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Identification of a secreted superoxide dismutase (SOD) from *Nocardia seriolae* which induces apoptosis in fathead minnow (FHM) cells

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Abstract

Fish nocardiosis is a chronic systemic granulomatous disease, and *Nocardia seriolae* is the main pathogen. The pathogenesis and virulence factors of *N. seriolae* are not fully understood. Secreted superoxide dismutase (SOD) may be a virulence factor found by a comparative bioinformatics analysis of the whole genome sequence of *N. seriolae* and the virulence factor database (VFDB). In order to determine the subcellular localization and study the preliminary function of SOD from *N. seriolae* (NsSOD), gene cloning, secreted protein identification, subcellular localization in fish cells, and apoptosis detection of NsSOD were carried out in this study. Subcellular localization research revealed that NsSOD-GFP fusion proteins were evenly distributed in the cytoplasm. Furthermore, apoptotic bodies were observed in the transfected FHM cells by the overexpression of protein NsSOD. Then, assays of mitochondrial membrane potential ($\Delta\Psi m$) value, caspase-3 activity and apoptosis-related genes (Bax, Bid, Bad and Bcl-2) mRNA expression were conducted. The results showed that $\Delta\Psi m$ was decreased, and caspase-3 was significantly activated. The mRNA expression of the *Bad* gene showed significant up-regulated expression at 24 h.p.t., while *Bid* and *Bax* genes showed significant up-regulated expression at 72 and 96 h.p.t. and anti-apoptotic gene (Bcl-2) was down-regulated in NsSOD overexpressed cells. Taken together, the results indicated that the protein NsSOD might be involved in apoptosis regulation. This study may lay the foundations for further studies on the function of NsSOD and promote the understanding of the virulence factors and the pathogenic mechanisms of *N. seriolae*.

KEYWORDS

cell apoptosis, *Nocardia seriolae*, overexpression, subcellular localization, superoxide dismutase

1 | INTRODUCTION

Nocardia spp. are opportunistic pathogens that can infect a wide range of cultured fish with high economic value, such as largemouth bass (*Micropterus salmoides*), snakehead (*Channa maculata*), amberjack (*Seriola dumerili*), yellowtail (*S. quinqueradiata*), golden pompano (*Trachinotus ovatus*), snubnose pompano (*T. blochii*), large yellow croaker (*Larimichthys crocea*) (Shimahara et al., 2008; Wang et al., 2009; Xia, Cai, et al., 2015) and other 30 kinds of fishes. *Nocardia* spp. has increased economic losses in the aquaculture industry. Fish nocardiosis is a chronic systemic granulomatous disease, and the clinical symptoms of *Nocardia*-infected fish are skin ulcers and numerous white nodular structures on the gills and in the head kidney, trunk kidney, spleen and liver, which has led to serious effects on Asian aquaculture systems (Maekawa, Yoshida, Wang, & Chen, 2000; Ping-Yueh et al., 2016; Vu-Khac et al., 2016).

Nocardia seriolae is a Gram-positive facultative intracellular pathogen (Nayak, Shibasaki and Nakanishi, 2014) which has been identified as the main pathogen of fish nocardiosis in recent years. It was reported that *N. seriolae* has effective strategies for surviving and colonizing in the host, which facilitates it to evade intracellular killing after being engulfed by macrophages, and then disseminate in the host (Beaman & Beaman, 1994) along with the movement of macrophages.

The reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) constitute an important component of the innate active defence response against invading microorganisms by fish phagocytic cells. It was well demonstrated that bacterial pathogens overcome the toxic effects of ROS to establish infections. Some pathogenic actinomycetes including *Nocardia* spp. have developed a system to protect themselves from elimination by these oxidative radicals (Beaman & Beaman, 1994).

Previous studies reported that enzymes involved in antioxidant defences in pathogens were associated with microbial virulence. Superoxide dismutase (SOD), a metalloenzyme capable of disproportionating O_2^-, was revealed to be an important virulence factor in some pathogenic bacteria such as *N. asteroids*, *Mycobacterium tuberculosis* (Beaman & Beaman, 1984), *Edwardsiella tarda* (Cheng, Zhang, & Sun, 2010) and *Brucella abortu* (Zhou et al., 2018). Studies on the survival strategy of *Nocardia* spp. in polymorphonuclear leukocytes have demonstrated that bactericidal activity at early time points heavily relies on its oxidative metabolism. Beaman et al. found that the surface-associated SOD modified by the *N. asteroides* GUH-2 played a significant role in resisting oxidative killing mechanisms of phagocytes (Beaman & Beaman, 1984). Similar to *N. asteroides* GUH-2, *M. tuberculosis* also secretes SOD into the medium and may be associated with its surface. In contrast, non-pathogenic mycobacteria hardly secrete SOD, and the SOD is cytoplasmic in the bacterial cells (Beaman & Beaman, 1984). Therefore, the secreted and surface-associated SOD of these microorganisms may interact with leukocytes or mononuclear phagocytes and help pathogens escape from the immune response of the host. It is conceivable that the presence of SOD on the cell surface can give the organism the advantage of resisting the microbicidal action of these oxidative metabolites. However, the effect of *N. seriolae* secreted SOD on host cells is still unclear.

In our study, shotgun mass spectrometry of the extracellular products showed that *N. seriolae* stain ZJ0503 has a secreted SOD protein (NsSOD). Gene cloning, secreted protein identification, subcellular localization and apoptosis detection of NsSOD were carried out in this study. It may lay the foundations for further studies on the function of NsSOD and promote the understanding of the pathogenic mechanisms of *N. seriolae*.

2 | MATERIALS AND METHODS

2.1 | Bacterial strains, cell and plasmids

Nocardia seriolae ZJ0503, isolated from diseased *Trachinotus ovatus* in China, was cultured at 28°C in an optimized medium (Xia, Cai, et al., 2015). *Escherichia coli* DH5α was used for gene cloning, and it was grown in Luria-Bertani (LB) medium with vigorous shaking at 37°C. Fathead minnow (FHM) epithelial cells (ATCC CCL-42, Manassas, VA, USA) (Gravell & Malsberger, 1965) were cultured in Leibovitz's L15 medium containing 10% foetal bovine serum (Invitrogen, Carlsbad, CA, USA) at 25°C. Plasmid pEGFP-N1 (Clontech, Mountain View, CA, USA) was used for subcellular localization. Plasmid pcDNA3.1/His A (Invitrogen) was used for overexpression.

2.2 | Preparation and identification of the *N. seriolae* extracellular products

N. seriolae was cultured on optimized medium plates for 3-5 days, and the bacterial suspension was prepared using single colonies. 100 μl of bacterial suspension was evenly spread on the cellophane-covered medium and cultured at 28°C for 3-5 days. The grown colonies on cellophane were collected, resuspended in sterile PBS, and then centrifuged at 8000 ×*g*, 4°C for 20 minutes. The supernatant containing extracellular products was sterilized through a 0.2-μm membrane filter and dialysed in ultrapure water at 4°C for 10-16 h, and the dialysate was changed 3× during the process. The extracellular products after dialysis were frozen and vacuum dried to obtain protein dry powder. Finally, it was identified via shotgun MS.

2.3 | Cloning of *NsSOD* and plasmid construction

According to the instructions of the bacterial genomic DNA extraction kit (Tiangen), the total DNA of *N. seriolae* ZJ0503 was extracted as a PCR amplification template. The PCR primers pEGFP-F/R and pcDNA-F/R (Table 1) were used to amplify the NsSOD gene. The PCR amplification conditions were as follows: 98°C pre-denaturation for 2 min; 98°C 10 s, 55°C 15 s, 68°C 1 min, a total of 30 cycles; 68°C for 5 min. The PCR product was detected

TABLE 1 Primers used in this study

Primer name	Sequence 5′-3′	Restriction endonucleases
pEGFP-F	5′-CGGGATCCATGGCTGTCTACACGCTGCC-3′	*BamHI*
pEGFP-R	5′-GGAATTCGCCGAAGATCAGGCCTTTGC-3′	*EcoRI*
pcDNA-F	5′-GGAATTCaTGGCTGTCTACACGCTGCCTG -3′	*EcoRI*
pcDNA-R	5′-CGGGATCCCGGCCGAAGATCAGGCCTTTG-3′	*BamHI*
Bad-F	5′-TGATCCTTTCAGGCGGAGATCT	GC-3′
Bad-R	5′-CAGACTCTTTGTGACTCCAAAGGAA-3′	
Bid-F	5′-CTGCTTCTCCTTTCCTTCTTTGAGC-3′	
Bid-R	5′- GATCAACTCAGCAGCCATATCCCTT-3′	
Bax-F	5′-TGGCACTGTTTCACCTCG-3′	
Bax-R		
Bcl-2-F	5′-TGGGACTGTTTGCCTTCG-3′	
Bcl-2-R	5′-TCTGCCGCTGCATCTTTT-3′	

by 1% agarose gel electrophoresis and purified with a DNA fragment purification kit. After double digestion with plasmid pEGFP-N1 and pcDNA3.1 His A, the ligation product was transformed into *E. coli* DH5α. After sequencing, the successfully constructed fusion expression vectors were named as pEGFP-NsSOD and pcDNA3-NsSOD.

2.4 | Bioinformatics analysis, sequence alignments and phylogenetic analysis

Based on the whole genome sequence data of *N. seriolae* ZJ0503 (Xia,Cai, et al., 2015), the potentially secreted proteins were predicted using LocTree3 and ExPASy-PROSITE. Then, the secreted virulence factors were inferred by comparative bioinformatics analysis of the potentially secreted proteins of *N. seriolae* and the virulence factor database (VFDB) (http://www.mgc.ac.cn/VFs/). Subcellular localization was predicted by using SignalP 4.1 and LocTree3. The amino acid sequence of NsSOD was analysed with the online Protein BLAST program and DNASTAR software. Eight SOD sequences from different bacteria were selected for multiple sequence alignments using ClustalX 2.0 and GeneDoc, and different bacterial SOD sequences were also used for phylogenetic tree analysis with MEGA5.0 by the neighbour-joining method (Tamura et al., 2011).

2.5 | Subcellular localization of NsSOD in FHM cells

According to the instructions of the E.Z.N.A™ Endotoxin-Free Plasmid Extraction Kit, the endotoxin-free plasmids pEGFP-NsSOD and pEGFP-N1 and plasmids were extracted. FHM cells were cultured in 24-well cell culture plates and transfected when the cell coverage was about 70% confluency (for subcellular localization) or 90% confluency (for overexpression). According to the instructions of Lipofectamine 2000, the FHM cells were transfected with pEGFP-NsSOD and pEGFP-N1, respectively. After 48 hours post-transfection (h.p.t.), the mitochondria were stained with MitoTracker Red CMXRos. The cells were fixed with 3.7% paraformaldehyde preheated at room temperature, and the nuclei were labelled with 4,6-diamidino-2-phenylindole (DAPI 1 μg/ml) in the dark for 10 min, after which the cells were observed and photographed under a fluorescent microscope (Leica).

2.6 | Detection of cell apoptosis

To detect whether the overexpression of NsSOD is involved in fish cell apoptosis, pcDNA-NsSOD was transfected into FHM cells and pcDNA3.1 His A was used as a negative control. Then, the FHM cells were stained with DAPI at 48 h.p.t. and observed by microscopy. Moreover, the mitochondrial membrane potential (ΔΨm) was measured with a JC-1 assay kit (Beyotime, Shanghai, China) and caspase-3 activity was assessed with a caspase-3 colorimetric assay kit (BioVision, Milpitas, CA, USA). At 24, 48 and 72 h.p.t., 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, Waltham, MA, USA). The caspase-3 activity detection was performed as described previously (Zhao et al., 2010) at 24 and 48 h.p.t. To confirm the NsSOD expression in pcDNA-NsSOD transfected FHM cells, Western blot analysis was performed as described previously (Xia et al., 2010). Briefly, total RNA and protein were isolated from the pcDNA-NsSOD or pcDNA 3.1 His A transfected FHM cells at 48 h.p.t. Western blot analysis was carried out by using mouse anti-His monoclonal antibody (Sigma-Aldrich, St Louis, MO, USA) as the primary antibody at a dilution of 1:1000 and horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma-Aldrich) as the secondary antibody at a dilution of 1:2000.

2.7 | Apoptosis-related gene mRNA expression

Two groups of pcDNA-NsSOD and pcDNA3.1/His A transfected FHM cells were harvested at 0, 24, 48, 72 and 96 h.p.t. to extract the total RNA, and quantitative real-time PCR (qRT-PCR) was performed following the synthesis of cDNA. Effects on the expression of apoptosis-related genes after transfection were investigated using real-time SYBR Green PCR Master Mix on Applied Biosystems 7500 Real-Time PCR system (ABI). Each assay was performed in triplicate with β-actin gene as the internal control. According to the sequences of apoptosis-related genes, four pairs of specific primers (Table 1) were carefully designed for qRT-PCR investigation. The PCR was performed in a 10 μl reaction volume containing 1 μl of each primer (10 μM), 1 μl of cDNA, 2 μl of PCR-grade water and 5 μl of SYBR® Select Master Mix (ABI) according to the manufacturer's protocol. The PCR procedure for four apoptosis-related genes and β-actin was as follows: 95°C for 2 min, 40 cycles of 95°C for 15 s and 55°C for 1 min. Melt curve analysis of the amplification products was performed over a range of 60-95°C at the end of each PCR to confirm the single product generation. The relative expression levels of the four apoptosis-related genes were calculated using the comparative Ct $2^{(-\triangle\triangle Ct)}$ method (Gast, Kuehner, Sobekb, & Mizaikoff, 2010).

2.8 | Statistical analysis

Data were presented as the means ± standard deviation (SD). Statistical analysis was performed with one-way ANOVA with the SPSS statistics 21.0 software, and the data were edited by GraphPad Prism software. Data represent the means for three independent experiments, and statistically significant is highlighted with asterisks in the figures as follows: $p > .05$, not significant; $p < .05$ (*), significant; and $p < .01$ (**), extremely significant.

3 | RESULTS

3.1 | Identification of NsSOD as a secreted protein

The extracellular products of *N. seriolae* were obtained, and the secreted proteins were identified using shotgun MS. Results showed that four peptide sequences of NsSOD (AKDDHAAIFLNEK, DGGDKPVGDLAAAVDEEFGSFDK, AFWNVVNWAEIQER, AVYTL PELDYDYSALEPFISGQINEIHHTK) were detected with confidence greater than or equal to 99%, which confirmed that NsSOD was a secreted protein.

3.2 | Cloning and sequence analysis of *NsSOD*

The *NsSOD* gene of *N. seriolae* strain ZJ0503 was cloned, and recombinant plasmids of pEGFP-NsSOD and pcDNA-NsSOD were successfully constructed. Sequence analysis revealed that the open reading frame (ORF) of the *NsSOD* gene is 624 bp and deduced 207 amino acids. The predicted molecular weight of NsSOD protein was 22.99 kDa, and the theoretical isoelectric point was 5.39. The instability index (II) of NsSOD was 22.04, indicating that the protein is relatively stable. The total average value of hydrophilicity was −0.179, indicating that NsSOD is hydrophilic. NsSOD was predicted to be a secreted protein in bacteria, co-localized with mitochondria in eukaryotic cells by LocTree3. Interpro predicted that NsSOD belongs to the Mn/Fe-SOD superfamily, with two Mn/Fe_SOD domains at the N-terminus (4-84 bp) and C-terminus (91-193 bp), and 160-167 bp for the manganese/iron binding site.

Protein BLAST showed that the deduced amino acid sequence of NsSOD displayed high homology with other SOD sequences from actinomycetes, ranging from 97.58 % identity with SOD from *N. concava* to 78.82 % identity with SOD from *Rhodococcus fascians*.

The alignment of NsSOD and other bacterial SOD sequences is shown in Figure 1a. The eight amino acids (DMWEHAFY) are the manganese/iron binding site of SOD. From the picture, it can be seen that the manganese/iron binding site of different bacterial SODs is absolutely conservative. A phylogenetic tree was constructed based on the amino acid sequences of eight bacterial SODs. As shown in Figure 1b, NsSOD and other actinomycetes SODs are clustered in a single-line branch with 100% bootstrapping.

3.3 | Subcellular localization of NsSOD in FHM cells

Subcellular localization of NsSOD in fish cells was determined by NsSOD-green fluorescent protein (GFP) fusion protein expression. The NsSOD-GFP fusion protein exhibited a strong green fluorescence signal at 48 h.p.t. The nucleus is marked with blue fluorescence, and the mitochondria are marked with red fluorescence. Compared with the position of nucleus and mitochondria in pEGFP-NsSOD transfected cells, the distribution of NsSOD-GFP fusion protein and mitochondria does not coincide, indicating that the protein NsSOD is not co-localized with mitochondria. They are distributed in the cytoplasm (Figure 2). Additionally, apoptotic characteristics such as nuclear shrinkage were observed in cells expressing recombinant protein which indicated that apoptosis may occur in FHM cells transfected with pEGFP-NsSOD. The green fluorescence in the control group transfected with pEGFP-N1 showed whole cell distribution, smooth nuclear edge, uniform staining and no apoptosis.

3.4 | Apoptosis induced in FHM cells by the overexpression of NsSOD

In order to investigate whether NsSOD is involved in cell apoptosis, the overexpression plasmid pcDNA-NsSOD was transfected into FHM cells, and the activities of ΔΨm and caspase-3 were detected.

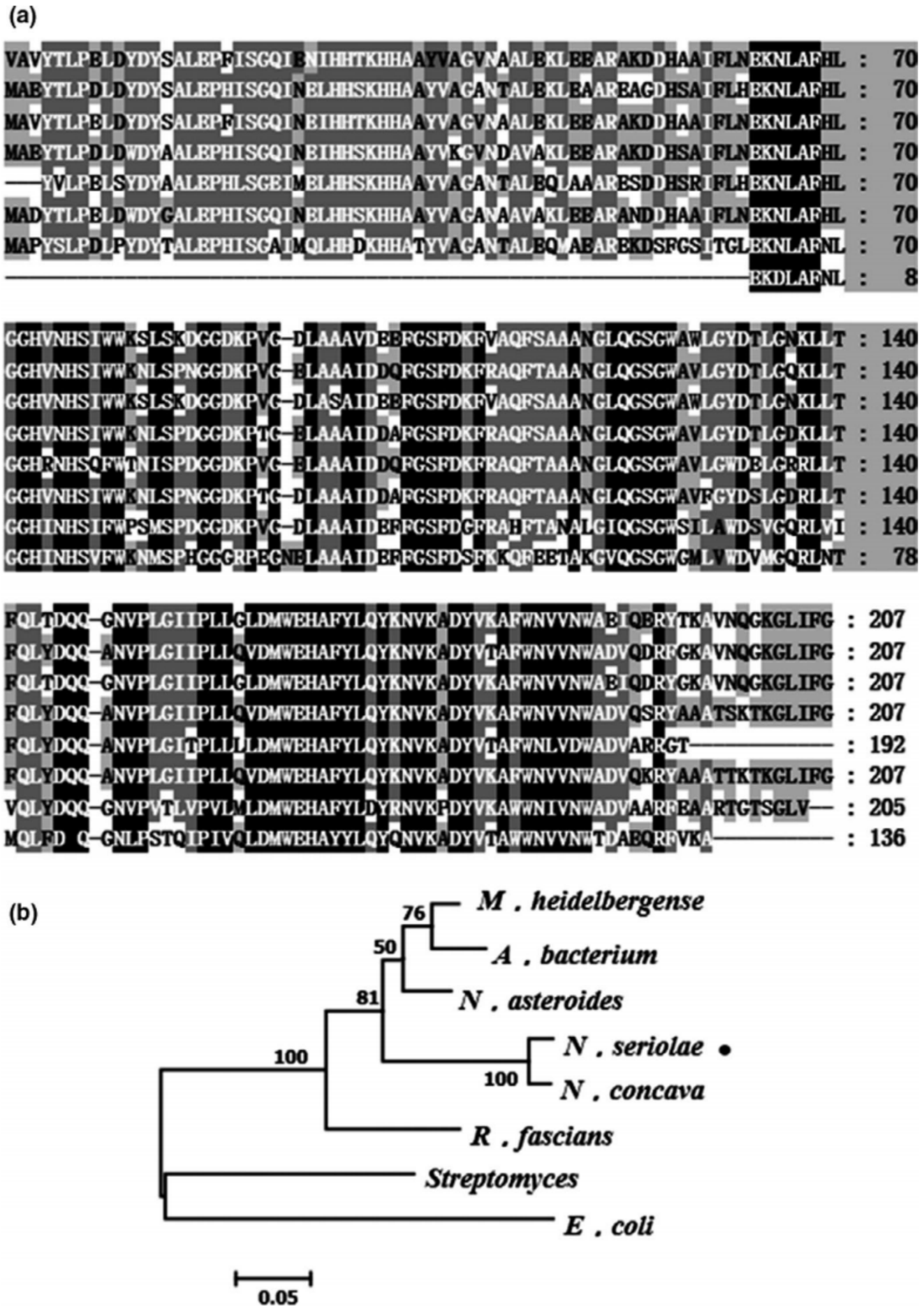

FIGURE 1 Multiple sequence alignment and construction of phylogenetic tree. (a) Multiple alignment of the deduced amino acid sequences of the NsSOD protein among different species. Shaded regions indicate residues sharing homology, black regions indicate 100% homology, and dark grey regions indicate homology higher than 75%. (b) Construction of phylogenetic tree among *N. seriolae* and other species with SOD protein homology sequences. Protein sequences were aligned with Clustal W, and the nonrooted neighbour-joining tree was generated by MEGA (version 5.0) program. Number at branch points indicates bootstrap support. GenBank accession numbers are shown as follows: *Nocardia asteroides* (*P53651.2*) *Nocardia concava* (*WP_040802278.1*) *Mycobacterium heidelbergense* (WP_083077568.1) *Rhodococcus fascians* (WP_052061919.1) *Actinobacteria bacterium* (MSW82911.1) *Streptomyces* (WP_139711406.1) *Escherichia coli* (WP_158132817.1)

The NsSOD expression in pcDNA-SOD transfected FHM cells was confirmed by the presence of a specific band on Western blot analysis (Figure 3a). At 48 h.p.t., apoptotic bodies were observed in NsSOD-overexpressing cells by DAPI staining (Figure 3b, lower panel), while cells transfected with the control plasmid remained intact at the same time (Figure 3b, upper panel). Moreover, several typical apoptotic features were observed in NsSOD-overexpressing cells, such as JC-1 polymer/monomer fluorescence ratio dropped obviously, the $\Delta\Psi m$ level in NsSOD-overexpressing cells was about 1-fold lower than the control group at 48 and 72 h.p.t. (Figure 3c), and the caspase-3 was activated at 48 h.p.t. (Figure 3d). These apoptosis features indicated that apoptosis can be induced by the overexpression of NsSOD in FHM cells.

3.5 | Apoptotic detection of NsSOD-overexpressing FHM cells

The mRNA expression of three pro-apoptotic genes (*Bax*, *Bad* and *Bid*) and an anti-apoptotic gene (*Bcl-2*) was investigated at 0, 24, 48, 72 and 96 h.p.t. by qRT-PCR. The results showed that after NsSOD was overexpressed, the expression levels of pro-apoptotic genes (*Bad*, *Bid* and *Bax*) were up-regulated. Among them, the *Bad* gene showed significant up-regulated expression at 24 h.p.t., while *Bid* and *Bax* genes showed significant up-regulated expression at 72 and 96 h.p.t., respectively. At the same time, the expression of anti-apoptotic gene *Bcl-2* suddenly decreased at 48 h.p.t. and was significantly lower than that of the control group (Figure 4).

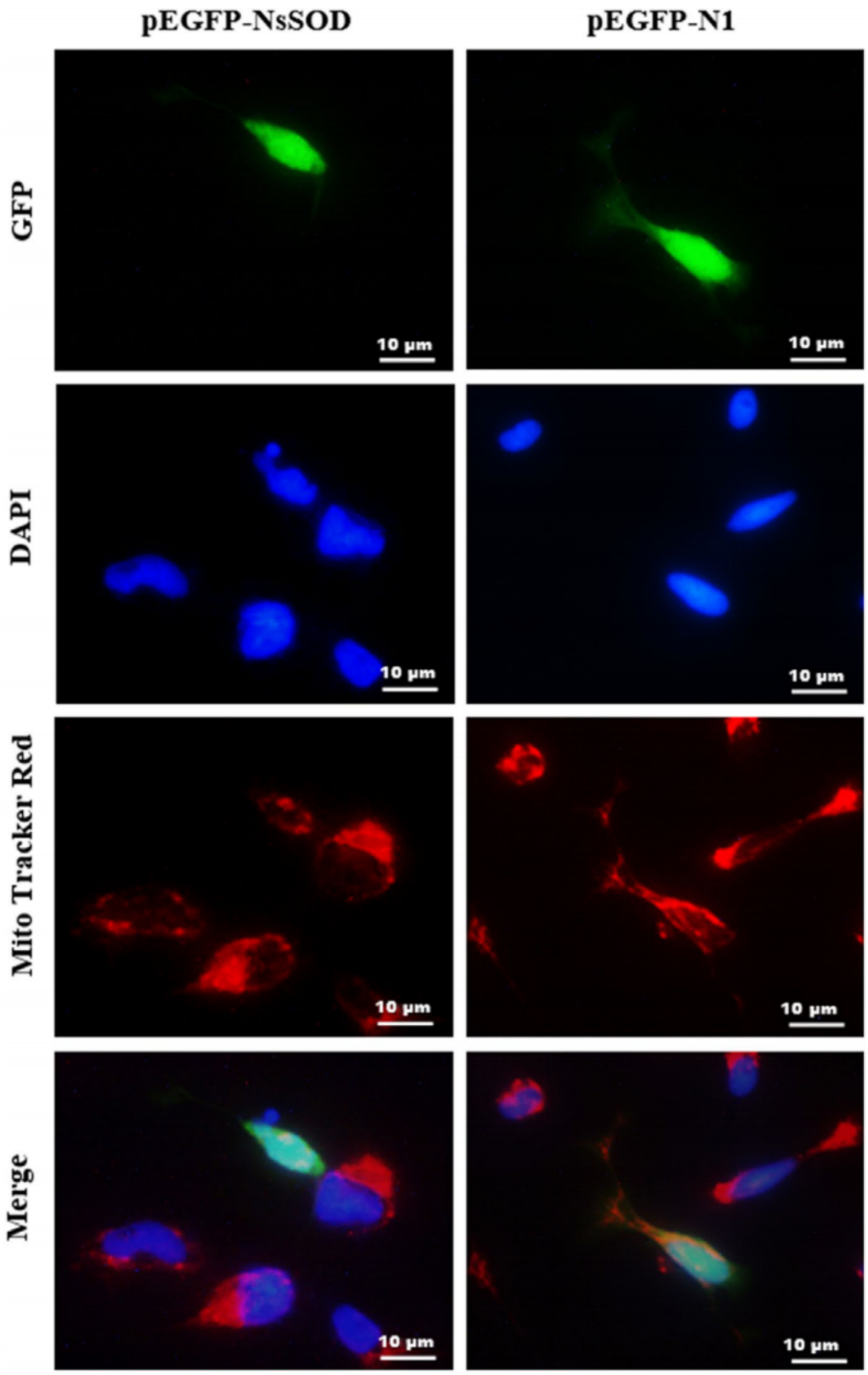

FIGURE 2 Subcellular localization of the NsSOD protein in FHM cells. Green fluorescence showed the NsSOD-GFP or GFP, red fluorescence showed the mitochondria, and blue fluorescence showed the nucleus. The left panels are presented as pEGFP-SOD, and the right panels are pEGFP-N1 [Colour figure can be viewed at wileyonlinelibrary.com]

4 | DISCUSSION

SOD constitutes a ubiquitous class of antioxidant defence metalloenzymes that catalyses the conversion of $O_2^{\cdot}$ into dioxygen (CO_2) and H_2O_2 to protect cells from the damage of superoxide radical ions (Broxton & Culotta, 2016). According to different types of binding metals, SOD can be divided into manganese (Mn-SOD), iron (Fe-SOD) and nickel (Ni-SOD), copper–zinc (Cu/Zn-SOD) and manganese–iron (Mn/Fe-SOD) (Lynch & Kuramitsu, 2000). In this study, the *NsSOD* gene was successfully cloned and protein blast revealed it belongs to the Mn/Fe-SOD superfamily with a highly conserved manganese/iron binding site (Parker and Blake, 1988).

Cellular distribution of SODs in pathogens has been reported to influence the ability of bacteria to survive oxidative stress in the host (Gregroy, Yost, & Fridovich, 1973). The SOD is distributed in the periplasmic space of the bacterial cell, possibly contributing to the elimination of $O_2^{\cdot}$ outside the bacteria (Barnes, Balebona, Horne, & Ellis, 1999). By identifying the extracellular products of *N. seriolae* with MS, the NsSOD was proved to be a secreted protein. It is conceivable that the host phagocytic cells can generate

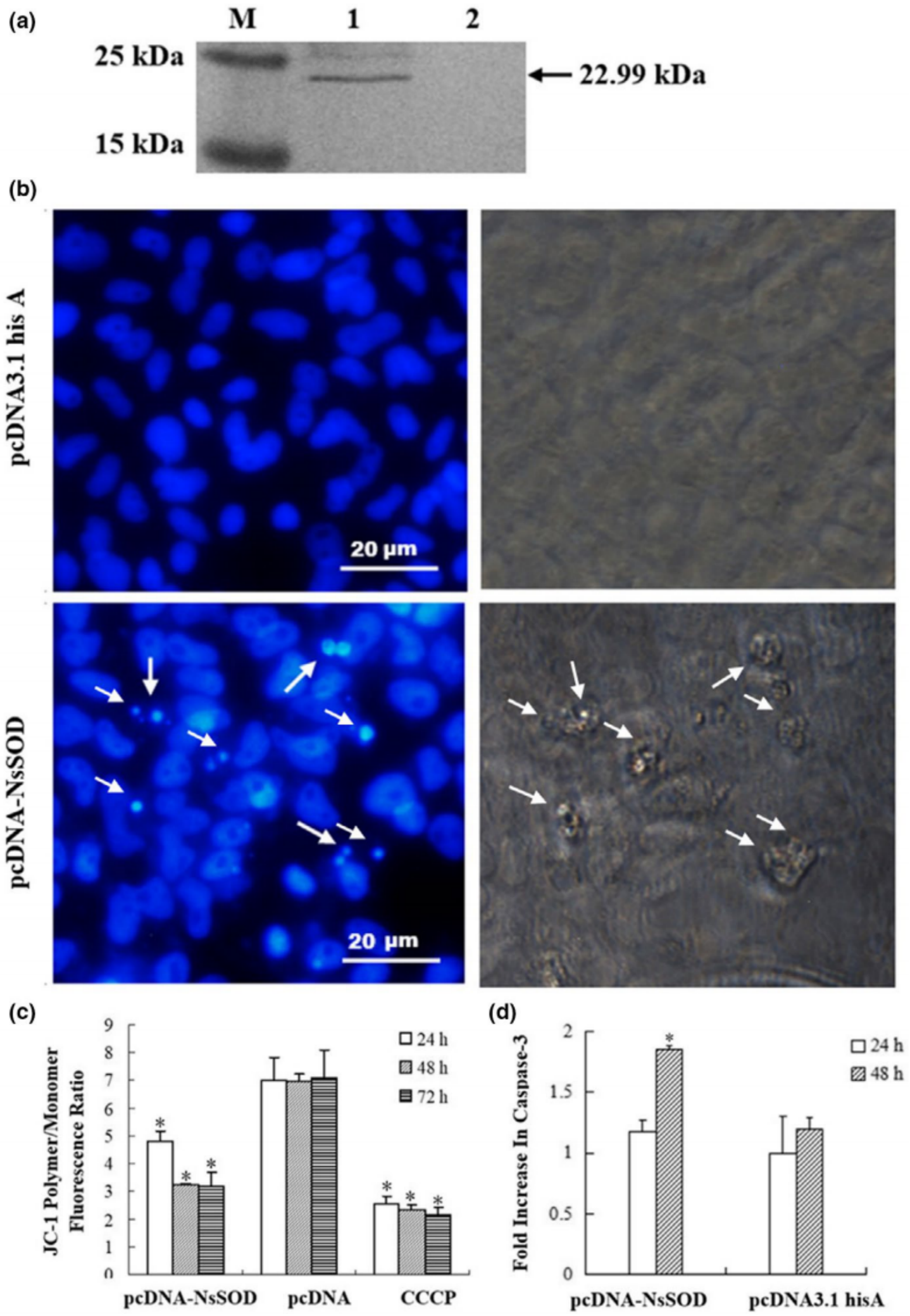

FIGURE 3 Western blot and observation of the apoptosis bodies in transfected FHM cells. (4A) Western blot analysis of NsSOD proteins. Abbreviations: M: marker; 1: pcDNA-NsSOD transfected FHM cells; 2: control. (4B) Observation of the nucleus in FHM cells transfected with either pcDNA-NsSOD or pcDNA3.1 hisA. Arrows indicate apoptotic bodies (fragmented nucleus). (4C) Mitochondrial membrane potential assay of FHM cells transfected with pcDNA-NsSOD and pcDNA3.1 hisA. Significant differences were indicated by *($p < .05$). (4D) Measurement of caspase-3 activity assay in transfected FHM cells. Significant differences were indicated by *($P < .05$) [Colour figure can be viewed at wileyonlinelibrary.com]

a high level of free radicals to inactivate pathogens. The secretion of SOD by these pathogens can provide a defence mechanism against the hostile environment in the host cells and hence may be required for bacterial virulence (Wu et al., 1998). The absence or attenuation of SOD can reduce the survivability of *M. tuberculosis* in the host cells, which is a key immune evasion strategy for its survival (Dan, Fan, & Bao, 2003). Beaman et al indicated that the surface-associated SOD elaborated by the cells of *N. asteroides* GUH-2 appears to play a significant role in protecting these bacteria from the oxidative killing mechanisms of phagocytes (L. Beaman & Beaman, 1984). Coincidentally, the *Aeromonas salmonicida* expression periplasmic Mn-SOD survived for the duration of the superoxide attack (Barnes, Horne, & Ellis, 1996). By analogy, Mn/Fe-SOD of *N. seriolae* may play a similar role in its survival strategy.

It is well known that *N. seriolae* can survive and replicate in the host cells, but its survival strategy still lacks direct evidence. Exploring the localization and function of NsSOD in host cells is significant to further understand the role of NsSOD in the survival strategy of *N. seriolae*. NsSOD was predicted to co-localize with mitochondria by bioinformatics analysis. In this study, the subcellular localization of NsSOD in fish cells was distributed throughout the cell, not just co-localized with mitochondria. Little is known about the distribution of secreted bacterial SODs within host cells, but the subcellular localization of mammalian SODs has been studied quite a lot. Previous studies showed that mammalian SODs mainly localize

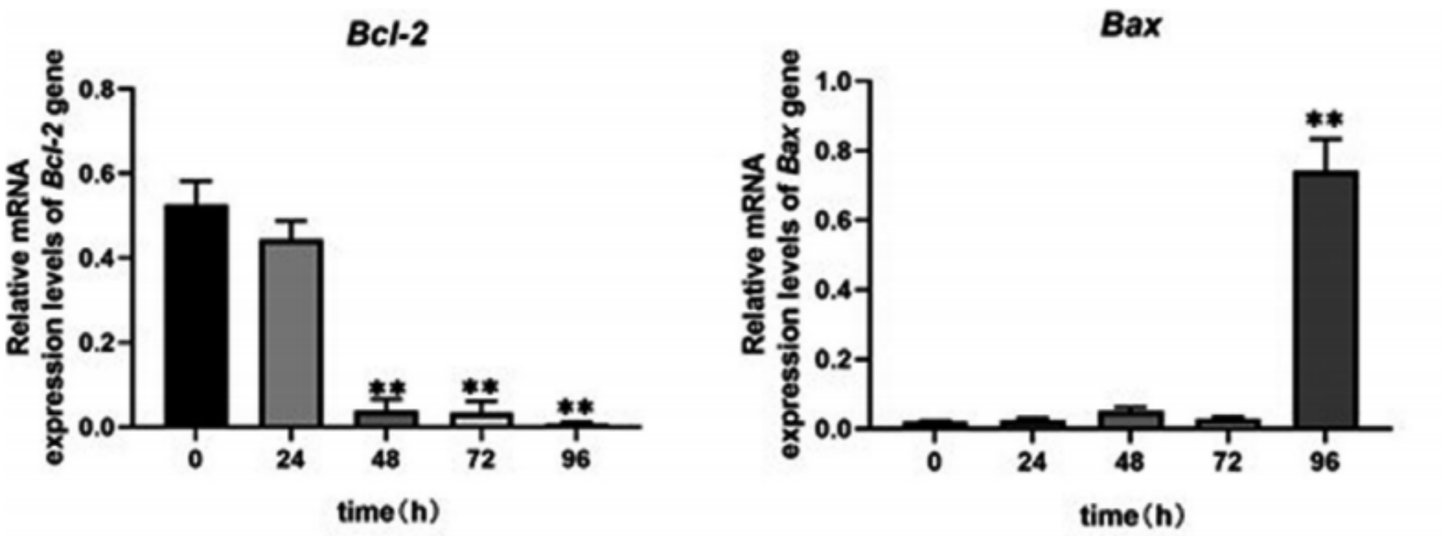

FIGURE 4 qRT-PCR analysis of the expression of four apoptosis-related genes in NsSOD-overexpressing FHM cells. Significant differences were indicated by *($p < .05$), **($P < .01$)

in the mitochondria or cytosol. In brief, the SOD located in the mitochondria of mammalian cell usually belongs to the Cu and Zn binding type, which is related to the apoptosis reaction triggered by cytochrome C (Li, Sato, Zhu, & Inoue, 2009).

Apoptosis or programmed cell death is essential for the proper development and health of multicellular eukaryotes, accompanied by typical morphological features such as nuclear fragmentation (karyorrhexis), chromatin condensation (pyknosis) and cell shrinkage, ultimately breaking down into apoptotic bodies (Kerr, Wyllie, & Currie, 1972). Apoptosis can safely eliminate senescent cells and regulate the normal development of the immune system and organs (Ekert & Vaux, 1997; Hipfner & Cohen, 2004; Monti, Grassilli, & Troiano, 1992). It has been reported that multiple species of pathogenic microorganisms can interfere with this strictly controlled system and cause inopportune apoptotic death of cells in their eukaryotic hosts which is an effective strategy for establishing infections (Moss & Antonios, 1999). Previous studies have shown that some secreted products of *Nocardia* spp. have the ability to cause apoptosis (Camp et al., 2003; Hou et al., 2020; Loeffler et al., 2004; Xia, Chen, & Liao, 2018; Xia, Tong, & Xu, 2017). Given that secreted protein of *Nocardia* spp. may participate in cell death, an experiment to determine whether NsSOD is related to cell apoptosis was performed in this study. The detection of $\Delta\Psi m$, caspase-3 activity and development of apoptotic bodies showed that the overexpression of NsSOD can induce apoptosis of FHM cells.

In addition, the apoptosis signalling pathway related to apoptosis regulation is quite complicated. The published data indicate that the main apoptotic pathways in fish are the extrinsic/death-receptor pathway (Mehlen & Bredesen, 2004) and the intrinsic/Bcl-2-regulated/mitochondrial pathway (Reed, 1997). That fish has similar functionally conserved Bcl-2 family members and an intrinsic apoptotic pathway as mammals has been shown by several lines of evidence. Pro-apoptotic members can be subdivided into BH3-only proteins or multidomain pro-apoptotic proteins (Dos Santos, Vale, Reis, & Silva, 2008). In our study, the expression of pro-apoptotic BH3-only proteins Bax, Bid and Bad was all significantly activated in NsSOD-expressing FHM cells. It is suggested that NsSOD can induce apoptosis of host cells through the intrinsic apoptotic pathway.

In summary, we have shown that the NsSOD is a secreted protein of *N. seriolae* and distributes in the whole FHM cells. The apoptosis detection proved that NsSOD can promote apoptosis through the intrinsic pathway in host cells. This subcellular localization and preliminary functional study of NsSOD may lay the foundation for further studies on the function of this gene and promote the understanding of the virulence factors and pathogenic mechanisms of *N. seriolae*.

5 | Ethics

This experiment did not involve animal experiments. But if there are animal experimental procedures involved, all the experimental procedures were carried out in accordance with the Regulations for Animal Experimentation of Guangdong Ocean University, and the animal facility was based on the National Institutes of Health Guide for the Care and Use of Laboratory (NIH Publications No. 8023).

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CONFLICT OF INTEREST

There were no conflicts of interest.

AUTHOR CONTRIBUTIONS

L. Xia and Y. Lu designed the experiments. G. Chen, W. Wang and S. Hou performed the experiments. S. Hou and G. Chen collected clinical data. SH and WW contributed to data analysis. S. Hou and G. Chen wrote the paper. L. Xia polished the paper.

DATA AVAILABILITY STATEMENT

I confirm that my article contains a Data Availability Statement even if no data are available (list of sample statements) unless my article type does not require one. I confirm that I have included a citation for available data in my references section, unless my article type is exempt.

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