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Research paper

KatG plays an important role in *Aeromonas hydrophila* survival in fish macrophages and escape for further infection

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ABSTRACT

The success of the pathogenic bacteria is partly attributable to their ability to thwart host innate immune responses, which includes resisting the antimicrobial functions of macrophages. And reactive oxygen species (ROS) is one of the most effective antimicrobial components of macrophages to kill invading bacteria. Our previous studies found that *Aeromonas hydrophila* can survive in fish macrophages, which suggested that this bacterium might take fish macrophages as their shelters to resist drug killings and other immune damage. But how *A. hydrophila* survive in host macrophages remains unknown. Since KatG has been reported to have not only catalase activity but also peroxidase and peroxynitritase activity, the amino acid sequence and protein structure of KatG was analyzed in this study, the function of KatG in *A. hydrophila* survival in and escape from host macrophages was also carried out. The bioinformatics analysis displayed that KatG of *A. hydrophila* B11 showed > 93% homologous to that of KatG in other *Aeromonas*. *KatG* of *A. hydrophila* was stable silenced by shRNA and RT-qPCR confirmed the expression of *KatG* in *KatG*-RNAi was significantly reduced. The survival rate of intracellular *KatG*-RNAi decreased by 80% compared to that of the wild type strain B11, while the intracellular ROS level of the macrophages that phagocytosed *KatG*-RNAi increased 65.9% when compared to that of the macrophages phagocytosed wild-type strain. The immune escape rate of *A. hydrophila* decreased by 85% when the expression of *KatG* was inhibited. These results indicated that (1) The amino acid sequence and protein structure of KatG of *A. hydrophila* is conserved; (2) *KatG* helped *A. hydrophila* to survive in fish macrophages by eliminating the harm of intracellular H_2O_2 and inhibiting intracellular ROS levels increased; (3) A small portion of intracellular *A. hydrophila* could escape from host macrophages for further infection, in this process *KatG* also played important role.

1. Introduction

Aeromonas hydrophila, a Gram-negative bacterium widely distributed in aquatic environments (Lin et al., 2017), is the causative agent of motile aeromonad septicemia (MAS) (Harikrishnan et al., 2003), which associated with illness in a broad spectrum of hosts such as mammals, amphibians, reptiles, and fish (Vivas et al., 2004). Studies reported that *A. hydrophila* caused fish outbreaks in aquaculture farms with high mortality rates and severe economic losses to the aquaculture industry worldwide (Fang et al., 2004; Hu et al., 2016; Jiang et al., 2017; Pridgeon et al., 2011; Vivas et al., 2004) and even caused gastrointestinal infections in humans through the food chain, leading to gastroenteritis (Daskalov, 2006).

The prevalence of *A. hydrophila* in various water and food sources represents a significant public health threat (Wu et al., 2007). But till now, pathogenesis mechanism of *A. hydrophila* has not been fully elucidated and antibiotic is still the most popular treatment to control aquaculture animal diseases caused by *A. hydrophila* (Abdel-Tawwab et al., 2017; Gobi et al., 2018). However, the efficacy of antibiotics is not ideal, on the contrary, more and more drug-resistant strains appear to lead to the disease become more difficult to control (Ebrahimi et al., 2012; Parker and Shaw, 2011). The *A. hydrophila* B11 strain was isolated from diseased *Anguilla japonica* and was found to resist Streptomycin, Ampicillin, Penicillin, Gentamicin and so on (Xu et al., 2013). So it's urgent to further reveal *A. hydrophila* pathogenicity and look for safe and effective measures to control *A. hydrophila* infection in aquaculture

Abbreviations: A. hydrophila, *Aeromonas hydrophila*; bp, base pair(s); *C. jejuni*, *Campylobacter jejuni*; Cm, chloramphenicol; DMSO, dimethyl sulphoxide; *E. coli*, *Escherichia coli*; FCS, foetal calf serum; *F. tularensis*, *Francisella tularensis*; H_2O_2, hydrogen peroxide; LB, Luria-Bertani (medium); MAS, motile aeromonad septicemia; MBT, *Mycobacterium tuberculosis*; MIC, minimum inhibitory concentration; MOI, multiplicity of infection; NBT, nitroblue tetrazolium; OD, optical density; ORF, open reading frame; p, plasmid; PBS, phosphate-buffered saline; R, (superscript) resistance/resistant; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; RNS, reactive nitrogen species; ROS, reactive oxygen species; Sm, streptomycin; Tc, tetracycline; TSA, tryptic soy agar; TSB, trypticase soy broth; −, denotes connection; +, denotes complementation; △, deletion

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industry.

Previous studies have revealed that *A. hydrophila* (Leung et al., 1995a; Leung et al., 1995b) is resistant to both serum- and phagocyte-mediated killing, which easily leads to chronic and recurrent infection (Srinivasa-Rao et al., 2001). Our studies also confirmed that *A. hydrophila* can survive in fish macrophages at least 24 h, which suggested that this bacterium could take fish macrophages as their shelters to resist drug killings after infection (Qin et al., 2014). This means traditional antibiotic treatment is not easy to remove infected *A. hydrophila* completely. Studies on molecular mechanism of intracellular survival of *A. hydrophila* will help to understand the pathogenesis of this bacteria from a new perspective and find more safe and effective treatment.

It has been well known that macrophages can be activated to produce several cytotoxic antimicrobial components to kill invading bacteria, among them reactive oxygen species (ROS) is one of the key intermediate (Grayfer et al., 2014; Klionsky, 2007). Thus, exposure to ROS is an unavoidable consequence of pathogenic bacteria (Tondo et al., 2010). Therefore, to cope with the harmful effects of ROS, most pathogenic bacteria have evolved an arsenal of enzymes involved in either direct detoxification of ROS or repair processes of oxidatively damaged cellular components to persist inside host macrophages and even grow and replicate (Imlay, 2008). Among antioxidant enzymes, catalase is central components of the detoxification pathways (Tondo et al., 2010). At least three genes *KatA*, *KatB* and *KatG* have been found in bacteria that encode catalase. KatA and KatB have only catalase activity. KatG was found to have not only catalase activity but also peroxidase and peroxynitritase activity, which had been confirmed to help *Mycobacterium tuberculosis* and *Francisella tularensis* resist ROS and reactive nitrogen species (RNS) and play important role in bacterial replication and persistence in host macrophages (Lindgren et al., 2007; Ng et al., 2004). Maybe *KatG* plays more important role in bacteria virulence.

The genome annotation of *A. hydrophila* B11 isolated from aquaculture fish by our team indicated that this bacterium only had one gene *KatG* encode catalase. However, the precise role of *KatG* in *A. hydrophila* virulence had not been elucidated. So in this study, we tried to reveal the function and possible mechanisms of *KatG* in the survival of *A. hydrophila* in fish macrophages and their escape from macrophages for further infection. Our results demonstrated that *KatG* of *A. hydrophila* can help bacteria to evade or counter oxidative stress and keep survival in fish macrophages and some of them may even escape to trigger further infections.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Escherichia coli was grown at 37 °C in Luria–Bertani (LB) with shaking at 220 rpm. *A. hydrophila* grown at 28 °C in trypticase soy broth (TSB) with shaking at 220 rpm. The bacteria were harvested and re-suspended in phosphate-buffered saline (PBS, pH 7.4) after overnight incubation. The density of bacterial suspension was adjusted according to the OD_{550}. The medium needs to add the appropriate concentration of antibiotics as shown below: 100 μg mL^{-1} streptomycin (Sm); 34 μg mL^{-1} chloromycetin (Cm).

Table 1 lists the strains and plasmids used in this study.

2.2. Bioinformatics analysis of amino acid sequence and protein structure predicted by KatG

The sequence of *KatG* (accession number: MH198046) was obtain by genome scan and analysis. The conserved domain and active site of the protein encoded by *KatG* gene were estimated using Blast and smartBlast. The 3-dimensional structure of the protein was analyzed by Swiss-Model and PyMOL.

Table 1
Strains and plasmids used in this study.

Strain or plasmid	Genotype and/or phenotype	Source or reference
Plasmid		
pACYC184	(Cm^R Tc^R)	Provided by Prof. Nie
pACYC184-*KatG*	pACYC184 derivative containing 60 bp fragment of one short-hairpin RNA sequence targeting the coding region of *KatG* mRNA and ORF (Cm^R)	This study
Strains		
B11	Wild-type strain (Sm^R), isolated from diseased *Anguilla japonica*	Guo, 2006
KatG-RNAi	*KatG* was silenced by shRNA (Sm^R, Cm^R)	This study
E. coli DH5α	F^-, φ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deoR*, *recA1 endA1*, *hsdR17* (rK^-, mK^+), *phoA*, *supE44*, λ^-, *thi-1*, *gyrA96*, *relA1*	Takara

2.3. Stable gene silencing

Stable gene silencing was performed following the method described by Tokunaga et al. (2015). One short-hairpin RNA sequence targeting the coding region of *KatG* mRNA was synthesized by Shanghai Generay Biotech Co. Ltd. (Shanghai, China) (Table 2). The annealed oligonucleotides were ligated using T4 DNA ligase (TaKaRa, Kusatsu, Japan) into the Tc operon of pACYC184 vector double digested with *Bam*HI and *Sph*I (Qin et al., 2014). The recombinant plasmids pACYC184-*KatG* were identified by DNA sequencing and transformed into *A. hydrophila* via electroporation. The clones of *KatG*-RNAi were screened by chloramphenicol and qPCR was used to evaluate the expression of *KatG*.

2.4. RNA extraction and reverse transcription

Total RNA was extracted from the bacteria using TRIzol (Invitrogen, Carlsbad, CA, USA) following the manufacturer's recommended protocol. First-strand cDNA was synthesized from the total RNA using a Revert Aid Mu-MLV cDNA synthesis kit following the manufacturer's recommended protocol.

2.5. qRT-PCR

qRT-PCR was performed on a QuantStudio™ 6 Flex real-time PCR system (ABI, Carlsbad, CA, USA) using Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA). The reactions were performed in a 10-μL volume mix containing 0.2-μL Power SYBR Green PCR Master Mix, 5 pmol/L specific primers, and approximately 50 ng cDNA. The cycling parameters were 95 °C for 10 min, followed by 45 cycles of 95 °C for 20 s, 56 °C for 20 s, and 72 °C for 20 s. Threshold cycles and dissociation curves were determined with QuantStudio™ 6 Flex software, to confirm that only one PCR product was amplified and detected. Gene expression levels were normalized to 16S rRNA. The Relative Expression Software Tool (version 2, REST 2008) was used to calculate the relative expression of genes in qRT-PCR using the Pair Wise Fixed Reallocation Randomization Test (Pfaffl et al., 2002). The mathematical model used was based on the mean crossing point deviation between the sample and the control group, normalized by the mean crossing point deviation of the reference genes. Specific amplification efficiencies were included in the correction of the quantification ratio. Significant differences between groups were determined by ANOVA followed by the Tukey's LSD. The primers are listed in Table 3.

2.6. Preparation of fish macrophages

Healthy Tilapia (1098.1 ± 43.4 g) individuals were obtained from

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