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G-protein coupled receptor 18 (GPR18) in channel catfish: Expression analysis and efficacy as immunostimulant against *Aeromonas hydrophila* infection

Julia W. Pridgeon*, Phillip H. Klesius

Aquatic Animal Health Research Unit, USDA-ARS, 990 Wire Road, Auburn, AL 36832, USA

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ABSTRACT

The objectives of this study were: 1) to determine the transcriptional profiles of G-protein coupled receptor 18 (GPR18) in channel catfish after infection with *Aeromonas hydrophila* compared to that in healthy catfish; 2) to determine whether over-expression of GPR18 in catfish gill cells will offer protection against infection of *A. hydrophila*; 3) to determine whether recombinant pcDNA-GPR18 could be used as an immunostimulant to protect channel catfish against *A. hydrophila* infection. Quantitative PCR revealed that the transcription levels of GPR18 in all tissues of infected catfish were significantly ($P < 0.05$) induced except in the intestine. When pcDNA3.2-vectored recombinant GPR18 was transfected in catfish gill cells G1B, the over-expression of pcDNA-GPR18 offered significant ($P < 0.05$) protection to G1B cells against *A. hydrophila* infection. When channel catfish were intraperitoneally injected with QCDCR adjuvant formulated pcDNA-GPR18 and challenged with a highly virulent *A. hydrophila* strain at 1-, 2-, 14-, and 28-days post treatment, pcDNA-GPR18 offered 50%, 100%, 57%, and 55% protection to channel catfish, respectively. Macrophages of fish treated with pcDNA-GPR18 produced significantly ($P < 0.05$) higher amounts of reactive oxygen species and nitric oxide than that of fish treated with pcDNA vector alone. In addition, serum lysozyme activity of catfish injected with pcDNA-GPR18 was significantly ($P < 0.08$) increased. Taken together, our results suggest that pcDNA-GPR18 could be used as a novel immunostimulant to provide immediate protection to channel catfish against *A. hydrophila* infection.

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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 against homologous *A. hydrophila* challenge [14,15]. In addition, live

* Corresponding author. Aquatic Animal Health Research Unit, 990 Wire Road, Auburn, AL 36832, USA. Tel.: +1 334 887 3741; fax: +1 334 887 2983.
E-mail addresses: Julia.Pridgeon@ars.usda.gov, juliapridgeon@yahoo.com (J.W. Pridgeon).

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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 vaccine that could offer immediate protection is urgently needed.

Innate immune system plays an essential role in the early defense against pathogen infection. It was reported that the transcriptional levels of several G-protein-coupled receptors (GPRs) in the anterior kidney of channel catfish was significantly induced by infection with *Edwardsiella ictaluri* [17]. Quantitative PCR array of 94 human GPRs revealed that GPR18, the most abundantly over-expressed orphan GPR, is able to inhibit apoptosis [18]. However, it was unknown whether GPR18 was up-regulated in tissues of channel catfish following infections of *A. hydrophila*. In addition, it was unknown whether recombinant channel catfish GPR18 plasmid DNA could be used as immunostimulant to protect catfish against *A. hydrophila* infection. Therefore, the objectives of this study were: 1) to determine the transcriptional profiles of GPR18 in channel catfish after infection with *A. hydrophila* compared to that in healthy catfish; 2) to determine whether over-expression of GPR18 in catfish gill cells will offer protection against infection of *A. hydrophila*; and 3) to determine whether recombinant pcDNA-GPR18 could be used as an immunostimulant to protect channel catfish against *A. hydrophila* infection.

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). *Escherichia 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 transcription level of GPR18 in different tissues under normal physiological conditions, eight tissue or cells (skin, intestine, liver, spleen, anterior kidney, posterior kidney, brain, and blood) were collected from ten untreated fish. Anterior kidney tissues of 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). The anterior kidney tissue was chosen based on the results of GPR18 expression analysis (the tissue that expressed the most GPR18 after infection with *A. hydrophila*). Fish were anesthetized in a 300 mg/L solution of MS-222 before collection of anterior kidney. All samples were flash frozen in liquid nitrogen and then stored at −80 °C until RNA extraction.

2.3. RNA extraction, cDNA synthesis, cloning, and bioinformatics

RNA extraction and cDNA synthesis were performed using published procedures [19]. For the cloning of full length channel catfish GPR18, *I. punctatus* GPR18 sequence (GenBank accession No. NP_001187728) was used to design primers (Table 1). PCR products 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 identity search using NCBI Blast program. Multiple sequence alignment and phylogenetic tree was created using ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Prediction of protein sorting signals and localization sites was performed using PSORT program (http://psort.hgc.jp/). Protein molecular weight was calculated using the following program: http://bioinformatics.org/sms/prot_mw.html.

2.4. Expression of GPR18 in various tissues under normal and infected condition

To determine the transcription level of GPR18 in different tissues under normal physiological conditions, eight tissue or cells (skin, intestine, liver, spleen, anterior kidney, posterior kidney, brain, and blood) were collected from ten untreated fish. To determine the transcription level of GPR18 in different tissues in response to *A. hydrophila* infection, the eight tissues 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. Tissues of normal and infected fish collected at 48 h post bath immersion treatment were used for RNA extraction and cDNA synthesis. QPCR were performed using published procedures [19]. Relative transcriptional level of GPR18 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: $\Delta C_t = C_t$ (GPR18) − C_t (18S rRNA). Relative expression level of GPR18 in *A. hydrophila* infected fish compared to that in TSB-treated fish was then calculated by the formula $2^{-\Delta\Delta Ct}$ where $\Delta\Delta C_t = \Delta C_t$ (TSB) − ΔC_t (*A. hydrophila*) as described previously [19].

2.5. Expression kinetics of GPR18 in anterior kidney of channel catfish

To determine the transcription level of GPR18 in different tissues in response to *A. hydrophila* infection, the eight tissues 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-immersion. To

Table 1
Primers used in this study.

Name	Forward primer (5′–3′)	Reverse primer (5′–3′)
For full length cloning		
GPR18	AGAGCTTGATGCGAGATGGT	GACTAGCTCGCAGTCCTCTCA
For recombinant DNA work		
r-GPR18	CACCATGGAACAGAACACATCTTTGACC	GATCATGGCACTGGTAAGG
For QPCR		
18S	ATGGCCGTTCTTAGTTGGTG	TAGGTAGCACACGCTGATCG
GPR18Q	GTGGGGATTATTGGCCTTTT	CAAATTCCCACACACGACAG