Subcellular localization and function study of a secreted phospholipase C from *Nocardia seriolae*

Liqun Xia [1, 2 ‡], Haiying Liang [1 ‡], Liang Xu [1], Jianlin Chen [1], Michaël Bekaert [4], Honglian Zhang [1], Yishan Lu [1, 2, 3 *]

（1. College of Fisheries, Guangdong Ocean University, Zhanjiang 524088, PR China; 2. Shenzhen Research Institute of Guangdong Ocean University, Shenzhen 518108, PR China; 3. Guangdong Provincial Key Laboratory of Pathogenic Biology and Epidemiology for Aquatic Economic Animals, Zhanjiang 524088, PR China; 4. Institute of Aquaculture, University of Stirling, Stirling, FK9 4LA, UK）

* Corresponding author. College of Fisheries, Guangdong Ocean University, 1 Haida Road, Mazhang District, Zhanjiang 524088, PR China. Tel: +86-755-23250536; Fax: +86-755-28380068; E-mail address: fishdis@163.com.

‡ These authors contributed to the work equally and should be regarded as co-first authors.

One sentence summary: The NsPLC is a secreted protein which exhibits a punctate distribution near the nucleus in FHM cells and may participate in the cell apoptosis regulation.

ABSTRACT

Fish nocardiosis is a chronic systemic granulomatous disease, and *Nocardia seriolae* is the main pathogen that causes this disease. But the pathogenesis and virulence factors of *N. seriolae* are not fully understood. A phospholipase C (PLC), which was likely to be a secreted protein targeting host cell mitochondria, was found by the bioinformatics analysis on the whole genome sequence of *N. seriolae*. In order to determine the subcellular localization and study the preliminary function of PLC from *N. seriolae* (NsPLC), the gene cloning, secreted protein identification, subcellular localization in host cells and apoptosis detection of NsPLC were carried out in this study. The results showed that NsPLC was a secreted protein by mass spectrometry analysis of extracellular products from *N. seriolae*. Subcellular localization of NsPLC-GFP fusion protein in FHM cells revealed that the green fluorescence exhibited a punctate distribution near the nucleus and did not co-localize with mitochondria. In addition, apoptosis assay suggested that apoptosis was induced in FHM cells by the overexpression of NsPLC. This study may lay the foundation for further study on the function of NsPLC and promote the understanding of the virulence factors and pathogenic mechanism of *N. seriolae*.

Keyword: *Nocardia seriolae*; phospholipase C; subcellular localization; secreted protein; cell apoptosis; overexpression

INTRODUCTION

Fish nocardiosis is a chronic systemic granulomatous disease, which has influenced the Asian aquaculture systems seriously (Ho, et al. 2016, Vu-Khac, et al. 2016). Three species of Nocardia have been identified as the pathogen of fish nocardiosis. They are *Nocardia seriolae*, *N. salmonicida* and *N. asteroides* (Xia, et al. 2015b). Notably, *N. seriolae* was shown to be the main pathogen of fish nocardiosis in recent years. It is reported that *N. seriolae* can infect over 20 kinds of freshwater fish and sea fish, such as largemouth bass (*Micropterus salmoides*), snakehead (*Channa maculata*), amberjack (*Seriola dumerili*), yellowtail (*S. quinqueradiata*), golden pompano (*Trachinotus ovatus*), snubnose pompano (*T. blochii*) and large yellow croaker (*Larimichthys crocea*) (Shimahara, et al. 2008, Wang, et al. 2009, Xia, et al. 2015a) .

N. seriolae is a Gram-positive facultatively intracellular pathogen (Nayak, et al. 2014), which can evade intracellular killing after being engulfed by the macrophages. Then macrophages may help *N. seriolae* to disseminate in the host and cause infection. But the mechanisms involved in *N. seriolae*-host interaction are not fully understood and the virulence factors of *N. seriolae* are not well studied. Nocardia have effective strategies to survive and colonize in the host, such as resisting oxidative killing by phagocytes, inhibiting phagosome-lysosome fusion, blocking phagosomal acidification, modulating phagosomal function and lysosomal content (Beaman and Beaman 1994). It was shown that the secreted proteins of *N. asteroides* strain GUH-2 can induce the apoptotic death of host cells (Barry and Beaman 2007, Camp, et al. 2003, Loeffler, et al. 2004). The secretome researches of pathogenic actinomycetes have revealed that secreted proteins are closely related to their pathogenicity, especially some mitochondria-targeted proteins may play an important role in modulation of cell death and bacterial pathogenesis (Lartigue and Faustin 2013,

Rudel, et al. 2010, Saint-Georges-Chaumet and Edeas 2016, West, et al. 2011).

According to the bioinformatics analysis of *N. seriolae* ZJ0503 genome (Xia, et al. 2015a), ORF5028 encodes a phospholipase C (PLC) homolog which is likely to be a secreted protein targeting host cell mitochondria. Phospholipases C (PLCs) have been defined to be important virulence factors of many bacterial pathogens including *M. tuberculosis*, *Pseudomonas aeruginosa*, *Clostridium perfringens*, *Bacillus cereus*, *Legionella pneumophila*, *Staphylococcus aureus* and *Listeria monocytogen* (Flores-Diaz, et al. 2016, Goldfine, et al. 1998, Raynaud, et al. 2002). The toxic PLC can interact with eukaryotic cell membranes and hydrolyze phosphatidylcholine and sphingomyelin, leading to cell lysis (Moigne, et al. 2015). The gene cloning, secreted protein identification, subcellular localization and apoptosis detection of *N. seriolae* PLC (NsPLC) were carried out in this study. It may lay the foundation for further study on the function of this gene and promote the understanding of molecular pathogenic mechanism of *N. seriolae*.

MATERIALS AND METHODS

Bacterial strains, cell and plasmids

N. seriolae ZJ0503, isolated from diseased *T. ovatus* in China, was cultured at 28°C in an optimized medium (Xia, et al. 2015b). *Escherichia coli* DH5α was grown in Luria-Bertani (LB) broth at 37 °C with vigorous shaking. Fathead minnow (FHM) epithelial cells (ATCC CCL-42, Manassas, VA)(Gravell and Malsberger 1965) were cultured in Leibovitz's L15 medium containing 10% fetal bovine serum (FBS, Invitrogen, USA) at 25 °C. Plasmid pEGFP-N1 (Clontech, Mountain View, CA) was used for subcellular localization. Plasmid pcDNA3.1/His A (Invitrogen, Carlsbad, CA) was used for overexpression.

Cloning of *NsPLC* and plasmid construction

Genomic DNA extraction from *N. seriolae* was performed as described previously (Xia, et al. 2015b). The PCR primers pEGFP-F/R and pcDNA-F/R (Table 1) were used to amplify the *NsPLC* gene. The PCR was performed with KOD-plus-Neo DNA polymerase (Toyobo, Osaka, Japan) using the following PCR procedure: pre-denaturation at 98 ℃ for 2 min, 30 cycles at 98 ℃ for 10 sec, 55 ℃ for 15 sec, and 68 ℃ for 15 sec, a final extension at 68℃ for 5 min. Respectively, the amplified fragments were digested by corresponding restriction enzymes and cloned into eukaryotic vectors pEGFP-N1 and pcDNA3.1/His A. These different constructs were confirmed by restriction enzyme digestion and DNA sequencing, then named as pEGFP-PLC and pcDNA-PLC.

Bioinformatics analysis, sequence alignments and phylogenetic analysis

Based on the whole genome sequence data of *N. seriolae* ZJ0503 (Xia, et al. 2015a), the potentially excreted proteins were predicted using LocTree3 and ExPASy-PROSITE. Subcellular localization was predicted by using SignalP 4.1 and LocTree3. The amino acid sequence of NsPLC was analyzed with the online Protein BLAST program and DNASTAR software. 14 PLC sequences from different bacteria were selected for multiple sequence alignments using ClustalX 2.0 and GeneDoc, and different bacterial PLC sequences were also used for phylogenetic tree analysis with MEGA5.0 by the neighbor-joining method (Tamura, et al. 2011).

Identification of sereted proteins

The extracellular products of *N. seriolae* were obtained by a cellophane overlay method with modification (Sudheesh, et al. 2007). Briefly, *N. seriolae* was cultured on optimized medium agar plates and bacterial cell suspension was prepared with single colonies. 200 μL of the bacterial cell

suspension was spread on optimized medium plates covered with sterile cellophane sheet and incubated at 28 ℃ for 3-5 d. Cells of *N. seriolae* grown on the cellophane sheet were washed with PBS (0.01M, pH 7.2) and centrifuged at 8000 g for 20 min. Then the supernatant containing extracellular products was filter sterilized with a 0.2 μm membrane filter. Following filtering, the sterilized supernatant was transfered into dialysis tubing (3.5k MW) and dialysed in ultra pure water at 4 ℃ for 16-24 h (water was changed for three times). The supernatant was transfered into a centrifuge tube after dialysis, freezed under -80 ℃, and lyophilized using a vacuum freeze dryer to get the protein dry powder. The powder of extracellular products was identified using shotgun mass spectrometry (MS).

Cell culture, transfection and staining

Plasmids were prepared using an endotoxin-free plasmid purification kit (Qiagen Inc., Chatsworth, CA). FHM cells were cultured in 24-well plates and grown to 70% confluency (for subcellular localization) or 90% confluency (for overexpression). Different plasmids were transfected into FHM cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The mitochondria of FHM cells were stained with 300 nM MitoTracker Red CMXRos dye (Molecular Probes, Carlsbad, CA) at 28 ℃ for 45min, and then the FHM cells were fixed with 4% paraformaldehyde for 30 min. Finally, the nucleuses of FHM cells were labeled with 1 μg/mL diamidino-2-phenylindole (DAPI) at room temperature for 10 min. The fluorescence exhibited in FHM cells were observed using fluorescence microscope (Leica DM IRB).

Subcellular localization in host cells

To determine the subcellular localization of NsPLC in host cells, FHM cells were transiently transfected with pEGFP-PLC and the control plasmid pEGFP-N1, respectively. Being stained with

both MitoTracker Red and DAPI at 48 h posttransfection, the FHM cells were microscopically observed.

Detection of cell apoptosis

To test whether the overexpression of NsPLC induces apoptosis in fish cells, FHM cells were transiently transfected with pcDNA-PLC or the control plasmid pcDNA3.1 His A. Then the FHM cells were stained with DAPI at 48 h posttransfection and microscopically observed. Moreover, the mitochondrial membrane potential (ΔΨm) was measured with a JC-1 assay kit（Beyotime, Shanghai, China)）and the caspase-3 activity was assessed with a caspase-3 colorimetric assay kit (BioVision, Milpitas, CA). At 24 h, 48 h and 72 h posttransfection, the FHM cells were collected and the ΔΨm was determined by the method described previously with minor modification (Sun, et al. 2014). As a positive control for low ΔΨm, FHM cells were treated with carbonyl cyanide 3-chlorophenylhydrazone (CCCP, 10 μM) at 25 °C for 20 min. ΔΨm was measured by the changes in the 590/530 (nm) JC-1 emitted fluorescence with an Enspire 2300 Multilabel Reader (Perkin Elmer, MA, USA). The caspase-3 activity detection was performed as described previously (Zhao, et al. 2010) at 24 h and 48 h posttransfection. Statistical analysis was carried out with SPSS Statistics 15.0 and differences were considered significant when the *p* values were less than 0.05. To confirm the NsPLC expression in pcDNA-PLC transfected FHM cells, RT-PCR and western blot analysis were performed as described previously (Xia, et al. 2010). Briefly, total RNA and protein were isolated from the pcDNA-PLC or pcDNA 3.1 His A transfected FHM cells at 48 h posttransfection. RT-PCR was then performed using primers pcDNA-F/pcDNA-R (Table 1) fellowing the synthesis of cDNA. Western blot analysis was carried out by using mouse anti-His monoclonal antibody (Sigma, St. Louis, MO) as the primary antibody at a dilution of

1:1000 and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Sigma, St. Louis, MO) as the secondary antibody at a dilution of 1:5000.

Sequence analysis and characterization of NsPLC

The *NsPLC* gene was obtained from the genome DNA of *N. seriolae* strain ZJ0503 by PCR. Sequence analysis revealed that the NsPLC was 1524 bp, which encoded 507 amino acids. The calculated molecular weight of NsPLC was 53.7 kDa and the theoretical isoelectric point (pI) was 4.95. The NsPLC was predicted to be a secreted protein by using SignalP 4.1 and LocTree3 and it may co-localize with mitochondria. Functional motifs search for NsPLC with ExPASy-PROSITE revealed that amino acid 1-38 (MGVFAGINRRDFLAKAMAAGGAASLASLAGPIIERAYA) in the N-terminus of this protein is a twin arginine translocation (Tat) signal (Fig. 1).

Protein BLAST showed that the deduced amino acid sequence of NsPLC displayed high homology with other PLC sequences from actinomycetes, ranging from 92% identity with PLC from *N. concava* to 59% identity with membrane-associated PLC from *Mycobacterium tuberculosis* H37Rv. Alignment among NsPLC and other PLC sequences from actinomycetes was shown in Fig. 1, and 5 amino acids (ETHDE) marked with asterisks in Fig. 1 were putative active sites on conserved domain in PLCs. A phylogenetic tree was constructed with amino acid sequences of 14 bacterial PLCs. As shown in Fig. 2, the NsPLC and other PLCs in actinomycetes were clustered within a monophyletic clade with a 100% bootstrap.

Identification of NsPLC as a secreted protein

The extracellular products of *N. seriolae* were obtained and the secreted proteins were identified using shotgun MS. Results showed that three peptide sequences of NsPLC (QAADPNSELAR, FEGITGPIGLGYR, GGLVASETFDHTSQLR) were detected with

confidence greater than or equal to 99%, which proved that NsPLC was a secreted protein.

Subcellular localization of NsPLC in FHM cells

Subcellular localization of NsPLC in fish cells was determined by NsPLC-GFP fusion protein expression. The NsPLC-GFP fusion protein was detected with a strong green fluorescence signal and exhibited a punctate distribution at 48 h posttransfection. The nucleus were shown with blue fluorescence and the mitochondria were displayed with red fluorescence. Comparing with the location of nucleus and mitochondrion in the pEGFP-PLC transfected cells, the NsPLC-GFP fusion protein was found mainly aggregate near the nucleus in the cytoplasm and did not co-localize with the mitochondria (Fig. 3 left). The results demonstrated that NsPLC did not target to mitochondria in FHM cells. Whereas the signal of GFP was distributed in both cytoplasm and nucleus in control cells transfected with pEGFP-N1, and no specific fluorescence co-localized with the mitochondria (Fig. 3 right).

Apoptosis induced in FHM cells by overexpression of NsPLC

To show whether NsPLC involves in apoptosis of fish cells, the plasmid pcDNA-PLC was transfected in FHM cells, and then the ΔΨm and caspase-3 activity in transfected cells were assayed. At 48 h posttransfection, apoptotic bodies were observed in NsPLC overexpressed cells by DAPI staining (Fig. 4A down). While the nucleus remained intact in the control plasmid transfected cells at the same time (Fig. 4A up). ΔΨm detection revealed that ΔΨm showed with JC-1 polymer/monomer fluorescence ratio dropped obviously in NsPLC overexpressed cells (Fig. 4B). The ΔΨm level in NsPLC overexpressed cells was about 0.5 fold lower than that in the control plasmid transfected cells at 48 h and 72 h posttransfection (Fig. 4B). Furthermore, measurement of caspase-3 activity showed that caspase-3 was activated in NsPLC overexpressed

cells (Fig. 4C). The caspase-3 activity value in NsPLC overexpressed cells was approximately 1.5 fold higher than that in control cells at 48 h posttransfection. Both the ΔΨm and caspase-3 activity assay indicated that apoptosis can be induced by the overexpression of NsPLC in FHM cells. The NsPLC expression in pcDNA-PLC transfected FHM cells were confirmed by the presence of a specific band on RT-PCR and western blot analysis (Fig. 4D).

DISUSSION

The NsPLC was successfully cloned in this study, and protein blast revealed it belonging to the PlcC superfamily. PLCs exist in prokaryotic and eukaryotic organisms widely. PLCs are found to be virulence factors in many pathogenic bacteria. Bacterial PLCs are known to generate a signaling lipid molecule sn-1,2-diacylglycerol (DAG), which can activate protein kinase C in macrophages and other cell types (Schmiel and Miller 1999). The *NsPLC* gene was predicted to encode a secreted protein with a Tat signal. By identifying the extracellular products of *N. seriolae* with MS, the NsPLC was proved to be a secreted protein in this research. It has been reported that *P. aeruginosa* and other bacterial pathogens express extracellular PLCs that are secreted through the inner membrane via the Tat pathway (Barker, et al. 2004, Smith, et al. 1995, Tuckwell, et al. 2006). Since PLCs were proved to play a critical role in the virulence of many bacterial pathogens, it was reasonable to presume that NsPLC was an extracellular enzyme and a pivotal virulence factor of *N. seriolae*.

Recent researches about the secreted microbial proteins revealed that most pathogenic bacteria deliver secreted proteins to the host cell cytoplasm, where they may target to mitochondria, golgi, lysosomes, peroxisomes and autophagosomes, affecting the physiology, signaling pathway and

innate immune response of the target cells (Bischofberger and van der Goot 2008, Lobet, et al. 2015, Moreno-Altamirano, et al. 2012). The NsPLC was predicted to co-localize with mitochondria by bioinformatics analysis. In this study, the subcellular localization of NsPLC in host cells exhibited a punctate distribution near the nucleus and did not co-localize with the mitochondria. Little is known about the distribution of secreted bacterial PLCs within host cell. But the subcellular localization of mammalian PLCs was studied quite a lot. Previous studies showed that the subcellular localization of mammalian PLCs mainly localized in the cytoplasm with diverse distribution, such as in cytoplasmic vesicles, in perinuclear halo, in a punctuate distribution, in Golgi apparatus, in granules and in nanotubes extending among cells. In brief, the subcellular localization of mammalian PLCs differed in quiescent cells compared to the pathological counterpart, depending on the isoform and may vary under different conditions (Di Raimo, et al. 2016, Lo Vasco, et al. 2010).

For the function of PLCs from Gram-positive bacteria, studies mainly focus on *C. perfringens* alpha-toxin, *M. tuberculosis* PLCs, *B. cereus* PC-PLC and *L. monocytogenes* PlcA (Flores-Diaz, et al. 2016). Notably, the bacterial PLCs are revealed to be associated with cell death in these studies. Multiple data indicate that alpha-toxin activates cell death, stimulating the release of cytochrome C from mitochondria and the consequent activation of caspases-3 (Manni, et al. 2017, Monturiol-Gross, et al. 2012). In *M. tuberculosis*, PLCs exhibit cytotoxic effects on macrophages and involve in cell death (Assis, et al. 2014). Given that bacterial PLCs may participate in cell death, experiment to determine whether NsPLC is related to the cell apoptosis was performed in this study. Both the ΔΨm detection and the caspase-3 activity assay showed the cell apoptosis was induced in FHM cells by the overexpression of NsPLC. *N. asteroides* is one of the pathogens of

fish nocardiosis, and previous studies have shown that *N. asteroides* strain GUH-2 has the ability to induce apoptotic death in the murine brain, PC12 cells and HeLa cells and some secreted product of nocardiae is capable of causing apoptosis (Barry and Beaman 2007, Camp, et al. 2003, Loeffler, et al. 2004). As a secreted protein of *N. seriolae*, NsPLC may participate in the cell apoptosis regulation.

The subcellular localization and preliminary function study of NsPLC may lay the foundation for further study on the function of this gene and promote the understanding of the virulence factors and pathogenic mechanism of *N. seriolae*. Further studies are required to verify the mechanisms involved in NsPLC-induced cell death. It remains to be clarified whether the NsPLC is the major virulence factor of *N. seriolae* by constructing a ΔPLC mutant attenuated *N. seriolae*. And the relationship among the interaction between NsPLC and macrophage also need to be highlighted in future studies.

FUNDING

This work was supported by National Natural Science Foundation of China (41406172, U1401211, 31402335 and 31472306), International S&T Cooperation Projects of Guangdong Province (2016A050502061), Shenzhen science and technology project (JCYJ20170306161613251), Natural Science Foundation of Guangdong Province (2014A030313602), Shenzhen Dapeng New District special fund for industry development (KY20160207), Special project of harbor construction and fishery industry development (A201608B15).

Conflict of interest. None declared.

REFERENCES

Assis PA, Espindola MS, Paula-Silva FW *et al. Mycobacterium tuberculosis* expressing phospholipase C subverts PGE2 synthesis and induces necrosis in alveolar macrophages. BMC Microbiol 2014;**14**: 128.

Barker AP, Vasil AI, Filloux A *et al.* A novel extracellular phospholipase C of *Pseudomonas aeruginosa* is required for phospholipid chemotaxis. Mol Microbiol 2004;**53**: 1089-98.

Barry DP, Beaman BL. *Nocardia asteroides* strain GUH-2 induces proteasome inhibition and apoptotic death of cultured cells. Res Microbiol 2007;**158**: 86-96.

Beaman BL, Beaman L. Nocardia species: host-parasite relationships. Clin Microbiol Rev 1994;**7**: 213-64.

Bischofberger M, van der Goot FG. Exotoxin secretion: getting out to find the way in. Cell Host Microbe 2008;**3**: 7-8.

Camp DM, Loeffler DA, Razoky BA *et al. Nocardia asteroides* culture filtrates cause dopamine depletion and cytotoxicity in PC12 cells. Neurochem Res 2003;**28**: 1359-67.

Di Raimo T, Leopizzi M, Mangino G *et al.* Different expression and subcellular localization of Phosphoinositide-specific Phospholipase C enzymes in differently polarized macrophages. J Cell Commun Signal 2016;**10**: 283-93.

Flores-Diaz M, Monturiol-Gross L, Naylor C *et al.* Bacterial sphingomyelinases and phospholipases as virulence factors. Microbiol Mol Biol Rev 2016;**80**: 597-628.

Goldfine H, Bannam T, Johnston NC *et al.* Bacterial phospholipases and intracellular

growth: the two distinct phospholipases C of *Listeria monocytogenes*. Symp Ser Soc Appl Microbiol 1998;**27**: 7S-14S.

Gravell M, Malsberger RG. A permanent cell line from the fathead minnow (*Pimephales promelas*). Ann N Y Acad Sci 1965;**126**: 555-65.

Ho PY, Byadgi O, Wang PC *et al.* Identification, molecular cloning of IL-1beta and its expression profile during *Nocardia seriolae* infection in largemouth bass, *Micropterus salmoides*. Int J Mol Sci 2016;**17**.

Lartigue L, Faustin B. Mitochondria: metabolic regulators of innate immune responses to pathogens and cell stress. Int J Biochem Cell Biol 2013;**45**: 2052-6.

Lo Vasco VR, Fabrizi C, Panetta B *et al.* Expression pattern and sub-cellular distribution of phosphoinositide specific phospholipase C enzymes after treatment with U-73122 in rat astrocytoma cells. J Cell Biochem 2010;**110**: 1005-12.

Lobet E, Letesson JJ, Arnould T. Mitochondria: a target for bacteria. Biochem Pharmacol 2015;**94**: 173-85.

Loeffler DA, Camp DM, Qu S *et al.* Characterization of dopamine-depleting activity of *Nocardia asteroides* strain GUH-2 culture filtrate on PC12 cells. Microb Pathog 2004;**37**: 73-85.

Manni MM, Valero JG, Perez-Cormenzana M *et al.* Lipidomic profile of GM95 cell death induced by *Clostridium perfringens* alpha-toxin. Chem Phys Lipids 2017;**203**: 54-70.

Moigne VL, Rottman M, Goulard C *et al.* Bacterial phospholipases C as vaccine candidate antigens against cystic fibrosis respiratory pathogens: The *Mycobacterium abscessus*, model. vaccine 2015;**33**: 2118-24.

Monturiol-Gross L, Flores-Diaz M, Araya-Castillo C *et al.* Reactive oxygen species and the MEK/ERK pathway are involved in the toxicity of clostridium perfringens alpha-toxin, a prototype bacterial phospholipase C. J Infect Dis 2012;**206**: 1218-26.

Moreno-Altamirano MM, Paredes-Gonzalez IS, Espitia C *et al.* Bioinformatic identification of *Mycobacterium tuberculosis* proteins likely to target host cell mitochondria: virulence factors? Microb Inform Exp 2012;**2**: 9.

Nayak SK, Shibasaki Y, Nakanishi T. Immune responses to live and inactivated *Nocardia seriolae* and protective effect of recombinant interferon gamma (rIFN gamma) against nocardiosis in ginbuna crucian carp, *Carassius auratus* langsdorfii. Fish Shellfish Immunol 2014;**39**: 354-64.

Raynaud C, Guilhot C, Rauzier J *et al.* Phospholipases C are involved in the virulence of *Mycobacterium tuberculosis*. Mol Microbiol 2002;**45**: 203-17.

Rudel T, Kepp O, Kozjak-Pavlovic V. Interactions between bacterial pathogens and mitochondrial cell death pathways. Nat Rev Microbiol 2010;**8**: 693-705.

Saint-Georges-Chaumet Y, Edeas M. Microbiota-mitochondria inter-talk: consequence for microbiota-host interaction. Pathog Dis 2016;**74**: ftv096.

Schmiel DH, Miller VL. Bacterial phospholipases and pathogenesis. Microbes Infect 1999;**1**: 1103-12.

Shimahara Y, Nakamura A, Nomoto R *et al.* Genetic and phenotypic comparison of *Nocardia seriolae* isolated from fish in Japan. J Fish Dis 2008;**31**: 481-8.

Smith GA, Marquis H, Jones S *et al.* The two distinct phospholipases C of *Listeria monocytogenes* have overlapping roles in escape from a vacuole and cell-to-cell spread. Infect Immun 1995;**63**: 4231-7.

Sudheesh PS, LaFrentz BR, Call DR *et al.* Identification of potential vaccine target

antigens by immunoproteomic analysis of a virulent and a non-virulent strain of the fish pathogen *Flavobacterium psychrophilum*. Dis Aquat Organ 2007;**74**: 37-47.

Sun YS, Lv LX, Zhao Z *et al.* Cordycepol C induces caspase-independent apoptosis in human hepatocellular carcinoma HepG2 cells. Biol Pharm Bull 2014;**37**: 608-17.

Tamura K, Peterson D, Peterson N *et al.* MEGA5 : molecular, evolutionary, genetics, analysis, using maximum, likelihood, evolutionary, distance, and maximum, parsimony, methods. Mol Biol Evol 2011;**28**: 2731-9.

Tuckwell D, Lavens SE, Birch M. Two families of extracellular phospholipase C genes are present in aspergilli. Mycol Res 2006;**110**: 1140-51.

Vu-Khac H, Duong VQ, Chen SC *et al.* Isolation and genetic characterization of *Nocardia seriolae* from snubnose pompano *Trachinotus blochii* in Vietnam. Dis Aquat Organ 2016;**120**: 173-7.

Wang PC, Chen SD, Tsai MA *et al. Nocardia seriolae* infection in the three striped tigerfish, *Terapon jarbua* (Forsskal). J Fish Dis 2009;**32**: 301-10.

West AP, Shadel GS, Ghosh S. Mitochondria in innate immune responses. Nat Rev Immunol 2011;**11**: 389-402.

Xia LQ, Liang HY, Huang YH *et al.* Identification and characterization of Singapore grouper iridovirus (SGIV) ORF162L, an immediate-early gene involved in cell growth control and viral replication. Virus Res 2010;**147**: 30-9.

Xia L, Cai J, Wang B *et al.* Draft genome sequence of *Nocardia seriolae* ZJ0503, a fish pathogen isolated from *Trachinotus ovatus* in China. Genome Announc 2015a;**3**.

Xia L, Zhang H, Lu Y *et al.* Development of a loop-mediated isothermal

amplification assay for rapid detection of *Nocardia salmonicida*, the causative agent of nocardiosis in fish. J Microbiol Biotechnol 2015b;**25**: 321-7.

Zhao Z, Chen C, Hu CQ *et al.* The type III secretion system of *Vibrio alginolyticus* induces rapid apoptosis, cell rounding and osmotic lysis of fish cells. Microbiology 2010;**156**: 2864-72.

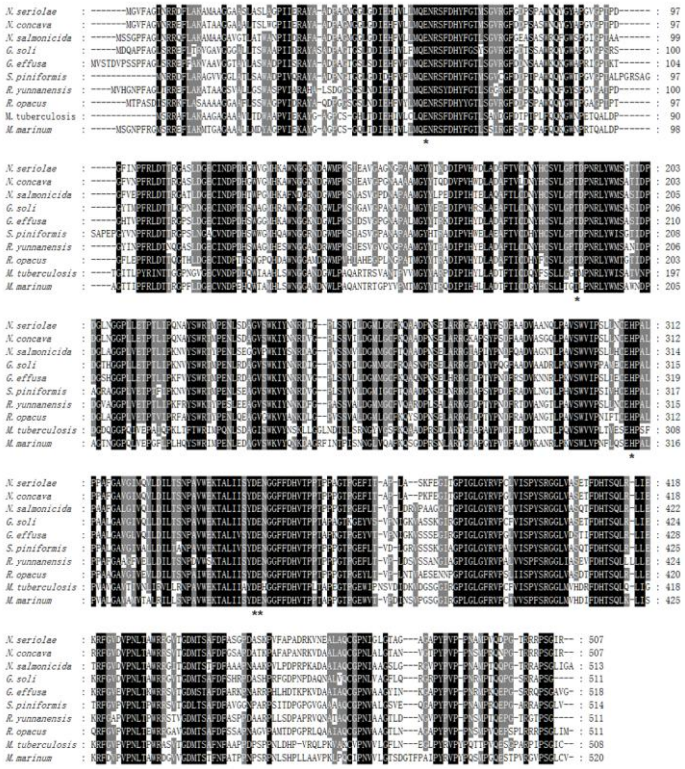

Figure 1. Multiple alignment of the deduced amino acid sequences of PLC among different species. GenBank accession numbers are shown in Figure 2. Black shading shows identical amino acids, whereas gray shading is used for regions with more than 75% identity. The asterisks indicate amino acids that are putative active sites on conserved domain in PLCs.

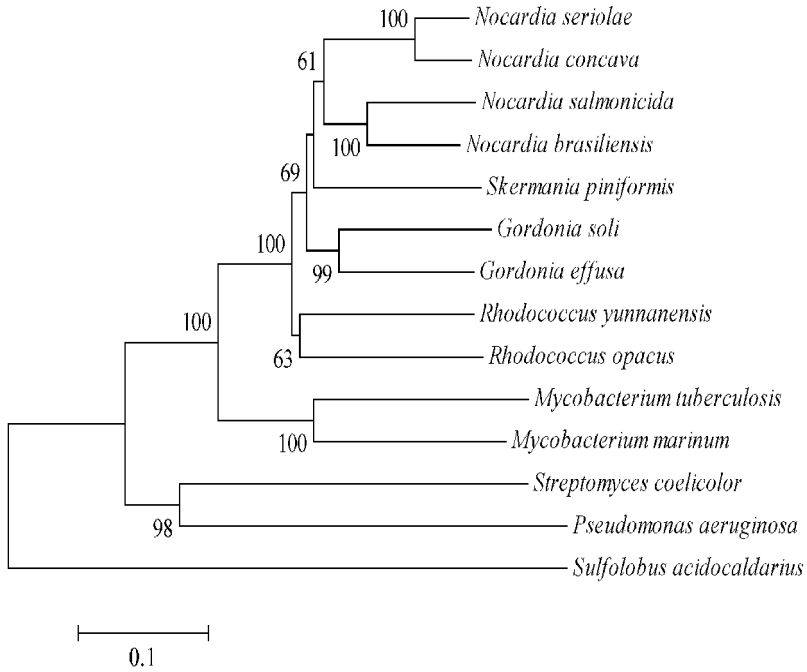

Figure 2. Phylogenetic tree of PLC based on the homologous amino acids. The numbers next to the branches indicate percentage values for 1000 bootstrap replicates. GenBank accession numbers are *Nocardia concava* (WP_040807264.1), *Nocardia salmonicida* (WP_062985526.1), *Nocardia brasiliensis* (WP_042255962.1), *Skermania piniformis* (WP_066470704.1), *Gordonia soli* (WP_007624473.1), *Gordonia effusa* (GAB20429.1), *Rhodococcus yunnanensis* (WP_072805611.1), *Rhodococcus opacus* (WP_012689406.1), *Mycobacterium tuberculosis* (NP_216865.1), *Mycobacterium marinum* (ACC42073.1), *Streptomyces coelicolor* (NP_733712.1), *Pseudomonas aeruginosa* (NP_249535.1), *Sulfolobus acidocaldarius* (WP_011278577.1).

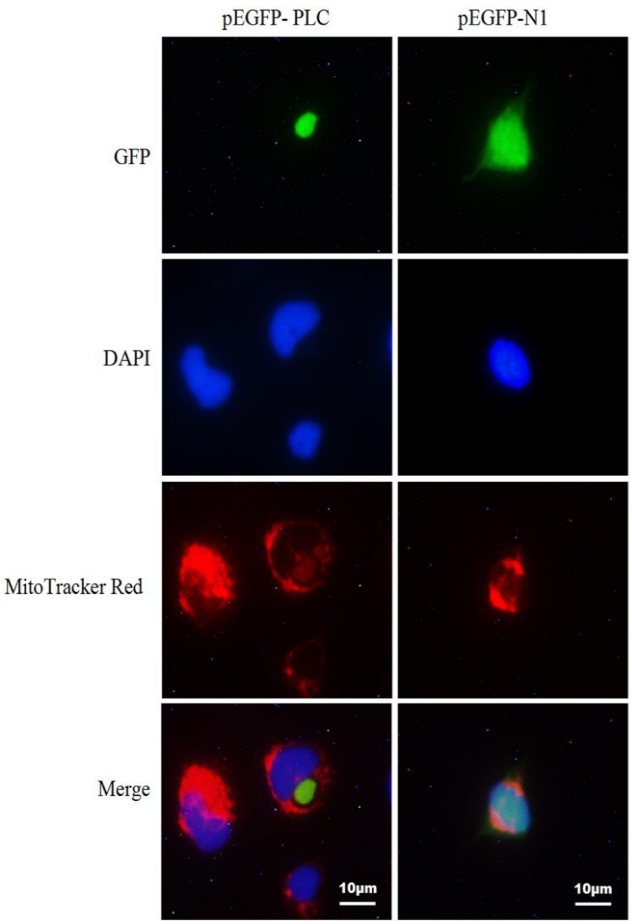

Figure 3. Subcellular localization of NsPLC in FHM cells. FHM cells were transiently transfected with pEGFP-PLC and pEGFP-N1. At 48 h posttransfection, the cells were stained with MitoTracker Red CMXRos and DAPI, and then observed under fluorescence microscope. Green fluorescence showed the localization of PLC-GFP fusion protein or GFP, red fluorescence showed the location of mitochondrion, and blue fluorescence showed the nucleus.

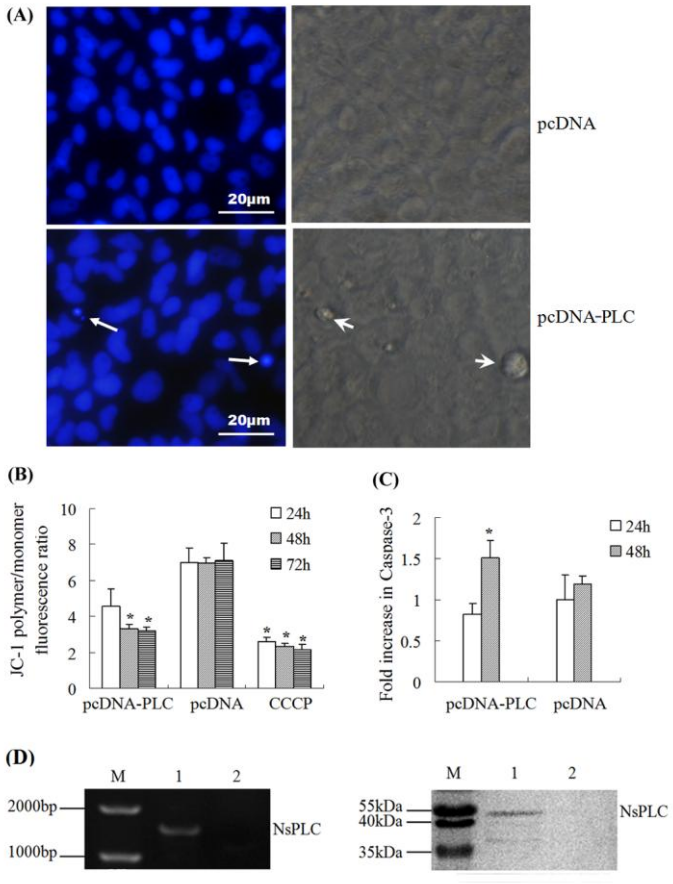

Figure 4. Apoptosis assay in transfected FHM cells. (A) Observation of apoptotic body. The transfected cells were fixed at 48 h posttransfection staining by DAPI. Arrows indicated the apoptotic bodies (fragmented nucleus), arrow heads indicated the apoptotic cells. (B) Mitochondrial membrane potential assay. FHM cells transfected with pcDNA-PLC or pcDNA plasmid were collected at indicated time points after transfection and the mitochondrial membrane potential were assessed using the JC-1. Untransfected cells treated with CCCP was positive control. The data were expressed as the JC-1 polymer/monomer fluorescence ratio. Significant differences were indicated by * ($p < 0.05$). (C) Measurement of caspase-3 activity. FHM cells transfected with pcDNA-PLC and pcDNA plasmid were collected at indicated time points after transfection and the levels of cleaved caspase-3 were measured. The data were expressed as fold increase compared to the corresponding caspase-3 activity values in untransfected cells. Significant differences were indicated by * ($p < 0.05$). (D) Confirmation of the NsPLC expression in pcDNA-PLC transfected FHM cells by RT-PCR and Western blot. Lane M, DNA or protein markers; lane 1, FHM/pcDNA-PLC; lane 2, FHM/pcDNA. NsPLC was expressed in the FHM cells transfected with plasmid of pcDNA-PLC.

Table 1. Primers used in this study.

Primer Name	Sequence 5'-3'	Restriction Enzyme
pEGFP-F	CCCAAGCTTATGGGCGTGTTCGCCGGCATC	*Hin*d III
pEGFP-R	CCGACGTCGACTGCCGGATACCGCTGGGCCGAC	*Sal* I
pcDNA-F	GGGGTACCATGGGCGTGTTCGCCGGCATC	*Kpn* I
pcDNA-R	GCTCTAGACCGGATACCGCTGGGCCGAC	*Xba* I