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Chicken-type lysozyme in channel catfish: Expression analysis, lysozyme activity, and efficacy as immunostimulant against *Aeromonas hydrophila* infection

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ABSTRACT

To understand whether chicken-type lysozyme (Lys-c) in channel catfish was induced by infection of *Aeromonas hydrophila*, the transcriptional levels of Lys-c in skin, gut, liver, spleen, posterior kidney, and blood cells in healthy channel catfish was compared to that in channel catfish infected with *A. hydrophila* by bath immersion. Quantitative PCR revealed that the transcription levels of Lys-c in infected catfish were significantly ($P < 0.05$) induced in all five tissues tested as well as in blood cells. Recombinant CC-Lys-c produced in *Escherichia coli* expression system (R-CC-Lys-c) exhibited significant ($P < 0.05$) lytic activity to Gram-positive *Micrococcus lysodeikticus* and Gram-negative *A. hydrophila*. When pcDNA3.2-vectored recombinant channel catfish lysozyme-c (pcDNA-Lys-c) was transfected in channel catfish gill cells G1B, the over-expression of pcDNA-Lys-c offered significant ($P < 0.05$) protection to G1B against *A. hydrophila* infection. When channel catfish were intraperitoneally injected with QCDCR adjuvant formulated pcDNA-Lys-c and challenged with a highly virulent *A. hydrophila* strain AL-09-71 at 1-, 2-, 14-, and 28-days post treatment, pcDNA-Lys-c offered 75%, 100%, 60%, and 77% protection to channel catfish, respectively. Macrophages of fish treated with pcDNA-Lys-c produced significantly ($P < 0.05$) higher amounts of reactive oxygen species and nitric oxide than that of fish treated with pcDNA vector alone. Taken together, our results suggest that pcDNA-Lys-c could be used as a novel immunostimulant to protect channel catfish against *A. hydrophila* infection.

1. Introduction

Aeromonas hydrophila, a Gram-negative motile bacillus widely distributed in aquatic environments, is a causative agent of motile aeromonad septicemia (MAS) [1]. In West Alabama, a disease outbreak caused by *A. hydrophila* in 2009 and 2010 has led to an estimated loss of more than $3 million annually [2,3]. Virulence studies have revealed that these West Alabama isolates of *A. hydrophila* are highly virulent to channel catfish [3]. To control disease outbreaks caused by *A. hydrophila*, feeding infected fish with antibiotic-medicated feed is a general practice [4]. However, this practice is expensive and usually ineffective as sick fish tend to remain off feed. In addition, MAS diseases caused by *A. hydrophila* such as the 2009 West Alabama isolates can be very acute, causing mortality within 48 h [2,3]. Furthermore, currently in the US, there are only three FDA approved antibiotics for use in aquaculture: oxytetracycline (Terramycin), sulfadimethoxine (Romet-30), and florfenicol (Aquaflor). The widespread use of the limited number of antibiotics for treating bacterial diseases in aquaculture has led to the development of antibiotic resistance in many fish pathogens worldwide [5,6]. Therefore, alternative control methods are urgently needed for the aquaculture industry.

Use of vaccine is an alternative control method to prevent MAS. The most extensively studied *A. hydrophila* vaccines are bacterins consisting of formalin or heat-killed bacteria of pathogenic *A. hydrophila* strains [7–9]. In addition, recombinant protein vaccines such as *A. hydrophila* outer membrane proteins and bacterial lysate have been demonstrated to elicit protection against *A. hydrophila* challenges [10–13]. Furthermore, live attenuated vaccines such as *aroA* mutant and transposon Tn916-generated mutant have been reported to confer significant protection

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against homologous *A. hydrophila* challenge [14,15]. In addition, live attenuated *A. hydrophila* vaccines were developed specifically against the highly virulent west Alabama isolates through selection for resistance to both novobiocin and rifampicin [16]. However, whether these vaccines could offer immediate protection to fish is currently unknown. It is well known that MAS diseases caused by *A. hydrophila* such as the west Alabama isolates can be very acute, causing mortality within 48 h [2,3]. Therefore, a treatment that could offer immediate protection such as within two days post treatment is urgently needed.

Innate immune system plays an essential role in the early defense against pathogen infection and lysozyme is a key component of the innate immune system. There are two types of lysozymes in fish: chicken-type or goose-type [17]. It was reported that the transcriptional level of lysozyme c in the anterior kidney was significantly induced by infection or vaccination with live *A. hydrophila* [18]. However, it was unknown whether lysozyme c was up-regulated in other tissues of channel catfish following infections of *A. hydrophila*. In addition, it was unknown whether channel catfish lysozyme c possesses any lysozyme activity. Furthermore, it was unknown whether channel catfish lysozyme c could be used as an immunostimulant to protect against *A. hydrophila* infection. Therefore, the objectives of this study were: 1) to determine whether lysozyme c in other tissues of channel catfish besides anterior kidney was induced by infection of *A. hydrophila*; 2) to investigate whether recombinant channel catfish lysozyme c produced in *Escherichia. coli* expression system possess any lysozyme activity; 3) to evaluate whether channel catfish lysozyme c could be used as an immunostimulant to protect catfish against *A. hydrophila*.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Bacterial isolate of *A. hydrophila* AL-09-71 [16] was cultured from the kidney of diseased channel catfish during the 2009 west Alabama disease outbreak. The Gram-positive strain *Micrococcus lysodeikticus* ATCC 4698 was purchased from Sigma—-Aldrich (St. Louis, MO). *E. coli* One Shot TOP10 and BL21 (DE3) chemically competent cells used for recombinant DNA transformation and protein expression were purchased from Life Technologies (San Francisco, CA). Tryptic soy broth (TSB, Becton Dickinson, Sparks MD) was used to culture *A. hydrophila* at 28 °C, whereas Luria—-Bertani Miller broth (LB, Becton Dickinson) was used to culture *E. coli* at 37 °C.

2.2. Experimental fish and sample collection

Fingerling channel catfish (*Ictalurus punctatus* industry pool strain) with mean weight of 30 ± 2 g were selected from stocks maintained at the USDA-ARS-Aquatic Animal Health Research Unit at Auburn, AL. All fish were acclimated for 14 days prior to challenge. Acclimated fish were maintained in 185 L glass aquaria with flow-through de-chlorinated tap water and constant aeration with water temperature at 28 °C. To determine the transcriptional level kinetics of Lys-c in response to *A. hydrophila* infection, thirty fish were sampled after injected with 100 μl of TSB or 10^5 CFU/ml of virulent *A. hydrophila* AL-09-71. The dose of 100 μl of 10^5 CFU/ml was chosen because that was the dose that caused no mortality in infected fish. Five fish per time point per treatment (TSB or *A. hydrophila* injection) were sampled at 0, 3, 6, 12, 24, and 48 h post-injection (hpi). Fish were anesthetized in a 300 mg/L solution of MS-222 before collection of posterior kidney. To determine the transcription level of Lys-c in different tissues under normal physiological conditions, skin, gut, liver, spleen, posterior kidney, and blood cells were collected from ten untreated fish. To determine the transcription level of Lys-c in different tissues in response to *A. hydrophila* infection, skin, gut, liver, spleen, posterior kidney, and blood cells were collected from ten fish after immersion with 100 ml TSB in 1 L water for 1 h or with 100 ml of 10^9 CFU/ml of *A. hydrophila* AL-09-71 in 1 L water for 1 h. The immersion condition of 100 ml of 10^9 CFU/ml of *A. hydrophila* AL-09-71 in 1 L water for 1 h was chosen because that was the immersion condition that caused no mortality in infected fish. All ten fish were sampled at 48 h post-treatment based on the results obtained from the time course study that Lys-c was up-regulated the most at 48 h post infection. All samples were flash frozen in liquid nitrogen and then stored at −80 °C until RNA extraction.

2.3. RNA extraction, cDNA synthesis, and cloning of full length Lys-c from catfish

RNA extraction and cDNA synthesis were performed using published procedures [19]. For the cloning of full length channel catfish Lys-c, *I. punctatus* lysozyme c sequence (GenBank accession No. GU589078) was used to design primers (Table 1). All PCR products and recombinant DNAs were subjected to sequencing at USDA-ARS Mid-South Area Genomic Laboratory (Stoneville, MS) with an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA). Full length cDNA sequence was then subjected to conserved domain search using NCBI program. Multiple sequence alignment and phylogenetic tree was created using ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

2.4. Expression kinetics of Lys-c in posterior kidney of channel catfish

Quantitative PCR (QPCR) was performed using published procedures [19]. Primers used in QPCR are listed in Table 1. All QPCR was run in duplicate for each cDNA sample. The relative transcriptional level of Lys-c was determined by subtracting the cycle threshold (C_t) of the sample by that of the 18S rRNA, the calibrator or internal control, as per the formula: $\Delta C_t = C_t$ (Lys-c) − C_t (18S rRNA). Relative expression level of Lys-c gene at certain time point post injection of TSB or *A. hydrophila* was then calculated by the formula of $2^{\Delta\Delta Ct}$ where $\Delta\Delta C_t = \Delta C_t$ (time point 0) − ΔC_t (time point *x*) as described previously [19]. Relative expression level of Lys-c in *A. hydrophila* infected fish compared to that in TSB-treated fish was then calculated by the formula $2^{-\Delta\Delta Ct}$ where $\Delta\Delta C_t = \Delta C_t$ (*A. hydrophila*) − ΔC_t (TSB) as described previously [19].

2.5. Expression of Lys-c in various tissues under normal and infected condition

Skin, gut, liver, spleen, posterior kidney, and blood cells of normal and infected fish collected at 48 h post bath immersion treatment were used for RNA extraction and cDNA synthesis. QPCR

Table 1
Primers used in this study.

Name	Forward primer (5′–3′)	Reverse primer (5′–3′)
For full length cloning		
Lys-c-full	TTCGGTCTCGAACTGTAGCA	TGCATTGTTGATATTGCTGGA
For recombinant DNA work		
r-Lys-c	CACCATGGAGGCTTTGGT GTTCCTTCTGC	AACTCCACAGCCTGCAGTGTA AGAGCC
For QPCR		
18S	ATGGCCGTTCTTAGTTGGTG	TAGGTAGCACACGCTGATCG
Lys-c	TCTGGCTAACTGGGTTTGCT	TGCCCTGCTGTCTCACTATG

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