrophobic regions.

3.1.3 Secondary and Tertiary structure analysis of EhV99B1-SPT deduced amino acid sequence

Secondary structure of EhV99B1-SPT showed that it was composed of 26 α -helices, 27 β -strands, and 19 β -turns connected by random coil (Fig. 3). In the residues 260–310 of EhV99B1-LCB2, it contains a central motif, TFTKSFGSVG, which was distributed symmetrically by α -helices and β -strands (α 9- β 10- β 11-motif- β 12- β 13- α 10) like a “pocket” (Fig. 4). We also observed Cys 535 of EhV99B1-LCB1 resided between one face of the pyridinium ring of PLP and central motif (TFTKSFGSVG).

EhV99B1-LCB2 has 30.184% identity (evalue: 2e-52) with *Sphingobacterium multivorum* SPT (PDB code 3a2b) and EhV99B1-LCB1 has 22.1% identity (evalue: 2e-27) with *Sphingomonas paucimobilis* SPT (PDB code 2w8w). The 3D structure of EhV99B1-SPT was constructed by Swiss PDB viewer

(<http://spdbv.vital-it.ch/>) based on combination of both the above results and algorithm of homology models (Liu et al., 2010). This 3D structure was highly similar to other members of the AOS family (such as 8-amino-7-oxononanoate synthase, 5-aminolevulinate synthase and 2-amino-3-ketobutyrate coenzyme) (data not shown). The central motif, TFTKSFG was composed of two α -helices and four β -Strands (“pocket”). The EhV99B1-LCB2 carried a lys 307 residue and EhV99B1-LCB1 carried a cys 535 residue expected to form a Schiff base with PLP. The His 197, Ala 198 and Ser 199 residues were involved in cofactor binding (Figs 4 and 5).

3.2 Expression and characterization of recombinant EhV99B1-LCB2

PCR with recombinant EhV99B1-SPT as a template resulted in amplification of LCB2 fragment with the size of 1 416 bp. To elucidate the catalytic function of the EhV99B1-LCB2 protein, IPTG-induced cultures of transformed *E. coli* cells were used to prepare sphingoid bases. The fusion protein migrated on SDS-PAGE with an apparent molecular mass of 78.2 kDa (Fig. 6). GST-LCB2 fusion protein showed about 50% soluble protein production and about 50% of the products appeared in the cellular supernatant fraction (Fig. 6 and Lines 5 to 6). When the recombinant strain was induced with 1 mmol/L IPTG at 37°C for 5 h, recombinant EhV99B1-LCB2 was capable of forming new sphingoid bases (Fig. 7), confirming the capability of expressing a LCB2 of virus origin.

4 Discussion

The phylogeny and microarray approach have been used to assess the diversity among the *coccolithovirus* and showed both geographical and temporal patterns (Allen et al., 2007; Dunigan et al., 2006). Our research also supports this result and shows that 12 EhV isolates were relatively small genetic variations and closely related evolutionary relationships. However, it is limited for determining evolutionary relationships between *coccolithovirus* and other members of *Phycodnaviridae* families by using single gene for phylogeny analysis. Three groups of proteins (such as ehv 030, 072, 085, 104, 128, 141, 438 and 459 belonging to Group I and further other genes belonging to Groups II and III) from members of the nucleocytoplasmic large DNA viruses (NCLDV) were concatenated for phylogenetic analysis and it showed that

Fig.3. Secondary structural elements of EhV99B1-SPT model. a. An alignment of residues 60–385 of the EhV99B1-SPT with other species' LCB2, and b. an alignment of residues 461–753 of the EhV99B1-SPT with other species' LCB1. The black-boxed sequences are conserved catalytic residue of EhV99B1-SPT (indicated with pentagram). The blue-boxed sequences are possible transmembrane domain (indicated with TMD). The green-boxed residues are potential glycosylation sites (indicated with ▲). The alpha helices (squiggles), beta turns (TT letters) and beta strands (arrows) are marked on the top of alignment sequence.

Fig.4. The enzyme active center of EhV99B1-SPT. Overlay of key conserved residue: His290, His197, Ala198, Ser199, Lys307 and Cys53(LCB1). The conserved (His, Ala, Ser) motif interacts with N1 of PLP and Serine. The catalytic residue is TFTSFG.

Fig.5. The 3-dimensional structures of EhV99B1-SPT. The catalytic residue is marked with black-box. The helices, sheets and loops are highlighted with red, yellow and green, respectively.

Phycodnaviridae (including *coccolithovirus*) most likely diverged from the common ancestral NCLDV (Allen et al., 2006). Since SPT has never been identified in *Phycodnaviridae* before *Coccolithoviridae*, SPT gene loss within the *Phycodnaviridae* families

is thought to contribute to the divergence between *Coccolithoviridae* and other members of *Phycodnaviridae* families (Allen et al., 2006; Wilson et al., 2005; Wilson et al., 2009).

Coccolithovirida as a reservoir of genetic diversity

Fig.6. Analysis of GST-LCB2 fusion protein expressed in *E. coli* by SDS-PAGE. The expression was induced with 1.0 mmol/L IPTG at 37°C for 5 h. Lane M represents MR markers, sizes of which are indicated to the left. Lanes 1–2 represent noninduced and induced *E. coli* (pGEX4T-3) culture, Lanes 3–4 noninduced and induced whole cell lysates of *E. coli* (pGEX4T-3-LCB2) cultures, and Lanes 5–6 induced cell extract supernatant and sediment of *E. coli* (pGEX4T-3-LCB2) cultures.

Fig.7. In vivo production of sphingoid bases expressed in *E. coli*. Lipid extracts were separated by silica-gel TLC. Control means empty vector (pGEX4T-3); +IPTG LCB2-carrying pGEX4T-3 was induced by IPTG; -IPTG LCB2-carrying pGEX4T-3 was not induced by IPTG.

could move host-derived genetic material from one organism to another, even from one ecosystem to another (Rohwer et al., 2009). It is called “horizontal gene transfer”. It has been documented that SPT gene was readily identified in *E. huxleyi* and EhV genome

(Monier et al., 2009) and they are likely to share a common “domain architectures” which are the presence of a single open reading frame (ORF). The ORF encodes a eukaryotic-like SPT in which its N-terminal domain most closely resembled the LCB2 subunit and the C-terminal domain most closely resembled the LCB1 subunit (Monier et al., 2009). Thus, we hypothesized that EhV-SPT might be derived from its host genome. To verify this assume, we constructed maximum likelihood phylogenetic tree based on the amino acid sequences of SPT-LCB1 and SPT-LCB2 shared by EhV99B1, *E. huxleyi* and the widest taxonomic range of homologs available in GenBank (Fig. 7). In this case, 99% bootstrap values for the LCB1 domain supported clustering of EhV-99B1 and *E. huxleyi* protein sequences. Curiously, both LCB2 of EhV-99B1 and *E. huxleyi* shared only 46% identity. It is difficult to illustrate that a deep phylogenetic origin between EhV99B1-SPT and *E. huxleyi*-SPT within eukaryotes. We hypothesized that EhV99B1-SPT was not directly derived from *E. huxleyi*, and was horizontally transferred between virus and eukaryote or its host (simultaneity or individually), ultimately leading to form *E. huxleyi*-SPT and EhV99B1-SPT, respectively.

EhV99B1-SPT was predicted to be an integral membrane protein. This is supported by one study showing that recombinant EhV86-SPT is a membrane-associated protein (Han et al., 2006). It has shown that ceramide has been obtained from plasmalemma (such as cytomembrane, microsome and endoplasmic reticulum) (Hannun et al., 2001). As a result, it could be concluded that EhV99B1-SPT is more likely to be resided in plasmalemma. Moreover, several potential N-linked glycosylation sites were presented in EhV99B1-LCB2. These results suggest that SPT was localized in endoplasmic reticulum (ER). However, subcellular localization prediction suggests that EhV99B1-LCB1 resided in cytoplasm. These seemingly contradictory results are possibly true, because these results were consistent with previous studies on the LCB1 of mammalian SPT which was proved to localize in cytoplasm (Yasuda et al., 2003) and the LCB2 of *Arabidopsis* SPT and yeast SPT was in the ER (Tamura et al., 2001; Han et al., 2004). It is known that eukaryotic SPTs are membrane bound and the active site facing the cytoplasm (Futerman et al., 2005). It is presumed to be an ER multimeric protein that its one subunit extended through the cytoplasm and was contiguous with TMD. As evidence aliunde, the substrates of SPT, serine and palmitoyl-CoA, with

cytoplasmic localization can move between cytoplasm and Golgi through vesicular transport (Han et al., 2004). Thus, we hypothesized that the C-terminus of EhV99B1-SPT was the binding site of substrates and the catalytic center of the N-terminus of EhV99B1-SPT interact with the cytoplasm, as well as within the lumen of ER. This “conjugated heterodimer-like enzyme” employs a pump to drive sphingolipid from cytoplasm to endoplasmic reticulum. This process would play an important role in subcellular infection dynamics. EhV99B1-LCB2 most closely resembles catalytic subunit. Two lines of evidence support this view: first, mutation of the EhV86-LCB2 was found to inactivate this enzyme (Han et al., 2006); second, our study has shown that recombinant EhV99B1-LCB2 was capable of forming new sphingoid bases and encoded an active catalytic subunit. In addition, it has been shown that EhV86-SPT exhibits a unique substrate preference to *de novo* synthesized novel ceramide or GSL (Jermy, 2010; Kolesnick et al., 2000). Recombinant EhV99B1-LCB2 was capable of producing sphingoid bases, which have never been identified in *E. coli* before. It suggests that recombinant EhV99B1-LCB2 is functionally similar to recombinant EhV86-SPT without co-expressing with EhV99B1-LCB1. These results have indicated that EhV-SPT as single-chain fusion proteins are novel enzymatically active in prokaryotic or eukaryotic expression system. It can be concluded that native EhV-LCB2 may be involved in infection process. However, mutations in the human SPTLC1 gene (LCB1) could cause decreased activity, leading to hereditary sensory neuropathy type I (Bejaoui et al., 2001; Gable et al., 2010) and the human Lcb1p at 180 cysteine is in a potential active site for N-myristoylation and membrane association of SPT (Gable et al., 2002). This site can also be found in EhV99B1-LCB1. It could be concluded that the EhV99B1-LCB1 had the similar function with SPTLC1 gene. Indeed, EhV99B1-LCB1 most closely resembles regulatory subunit. In addition, it has shown that Tsc3p or ssSPT, these small subunits are required for optimal SPT activity in yeast or mammalian and do not seem to have effects on EhV86-SPT (Gable et al., 2000; Han et al., 2009). However, it is unknown whether EhV99B1 contains one gene like Tsc3p or ssSPT throughout the genome. If these putative genes are present, EhV99B1-SPT would be dependent on them for intact activity. Thus, this intriguing domain architecture of EhV99B1-SPT was probably one way to allow viruses to manipulate and contribute

to the synthesis of ceramide, and this process eventually causes the host cell to die.

The EhV-encoded SPT gene is expressed and host SPT is reduced to low transcriptional levels during infection (Pagarete et al., 2009). It indicates that there could exist potential relationship between viral and host genes, and this host-virus interaction is one of the features of co-evolutionary strategy, which is called “Red Queen” hypothesis in action. It argues for an intense “arms race” between viruses, trying to turn over host apoptotic pathway for replication and proliferation of itself, and host, trying to trigger apoptotic pathway to avoid the spread of viruses and colony collapse. In this process, SPT encoded by EhV exhibits a unique substrate preference to *de novo* synthesized novel ceramide or GSL (Jermy, 2010; Kolesnick et al., 2000). These viral ceramide or GSL are distinct from host-specific ceramide or GSL involving in the intra- and intercellular signal transduction apoptosis pathways in *E. huxleyi*. Clearly, EhV-SPT plays an obvious role in this co-evolutionary strategy.

Natural mortality of phytoplankton induced by virus has been recognized as an important factor that caused the loss of marine microalgae, especially in ecological dynamics between EhV and *E. huxleyi* (Liu et al., 2009). EhV99B1-SPT implicates in the regulation of apoptosis that plays important roles in bloom dynamics of *E. huxleyi*. It has been demonstrated that *E. huxleyi* displayed apoptosis characteristics of morphology and biochemistry during EhV infection (Bidle et al., 2011). However, it is unknown whether the expression of EhV99B1-SPT activated or inhibited the process of apoptosis in *E. huxleyi*. One study suggests that ceramide prevents PCD induced by nerve growth factor (NGF) deprivation (Ito et al., 1995). EhV99B1-SPT also was thought to take over the sphingolipid metabolic pathway in host and can be termed the viral shunt (Wilhelm et al., 1999). However, Sequence analysis of this viral genome has shown that it lacks a critical enzyme (3-ketosphinganine reductase) for sphingolipid biosynthetic activity. An unusual glucosylceramide from *E. huxleyi* infected with EhV was isolated, implicating sphingolipids in the lysis of this alga. It suggests that the novel sphingolipids are not the only product of viral genomes, because EhV-encoded genes contain only a subset of the activities (such as dihydroceramide synthase, fatty acid desaturase, lipid phosphate phosphatase, transmembrane fatty acid elongation protein, sterol desaturase, fatty acid desaturase) required to generate the novel

sphingolipid, implying that its synthesis is the result of coordinated interactions between algal- and viral-encoded biosynthetic enzymes. The 3-ketosphinganine reductase is dispersed in the *E. huxleyi* genome, which does not encode any obvious homologs for transmembrane fatty acid elongation protein and sterol desaturase. It suggests the metabolic complementation of

EhV99B1 with its host in the entire sphingolipid synthesis pathway. This complementation and expression of EhV-SPT lead to *de novo* sphingolipid biosynthesis pathway (SBP) (Pagarete et al., 2009). *De novo* fatty acid may play an important role in an enveloped virus release, intra- and intercellular signal transduction pathways and host apoptosis (Evans et al., 2009).

Fig.8. Maximum likelihood phylogenetic trees based on the amino acid sequences of serine palmitoyl-transferase LCB1 and LCB2 domain sequences and their homologs. The number of substitutions per site is indicated under the scale bar. Sequences are best hitting to EhV99B1-SPT (GeneBank accession No. JF429838) after NCBI/CDD searches.

In conclusion, this work contributed to our understanding of the gene composition, evolution and horizontal transfer between marine virus and its host, their ecological significance, as well as host-virus interactions. It can provide important insights into the role of this gene in its native host. The viral-encoded SPT as sufficient novelty and importantly gene storing in DNA library would facilitate gene manipulation and reform in the future. Besides, this suggests that trans-dominant nature of the viral-encoded sphingolipid biosynthetic activities also make the EhV99B1-SPT gene serve as a useful paradigm for metabolic

engineering and studies and exploit a novel way for ceramide production in the future.

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References

- Allen M J, Howard J A, Lilley K S, et al. 2008. Proteomic analysis of the EhV-86 virion. *Proteome Science*, 6(11): 1–6

- Allen M J, Schroeder D C, Holden M T, et al. 2006. Evolutionary history of the *Coccolithoviridae*. *Molecular Biology and Evolution*, 23(1): 86–92
- Allen M J, Martinez-Martinez J, Schroeder D C, et al. 2007. Use of microarrays to assess viral diversity: from genotype to phenotype. *Environmental Microbiology*, 9(4): 971–982
- Baumann K H, Bockel B, Frenz M. 2004. Coccolith contribution to South Atlantic carbonate sedimentation. In: Thierstein H R, Young J R, eds. *Coccolithophores: From Molecular Processes to Global Impact*. Berlin, Heidelberg, New York: Springer, 367–402
- Bejaoui K, Wu C, Scheffler M D, et al. 2001. SPTLC1 is mutated in hereditary sensory neuropathy, type 1. *Nature Genetics*, 27(3): 261–262
- Bidle K D, Vardi A. 2011. A chemical arms race at sea mediates algal host-virus interactions. *Current Opinion in Microbiology*, 14(4): 449–457
- Bidle K D, Falkowski P G. 2004. Cell death in planktonic, photosynthetic microorganisms. *Nature Reviews Microbiology*, 2: 643–655
- Bligh E G, Dyer W J. 1959. A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology*, 37(8): 911–917
- Bratbak G, Egge J K, Heldal M. 1993. Viral mortality of the marine alga *Emiliania huxleyi* (*Haptophyceae*) and termination of algal blooms. *Marine Ecology Progress Series*, 93: 39–48
- Brussaard C P D. 2004. Viral control of phytoplankton populations—a review. *The Journal of Eukaryotic Microbiology*, 51(2): 125–138
- Buede R, Rinker-Schaffer C, Pinto W J, et al. 1991. Cloning and characterization of LCB1, a *Saccharomyces* gene required for biosynthesis of the long-chain base component of sphingolipids. *Journal of Bacteriology*, 173(14): 4325–4332
- Castberg T, Thyraug R, Larsen A, et al. 2002. Isolation and characterization of a virus that infects *Emiliania huxleyi* (*haptophyta*). *Journal of Phycology*, 38(4): 767–774
- Charlson R J, Lovelock J E, Andreae M O, et al. 1987. Oceanic phytoplankton, atmospheric sulphur, cloud albedo and climate. *Nature*, 326: 655–661
- Dunigan D D, Fitzgerald L A, Van Etten J L. 2006. Phycodnaviruses: a peek at genetic diversity. *Virus Research*, 117(1): 119–132
- Evans C, Pond D W, Wilson W H. 2009. Changes in *Emiliania huxleyi* fatty acid profiles during infection with *E. huxleyi* virus 86: physiological and ecological implications. *Aquatic Microbial Ecology*, 55(3): 219–228
- Field C B, Behrenfeld M J, Randerson J T, et al. 1998. Primary production of the biosphere: integrating terrestrial and oceanic components. *Science*, 281(5374): 237–240
- Futerman A H, Riezman H. 2005. The ins and outs of sphingolipid synthesis. *Trends in Cell Biology*, 15(6): 312–318
- Gable K, Gupta S D, Han G, et al. 2010. A disease-causing mutation in the active site of serine palmitoyltransferase causes catalytic promiscuity. *Journal of Biological Chemistry*, 285(30): 22846–22852
- Gable K, Han G, Monaghan E, et al. 2002. Mutations in the yeast LCB1 and LCB2 genes, including those corresponding to the hereditary sensory neuropathy type I mutations, dominantly inactivate serine palmitoyltransferase. *Journal of Biological Chemistry*, 277(12): 10194–10200
- Gable K, Slife H, Bacikova D, et al. 2000. Tsc3p is an 80-amino acid protein associated with serine palmitoyltransferase and required for optimal enzyme activity. *Journal of Biological Chemistry*, 275(11): 7597–7603
- Guillard R R L. 1975. Culture of phytoplankton for feeding marine invertebrates. In: Smith W L, Chanley M H, eds. *Culture of Marine Invertebrate Animals*. New York: Plenum Press, 29–60
- Han G, Gable K, Yan L, et al. 2006. Expression of a novel marine viral single-chain serine palmitoyltransferase and construction of yeast and mammalian single-chain chimera. *Journal of Biological Chemistry*, 281(52): 39935–39942
- Han G, Gable K, Yan L, et al. 2004. The topology of the Lcb1p subunit of yeast serine palmitoyltransferase. *Journal of Biological Chemistry*, 279(51): 53707–53716
- Han G, Gupta S D, Gable K, et al. 2009. Identification of small subunits of mammalian serine palmitoyltransferase that confer distinct acyl-CoA substrate specificities. *Proceedings of the National Academy of Sciences of the United States of America*, 106(20): 8186–8191
- Hannun Y A, Luberto C, Argaves K M. 2001. Enzymes of sphingolipid metabolism: from modular to integrative signaling. *Biochemistry*, 40(16): 4893–4903
- Holligan P M, Viollier M, Harbour D S, et al. 1983. Satellite and ship studies of coccolithophore production along a continental shelf edge. *Nature*, 304: 339–342
- Iglesias-Rodriguez M D, Halloran P R, Rickaby R E M, et al. 2008. Phytoplankton Calcification in a High-CO₂ World. *Science*, 320(5874): 336–340
- Ikushiro H, Hayashi H, Kagamiyama H. 2001. A water-soluble homodimeric serine palmitoyltransferase from *Sphingomonas paucimobilis* EY2395T strain. Purification, characterization, cloning, and overproduction. *Journal of Biological Chemistry*, 276(21): 18249–18256

- Ito A, Horigome K. 1995. Ceramide prevents neuronal programmed cell death induced by nerve growth factor deprivation. *Journal of Neurochemistry*, 65(1): 463–466
- Jermey A. 2010. Environmental microbiology: Viral lipid in bloom. *Nature*, 8(1): 6
- Kolesnick R N, Goni F M, Alonso A. 2000. Compartmentalization of ceramide signaling: physical foundations and biological effects. *Journal of Cellular Physiology*, 184(3): 285–300
- Larsen J B, Larsen A, Bratbak G, et al. 2008. Phylogenetic analysis of members of the Phycodnaviridae virus family, using amplified fragments of the major capsid protein gene. *Applied and Environmental Microbiology*, 74(10): 3048–3057
- Liu Guangfei, Zhou Jiti, Wang Jing, et al. 2010. Homology modeling and analysis of three-dimensional structure of azoreductase of *Rhodobacter sphaeroides*. *Journal of Dalian University of Technology (in Chinese)*, 50(2): 171–175
- Liu Jingwen, Dong Shuanglin, Zhang Zhilan, et al. 2009. Microecological relationship between microalgal virus and host. *Periodical of Ocean University of China*, 39(1): 26–34
- Michaelson L V, Dunn T M, Napier J A. 2010. Viral trans-dominant manipulation of algal sphingolipids. *Trends in Plant Science*, 15(12): 651–655
- Monier A, Pagarete A, De Vargas C, et al. 2009. Horizontal gene transfer of an entire metabolic pathway between a eukaryotic alga and its DNA virus. *Genome Research*, 19(8): 1441–1449
- Nagiec M M, Baltisberger J A, Wells G B, et al. 1994. The LCB2 gene of *Saccharomyces* and the related LCB1 gene encode subunits of serine palmitoyltransferase, the initial enzyme in sphingolipid synthesis. *Proceedings of the National Academy of Sciences*, 91(17): 7899–7902
- Pagarete A, Allen M J, Wilson W H, et al. 2009. Host-virus shift of the sphingolipid pathway along an *Emiliania huxleyi* bloom: survival of the fattest. *Environmental Microbiology*, 11(11): 2840–2848
- Rohwer F, Thurber R V. 2009. Viruses manipulate the marine environment. *Nature*, 459: 207–212
- Salzmann D, Christen P, Mehta P K, et al. 2000. Rates of evolution of pyridoxal-5'-phosphate-dependent enzymes. *Biochemical and Biophysical Research Communications*, 270(2): 576–580
- Sambrook J, Russell D W. 2001. *Molecular cloning: A Laboratory Manual*. New York: Harbor Laboratory, 1–96
- Stefels J, Steinke M, Turner S, et al. 2007. Environmental constraints on the production and removal of the climatically active gas dimethylsulphide (DMS) and implications for ecosystem modelling. *Biogeochemistry*, 83(1–3): 245–275
- Suttle C A. 2007. Marine viruses-major players in the global ecosystem. *Nature Reviews Microbiology*, 5(10): 801–812
- Tamura K, Mitsushashi N, Hara-Nishimura I, et al. 2001. Characterization of an *Arabidopsis* cDNA encoding a subunit of serine palmitoyltransferase, the initial enzyme in sphingolipid biosynthesis. *Plant and Cell Physiology*, 42(11): 1274–1281
- Weiss B, Stoffel W. 1997. Human and murine serine-palmitoyl-CoA transferase—cloning, expression and characterization of the key enzyme in sphingolipid synthesis. *European Journal of Biochemistry*, 249(1): 239–247
- Wilhelm S W, Suttle C A. 1999. Viruses and nutrient cycles in the sea. *Bioscience*, 49(10): 781–788
- Wilson W H, Schroeder D C, Allen M J, et al. 2005. Complete genome sequence and lytic phase transcription profile of a Coccolithovirus. *Science*, 309(5737): 1090–1092
- Wilson W H, Tarran G A, Schroeder D, et al. 2002. Isolation of viruses responsible for the demise of an *Emiliania huxleyi* bloom in the English Channel. *Journal of the Marine Biological Association of the United Kingdom (New Series)*, 82(3): 369–377
- Wilson W H, Van Etten J L, Allen M J. 2009. The Phycodnaviridae: the story of how tiny giants rule the world. *Curr Top Microbiol Immunol*, 328: 1–42
- Yasuda S, Nishijima M, Hanada K. 2003. Localization, topology, and function of the LCB1 subunit of serine palmitoyltransferase in mammalian cells. *Journal of Biological Chemistry*, 278(6): 4176–4183