Characterization of an Attenuated *Aeromonas hydrophila* Vaccine and Molecular Mechanisms of Channel Catfish Immunity against *Aeromonas hydrophila* infection

by

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Abstract

An attenuated *Aeromonas hydrophila* AL09-71 N+R vaccine strain was compared to its virulent parent strain *A. hydrophila* AL09-71. The attenuated AL09-71 N+R strain was developed through selection of resistance to both novobiocin and rifampicin. The attenuated AL09-71 N+R strain had smaller colony size, slower growth and weaker chemotactic response compared to AL09-71. The motility and invasion ability of AL09-71 N+R were found to be abolished whereas that of AL09-71 was retained. The fatty acid methyl ester profiles of the attenuated strain AL09-71 N+R were detected to be different from that of AL09-71. However, at genomic DNA level, AL09-71 N+R appeared to be similar to that of AL09-71.

To understand the molecular mechanisms of protection elicited by the attenuated AL09-71 N+R vaccine strain in catfish, suppression subtractive hybridization (SSH) was used to identify genes up-regulated by the vaccine. A total of 22 unique genes were identified at 12 h post vaccination. Of the 22, six were confirmed to be significantly induced by vaccination. In addition, 88 channel catfish genes that were reported to be associated with host immunity were included in the expression analysis. Of the 88 genes, 14 were found to be significantly up-regulated by the vaccination. Expression profiles of the 20 genes at different time points showed that the pattern of gene up-regulation in vaccinated fish was similar to that in infected fish.

To understand whether channel catfish response to secondary infection is similar to primary infection, SSH was used to identify genes up-regulated by secondary infection. Of the 28 unique genes identified by the library, eight were confirmed to be significantly induced by

secondary infection compared to that by primary infection at 6 h post infection. In addition to the eight genes identified by SSH, 94 genes known to be associated with host immunity were also subjected to expression analysis. Of the 94 genes, 22 were identified to be induced and differential regulated at different time points. These results suggest that channel catfish host response play an important role in its immunity against *A. hydrophila* infection.

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List of Abbreviations

AMPs	Antimicrobial peptides
bp	Base pair
BPI	Bactericidal permeability-increasing protein
cDNA	Complementray DNA
CELSR1	Cadherin EGF LAG seven-pass G-type receptor 1
CFU	Colony forming unit
*C*t	Cycle threshold
DNA	Deoxyribose nucleic acid
dpi	Day post infection
dpv	Day post vaccination
EST	Expressed sequence tags
FAMEs	Fatty acid methyl esters
hpi	Hour post infection
hpv	Hour post vaccination
Ig	Immunoglobulin
IL	Interleukin
IP	Intraperitoneally
LB	Luria–Bertani
LD_{50}	Lethal dosage necessary to kill 50% of a test population

LEAP2	Liver-expressed AMP 2
MAS	Motile aeromonad septicemia
NKL	NK-lysin
PADII	Protein-arginine deiminasae type II-like
PCR	Polymerase chain reaction
QPCR	Quantitative PCR
RPS	Relative percent of survival
SSH	Suppression subtractive hybridization
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
TNFAIP2	Tumor necrosis factor alpha, alpha-induced protein 2
TOPK	Lymphokine-activated killer T-cell-originated protein kinase-like
TSA	Tryptic soy agar
TSB	Tryptic soy broth
VLIG	Very large inducible GTPase 1

I. INTRODUCTION AND LITERATURE REVIEW

Aeromonas hydrophila

Aeromonas hydrophila is a gram-negative bacterium classified as a member of the family *Vibrionaceae* whose normal habitat is soil and water (Popoff and Veron, 1976). Historically, the genus *Aeromonas* has been divided into two groups: nonmotile group (psychrophilic species) and motile group (mesophilic species). The nonmotile group can be best represented by *Aeromonas salmonicida*, which are generally only associated with fish disease. However, the motile group is associated with human disease (Seshadri et al., 2006). *Aeromonas hydrophila* is in the motile group category, which has the unusual potential to infect both cold- and warm-blooded organisms. Thus, it causes devastating epidemics in fish, reptile, and amphibian populations (Schubert, 1967; Shotts et al., 1975) as well as sporadic water-associated human disease (Schubert, 1967). Channel catfish (*Ictalurus punctatus*) is the most important cultured fish species in the United States. In West Alabama, a motile aeromonad septicemia (MAS) disease outbreak caused by *A. hydrophila* in 2009 alone led to an estimated loss of more than 3 million pounds of food size channel catfish (Pridgeon and Klesius, 2011a). Virulence studies have revealed that AL09-71, a 2009 West Alabama isolate of *A. hydrophila*, is highly virulent to channel catfish, killing fish within 24 h post exposure (Hemstreet, 2010; Pridgeon and Klesius, 2011b). In 2010, *A. hydrophila* continued to cause disease outbreaks in channel catfish grown in Alabama (Hemstreet, 2010).

Motile aeromonad septicemia

Aeromonas hydrophila is the causative agent of MAS. This disease occurs mostly from February to July, with some outbreaks occurring in September and November (Woo and Bruno, 2002). It was the most frequently diagnosed bacterial fish disease and the most severe disease problem among cage cultured channel catfish in the USA between 1972 and 1980 (Plumb, 1994). Subsequently, it became one of the most common bacterial infections (1987-1991) among cage-cultured channel catfish in the USA, accounting for 13-22% of disease outbreaks (Duarte et al., 1993).

MAS causes diverse pathological conditions including acute and chronic infections. The disease is usually acute in very young fish and chronic in adult fish (Plumb, 1994). Infected fish lose their appetite, become lethargic and swim near the surface. The clinical signs of *A. hydrophila* infections include swelling of tissues, dropsy, red sores, necrosis, ulceration, and hemorrhagic septicemia (Karunasagar et al., 1989, Azad et al, 2001). However, in the acute form of MAS, the fish can die so rapidly before they have time to develop clinical signs but a few gross signs of disease. The infection of *A. hydrophila* on channel catfish can be divided into three categories: (i) motile aeromonad septicemia with external signs; (ii) cutaneous, manifesting lesions that are limited to the skin and underlying muscle; and (iii) latent septicemia with no external signs (Grizzle and Kiryu, 1993). Internal clinical signs include oedema, haemorrhage, and necrosis (Woo and Bruno, 2002).

Prevention and control

MAS outbreaks are generally related to environmental stress such as elevated water temperature, a decrease in dissolved oxygen concentration, low pH, and increased levels of

ammonia and carbon dioxide (Walters and Plumb, 1980; Lio-Po et al., 1986). Environmental variables are monitored for stressful situations and possible avoidance of outbreaks.

Oxytetracycline (Terramycin) has been the drug of choice for treating MAS in fishes, which is approved for use with pond fishes, channel catfish, and salmonids (Cipriano and Austin, 2011). Medicated feed with 2–4 g oxytetracycline kg^{-1}feed (50–100 mg/kg fish) for 14 days is recommended (Plumb, 1994). However, drug-resistant strains of *A. hydrophila* may evolve. The antibiotic and chemical resistance development is a persistent problem in *A. hydrophila* management in aquaculture.

Vaccination is regarded as a good method to prevent MAS disease caused by *A. hydrophila* (Evelyn, 1997). Formalin or heat-killed bacteria of pathogenic *A. hydrophila* strains show limited success in protection (Areechon et al., 1992; Chandran et al., 2002; John et al., 2002). Vaccination with crude lipopolysaccharide (LPS) induced better protection against *A. hydrophila* infection in the common carp, *C. carpio*, than the formalin killed vaccine (Baba et al., 1988). Additionally, live attenuated vaccines have been studied to be effective in protection against homologous *A. hydrophila* challenge such as aroA mutant and transposon Tn916-generated mutant (Hernanz Moral et al., 1998; Liu et al., 2007). Furthermore, recombinant protein vaccines, developed by using protein OmpTS or S-layer protein have been reported to confer protection against *A. hydrophila* challenges (Khushiramani et al., 2007; Poobalane et al., 2010). However, it is well known that *A. hydrophila* is very heterogeneous biochemically and serologically, which is the biggest obstacle in developing effective commercial vaccine against *A. hydrophila* (Poobalane et al., 2010). Recently, an attenuated vaccine AL09-71 N+R specifically targeting *A. hydrophila* AL09-71 was developed to prevent future disease outbreaks

caused by the highly virulent West Alabama 2009 isolates of *A. hydrophila* (Pridgeon and Klesius, 2011c).

Important immune factors in channel catfish

Immune system has been divided into two parts: innate immunity and adaptive immunity. In teleosts, both innate and adaptive immune responses are initiated in against bacterial infection. The innate immune system is of prime importance in the immune defense of fish, which provides the first line of immune defense, whereas the adaptive immunity relies on the generation of random and highly diverse repertoires of T and B-lymphocyte receptors contributes to a more specific and efficient response against infections (McGuinness et al., 2003; Medzhitov, 2007). However, this dichotomy between innate and adaptive systems has been challenged by increasing evidences of the integration of different immune mechanisms into a multilevel network (Flajnik and Du Pasquier, 2004). The immune system not only protects an organism against diseases by identifying and eliminating the pathogen, but also play an important role in processes that maintain stable conditions (homeostasis) following inflammatory reaction or tissue damage (Magnadóttir, 2010). Numerous immune-relevant genes involved in innate and/or adaptive immunity have been characterized from channel catfish, include the following:

1) Toll-like receptors:

There is a growing interest in Toll-like receptors (TLRs), which is demonstrated by the 2011 Nobel Prize in medicine awarded to BA Beutler and JA Hoffmann for their studies on TLR role in physiology and pathology. Innate immune initiation relies on the recognition of pathogen-associated molecular patterns (PAMPs) by pathogen recognizing receptors (PRRs) that induces subsequent host immunity through multiple signaling pathways that contribute to the eradication of the pathogen (Janeway and Medzhitov, 2002). There are several functionally distinct classes

of PRRs, but the best characterized are Toll-like receptors (TLRs). All TLRs are type I transmembrane proteins which were composed by three parts: an N-terminal ectodomain containing leucine-rich repeats (LRR) that mediate the recognition of PAMPs, a transmembrane region with one a-helix, and a C-terminal intracellular Toll-IL-1 receptor (TIR) domain that activates downstream signaling pathways (Kawai and Akira, 2011). Following recognition of ligands, TLRs initiate both cell–cell interaction and signaling events that result in acute innate responses. TLRs are also responsible for initiation of adaptive immune responses against pathogen-derived antigens primarily through triggering dendritic cell activation (Pasare et al., 2004). At present, at least 17 TLRs (TLR1, 2, 3, 4, 5, 5S, 7, 8, 9, 13, 14, 18, 19, 20, 21, 22, 23) were identified in teleost species, among which TLR14, TLR19, TLR20, TLR21, TLR22, and TLR23 are non-mammalian TLRs and TLR5S is a soluble isoform of TLR5 that appears to be unique in fish (Palti, 2011). The best characterized ligand that TLRs recognize include: (1) lipoteichoic acid and lipoproteins by TLR2; (2) dsRNA by TLR3; (3) lipopolysaccharide (LPS) by TLR4; (4) bacterial flagellin by TLR5, (5) single stranded RNA (ssRNA) by TLR7, and (6) dsDNA by TLR9 (Baoprasertkul et al., 2007b; Iwasaki and Medzhitov, 2010). Additionally, TLRs were also reported to have multi-functions and act together in pathogen recognition and signaling (Ishii et al., 2005; Baoprasertkul et al., 2007a). Five TLRs (TLR2, TLR3, TLR5, TLR20 and TLR21) have been reported in channel catfish (Bilodeau and Waldbieser, 2005; Baoprasertkul et al., 2007a; Baoprasertkul et al., 2007b).

TLR2 belong to TLR1 family which was found to recognize lipopeptides (Rebl et al., 2010). Specific lipopeptide derivates are recognized by combinations of different members of the TLR1 family in mammals (Palsson-McDermott and O'Neill, 2007). Regarding teleosts, Chang and Nie (2008) reported a cooperation of TLR2 and the peptidoglycan recognition proteins

(PGRP). In adult zebrafish, the expressions of both TLR2 and TLR1 were induced by Gram-positive *Mycobacterium marinum* at eight weeks post infection (Meijer et al., 2004). In channel catfish and blue catfish (*Ictalurus furcatus*), TLR2-encoding mRNA concentration was found to increase one day post-infection by Gram-negative *Edwardsiella ictulari* (Baoprasertkul et al., 2007a). Recently, Pridgeon et al. (2010) reported that the expression of TLR2 in the head kidney was significantly induced by the infection of *E. ictulari* at 6 hour post infection (hpi). In addition, TLR2 expression was detected to be significantly induced at 12 hpi in the kidney of Indian major carp, *Cirrhinus mrigala*, infected by *A. hydrophila* (Basu et al., 2012a). Since the expression of TLR2 was shown to be up-regulated by infection of both Gram-positive and Gram-negative pathogens, TLR2 may function more widely in teleosts than previously assumed (Rebl et al., 2010).

Matsuo et al. (2008) showed that TLR3 can recognize relatively short dsRNA in the pufferfish. Several studies reported upregulation of fish TLR3s mRNA in response to infections with dsRNA viruses (Phelan et al., 2005; Rodriguez et al., 2005; Su et al., 2008). Interestingly, the increasing expression of TLR3 induced by Gram-negative bacteria was also been detected in zebrafish and channel catfish after infection with the Gram-negative *E. tarda* and *E. ictulari*, respectively (Phelan et al., 2005; Bilodeau and Waldbieser, 2005; Pridgeon et al., 2010).

TLR5 has been identified in bony fish and plays an important role in recognizing the flagellin of bacterial pathogens. In fish, TLR5 was identified to have two forms, membrane-bound TLR5 and soluble TLR5S. Although soluble forms of TLR4S and TLR2S have already been identified in mammals (Iwami et al., 2000; LeBouder et al., 2003), so far no soluble TLR5S was found in mammalian genomes (Rebl et al., 2010). In channel catfish, both, the membrane-bound TLR5 and the soluble TLR5S were characterized and found up-regulated in different

tissues by infection of *E. ictulari* (Bilodeau and Waldbieser, 2005; Baoprasertkul et al., 2007b; Pridgeon et al., 2010).

TLR20 and TLR21 belong to so-called 'fish-specific' TLR family, which were not identified in mammalian (Rebl et al., 2010). The channel catfish TLR20 and TLR21 were characterized by Baoprasertkul et al. (2007b). Although they appear to branch with the murine TLR11, 12 and 13 in phylogenetic analyses, they form distinct branches (Baoprasertkul et al., 2007b; Palti, 2011). No direct evidence of ligand specificity has been identified for TLR20 and TLR21 (Palti, 2011). The roles of TLR20 and TLR21 are also unknown in channel catfish. However, TLR20 and TLR21 in the head kidney were significantly upregulated in channel catfish infected by *Edwardsiella ictaluri* (Pridgeon et al., 2010), indicating that they may play an important role in immune response against Gram-negative bacteria pathogens.

2) Antimicrobial peptides (AMPs)

It is well known that fish is able to secrete a lot of different kinds of antimicrobial peptides (AMPs) that are positively charged short amino-acid-chain molecules. AMPs, also known as host defense peptides, play major roles in the innate immune system, and protect against a wide variety of bacterial, fungal, viral, and other pathogenic infections. To answer how AMPs works in the immune system, two different viewpoints about the anti-bacterial mechanisms of AMPs have been proposed: a) the amphiphilic structure of AMPs can selectively bind to the bacterial membrane and form transmembrane channels, which destruct of their membrane integrity and kill incursive bacteria; and b) AMPs can directly enter the bacterial cell to interact with specific intracellular targets to interfere with bacterial growth and metabolism, thus playing a role in bacterial death (Wimley, 2010; Zhu et al., 2012). Except for the central role in infection and inflammation, AMPs also have other important functions since they are

multifunctional molecules (Lai et al., 2007). For example, some AMPs influence diverse cellular processes including cytokine release, chemotaxis, antigen presentation, angiogenesis and wound healing through interacting with its receptors on the membrane or altering the properties of the mammalian membrane (Lai et al., 2007). These functions can indirectly support the immune system to eliminate bacteria pathogens and help the host maintain stable conditions (homeostasis) and repair damaged tissues. Now, an increasing number of AMPs have been isolated from fish and with their abundance in many tissues, they may represent the most important innate defense in fish (Noga et al., 2011).

Hepcidin, a cysteine-rich amphipathic peptide, is the most widely studied AMP in fish. Hepcidin has been found to play an important role in regulation of iron metabolism and indirect host defense by binding to ferroportin (a key iron exporter on macrophages) and inducing ferroportin-mediated endocytosis and proteolysis (Zhu et al., 2012). Recently, hepticdin were reported to be strikingly induced after challenged by *E. ictaluri* (Pridgeon et al., 2012). Transferrin also has a critical role in iron metabolism, maintaining low levels of extracellular free iron and transporting iron to tissues as required, which also participates in a wide variety of metabolic processes, including immune regulation, antimicrobial and antioxidant activity, DNA synthesis, cytoprotection, and electron transport (Stafford et al., 2003; Ong et al., 2006). In channel catfish, transferrin was identified, sequenced, and characterized by Liu et al. (2010). Six other AMPs [(NK-lysin type 1, NK-lysin type 2, NK-lysin type 3, bactericidal permeability-increasing protein (BPI), cathepsin D and liver-expressed AMP 2 (LEAP2)] have also been reported in channel catfish. The trancriptional profiles of the three types of NK-lysin gene and LEAP2 have been demonstrated to be different in various tissues of normal channel catfish (Bao et al., 2006; Wang et al., 2006ab). The expression profiles of cathepsin D and BPI in response to

pathogen infections at 24 h or longer post-infection have also been reported (Cho et al., 2002; Xu et al., 2005). Recently, the transcriptional files of these six AMPs were analyzed in the anterior kidney of channel catfish infected by *E. ictaluri* (Pridgeon et al., 2012).

3) Cytokines

Cytokines are a family of low molecular weight proteins, which are secreted by activated immune-related cells and related to both innate and adaptive responses (Salazar-Mather and Hokeness, 2006). They can be divided into five types: interferons (IFNs), interleukins (ILs), tumor necrosis factors (TNFs), colony stimulating factors, and chemokines (Savan and Sakai, 2006). Cytokines can modulate immune responses through an autocrine or paracrine manner upon binding to their corresponding receptors (Zhu et al., 2012).

Interleukin-1β (IL-1β) gene is a well studied gene in teleost, which belongs to interleukin-1 (IL-1) family. IL-1 is an important early response pro-inflammatory cytokine that regulate both innate and adaptive immune response. IL-1 could be secreted by monocytes, activated macrophages, granulocytes, endothelial cells, activated Tlymphocytes, and many other cell types (Zhu et al., 2012). So far, IL-1β genes have been identified in various teleost fish species, including rainbow trout (*Oncorhynchus mykiss*), carp (*Cyprinus carpio*), seabass (*Dicentrarchus labrax*), channel catfish (*I. punctatus*) and yellowfin sea bream (*Acanthopagrus latus*) (Zou et al., 1999; Fujiki et al., 2000; Scapigliati et al., 2001; Wang et al., 2006c; Jiang et al., 2008). In general, only one IL-1b gene seems to exist in fish. However, two IL-1-b-like genes encoding 280-amino acid peptides with high identity (94.3%) with each other have been cloned from channel catfish (Wang et al., 2006c).

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II. CHARACTERIZATION OF THE ATTENUATED *AEROMONAS HYDROPHILA* VACCINE STRAIN AL09-71 N+R COMPARED TO ITS PARENT STRAIN AL09-71

Introduction

Aeromonas hydrophila is the causative agent of motile aeromonad septicemia (MAS) (Harikrishnan et al., 2003) in fish which is also known as epizootic ulcerative syndrome (EUS) (Mastan and Qureshi, 2001). Although usually considered as a secondary pathogen, *A. hydrophila* can be a primary pathogen, which may cause outbreaks in fish farms with high mortality rates, resulting in severe economic losses to the aquaculture industry worldwide (Thore and Roberts, 1972; Nielsen et al., 2001; Fang et al., 2004). In Alabama, MAS disease outbreaks caused by *A. hydrophila* in 2009 led to an estimated loss of more than 3 million pounds of food size channel catfish (Pridgeon and Klesius, 2011a). Virulence studies have revealed that the AL09-71 strain, a 2009 Alabama isolate of *A. hydrophila*, is highly virulent to channel catfish, killing fish within 24 h post exposure (Hemstreet, 2010; Pridgeon and Klesius, 2011b). In 2010, virulent strains of *A. hydrophila* were also associated with major disease outbreaks in Alabama (Hemstreet, 2010).

Due to the multiple medications resistance of *A. hydrophila*, vaccination may be a better choice for the farmer to protect fish from the infection with *A. hydrophila*. An attenuated vaccine specifically against *A. hydrophila* AL09-71 was developed through selection for resistance to both novobiocin and rifampicin (Pridgeon and Klesius, 2011c).

The attenuated AL09-71 N+R vaccine provided 80–100% protection against challenges with virulent parent *A. hydrophila* AL09-71 (Pridgeon and Klesius, 2011c). However, the detailed morphological and growth profiles of this attenuated *A. hydrophila* strain has not been well characterized. In addition, whether there is a difference at genomic DNA level between the attenuated AL09-71 N+ R strain and its parent AL09-71 strain is currently unknown. Therefore, the objectives of this study were: 1) To characterize the morphological and biological characters of the attenuated AL09-71 N+R vaccine strain compared to its virulent parent AL09-71 strain; and 2) To understand whether the vaccine strain AL09-71 N+R is different from that of the parent strain AL09-71 at genomic DNA level.

Materials and methods

Bacteria source and growth conditions

The *A. hydrophila* AL09-71 was collected from diseased food-size channel catfish from West Alabama in August 2009. The isolate was cultured on tryptic soy agar (TSA) plates following the procedures described by Panangala et al. (2007). The isolate AL09-71 was then confirmed as *A. hydrophila* through biochemical analysis with standard biochemical tests as well as API-20E (Biomérieux, Durham, NC, USA) and molecular identification using gene-specific primers for four *A. hydrophila* genes: 16S–23S rDNA intergenic spacer region, 60 kDa chaperonin, DNA gyrase B subunit and RNA polymerase sigma factor RpoD (Pridgeon and Klesius, 2011a). The attenuated *A. hydrophila* AL09-71 N+R mutant was produced from the virulent parent strain of *A. hydrophila* AL09-71 through selection for resistance to both novobiocin and rifampicin (Pridgeon and Klesius, 2011c). The isolates were maintained on TSA plates or in tryptic soy broth (TSB) (Difco, Sparks, MD, USA) for

18–24 h at 28 ℃. Bacteria were stored as frozen cultures at -80 ℃ in TSB containing 25% (v/v) glycerol.

Colony forming unit and growth rate

Absorbance readings of overnight sample cultures were adjusted to 1.0 at 540 nm using Thermospectronic spectophotomer (Fisher Scientific, Pittsburgh, PA). Serial dilutions (1:10) of each samples were prepared in TSB and immediately 0.1 ml of dilutions in quadruplicate plated onto TSB plates. After incubating the plates overnight at 28 ℃, number of colonies was counted and the average number of colony forming unit (FU) ml^{-1} were calculated for both samples. For the determination of growth rate, same bacterial cultures above were used. Serial dilutions of 1:2 (starting from 1:10 dilution) up to 1:640 were made in triplicate using sterile 96-microtiter plates for assay. Plates were incubated at 28 ℃ with constant shaking and absorbance measured at time intervals at 540 nm for growth rate using ELISA spectrophotometer. Optical reading were blanked with 0 h readings and log plotted versus incubation time.

In vitro motility assay

Agar was used at a low concentration (2.5 g L^{-1}) as a solidifying agent for the differentiation of parent and mutant on the basis of motility. Tubes containing semi-solid agar were inoculated by stabbing through center of the medium with inoculating needle. Quadruplicate samples of test tubes were incubated at 28 ℃ for 24 h. Motility was observed visually by diffuse growth spreading from the line of inoculation.

In vitro chemotactic response of AL09-71 N+R and AL09-71 to catfish mucus

In vitro chemotaxis assays were performed according to procedures (Klesius et al., 2010) with slight modifications. Briefly, healthy channel catfish were anesthetized with 100 mg L^{-1} tricane methanesulfonate (Agent Chemicals, Redmond, CA). The anesthetized fish were held vertically and mucus was collected from the skin into a petri dish by pressing the edge of petri dish gently against the skin. Mucus samples were then transferred form petri dish into a 1.5 ml tube. The mucus samples were centrifuged at 6000 g for 15 min and the pellets (epithelium cells and cellular debris) were discarded. The mucus protein concentration was then adjusted to 0.2 mg ml^{-1} with PBS. The pooled mucus samples were stored at -20°C until use.

Chemotaxis assay was performed using blind-well chemotaxis chambers (Corning CoStar, Cambridge, MA). Briefly, the bottom chambers were filled with 200 µl of either *A. hydrophila* ($1x10^7$ CFU ml^{-1}) parent or mutant strain. The bottom chamber was separated from the upper chamber by an 8-µm pore diameter polycarbonate membrane filter (Nucleopore, Pleasontan, CA) and the chambers were assembled. Triplicate mucus (0.2 mg protein ml^{-1}) samples were added to the upper chamber of each parent and mutant. As negative controls, lower chamber with tryptic soy broth (TSB) and upper chamber with mucus samples was also included in the assay. The chambers were incubated for 3 h at 28°C. Following incubation, 100 µl mucus from the upper chambers were transferred to a flat-bottom 96-well microtiter plate (Thermo Scientific, Milford, MA). The number of viable bacterial cells in each well was then determined by CellTiter 96 ® AQ_{ueous} Non-Radioactive Cell Proliferation Assay (MTS) (Promega, Madison, WI). Soon after 20 µl of MTS was added to each well, the OD at 490 nm was recorded. The plate was then incubated at 28°C for

20 min. After the incubation, the OD was measured again at 490 nm. Relative increased OD value was calculated using the following formula: ∆ OD 490 nm value (sample) = OD 490 nm value (after incubation) - OD 490 nm value (0 h of the incubation). The relative chemotactic index of AL09-71 or AL09-71 N+R to fish mucus was calculated using the following formula: ∆ ∆ chemotactic index = ∆ OD 490 nm value of sample (AL09-71 or AL09-71 N+R) - ∆ OD 490 nm value of sample alone (without bacteria in the lower chamber, negative control). The experiments were repeated four times.

In vitro invasion of A. hydrophila to G1B catfish gill cells

Invasion assays were performed as described by Thiagarajan et al. (1996) with slight modifications made by Pridgeon et al. (2011). Briefly, G1B gill cells in a total volume of 1 ml were split into 96 –well tissue culture plates with final concentration of $5x10^4$ cells per well and grown at 25°C for 24 h. Overnight culture of *A. hydrophila* parent and mutant strains were adjusted to same OD reading at 540nm (0.80) and, then, were diluted to 1:10 and mixed with cells at ratio 1:5. Gill cells in the absence of any bacteria were used as negative control. Plates were incubated at 25°C for 1h.

For the invasion assay, a total volume of 0.2ml of culture media containing 5mg ml^{-1} gentamicin (Sigma-Aldrich, St. Louis, MO, USA) was added to each well to kill any extracellular bacteria. Plates were incubated at 25°C for 1h. The culture medium containing gentamicin and any extracellular bacteria were gently removed. The 0.2 new fresh medium were added into each well. The number of viable bacterial cells in each well was then determined by CellTiter 96 ® AQ_{ueous} Non-Radioactive Cell Proliferation Assay (MTS) (Promega, Madison, WI). Soon after 20 µl of MTS was added to each well, the OD at 490 nm

was recorded. The plate was then incubated at 28°C for 30 min. After the incubation, the OD was measured again at 490 nm. Relative increased OD value was calculated using the following formula: ∆ OD 490 nm value (sample) = OD 490 nm value (after incubation) - OD 490 nm value (0 h of the incubation). The relative invasion index of AL09-71 or AL09-71 N+R to G1B gill cells was calculated using the following formula: ∆ ∆ invasion index = ∆ OD 490 nm value of sample (AL09-71 or AL09-71 N+R) - ∆ OD 490 nm value of cell alone (negative control). The experiments were repeated four times.

Whole cellular fatty acid profile

Preparation of fatty acid methyl esters (FAMEs) from bacteria grown at 28 ℃ on sheep blood agar plates was done according to the Microbial Identifications Systems (MIS) (MIDI, Newark, DE, USA) version 4.5 (Shoemaker et al. 2005). Briefly, overnight culture of 25-30 mg of bacteria were harvested and placed in 13 mm x 100 mm glass tubes. Bacterial cells were saponifed in 1 ml of a sodium hydroxide and methanol solution while boiling in a water bath for 30 min. After cooling, the fatty acids were methylated in 2 ml of hydrochloric acid and methanol reagent for 10min in an 80 ℃ water bath. Then, the FAMEs were extracted in hexane and methyl tert-butyl ether (1.25 ml). Any residual fatty acids and reagents were removed from the organic extract by washing with 3.0 ml of a 0.3 M sodium hydroxide solution for 5 min. Final, top 2/3 phase was removed and transferred to GC vial and FAMEs were injected into an Agilent Technology 6850 gas chromatograph for analysis following the MIS rapid protocol (RCLN50). FAMEs were identified by comparison of their retention times with those of authentic standards obtained from Microbial ID, Inc. (Newark, DE, USA). FAMEs in hexane were used as calibration standards.

Suppression subtractive hybridization (SSH)

Bacterial genomic DNA was extracted according to the manual of DNeasy kit (Qiagen, Valencia, CA, USA). All DNAs were eluted with distilled water and quantified on a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Rockland, DE). SSH was carried out using PCR-Select Bacterial Genome Subtraction Kit (Clontech, Palo Alto, CA, USA). Genomic DNA of the most virulent 2009 isolate AL09-71was used as tester and that of attenuated AL09-71 N+R was used as driver. According to the manufacturer's instructions, equal amounts (2 mg) of genomic DNA from both tester and driver were digested by RsaI at 37 ℃ overnight. The digested tester DNAs were purified, subdivided equally and ligated with two different adaptors (adaptor 1 and adaptor 2R supplied by the kit) respectively. Two hybridizations were performed. In the first, an excess of driver was added to each adaptor-ligated tester sample followed by 98 ℃ for 1.5 min, 63 ℃ for 6 h. In the second hybridization, the denatured driver was added into two primary hybridization samples which were not denatured followed by 63 ℃ overnight. Finally, the ratio of driver DNA: tester DNA sample was 50:1. After filling in the adapter ends with DNA polymerase, the entire population of molecules was then subjected to PCR to amplify the tester-specific sequences as described in the manual. The secondary PCR amplification product was cloned into pGEM-T Easy vector (Promega, Madison, WI, USA) following manufacturer's instructions and transformed into one Shot® TOP10 chemically competent E. coli (Invitrogen, Carlsbad, CA) according to the manual. Transformed cells were then plated on Luria–Bertani (LB) plates containing ampicillin (100 μg/ml) and X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) (40 μg/ml).

Plasmid DNA isolation and sequencing

From the library, a total of 96 white colonies were subsequently picked and cultured overnight in LB broth in the presence of ampicillin (100 µg/ml) in the Innova™ 4000 Incubator Shaker (New Brunswick Scientific, Edison, NJ) at 37 ℃ and 235 rpm settings, respectively. Overnight cultures were then sent to USDA-ARS Mid South Genomics Laboratory in Stoneville, MS for plasmid DNA extraction and DNA sequencing was carried out with an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequences were trimmed and analyzed using the National Center for Biotechnology Information (NCBI) BlastX program to search for sequence homologies.

Primer design and polymerase chain reaction

Sequencing results of different clones were used to design gene-specific primers by using Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). PCR was performed in a 10 µl mixture consisting of 5 µl of Taq PCR Master Mix (Qiagen, Valencia, CA), 3 µl of nuclease-free H_2O, 1 µl of A. hydrophila genomic DNA (10 ng/µl), 0.5 µl of forward primer (5 µM), and 0.5 µl of reverse primer (5 µM). All PCRs were carried out in a Biometra T Gradient thermocycler (Biometra, Goettingen, Germany). PCR products were analyzed on 1% agarose gel by electrophoresis.

Gel purification and sequencing analysis

PCR products were analyzed on 1% agarose gel by electrophoresis and purified with QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). The purified DNA products and correlated primers were then sent to USDA-ARS Mid South Genomics Laboratory in Stoneville for DNA with an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City,

CA). Sequences were analyzed by using the National Center for Biotechnology Information (NCBI) BlastT, BlastX program to search for sequence homologies and CLUSTALW (Thompson et al., 1994) to do the alignment.

Statistical analysis

Growth rate data and cellular fatty acid profile of parent and mutant strains were analyzed by ANOVOA using Student's t test with a significance level of $P<0.05$ (SAS, version 9.2, Cary, NC).

Results

Colony, growth rate and motility

The colony morphology of parent strain (AL09-71) and attenuated mutant strain (AL09-71 N+R) are shown (Fig. 1). AL09-71 N+R had smaller colony size than its parent, however, no other obvious difference was observed. A comparison on the growth of the attenuated mutant and its parent was determined by turbidity at 540nm. The results showed that the parent strain grew faster than that of AL09-71 N+R at all dilutions tested (1:10, 1:20, 1:40, 1:80, 1:160, 1:320, and 1:640) (Fig. 2). The vitro motility test revealed that the parent strain was motile whereas the vaccine strain was not motile (Fig. 3).

In vitro chemotactic response of AL09-71 N+R and AL09-71 to catfish mucus

The results of the *in vitro* chemotactic response of AL09-71 N+R and AL09-71 to catfish mucus are summarized in Figure 7. The average chemotactic index of AL09-71 to catfish mucus was 1.17 ± 0.14, which was significantly ($P<0.05$) higher than that of AL09-71 N+R.

In vitro invasion of A. hydrophila to G1B catfish gill cells

The results of the *in vitro* invasion of AL09-71 N+R and AL09-71 to G1B catfish gill cells are summarized in Figure 7. The average invasion index of AL09-71 to G1B catfish gill cells was 0.31 ±0.056, which was significantly ($P<0.05$) higher than that of AL09-71 N+R. The average invasion index of AL 09-71 N+R was only 0.016 ±0.009, which was almost zero.

Whole cellular fatty acid profile

The major cellular fatty acids associated with the parent (AL09-71) and attenuated mutant (AL09-71 N+R) strain are shown in Table1. The most abundant fatty acids present in both were 16:0, 18:1 w7c, summed feature 2 and summed feature 3, which collectively accounted for over 80% of total peak area (Fig 4.). The percentage of 16:00 and18:1 w7c fatty acid was significantly higher whereas the percentage of summed feature 3 was significantly lower in total cellular lipid of attenuated mutant compared to patent strain (Fig 4.). No significant differences were detected in the saturated cellular fatty acids between parent and attenuated AL09-71 N+R on dodecanoic acid and tetradecanoic acid.

Characteristics of the subtractive genomic DNA library

A total of 96 clones were obtained from the subtractive genomic DNA library using the highly virulent 2009 isolate of *A. hydrophila* AL09-71 as tester and attenuated vaccine AL09-71 N+R as driver. All 96 clones were subjected to sequencing. Of the 96 clones, 94 contained inserts. After amputation of the vector sequences, BlastX sequence homology analyses were performed by using the BlastX network service of the National Center for Biotechnology Information (NCBI). Of these 94 sequences, 62 sequences were discarded due to redundancy. A total of 32 unique DNA sequences (34%) were obtained from the 94 clones (Table 2). The insert sizes of the 32 unique sequences ranged in size from 147 bp to

840 bp. The average insert size was 406 bp (Table 2). According to the BlastX result unique sequences (94%) in 32 unique DNA were found had an e-value lower than 10^{-20}(Table 2). Twenty-four of the 32 sequences shared high homologies with the genome of *A. hydrophila* ATCC7966 strain deposited at GenBank.

PCR analysis, gel purification and sequence analysis of the 32 genome sequences obtained from the subtractive library

In order to determine the specificity of 32 unique DNA sequences to the highly virulent 2009 isolate of *A. hydrophila* AL09-71, one set of primers (forward primer and reverse primers) was designed for each DNA sequence. Primers used in PCR are listed in Table 3. Genomic DNA from AL09-71 and AL09-71 N+R was used as template in PCR. However, all PCR products using AL09-71 as template yielded similar size to that using AL09-71 N+R as template. The PCR products using the genomic DNA of AL09-71 or AL09-71 N+R as templates were then subjected to sequencing. Sequence analysis of the 64 PCR products revealed that there was no difference between AL09-71 and AL09-71 N+R (Fig 5.).

Discussion

In this study, the attenuated vaccine strain had smaller colony size and significantly ($P<0.05$) lower growth rate compared to the parent strain. The reduced bacterial growth rate is typically regarded as a fitness cost of most antibiotic resistance mechanisms (Andersson and Hughes, 2010). Rifampicin resistance is caused by mutations in DNA-directed RNA polymerase subunit-β. It has been reported that the tested rifampicin-resistant mutants have a reduced fitness on growth rate (Reynolds, 2000; Enne et al., 2004). The slower growth has also been reported as a fitness cost in novobiocin-resistant *Streptococcus iniae* (Pridgeon and

Klesius, 2011d). In addition, resistant mutants usually show decreased fitness as well as decreased virulence (Cohen et al., 2003; Andersson and Hughes, 2010). For example, mutant strains of *Mycobacterium bovis* and *M. tuberculosis* that are resistant to isoniazid was shown to have significantly reduced virulence as measured by host killing and by histopathology (Wilson et al., 1995; Li et al., 1998). Furthermore, Andersson and Levin (1999) reported that mutations that confer antibiotic resistance resulted in reduce fitness, such as decreased virulence and slowed growth rate. Recently, the novobiocin-resistant *Streptococcus iniae* strain was reported to be attenuated with smaller colony size and slower growth rate compared to its parent (Pridgeon and Klesius, 2011d). Taken together, our results suggest that the smaller colony size and slower growth rate associated with the attenuation of virulence of AL09-71 N+R may be regarded as fitness costs related to its resistance to novobiocin and rifampicin.

The ability of a pathogen to attach to, invade and subsequently infect a susceptible host is directly related to its virulence. Motility and the presence of flagella have been related to different early aspects of bacterial pathogenesis, predominantly adherence to and invasion to eukaryotic cells (Yao et al., 1994). Motility is considered to be an important virulence factor in the pathogenesis of *Aeromonas*-associated infections, as it facilitates pathogens to adhere and invade the host cells (Kirov et al., 2002; Khajanchi et al., 2012). Studies have demonstrated the requirement of motility for the virulence of *Helicobacter felis* (Josenhans et al., 1999) and *Pseudomonas aeruginosa* (Feldman et al., 1998). In addition, Merino et al. (1997) reported that motility in *A. hydrophila* of serogroup O:34 strains was important for its adherence to host. Furthermore, a mutant *A. hydrophila* was found to be diminished motility

and attenuated (Khajanchi et al., 2012). In the present study, our result revealed that the motility of AL09-71 N+R strain was impaired, which might, at least, partially explain why AL09-71 N+R strain does not show virulence to channel catfish.

In order to act as a pathogen, bacteria need to have contact with the host. This can be coincidental or the result of directed chemotactic movement. Hazen et al (1982) reported that skin mucus was a chemoattractant for *A. hydrophila*. Based on the difference of chemotactic behaviour of the isolates, it was suggested that there could be a difference in pathogenicity between *A. hydrophila* strains (Hazen et al.,1982). In addition, Ascencio et al. (1998) stated that mucus can serve as a carbon and nitrogen source for *A. hydrophila.* In this study, the *in vitro* chemotaxis assays revealed that AL09-71 had significantly higher chemotactic response to catfish mucus than AL09-71 N+R. Furthermore, our *in vitro* invasion studies revealed that the invasion rate of AL09-71 to G1B gill cells was significantly higher than that of AL09-71 N+R. The attenuated vaccine strain almost lost their invasion ability with much lower invasion index which is near zero. Although cell motility and invasion ability are well known virulence factors (Josenhans and Suerbaum 2002; Zakikhany et al. 2008), the importance of chemotaxis affecting the virulence of pathogens are not extensively studied. It has been suggested that chemotaxis is not necessary for *A. hydrophila* to become pathogenic to common carp, but may be a necessary parameter for *A. hydrophila* to become an obligate pathogen (Van der Marel et al. 2008).

The fatty acid profiles of *Aeromonas* species were described (Canonica and Pisano, 1988; Hansen et al., 1991; Huys et al., 1994). In the present study, the most abundant fatty acids in both strains were hexadecanoic acid (16:0), cis-7-Octadecenoic acid (18:1 w7c), and

summed feature 3(2-Hydroxypentadecanoic acid or cis-7-Hexadecenoic acid) (SF3). These three major components accounted for approximately 80% of the total cellular fatty acids, which confirms with the fatty acid profiles described before. Lambert et al. (1983) reported that *A. hydrophila* and *A. salmonicida* were differentiated from other members of the *Vibrionaceae* because they did not contain 13:l iso and contained only trace amounts of 12:0 3OH. In our study, the fatty acid profiles of both strains do not inculde 13:l iso, but, only AL09-71 N+R contains up to 0.18% 12:0 3OH of total fatty acids. In general, as long as the growth rate was relatively normal, the fatty acid composition is highly conserved (Campbell and Cronan, 2001; Heath et al., 2002). Slight but significantly ($P<0.05$) higher level of hexadecanoic acid (16:0), cis-7-Octadecenoic acid (18:1 w7c) and lower level of SF 3 in the attenuated AL09-71 N+R were found, suggesting that some kinds of mutants may exist on genomic or transcriptional level in AL09-71 N+R , which can affect the fatty acid biosynthesis.

Suppression subtractive hybridization (SSH) has been widely used to identify sequences that are unique to one genome but absent in another (Straus and Ausubel, 1990; Mahairas et al., 1996; Zhang et al., 2000; Olivares-Fuster and Arias, 2008; Dai et al., 2010). Pridgeon et al. (2011) identified 2 sequences only present in highly virulent *A. hydrophila* strains, but absent in avirulent strains by using SSH. However, in this study, no different sequence is detected out between the attenuated mutant and parent strain by SSH. The fact that no difference is identified and confirmed could be due to the following reasons. Firstly, when the samples under comparison have relatively small number of differential sequences and the background is high, it will be easier to get the false positive results (Buzdin and

Lukyanov, 2007). Secondly, the mutant on the genome level may be slight such as point mutation, which is not easier to detect with SSH. Finally, the difference between the AL09-71 and AL09-71 N+R may happen on the transcriptional level.

In conclusion, our results indicate that the attenuated vaccine strain *A. hydrophila* AL09-71 N+R has smaller colony size and slower growth rate compared to its parent AL09-71. *In vitro* motility assay revealed that AL09-71 N+R was immotile whereas AL09-71was motile. The chemotactic response of AL09-71 N+R to channel catfish mucus was significantly lower than that of AL09-71. The ability of AL09-71 N+R to invade catfish gill cells was significantly lower than that of AL09-71. Furthermore, significantly different cellular fatty acid profiles were detected between the vaccine strain and its virulent parent strain. However, at genomic DNA level, the vaccine strain and its virulent parent strain appeared to be similar to each other.

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Table 1. Nomenclature of fatty acid methyl esters of mutant strain AL09-71 N+R and parent strain AL09-71.

Shorthand name	Systematic name	Trivial name
Saturated fatty acids		
12:00	Dodecanoic acid	Lauric acid
14:00	Tetradecanoic acid	Myristic acid
16:00	Hexadecanoic acid	Palmitic acid
18:1ω7cis	cis-7-Octadecenoic acid	Unknown
SF*		
SF-2		
14:0 3-OH	3-Hydroxytetradecanoic acid	3-Hydroxymyristic acid
16:1 iso I	Hexadecenoic acid, isomer I	Palmitoleic acid
SF-3		
15:0 iso 2-OH	2-Hydroxypentadecanoic acid	Unknown
16:1 cis 7	cis-7-Hexadecenoic acid	Palmitoleic acid

*Summed feature (SF) denotes two peaks exhibiting overlapping retention times with fatty acids in each of two elution profiles.

Table 2. List of sequences isolated from mutant strain AL09-71 N+R vs parent strain AL09-71 bacterial genome subtractive library.

No.	Clone no.	Accessetion no.	Gene Name	Organism	E-value	Identity	Insert size
1	A03	YP_855339	sigma-E factor regulatory protein RseB	Aeromonas hydrophila	1.00E-38	100%	480
2	A06	YP_854878	TRAP transporter solute receptor TAXI family protein	Aeromonas hydrophila	1.00E-47	84%	636
3	A07	YP_856271	queuine tRNA-ribosyltransferase	Aeromonas hydrophila	1.00E-12	71%	916
4	A09	YP_855029	Mg2+ transporter	Aeromonas hydrophila	2.00E-46	100%	435
5	A12	YP_854606	ubiquinone/menaquinone biosynthesis methyltransferase UbiE	Aeromonas hydrophila	1.00E-31	97%	356
6	B03	YP_846969	ATPase central domain-containing protein	Syntrophobacter fumaroxidans	5.00E-28	67%	225
7	B04	YP_002479020	AraC family transcriptional regulator	Desulfovibrio desulfuricans	7.00E-77	67%	839
8	B08	YP_001141367	GGDEF domain-containing protein	Aeromonas salmonicida	7.00E-103	99%	1014
9	B09	YP_858309	type 4 fimbrial assembly protein PilC	Aeromonas hydrophila	6.00E-128	98%	583
10	B11	YP_857798	triosephosphate isomerase	Aeromonas hydrophila	2.00E-47	100%	247
11	C02	YP_004393797	SNF2 family protein	Aeromonas veronii	4.00E-156	98%	716
12	C04	ZP_08519501	two-component system response regulator, LuxR family protein	Aeromonas caviae	8.00E-27	96%	225
13	C07	YP_855648	cyclopropane-fatty-acyl-phospholipid synthase	Aeromonas hydrophila	7.00E-107	99%	482
14	C08	YP_854955	zinc protease	Aeromonas hydrophila	4.00E-58	97%	431
15	C09	YP_857681	type IV pilus secretin PilQ	Aeromonas hydrophila	4.00E-140	99%	752
16	D02	YP_857301	DNA-binding transcriptional activator UhpA	Aeromonas hydrophila	2.00E-82	84%	1077
17	D04	ZP_07779650	phage integrase family protein	Escherichia coli	1.00E-135	100%	1086
18	D05	YP_855854	4'-phosphopantetheinyltransferase family protein	Aeromonas hydrophila	3.00E-83	95%	442
19	D06	YP_855273	diguanylate cyclase/phosphodiesterase	Aeromonas hydrophila	4.00E-103	100%	843
20	D11	YP_001142237	ATP-dependent Clp protease, ATP-binding subunit ClpA	Aeromonas salmonicida	8.00E-94	100%	442
21	E01	YP_855297	2-nitropropane dioxygenase family oxidoreductase	Aeromonas hydrophila	3.00E-89	97%	801
22	E02	YP_855232	type IV pilus biogenesis protein	Aeromonas hydrophila	2.00E-53	100%	264
23	E03	YP_001141849	Poly(hydroxyalcanoate) granule associated protein	Aeromonas salmonicida	4.00E-31	95%	547
24	E05	YP_854909	histidine ammonia-lyase	Aeromonas hydrophila	3.00E-37	100%	253
25	E06	YP_854569	sensory box/GGDEF family protein, putative	Aeromonas hydrophila	4.00E-153	98%	888
26	F04	YP_856871	bifunctional fructose-specific PTS IIA/HPr protein	Aeromonas hydrophila	1.00E-151	99%	784
27	F09	YP_856204	maltose transporter membrane protein	Aeromonas hydrophila	2.00E-125	99%	579
28	F11	YP_854816	shikimate kinase	Aeromonas hydrophila	2.00E-64	87%	1013
29	G04	YP_856398	recombination factor protein RarA	Aeromonas hydrophila	0	99%	841
30	G08	ZP_08519441	DNA-binding transcriptional activator GcvA	Aeromonas caviae	2.00E-72	99%	1045
31	G10	YP_857763	large-conductance mechanosensitive channel	Aeromonas hydrophila	3.00E-82	99%	774
32	H01	YP_857314	putative transporter	Aeromonas hydrophila	1.00E-19	98%	438

Table 3. Gene-specific primers used in PCR.

Clone no.	Forward primer (5'–3')	Reverse primer (5'–3')
A03	CAGTCCAGCCGTATTTTGCT	GCCATCGAGATAGCTGAGGT
A06	TCCGGAAATGGTCTATCACC	AGCCGCGCTCTTTGTAGTAG
A07	AGTGGGTGAGGCGAAAGAG	GGTAGTTTTGATGCGGCAGT
A09	ACGACTGATTGGTGGGACAT	GATGGCGAGAAAGCAGTAGG
A12	CCTCGACCGAGAAGGAAAC	GAGCAGTCGATGGTGAAGC
B03	GCCGATCAGTAAGCCAAGAG	AGATAGACCGTGGGACAGGA
B04	CATTTTCACTGCATCGTGCT	GGCATCCTTGATTTTCCTCA
B08	ATGGACGCCTCAATACTGCT	CATAAAGGCCTGCAGGGTAA
B09	GCGCTCTGGAGACCATCTAC	AACTGGGGAATGACGAACAG
B11	AGCAGGCACAAGAGGTTCAT	GCCGCTTCAACAATTCCTAA
C02	GAAGGACCACAGGTTCTGGA	CAAGGAATCAACGCTTCACA
C04	GCAGCCTCAACAAGCAGAT	CGGTTCTTGACACCGAGTTT
C07	CTTGCTGCTGGAGGACTACC	AGCCACCGGGGAAGATATAG
C08	AACAGGCTGTCGGTCAAACT	GTTGAGCGCCTCTTCCAGAT
C09	CCCTGTCTCTCAGGTTGAGG	GAGATGGGTTTGCCCTGATA
D02	ACTCATCTCGAACCCGACTG	TGCACCGACAGCATCACTAC
D04	AAACGAAATTTGGCAACCAG	ATTTCTGCAGGCGACAAAGT
D05	GCAGTTCAATCTCAGCCACA	CGAGCAGCCACTCATATTCA
D06	CCTTCTTCGACCATCTGAGC	TCTCGTGGGTGATGTCGAT
D11	GCTGGACGAGATCGAAAAAG	ATCGTGGCTCATATCCTGCT
E01	GGGCCATTCAACATCAACTT	GGCAGTCCGAAATGAAAACT
E02	CGCTACGTCTATCCCAGAGC	GTTGTTGCTTGAGCGGGTAT
E03	ACCGACAACCAACTCACCTC	TCGAAGATTTTACCGCCTTC
E05	TTTGCCGAGGATGAAGACAT	CATGAAACCGGAGTTGACG
E06	CAGACACTGCTGGTGGAAGA	GAACAGCTGCTGGATCTTGG
F04	GGCATCTGGTGAAGAACACC	GCACCGGTCAGGATATTGAT
F09	GCATCGCCTTCACCAACTAT	ATTGGTCGTCCTGTTTCAGC
F11	TGGCGGGTAAGAGCATTATC	GTTCTTCTGCTCCAGGCTGT
G04	CCGGGTCTATCTGCTCAAAC	GCAGCTCCAGGTAGTTGAGG
G08	ATCTTCAGCCACTCCACCAT	TATCTTGCCCTGATCGGACT
G10	CGGTCGGTATCATCATTGGT	TCGATGATGGTCTGGATGAA
H01	TGCCGATAGTTTATTTTTCCTGA	CCATCACCTGCAACTTGATCT

Figure legends

Figure 1. Colony morhology of *Aeromonas hydrophila* parent AL09-71 and mutant AL09-71 N+R strains. Quadruplicate plates for per strain were incubated overnight at 28°C.

Figure 2. Growth curve of *Areomonas hydrophila* parent AL09-71 and mutant AL09-71 strains for 25 h. Four dilutions as follows: 1:40 (A), 1:80 (B), 1:160 (C), and 1:320 (D) with triplicate.

Figure 3. Motility assay of *Aeromonas hydrophila* parent AL09-71 strain showed diffuse growth throughout the entire medium and mutant AL09-71 N+R strain grown only along the line of inoculation. Quadruplicate tubes were inoculated for per strain.

Figure 4. Fatty acid composition (%) of total lipid from *Aeromonas hydrophila* parent AL09-71 and mutant AL09-71 N+R strains. Values are means of four replicates per strain. Significant deference between parent and mutant strain for the same fatty acid are labeled with a, b.

Figure 5. The alignment results of part sequences from parent AL09-71 (P) and mutant AL09-71 N+R (N+R) strains. * Clone no.

Figure 6. Chemotactic response of *A. hydrophila* AL09-71 and AL09-71 N+R to channel catfish mucus. Data were presented as mean ± standard deviation (S.D.) from four replicates. Significant difference ($P<0.05$) was marked by asterisk.

Figure 7. *In vitro* invasion of *A. hydrophila* AL09-71 and AL09-71 N+R to G1B catfish gill cells. Data were presented as mean ± standard deviation (S.D.) from four replicates. Significant difference ($P<0.05$) was marked by asterisk.

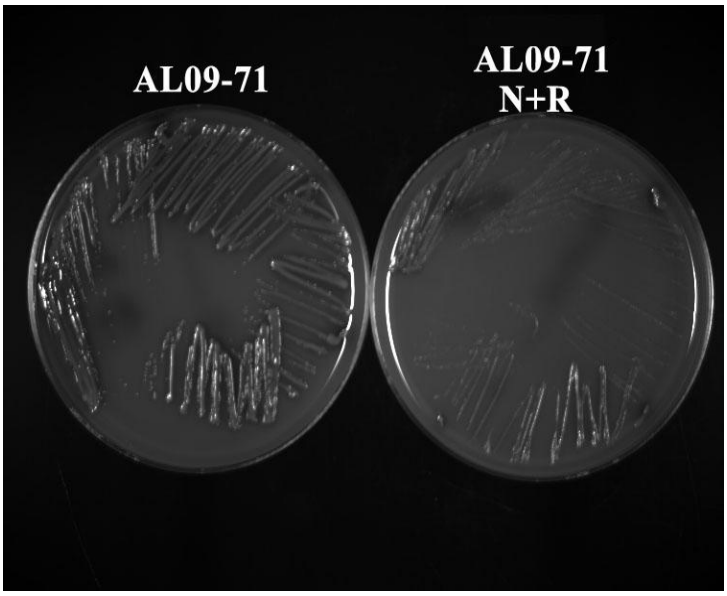

Figure 1.

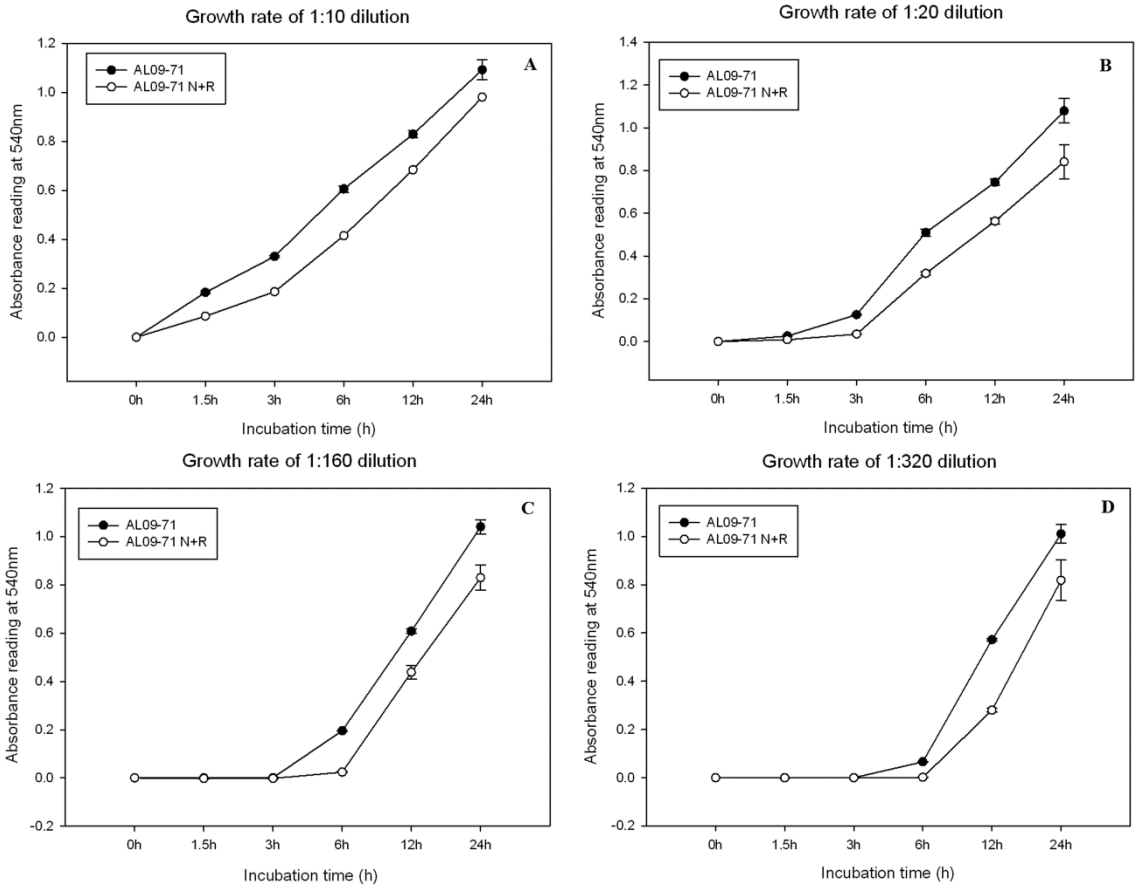
Growth rate of 1:10 dilution
A
AL09-71
AL09-71 N+R
Absorbance reading at 540nm
Incubation time (h)
0h
1.5h
3h
6h
12h
24h
Growth rate of 1:20 dilution
B
Growth rate of 1:160 dilution
C
Growth rate of 1:320 dilution
D

Figure 2.

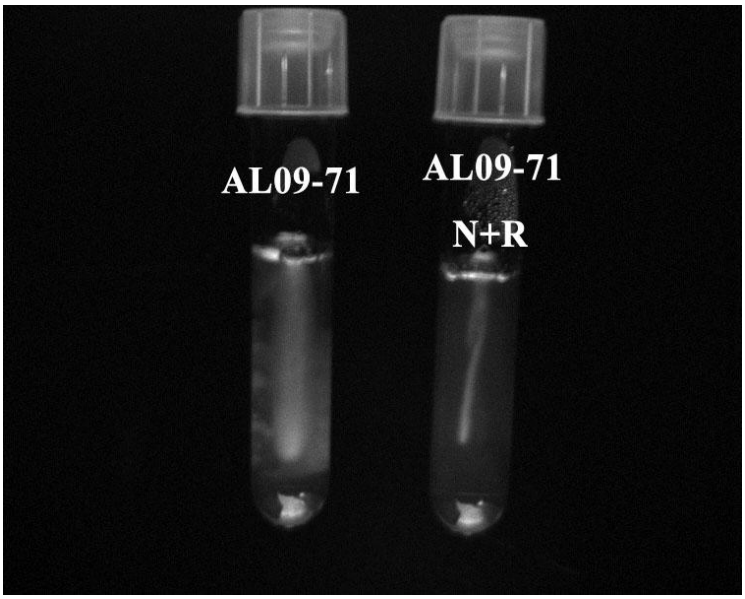

Figure 3.

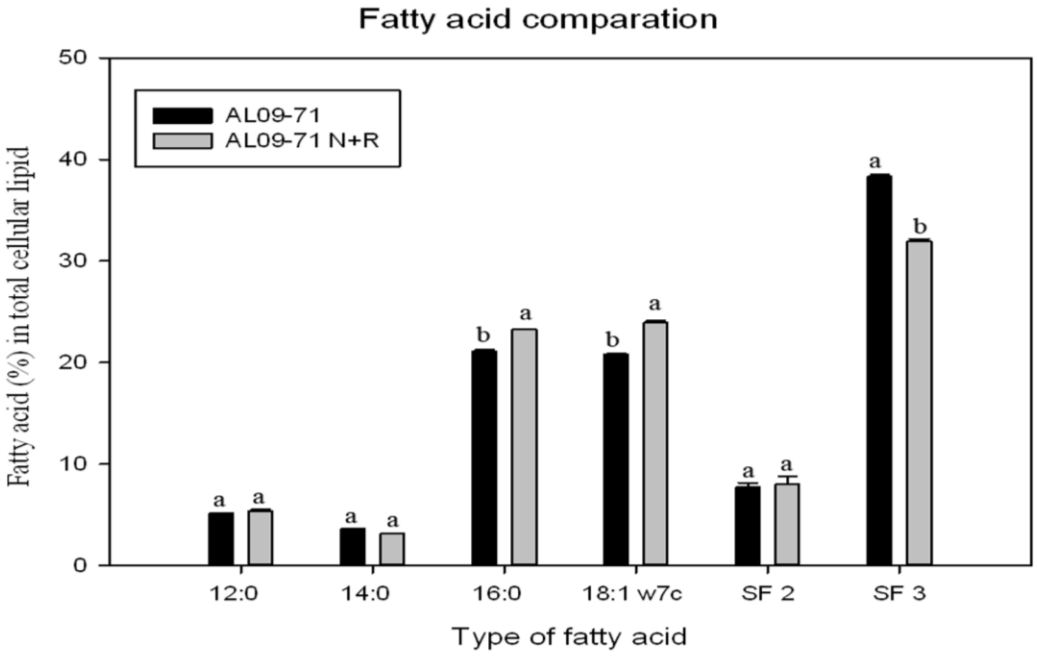

Figure 4.

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P_C07*            1 GAGATGATCGAAGCGGTGGGCCACGCCTTCCTGCCCGACTATTTCCGCCA     50
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
N+R_C07           1 GAGATGATCGAAGCGGTGGGCCACGCCTTCCTGCCCGACTATTTCCGCCA     50

P_C07            51 GCTGTCGCGGCTGCTCAAACCCGGTGGTCGCCTGCTCATTCAGGCCATCA    100
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
N+R_C07          51 GCTGTCGCGGCTGCTCAAACCCGGTGGTCGCCTGCTCATTCAGGCCATCA    100

P_C07           101 CCATCGCCGATCAACGCCATGCCCAGTATCTGCGCGGGGGTGGATTTCATC    150
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
N+R_C07         101 CCATCGCCGATCAACGCCATGCCCAGTATCTGCGCGGGGGTGGATTTCATC    150

P_C07           151 CAGCGCTATATCTTCCCCGGTGGCT    175
                    |||||||||||||||||||||||||
N+R_C07         151 CAGCGCTATATCTTCCCCGGTGGCT    175


P_E01*            1 CCGCTGGTTGCAGCGGCTGACGCCCTACTATGACGAATACGGCGTGCGCG     50
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
N+R_E01           1 CCGCTGGTTGCAGCGGCTGACGCCCTACTATGACGAATACGGCGTGCGCG     50

P_E01            51 ATGCTGCCGGCACGGCGGCTCCCAGTCGGGCCCCGTTCAATGCCGAGCAT    100
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
N+R_E01          51 ATGCTGCCGGCACGGCGGCTCCCAGTCGGGCCCCGTTCAATGCCGAGCAT    100

P_E01           101 GCCGCCATGGTGGCCGAGTTCAAACCGGCCGTGGTCAGTTTTCATTTCGG    150
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
N+R_E01         101 GCCGCCATGGTGGCCGAGTTCAAACCGGCCGTGGTCAGTTTTCATTTCGG    150


P_E06*            1 TGGAGAGCCGCAGCCACTTCGAGCTGTTGCTCGATGAGCGGCTGGCCAGC     50
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
N+R_E06           1 TGGAGAGCCGCAGCCACTTCGAGCTGTTGCTCGATGAGCGGCTGGCCAGC     50

P_E06            51 GAGAGCGGCAGCTTCAGCCTGCTGCAATTCAGCGTCGACCACAGGGCCAA    100
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
N+R_E06          51 GAGAGCGGCAGCTTCAGCCTGCTGCAATTCAGCGTCGACCACAGGGCCAA    100

P_E06           101 GATCCAGCAGCTGTTC    116
                    ||||||||||||||||
N+R_E06         101 GATCCAGCAGCTGTTC    116


P_G04*            1 TCGATCAGGCGATGCAGGACGCTCGCGGGCTCAATGATCCGGCGCTGACC     50
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
N+R_G04           1 TCGATCAGGCGATGCAGGACGCTCGCGGGCTCAATGATCCGGCGCTGACC     50

P_G04            51 TTCGCCCCCGGGGGTGAAGGAGGCGCTGGCCAAGGCGGTGGATGGGGACGG    100
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
N+R_G04          51 TTCGCCCCCGGGGGTGAAGGAGGCGCTGGCCAAGGCGGTGGATGGGGACGG    100

P_G04           101 GCGCAAGTCCCTCAACTACCTGGAGCTGC    129
                    ||||||||||||||||||||||||||||
N+R_G04         101 GCGCAAGTCCCTCAACTACCTGGAGCTGC    129
```

Figure 5

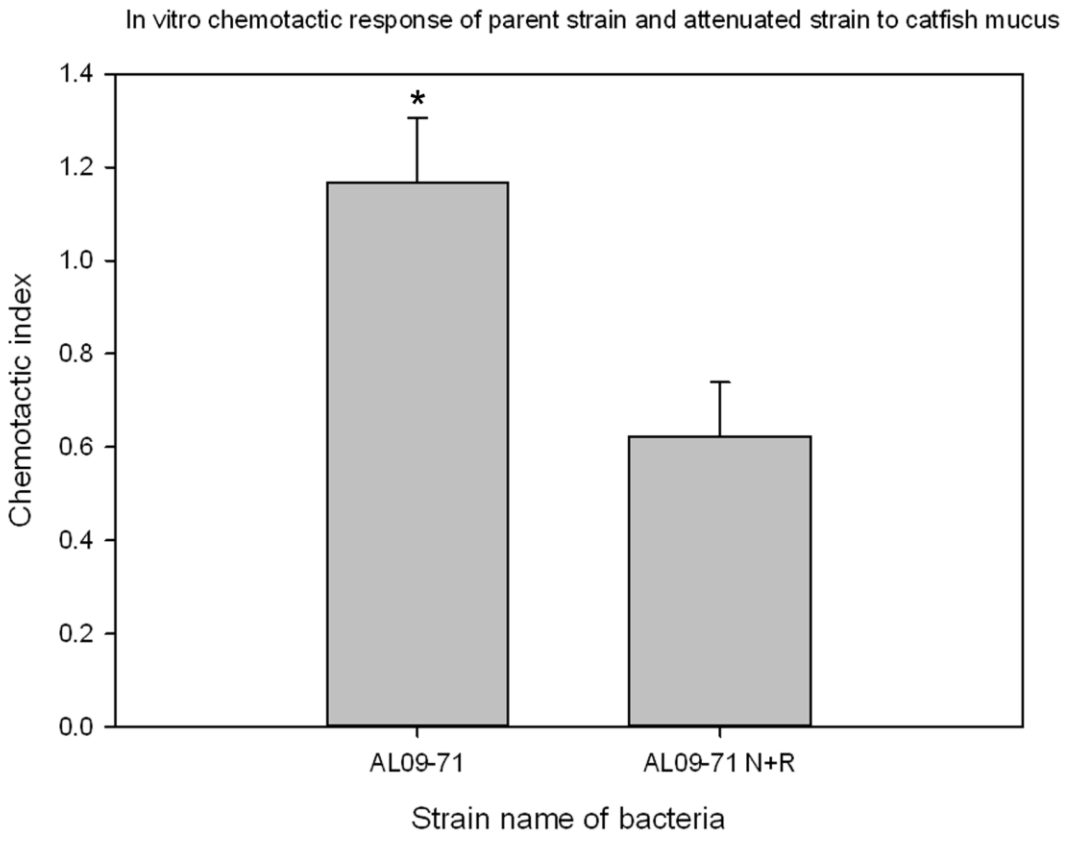
In vitro chemotactic response of parent strain and attenuated strain to catfish mucus
Chemotactic index
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
AL09-71
AL09-71 N+R
Strain name of bacteria

Figure 6

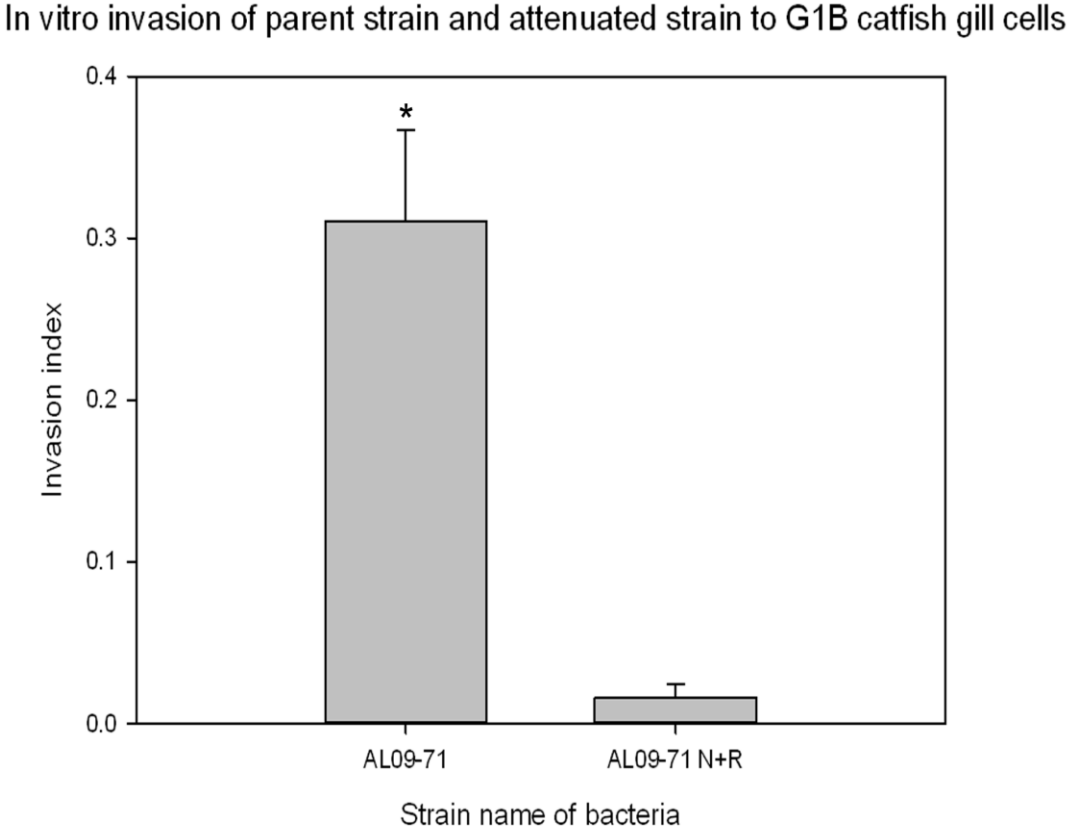
In vitro invasion of parent strain and attenuated strain to G1B catfish gill cells
0.4
0.3
0.2
0.1
0.0
Invasion index
*
AL09-71
AL09-71 N+R
Strain name of bacteria

Figure 7

III. TRANSCRITIONAL PROFILES OF MULTIPLE GENES IN THE ANTERIOR KIDNEY OF CHANNEL CATFISH VACCINATED WITH AN ATTENUATED *AEROMONAS HYDROPHILA*

Introduction

Aeromonas hydrophila, a Gram-negative motile bacillus widely distributed in aquatic environments, is a causative agent of motile aeromonad septicemia (MAS) (Harikrishnan et al., 2003). MAS is also known as epizootic ulcerative syndrome (EUS) (Mastan and Qureshi, 2001). The symptoms of *A. hydrophila* infections include swelling of tissues, dropsy, red sores, necrosis, ulceration, and hemorrhagic septicemia (Karunasagar et al., 1989; Azad et al., 2001). Fish species affected by MAS include tilapia (Abd-El-Rhman, 2009; Tellez-Ba ñuelos et al., 2010), catfish (Majumdar et al., 2007; Ullal et al., 2008), goldfish (Irianto et al., 2003; Harikrishnan et al., 2009), common carp (Yin et al., 2009; Jeney et al., 2009), and eel (Esteve et al., 1994). Although usually considered as a secondary pathogen associated with disease outbreaks, *A. hydrophila* mayalso become a primary pathogen, causing outbreaks in fish farms with high mortality rates (Thore and Roberts, 1972; Nielsen et al., 2001; Fang et al., 2004). In West Alabama, a MAS disease outbreaks cause by *A. hydrophila* in 2009 alone led to an estimated loss of more than 3 million pounds of food size channel catfish (Pridgeon and Klesius, 2011a). Virulence studies have revealed that AL09-71 strain, a 2009 West Alabama isolate of *A. hydrophila*, is highly virulent to channel catfish, killing fish within 24 h post exposure (Pridgeon and Klesius, 2011b).

To control disease outbreaks caused by *A. hydrophila*, feeding infected fish with antibiotic-medicated feed is a general practice (DePaola et al., 1995). However, this practice is expensive and usually ineffective as sick fish tend to remain off feed. Furthermore, currently in the US, there are only three FDA approved antibiotics for use in aquaculture: oxytetracycline (Terramycin), sulfadimethoxine (Romet-30), and florfenicol (Aquaflor). 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 (Chandran et al., 2002; John et al., 2002). 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 (Khushiramani et al., 2007; Poobalane et al., 2010). 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 (Hernanz Moral et al., 1998; Liu et al., 2007). However, it is well known that *A. hydrophila* is very heterogeneous biochemically and serologically, which is the obstacle in developing effective commercial vaccine against *A. hydrophila* (Khashe et al., 1996; Poobalane et al., 2010). To prevent disease outbreaks caused by the highly virulent West Alabama 2009 isolates of *A. hydrophila*, an attenuated vaccine AL09-71 N+R specifically targeting *A. hydrophila* AL09-71 strain was developed (Pridgeon and Klesius, 2011c).

Several studies have demonstrated that protective immunity elicited by attenuated bacterial vaccines in channel catfish is largely mediated by cellular immune responses with humoral antibodies having a secondary role (Shoemaker and Klesius, 1997; Ellis, 1999). Similarly, it has been reported that the antibody titres of rainbow trout vaccinated with an attenuated *A. hydrophila* were not significantly different from that of control fish, although the

attenuated vaccine provided 64% protection against challenges by virulent *A. hydrophila* (Vivas, et al., 2004), suggesting that components of immunity other than antibody play an essential role in combating *A. hydrophila*. The objectives of this study are to: 1) identify up-regulated genes in channel catfish after vaccination with attenuated *A. hydrophila*; and 2) determine the transcriptional regulation of genes identified in response to vaccination or infection of *A. hydrophila*. We used two approaches in this study to identify up-regulated genes. Firstly, we used suppression subtractive cDNA hybridization (SSH) technique to identify up-regulated genes in the anterior kidney of vaccinated channel catfish without any preconception of their identities. Secondly, we screened channel catfish genes reported in literatures as responses to either attenuated bacterial vaccines or virulent bacterial infections to identify genes induced by the attenuated *A. hydrophila*.

Materials and methods

Bacteria source and growth conditions

The AL09-71 isolate of *A. hydrophila* was obtained from diseased channel catfish in 2009 from West Alabama. The isolate has been confirmed to be *A. hydrophila* through biochemical and molecular identification (Pridgeon and Klesius, 2011a). The attenuated *A. hydrophila* AL09-71 N+R strain was obtained from the virulent parent strain of *A. hydrohila* AL09-71 through selection for resistance to both novobiocin and rifampicin (Pridgeon and Klesius, 2011c). Bacterial cultures were grown in tryptic soy broth (TSB) (Fisher Scientific, Pittsburgh, PA) for 24 h at 28 °C.

Experimental fish

Channel catfish (4.6 ± 1.3 g) were obtained from stocks maintained at USDA-ARS, Aquatic Animal Health Research Laboratory (Auburn, AL, USA). All fish were maintained in

dechlorinated water in 340 L tanks. Prior to experiments, fish were acclimated in flow-through 57-L aquaria supplied with ~ 0.5 L h^{-1} dechlorinated water for 14 days. Experimental fish were confirmed to be culture negative for bacterial infection by culturing posterior kidney tissues from representative groups of fish on tryptic soy agar plates. A 12:12 hour light:dark period was maintained and supplemental aeration was supplied by air stones. Mean dissolved oxygen was ~5.6 mg L^{-1} at water temperature ~27° C, with pH ~ 7.1 and hardness ~ 100 mg L^{-1}. Fish were fed ~3% body weight daily with commercial dry fish food.

Sample collection from A. hydrophila vaccinated or infected fish

Prior to vaccination or challenge, fish were moved to 57-L flow through aquaria and acclimated for 14 days. Vaccination dose of *A. hydrophila* AL09-71 N+R was 5×10^4 colony forming unit per fish (CFU/fish) based on published results (Pridgeon and Klesius, 2011c). Many studies have revealed that anterior kidney is an important immune organ involved in innate immunity against bacterial infections (Bao et al., 2005; Russo et al., 2009; Pridgeon et al., 2010a; Pridgeon and Klesius, 2010). Therefore, we decided to collect anterior kidney samples after vaccination with the attenuated *A. hydrophila*. At different time points (0 h, 3 h, 6 h, 12 h, 24 h, 48 h, and 14 days post vaccination,dpv), anterior kidney samples from five fish at each time point were collected and pooled together. Anterior kidney samples from five fish intraperitoneally (IP) injected with TSB at each time point were collected as control. The experiments were repeated three times. Using green fluorescent protein as a biomarker, the *in vivo* invasion pathway study of a virulent strain of *A. hydrophila* in Crucian carp (*Carassius auratus gibelio*) has revealed that the amount of bacteria in the kidney significantly increased at 12 h post challenge compared to that at 2 h post challenge (Chu and Lu, 2008). Therefore, we chose 12 h post vaccination as the time point to identify up-regulated genes induced by the

attenuated *A. hydrophila*. To understand whether infection by virulent *A. hydrophila* will have different effects on the transcription levels of genes at different time points (0 h, 3 h, 6 h, 12 h, 24 h, 48 h), the virulent parent strain of *A. hydrophila* AL09-71 was also IP injected to fish at a sublethal dose of 2×10^2 CFU/fish based on published virulence data of this strain (LD_{50} = 1.6×10^3 CFU/fish, (Pridgeon and Klesius, 2011b)). All anterior kidney tissues were flash frozen on dry ice during collection followed by storage at -80°C until RNA extraction.

Total RNA extraction and cDNA synthesis

Total RNA was isolated from anterior kidney tissues using TRIzol Reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. All RNAs were treated with DNase provided by the DNA-free kit (Ambion, Austin, TX) and quantified on a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Rockland, DE). The first strand cDNAs used for quantitative PCR were synthesized using 2 μg of total RNA, AMV reverse transcriptase, and Oligo-dT primer provided by the cloned AMV first strand cDNA synthesis kit (Invitrogen, Carlsbad, CA).

Construction of subtractive cDNA library

For subtractive library construction, total RNAs were extracted from pooled anterior kidney samples of five fish either vaccinated with the attenuated *A. hydrophila* AL09-71 N+R or injected with the TSB control at the 12 h time point. cDNAs were then synthesized using PCR-select cDNA Subtraction Kit (Clontech, Palo Alto, CA). Two-step subtractive hybridizations were performed according to procedures described previously (Pridgeon et al., 2010a). Briefly, two primary hybridization reactions (A and B) were formed by adding excess amounts of unmodified TSB control cDNA (driver) to *A. hydrophila* AL09-71 infected cDNA (tester) samples at a 50:1 ratio. The samples were denatured for 2 min at 98 ℃ and allowed to anneal for

8 h at 68 ℃. The remaining single-stranded, adaptor-ligated tester cDNAs were substantially enriched in each hybridization reaction for overexpressed sequences because non-target cDNAs present in the tester and driver could form hybrids. After filling in the adapter ends with DNA polymerase, over-expressed sequences (tester cDNA) had different annealing sites on their 3'- and 5'- ends. The molecules were then subjected to suppression subtraction PCR. The PCR products were then cloned into pGEM-T easy vector (Promega, Madison, WI). Plasmids were transformed into One Shot® TOP10 competent cells (Invitrogen, Carlsbad, CA). Transformed cells were plated on Luria-Bertani (LB) plates containing ampicillin (100 μg/ml) and X-Gal (5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside) (40 μg/ml).

DNA Sequencing

From the library, a total of 192 colonies were subsequently picked to grow overnight in Lysogeny broth (LB) in the presence of ampicillin (100 μg/ml) at 37 ℃ and 235 rpm in Innova™ 4000 Incubator Shaker (New Brunswick Scientific, Edison, NJ). Overnight cultures were then sent to USDA-ARS Mid South Genomics Laboratory in Stoneville, MS for plasmid DNA extraction and DNA sequencing with an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA). Raw sequence base calling and trimming was conducted at the Mid South Genomics Laboratory by using Phred with a cut-off score of Q20. Vector and adaptor sequences were then manually trimmed. Trimmed cDNA sequences were then analyzed using the National Center for Biotechnology Information (NCBI) BLAST program to search for sequence homologies.

Primer design and quantitative PCR

Sequencing results of different clones were used to design gene-specific primers by using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Quantitative

PCR (QPCR) was performed using Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). For each cDNA sample, channel catfish18S ribosomal RNA primers were included as an internal control to normalize the variation in cDNA amount as published previously (Pridgeon et al., 2010a). All QPCR was performed using Platinum® SYBR® Green qPCR SuperMix-UDG with ROX (Invitrogen, Carlsbad, CA) in a total volume of 12.5 µl. The QPCR mixture consisted of 1 µl of cDNA (input RNA of 10 ng), 0.5 µl of 5 µM gene-specific forward primer, 0.5 µl of 5 µM gene-specific reverse primer and 10.5 µl of 1× SYBR Green SuperMix. The QPCR thermal cycling parameters were 50 ℃ for 2 min, 95 ℃ for 10 min followed by 40 cycle of 95 ℃ for 15s and 60 ℃ for 1min. All QPCR was run in duplicate for each cDNA sample and three pooled cDNA samples were analyzed by QPCR. The fluorescence intensities of the control and treatment products for each gene, as measured by cycle threshold (*C*t) values, were compared and converted to fold differences by the relative quantification method (Pfaffl, 2001) using the Relative Expression Software Tool 384 v. 1 (REST) and assuming 100% efficiencies. Expression differences between control and treatment groups were assessed for statistical significance using a randomization test in the REST software. The mRNA expression levels of all samples were normalized to the levels of 18S ribosomal RNA gene in the same sample. Expression levels of 18S were constant between all samples (<0.30 change in *C*t). Each primer set amplified a single product as indicated by a single peak present for each gene during melting curve analysis.

Genes reported in literatures screened in this study

Previously we reported that the transcriptional levels of 43 channel catfish genes were induced by vaccination with attenuated *Edwardsiella ictaluri* vaccine (Pridgeon et al., 2010a). Similarly, the transcriptional levels of 28 genes have been reported to be up-regulated by

attenuated *Flavobacterium columnare* vaccine (Pridgeon and Klesius, 2010). In addition, the transcriptional levels of five toll-like receptors (TLRs) have been reported to be up-regulated by acute infection with *E. ictaluri* (Pridgeon et al., 2010b). Furthermore, the expression levels of tumor necrosis factor α (TNF-α) (Zhang and Wang, 1998), interleukin (IL)-1 β (Zhang and Wang, 1998), IL-10 (Zhang and Wang, 1998), chemokine CXCL10 (Baoprasertkul et al., 2004), hepcidin (Hu et al., 2007), bactericidal permeability-increasing protein (Xu et al., 2005), liver-expressed antimicrobial peptide-2 (Bao et al., 2006), transferrin (Elibol-Flemming et al., 2009), and cathepsin D (Feng et al., 2011) in channel catfish have been reported to be up-regulated by bacterial infections. In addition, three channel catfish NK-lysin genes have been reported to be expressed in the anterior kidney of uninfected fish (Wang et al., 2006). Therefore, primers for these total 88 selected genes were purchased from Sigma-Aldrich (St. Louis, MO) and used in this study to determine which genes were up-regulated by the *A. hydrophila* AL09-71 N+R vaccination.

Vaccination of channel catfish followed by challenge with AL09-71

The attenuated AL09-71 N+R vaccine was cultured in TSB broth at 28°C with shaking at 125 rpm overnight before vaccination. Channel catfish were vaccinated with *A. hydrophila* AL09-71 N+R at dose of 5×10^4 CFU/fish in a total volume of 100μl by intraperitoneal injection. As sham-vaccination controls, 100μl of TSB were injected into each fish. A total of 60 fish were used in each treatment group (20 fish per tank, three replicates). At 14 days post vaccination (dpv), fish were challenged with the parent isolate of *A. hydrophila* AL09-71 at dose of approximately 5×10^4 CFU/fish through IP injection. Mortalities were recorded for 14 days post challenge. Results of challenge were presented as relative percent of survival (RPS) according to

the following formula as described previously (Amend et al., 1981): RPS = (1-(vaccinated mortality ÷ control mortality)) × 100.

Data analysis

The relative transcriptional levels of different genes were 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: $\Delta C_t = C_t$ (sample) − C_t (calibrator). The relative expression level of a specific gene in TSB injected control fish or in bacteria injected fish were compared to that of average control fish by the formula $2^{-\Delta\Delta Ct}$ where $\Delta\Delta C_t = \Delta C_t$ (infected) − ΔC_t (control) as described previously (Pridgeon et al., 2010a). The relative expression data of a specific gene in control or infected fish were examined by unpaired t-test using SigmaStat statistical analysis software (Systat Software, San Jose, CA) and the differences were considered significant when the P value was less than 0.05. Differences in antibody titre and mortality were analyzed with Student t-test and the significance level was defined as $P < 0.05$.

Results

Characteristics of the subtractive cDNA library

A total of 192 clones were obtained from the subtractive library. Of the 192 clones, 149 contained inserts. Sequencing results revealed that these 149 clones represented 22 unique expressed sequence tags (ESTs) (Table 1). All ESTs listed in Table 1 have been deposited in the GenBank dbEST under accession numbers JK088411 to JK088432. Of the 22 unique ESTs identified from the subtractive library, 14 shared homology with deposited channel catfish (*Ictalurus punctatus*) proteins, six shared homology with zebrafish (*Danio rerio*) proteins, and one shared homology with deposited rainbow smelt (*Osmerus mordax*) and Atlantic salmon (*Salmo salar*) protein, respectively (Table 1). The biggest insert size was 549 bp (2E08) and the

smallest insert size was 135 bp (2B06). The average insert size of the 22 ESTs was 275 bp (Table 1).

Expression of the 22 ESTs and the 88 known genes at 12 hpv

To determine whether the expression levels of the 22 ESTs isolated from the subtractive library were up-regulated in *A. hydrophila* AL09-71 N+R vaccinated catfish, gene-specific primers for the 22 ESTs were designed (Table 2) for relative QPCR experiments. QPCR results revealed that, at 12 hpv, 6 ESTs were significantly ($P<0.05$) induced in the vaccinated fish compared to that in unvaccinated control fish. QPCR results also revealed that 14 of the 88 known genes were significantly ($P<0.05$) induced by the vaccination, including 7 genes that were reported to be induced by vaccination with attenuated *E. ictaluri* (Pridgeon et al., 2010a). The identities of the total 20 significantly induced genes are listed in Table 3.

Expression kinetics of the six genes identified by SSH in this study

To determine the expression kinetics of the six genes that were significantly induced by *A. hydrophila* vaccination at 12 hpv, QPCR analysis were performed using samples collected at different time points. To understand whether vaccination mimics infection, anterior kidney cDNA samples from infected fish were also included in the QPCR analysis. The expression kinetics of the six genes identified by SSH is summarized in Figure 1. Both vaccination and infection of *A. hydrophila* significantly ($P<0.05$) induced the transcriptional level of ADP/ATP translocase 2 at 12 h post treatment (Fig 1.A). Similarly, both vaccination and infection significantly ($P<0.05$) induced the transcriptional level of lymphokine-activated killer T-cell originated protein kinase at 12 h post treatment (Fig 1.B). In addition, vaccination also significantly induced its expression at 24 hpv (Fig 1.B). Of the six genes identified by SSH, lysozyme c at 24 h post vaccination was induced the most (Fig 1.C). For the remaining three

genes (motile sperm domain-containing protein 2, transcriptional regulator ATRX, and cadherin EGF LAG seven-pass G-type receptor 1), both vaccination and infection of *A. hydrophila* only induced their transcriptional levels at 12 h post treatment (Fig. 1D to 1F).

Expression kinetics of the seven reported genes induced by E. ictaluri vaccination

To determine the expression kinetics of the seven reported genes that were significantly induced by *A. hydrophila* vaccination at 12 hpv, QPCR analysis were performed using samples collected at different time points. Anterior kidney cDNA samples from infected fish were also included in the QPCR analysis. The expression kinetics of the seven reported genes is summarized in Figure 2. Both vaccination and infection of *A. hydrophila* significantly ($P<0.05$) induced the transcriptional level of Ring finger 144B at 12 h post treatment (Fig 2.A). Similarly, both vaccination and infection significantly ($P<0.05$) induced the transcriptional level of metacaspase-like protine at 12 h post treatment (Fig 2.B). The transcriptional level of complement C4a was significantly ($P<0.05$) induced at 6 h post treatment of both infection and vaccination (Fig 3.C). Of the seven reported genes, lysosomal-associated transmembrane protein 5 was induced the most by the infection at 6 h post treatment, followed by the vaccination or infection at 12 h post treatment (Fig 2.D). The transcriptional level of SET translocation B was only significantly induced at 6 hpi and 12 hpv (Fig 2.E). However, both vaccination and infection significantly induced the transcriptional level of uroporphyrinogen decarboxylase at 6 h post treatment (Fig 2.F). Of the seven reported genes, solute carrier family 25 member 3 isoform 3 was induced by both vaccination and infection as early as 3 h post treatment, with peaked level at 6 h post treatment (Fig 2.G).

Expression kinetics of the other seven reported genes

The expression kinetics of the other seven reported genes that were found to be significantly induced by *A. hydrophila* vaccination at 12 hpv is summarized in Figure 3. Infection of *A. hydrophila* significantly ($P<0.05$) induced the transcriptional level of IL-1 β at both 6h and12 h post treatment, whereas vaccination only significantly induced IL-1 β at 12 hpv (Fig 3.A). Similarly, infection of *A. hydrophila* significantly ($P<0.05$) and dramatically induced the transcriptional level of IL-10 at 6h post treatment, followed by a reduced but significant induction at 12 hpi (Fig 3.B). IL-10 was slightly but significantly induced by the vaccination at 6 hpv, with peaked up-regulation at 12 hpv (Fig 3.B). Chemokine CXCL10 was significantly induced by the infection at 6 h post treatment, followed by peaked induction at 12 hpi (Fig 3.C). However, chemokine CXCL10 was significantly induced by the vaccination at 12 h post treatment, followed by peaked induction at 24 hpv (Fig 3.C). Infection of *A. hydrophila* significantly and dramatically induced TLR5 at 6 hpi, followed by a reduced but significant induction at 12 hpi (Fig 3.D). However, vaccination only slightly but significantly induced TLR5 at 6 hpv, followed by peaked induction and 12 hpv (Fig 3.D). Similarly, infection of *A. hydrophila* significantly and dramatically induced hepcidin at 6 hpi, followed by a reduced but significant induction at 12 hpi (Fig 3.E). Vaccination only slightly but significantly induced hepcidin at 6 hpv, followed by a peaked induction at 12 hpv (Fig 3.E). Both vaccination and infection significantly induced the transcriptional level of NK lysine type-2 and sodium/potassium-transporting ATPase alpha subunit at 12 h and 3h post treatment, respectively (Fig 3.F, G).

Vaccination of channel catfish followed by challenge with AL09-71

When AL09-71 N+R vaccinated channel fish were challenged by its virulent parent AL09-71 at 14 dpv, no fish died. However, 40 to 50% fish died in the TSB-sham vaccination

group (Fig 4.). The relative percent of survival of vaccinated fish at 14dpv was 100%. At 14dpv, when channel catfish were challenged by virulent AL09-71, cumulative mortalities of AL09-71 N+R vaccinated fish at different time points were significantly ($P<0.05$) lower than that of TSB sham-vaccinated fish (Fig 4.), indicating that the vaccine provided significant protection against its virulent parent.

Expression of the 20 identified genes at 14dpv

The relative expression levels of the 20 genes in infected fish compared to that in vaccinated fish at 14dpv are summarized in Figure 5. At 14 dpv, only two genes (#17: TLR5; and #18: hepcidin) were significantly ($P<0.05$) up-regulated in vaccinated fish, with higher induction level of TLR5 compared to hepcidin (Fig 5.).

Discussion

Using the SSH technique, 22 unique ESTs were identified from a total of 192 clones. Of the 22 ESTs, only six were confirmed to be significantly up-regulated by the vaccination. The reasons for few genes were found to be significantly up-regulated may be due to the following.. First, only a small portion of the subtraction library was sequenced due to budget constraints. Although our library generated 192 clones, the library was generated from 3 μl PCR products from a total of 25 μl PCR reaction, which might have contributed to the limited discovery of well known genes related to immune response. Second, we used a pool of fish instead of single fish which might have eliminated the differences between individual fish. Third, we used total RNA instead of messenger RNA, which might have contributed to our limited discovery by the SSH. Fourth, only one set of specific primers were used to do the QPCR confirmation, which may have contributed to the unsuccessful confirmation of the SSH results.

Of the six up-regulated genes confirmed by QPCR, two of which (ADP/ATP translocase 2 and lysozyme c) have been previously reported to be up-regulated in fish infected by *Aeromonas*. ADP/APT translocase 2 is one of the four genes identified (from 4131 known genes by microarray) to be up-regulated in Atlantic salmon (*Salmo salar*) in response to *Aeromonas salmonicida* infection (Tsoi et al., 2003). Recently, significant transcriptional up-regulation of lysozyme c has been reported in the kidney of ray-finned fish (*Puntius sarana*) following *A. hydrophila* infection (Das et al., 2011).

Gene expression profiles at different early time points revealed that the pattern of gene up-regulation in vaccinated fish was similar to that in infected fish, confirming that vaccination of attenuated bacteria mimics live infection at molecular level. However, the extent of gene induction by the infection differed from that by the vaccination. For example, at 3 hpv, Na^+/K^+ ATPase α subunit was up-regulated less than 50 fold. However, the infection by the virulent *A. hydrophila* AL09-71 strain resulted in more than 200 fold of up-regulation. Other genes at other time points also showed this trend. For example, at 6 hpv, IL-1β was induced less than 10 fold by the vaccination, whereas the infection resulted in more than 100 fold up-regulation. Similarly, vaccination induced hepcidin less than 50 fold at 6 hpv, whereas infection up-regulated hepcidin more than 200 fold, suggesting that their regulation may be associated with severity of the infection. It has been reported that the induction of human β-defensin 3 (HBD-3) is associated with the severity of *Staphylococcus aureus* skin infection (Zanger et al., 2010). For example, immune genes such as IL-1β, TNFα1, serum amyloid A, and interferon-γ in rainbow trout (*Oncorhynchus mykiss*) are all significantly up-regulated at 24 h post infection by *Yersinia ruckeri* (the causative agent of enteric red mouth disease) at lethal doses injected, but not at sublethal doses (Wiens and Vallejo, 2010).

Time course studies revealed that Na^+/K^+ ATPase α subunit was highly and significantly up-regulated at 3 hpv or 3 hpi, indicating that it might play an early role in fish immune response to *Aeromonas* infection. Up-regulation of Na^+/K^+ ATPase has been reported as an early response in channel catfish fry at 10 min post vaccination with attenuated *F. columnare* (Pridgeon and Klesius, 2010). In addition, significant up-regulation of Na^+/K^+ ATPase α subunit has been reported in Atlantic salmon (*Salmo salar*) as an early response to *Aeromonas salmonicida* infection (Tsoi et al., 2003). Time course studies also revealed that solute carrier family 25 member 3 isoform 3 was significantly up-regulated at 3, 6, and 12 hpv. Significant up-regulation of solute carrier family 25 members 3 isoform 3 has been reported in channel catfish at 48h post immersion vaccination of attenuated *E. ictaluri* (Pridgeon et al., 2010a). Taken together, these results suggest that Na^+/K^+ ATPase α subunit and solute carrier family 25 member 3 isoform 3 may play important roles in the early immune response to *Aeromonas* infection.

Toll-like receptors (TLRs) are evolutionarily conserved receptors that function in innate immunity through recognition of the conserved pathogen-associated molecular patterns (PAMPs) of an invading pathogen and eliciting inflammatory immune responses (Medzhitov and Janeway, 2010). The best characterized ligand that TLRs recognize include: (1) lipoproteins by TLR2; (2) dsRNA by TLR3; (3) lipopolysaccharide (LPS) by TLR4; and (4) bacterial flagellin by TLR5 (Baoprasertkul et al., 2007). In mammals, inflammation will result in a cytokine cascade whereby tumor necrosis factor α (TNFα) is released, followed by IL-1β and IL-6. After the release of these cytokines, chemokines are released to serve as potent chemoattractants to induce migration of neutrophils and macrophages to the site of infection (Secombes et al., 2001). It has been reported that TLR5 in channel catfish is significantly up-regulated at 4 and 6 hpi after acute infection of *E. ictaluri* (Pridgeon et al., 2010a). Similarly, after infection of *A. hydrophila*, the

expression level of TLR5 increased at 3 hpi and significantly peaked at 6 hpi, suggesting that TLR5 plays an essential role in recognizing this flagellated bacteria. It has been demonstrated that TLR5 also functions as an endocytic receptor to enhance flagellin-specific adaptive immunity (Letran et al., 2011). Significant up-regulation of IL-1β has been reported in the kidney of ray-finned fish (*Puntius sarana*) at 1, 3, and 6 h post *A. hydrophila* infection (Das et al., 2011). In consistent with that report, our results also revealed that IL-1β was significantly up-regulated at 3 and 6 hpi, confirming that IL-1β plays an important role in the immune response to *Aeromonas* infection. In addition to IL-1β, IL-10 was also significantly up-regulated at 6 and 12 hpi. Since IL-10 was not significantly up-regulated at 3 hpi, whereas IL-1β was significantly up-regulated at 3 hpi, suggesting that IL-10 is the downstream cytokine of IL-1β. From 6 hpi to 12 hpi, the induced level of IL-10 was reduced from 40 fold to 11 fold, whereas the induced level of chemokine CXCL10 was increased from 3 fold to 5 fold, suggesting that CXCL10 is a downstream chemokine followed by the release of IL-10.

Antimicrobial peptides (AMPs) are evolutionarily ancient defensive weapons against bacteria, fungi and viruses (Zasloff et al., 2002). In channel catfish, overexpression of hepcidin (an AMP) after *E. ictaluri* infection has been reported at 1 to 3 dpi (Bao et al., 2005). Similarly, significant up-regulation of hepcidin in channel catfish has been reported at 4, 24, and 48 hpi of *E. icataluri* (Hu et al., 2007). In consistent with previous reports, our time course studies also revealed that hepcidin was significantly up-regulated at 6, 12, 24, and 48 hpi of *A. hydrophila*. In addition to hepcidin, NK-lysin-type 2 antimicrobial peptide was also significantly up-regulated at 6 and 12 hpi, further confirming that AMPs play important roles in host defense against bacterial infections. In addition to AMPs, lysozyme is also an important parameter in the immune defense of both invertebrates and vertebrates. Lysozyme works by hydrolysing the glycoside bonds of

bacterial cell wall, therefore resulting in the lysis of bacteria (Magnadóttir, 2006). In this study, lysozyme c and lysomal-associated transmembrane protein 5 were both found to be significantly up-regulated at 6, 12, and 24 hpv or hpi.. Transcriptional profile studies revealed that the transcriptional levels of TLR 5 and hepcidin at 14 dpv were significantly up-regulated in vaccinated fish. Efficacy studies revealed that the vaccination with the attenuated *A. hydrohila* provided 100% protection against challenges by the virulent parent at 14 dpv. In summary, a total of 22 ESTs were identified from channel catfish anterior kidney subtractive cDNA library at 12 h post vaccination with an attenuated *A. hydrophila* (AL09-71 N+R). Of the 22 ESTs, six were confirmed to be significantly ($P<0.05$) induced by the vaccination. Of the selected 88 channel catfish genes reported in literatures, 14 were found to be significantly ($P<0.05$) up-regulated by the vaccination. The transcriptional levels the total 20 genes induced by the vaccination were then compared to that by the virulent parent *A. hydrophila* (AL09-71) at different time points. At 3 hpv or hpi, Na^+/K^+ ATPase α subunit was up-regulated the most. At 6 and 12 hpv or hpi, hepcidin and interleukin-1β were induced the most. At 24 hpv or hpi, hepcidin was up-regulated the most, followed by lysozyme c. At 48 hpi, lysozyme c and hepcidin were significantly induced. When vaccinated fish were challenged by AL09-71, relative percent of survival of vaccinated fish were 100% at 14 dpv. Transcriptional levels of TLR5 and hepcidin were significantly up-regulated in vaccinated fish at 14 dpv.

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Table 1. List of the 22 genes isolated from the *Aeromonas hydrophila* AL09-71 N+R vaccinated *vs* non-vaccinated channel catfish anterior kidney subtractive cDNA library

Clone	Blast Search Homology (Putative Gene Name)	Accession No.	Organism	Identities (%)	E value	Insert (bp)
2B02	60S acidic ribosomal protein P0	NP_001187030	*Ictalurus punctatus*	99%	7.00E-79	456
2B03	40S ribosomal protein S12	NP_001187076	*Ictalurus punctatus*	100%	9.00E-31	195
2B06	ADP/ATP translocase 2	NP_001188059	*Ictalurus punctatus*	96%	2.00E-18	135
2D08	40S ribosomal protein SA	NP_001187066	*Ictalurus punctatus*	99%	1.00E-40	243
2E08	Lymphokine-activated killer T-cell-originated protein kinase	NP_001187345	*Ictalurus punctatus*	100%	5.00E-88	549
2G12	Transcriptional regulator ATRX	NP_956947	*Danio rerio*	73%	4.00E-06	198
2H10	Ribosomal protein L13a	AAK95140	*Ictalurus punctatus*	99%	8.00E-48	285
3F04	Retrotransposable element Tf2 155 kDa protein type 1-like	XP_003199901	*Danio rerio*	45%	1.00E-31	432
3H03	60S ribosomal protein L7a	NP_001187036	*Ictalurus punctatus*	99%	2.00E-80	492
4A02	40S ribosomal protein S25	NP_001187221	*Ictalurus punctatus*	100%	3.00E-22	198
4A09	Cadherin EGF LAG seven-pass G-type receptor 1	XP_001920772	*Danio rerio*	91%	4.00E-32	231
4B04	Lysozyme c	NP_001187718	*Ictalurus punctatus*	98%	5.00E-70	369
4C04	Sorbin and SH3 domain containing 2 isoform 1	BX537102	*Danio rerio*	88%	4.00E-21	144
4C06	Hemoglobin-beta	NP_001187115	*Ictalurus punctatus*	100%	2.00E-24	165
4E12	Cytochrome c oxidase subunit III	YP_004300503	*Osmerus mordax*	93%	2.00E-27	204
4G01	Ras-related protein Rab-35-like	XP_001339327	*Danio rerio*	93%	7.00E-42	270
4G11	motile sperm domain-containing protein 2	NP_001007294	*Danio rerio*	61%	4.00E-21	297
4H09	60S ribosomal protein L10a	NP_001187211	*Ictalurus punctatus*	98%	4.00E-26	183
5D09	Elongation factor 2	ACN58590	*Salmo salar*	96%	2.00E-70	408
5F08	40S ribosomal protein S16	NP_001187219	*Ictalurus punctatus*	100%	1.00E-19	144
5F09	Proteasome activator complex subunit 1	ADO29299	*Ictalurus punctatus*	100%	3.00E-33	213
6E03	60S ribosomal protein L19	ADO29213	*Ictalurus punctatus*	100%	5.00E-40	249

Table 2. Gene-specific primers used in qPCR

Clone No.	*w*ard Primer (5’ to 3’)	Reverse Primer (5’ to 3’)
2B02	CTTCCAGGCTTTGGGTATCA	TCACACCCTCCAGGAATCTC
2B03	CGAGCATCAAATCAACCTCA	CTCCTCGATGACATCCTTGG
2B06	ACCATCGACTGCTGGAAGAA	ATACAGGACCAGCACGAAGG
2D08	ATCAGATTCAGGCTGCCTTC	AGGGGATGGCAATATCAACA
2E08	CTTCTGCACGGAGACATGAA	GTGAGCCCATACGCAAAGAT
2G12	CAAACGGGGCAAAGTTAAAA	GTTCAAGCCGTTTGTCGTCT
2H10	TCTGGAGAGGCTGAAGGTGT	CCTGTGATGGCCTGGTACTT
3F04	AATGGGAACAGGAAGGGAAC	CAAGAGCGTAATCTGCCACA
3H03	ATGGGTGTCCCATACTGCAT	TGGGACCCATGATGTTACCT
4A02	TGGTGTCCGAGAGACTGAAG	TTTCTCTGGTGCTCCCTCAT
4A09	CCGTGACGGTGTCTTCATC	GCGGTTCAGGTAGATTTGCT
4B04	TCTGGCTAACTGGGTTTGCT	TGCCCTGCTGTCTCACTATG
4C04	CCCTCTCTCATCCCTCTCCT	CCAAACTCCAGCTCTGCAA
4C06	CAGCAACTTCACGCTTCTTG	GGAACTTCTGCCAAGTCTCG
4E12	GTCATCATCGGCTCAACCTT	CCTCATCAGTAAATAGAGACA
4G01	ATCCCGAGTCGTTTGTGAAC	ACGCCATGAACATCTCTTCC
4G11	TCTCGAGCCTCCGAAATCTA	GCCATCAGGACTGAAACCAT
4H09	GCTGCAGATCAGCTTGAAGA	AGAATTTGGGACGTGGAGTG
5D09	CTTCTCTGGCTGTGTGTCCA	GGTCCCAGTCTTCACCAAAA
5F08	CCTGCAGTCTGTCCAGGTCT	CAGTCCATTCCCTCGCTTAC
5F09	GGATGGGAACAACTTTGGTG	CGTGAACAAGCTGCCTGTAA
6E03	GCTGTGGCAAAAAGAAGGTC	TCTCTTACCGATGCCCATGT

Table 3. List of the 20 genes identified to be significantly upregulated at 12h post vaccination in the anterior kidney of channel catfish

Code No.	Blast Search Homology (Putative Gene Name)	Gene Source
1	ADP/ATP translocase 2	SSH library sequencing results of this study
2	Lymphokine-activated killer t-cell-originated protein kinase-like	SSH library sequencing results of this study
3	Lysozyme c	SSH library sequencing results of this study
4	Motile sperm domain-containing protein 2	SSH library sequencing results of this study
5	Transcriptional regulator ATRX	SSH library sequencing results of this study
6	Cadherin EGF LAG seven-pass G-type receptor 1	SSH library sequencing results of this study
7	Ring finger 144B	Pridgeon et al. 2010a
8	Metacaspase-like protein	Pridgeon et al. 2010a
9	Complement C4a	Pridgeon et al. 2010a
10	Lysosomal-associated transmembrane protein 5	Pridgeon et al. 2010a
11	SET translocation B	Pridgeon et al. 2010a
12	Uroporphyrinogen decarboxylase	Pridgeon et al. 2010a
13	Solute carrier family 25, member 3 isoform 3	Pridgeon et al. 2010a
14	Interleukin-1β	Zhang and Wang 1998
15	Interleukin-10	Zhang and Wang 1998
16	Chemokine CXCL10	Baoprasertkul et al. 2004
17	Toll-like receptor 5	Pridgeon et al. 2010c
18	Hepcidin	Bao et al. 2005
19	NK lysine type-2	Wang et al. 2006
20	Sodium/potassium-transporting ATPase, alpha subunit	Pridgeon et al. 2010b

Figure legends

Figure 1. Effect of *Aeromonas hydrophila* treatment on the transcriptional kinetics of the six genes identified by suppression subtractive hybridization. A: ADP/ATP translocase 2; B: Lymphokine-activated killer T-cell originated protein kinase; C: ADP/ATP translocase 2; C: Lysozyme c; D: Motile sperm domain-containing protein 2; E: Transcriptional regulator ATRX; F: Cadherin EGP LAG seven-pass G-type receptor 1. Data are presented as means ± S.D. from three replicates.

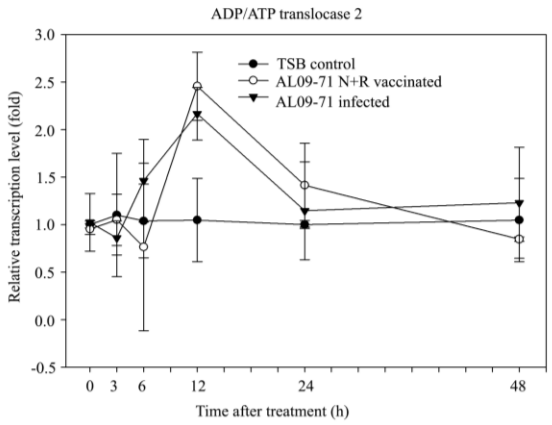
Figure 2. Effect of *Aeromonas hydrophila* treatment on the transcriptional kinetics of the seven genes induced by attenuated *Edwardsiella ictaluri*. A: Ring finger 144B; B: Metacaspase-like protein; C: Complement C4a; D: Lysosomal-associated transmembrane protein 5; E: SET translocation B; F: Uroporphyrinogen decarboxylase; G: Solute carrier family 25 member 3 isoform 3. Data are presented as means ± S.D. from three replicates.

Figure 3. Effect of *Aeromonas hydrophila* treatment on the transcriptional kinetics of the other seven genes selected from literature. A: Interleukin-1β; B: Interleukin-10; C: Chemokine CXCL10; D: Toll-like receptor 5; E: Hepcidin; F: NK lysine type 2; G: Sodium/potassium-transporting ATPase alpha subunit. Data are presented as means ± S.D. from three replicates.

Figure 4. Cumulative mortality of channel catfish intraperitoneally vaccinated with or without the *Aeromonas hydrophila* AL09-71 N+R and challenged with their respective virulent parent isolates of *A. hydrophila* through intraperitoneal injection at 14 days post vaccination. Data are presented as mean ± S.D. from three trials.

Figure 5. Relative transcriptional levels of the 20 genes in the anterior kidney of channel catfish at 14 days post vaccination. Data are presented as means ± S.D. from three replicates. Differences were considered statistically significant between vaccinated or infected and control fish when P value < 0.05. Significant difference is marked by an asterisk. TSB: tryptic soy broth; N+R: *A. hydrophila* AL09-71 N+R; parent: *A. hydrophila* AL09-71.

Figure 1A



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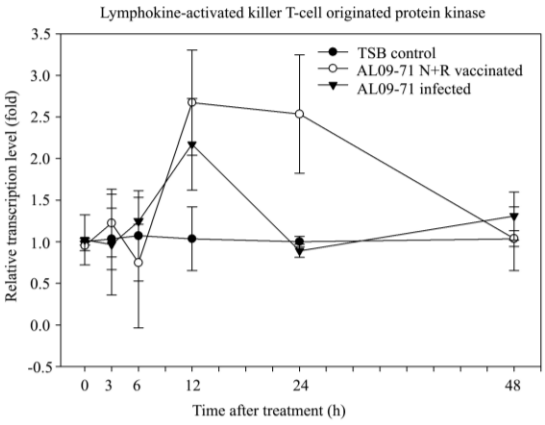

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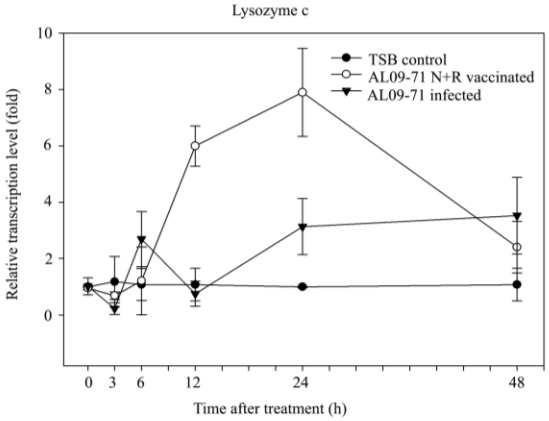

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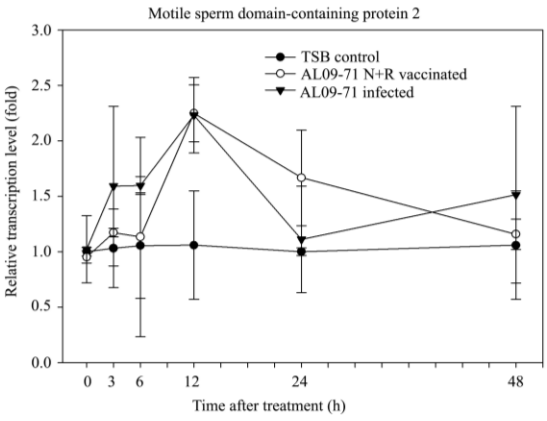

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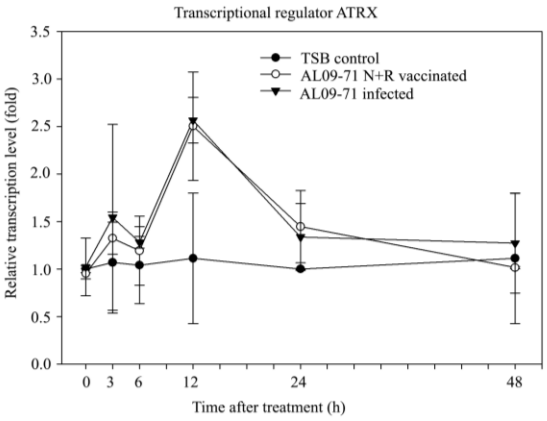

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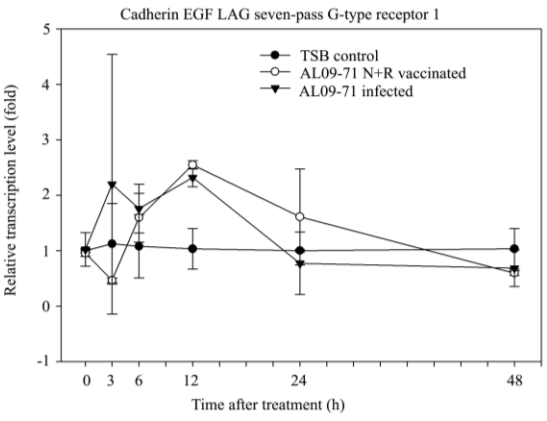
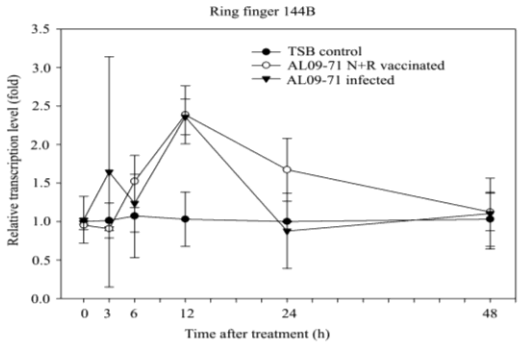


Figure 1

Figure 2A



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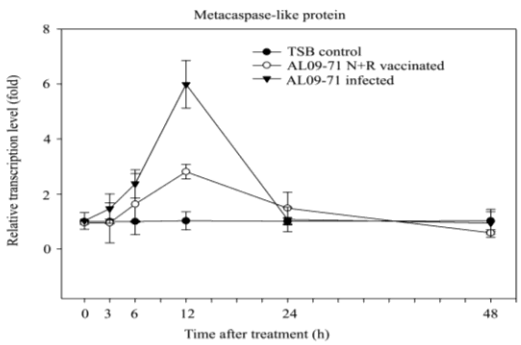

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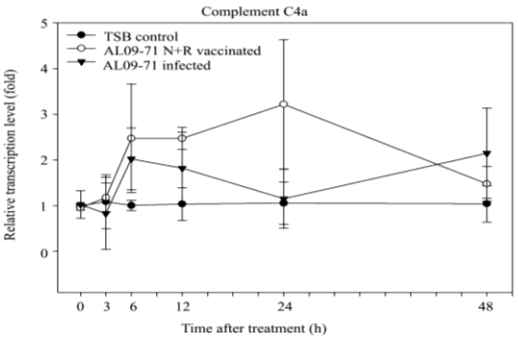

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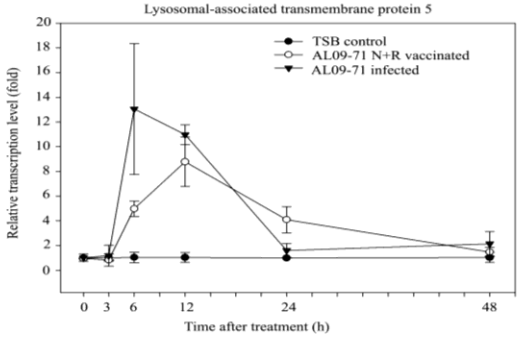

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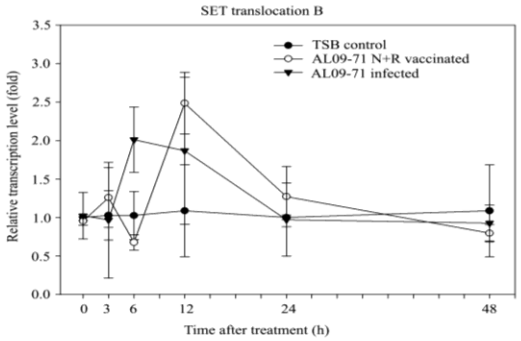

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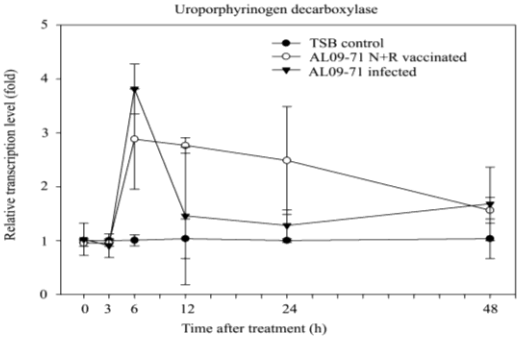

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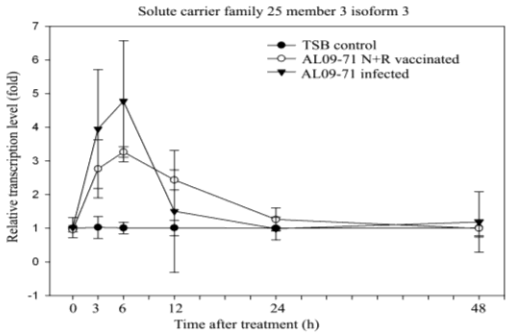
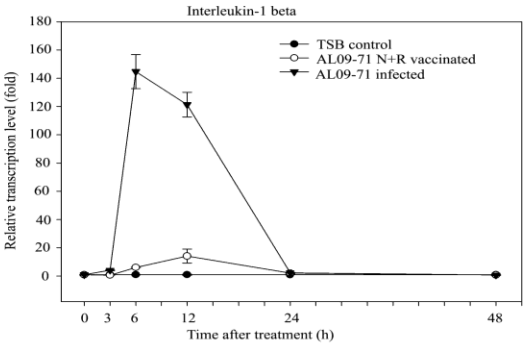


Figure 2

Figure 3A



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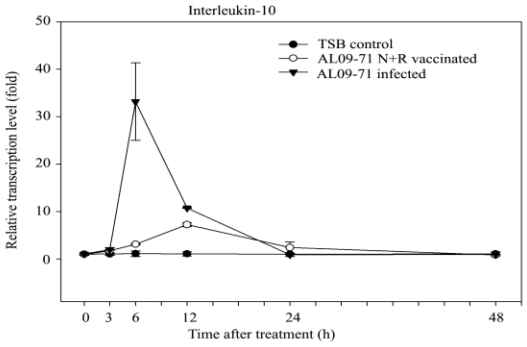

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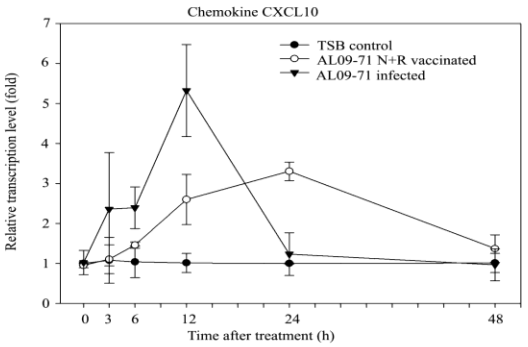

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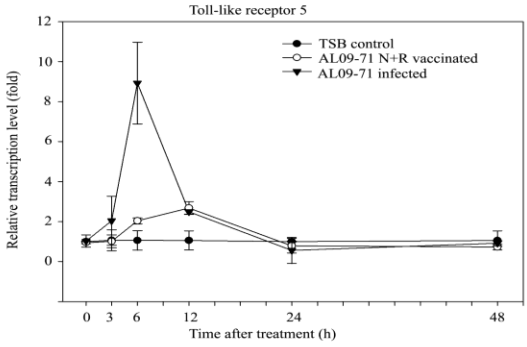

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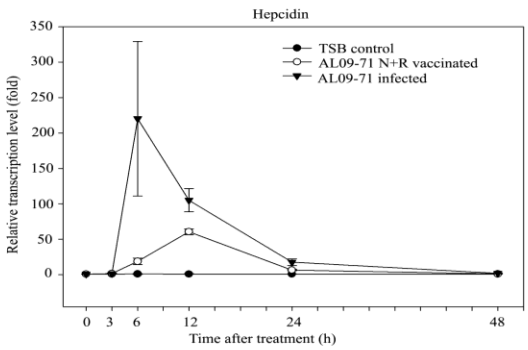

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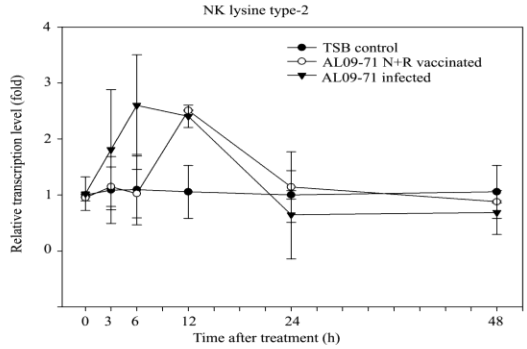

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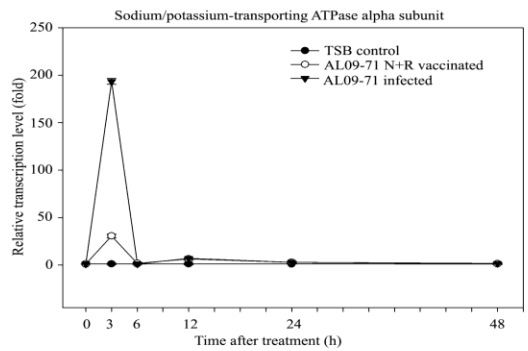


Figure 3

Figure 4

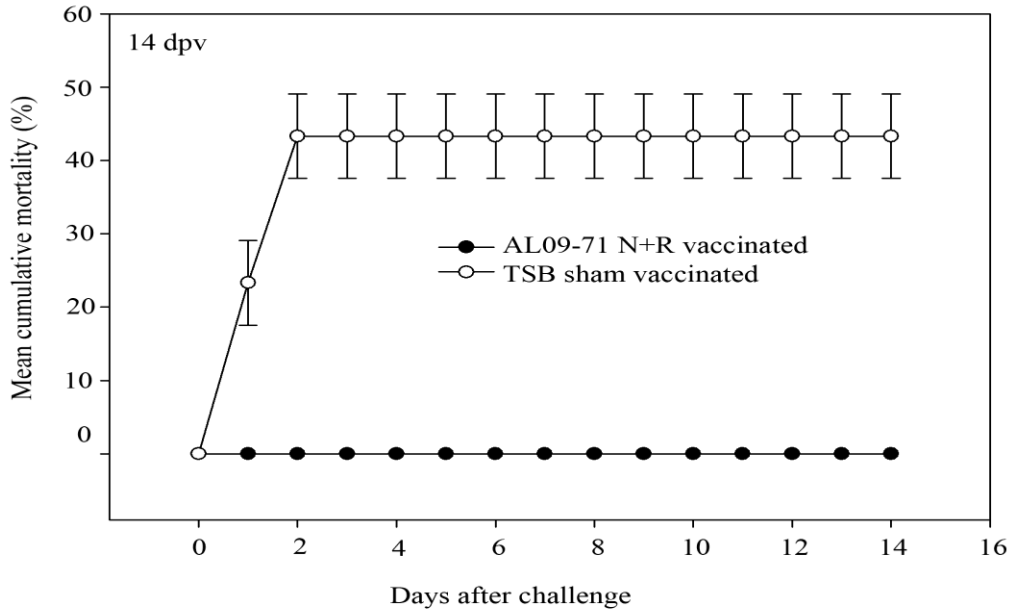
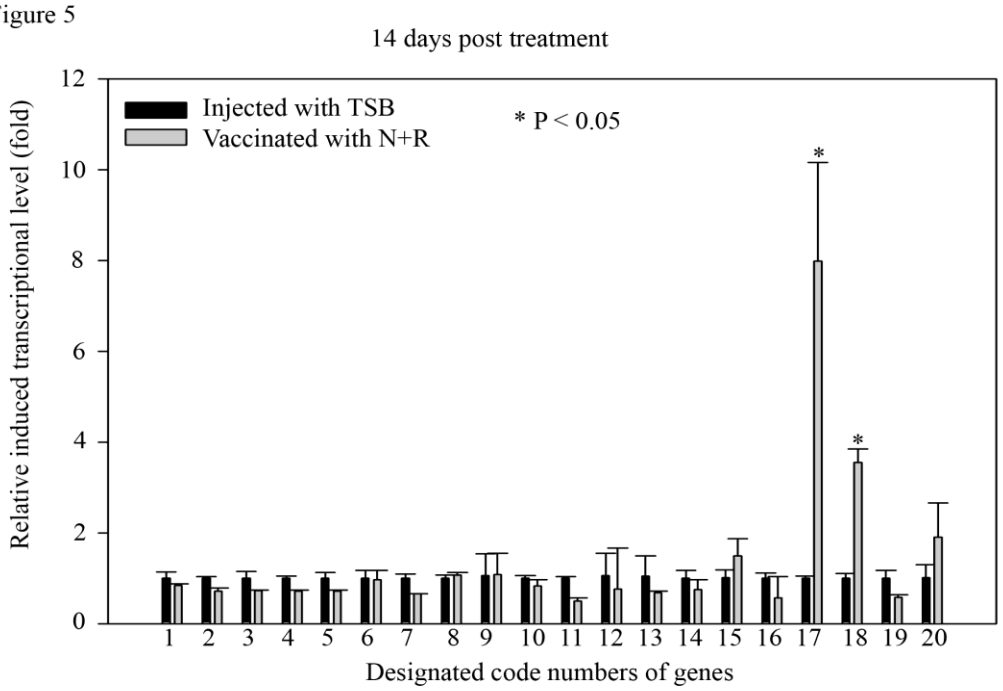


Figure 5



IV. USING SSH TO IDENTIFY GENES OVEREXPRESSED IN CHANNEL CATFISH AFTER SECONDARY EXPOSURE TO *AEROMONAS HYDROPHILA* COMPARED TO PRIMARY EXPOSURE

Introduction:

Aeromonas hydrophila is the causative agent of motile aeromonad septicemia (MAS) in channel catfish (*Ictalurus punctatus*). In 2009 and 2010, *A. hydrophila* is responsible for estimated loss of more than 3 million pounds of food size channel catfish (Pridgeon and Klesius, 2011a; Hemstreet, 2010). Virulence studies have revealed that AL09-71, a 2009 West Alabama strain of *A. hydrophila,* is highly virulent to channel catfish, killing fish within 24 h post exposure (Pridgeon and Klesius, 2011b).

In fish, the anterior kidney plays an important function in immune defense (Tort et al., 2003). The anterior kidney is responsible for phagocytosis (Danneving et al., 1994), antigen processing (Brattgjerd et al., 1996; Kaattari et al., 1985), formation of IgM and immune memory through melanomacrophagic centres (Herraez et al., 1986; Tsujii et al., 1990). It is also an important endocrine organ, homologous to mammalian adrenal glands, releasing corticosteroids and other hormones. Thus, the anterior kidney is an important organ that plays key regulatory functions for immune-endocrine interactions and neuroimmunoendocrine connections (Tort et al., 2003). The main white blood cells found in the anterior kidney are macrophages, which aggregate into structures called melanomacrophage centers, and lymphoid cells, which are found at all developmental stages and exist mostly as Ig+ cells (B cells) (Press et al., 1994).

The immune system in fish is divided into two parts: the innate (non-specific) and the adaptive (specific) immune systems. The innate immune system, which is characterized as being non-specific and therefore not depend on previous recognition of the surface structures of the pathogen, is of prime importance in the immune defense of fish (Tort et al., 2003). It is the first and basic defense line in fish, which is is generally subdivided into 3 parts: the epithelial/mucosal barrier, humoraland the cellular components. The innate immune systerm is composed of germ-line encoded, relatively non-specific recognition parameters, showing instant action but of short duration (Magnadóttir, 2010).

The adaptive immune system is composed of T- and B-lymphocyte, random and highly diverse receptors, which encoded by recombinant activation genes, and contribute to a more specific and efficient immune response against infections (Medzhitov, 2007; Alvarez-Pellitero, 2008). The innate and adaptive immune systems are connected in different ways and both form part of an integrated and efficient immune system (Fearon and Locksley, 1996; Medzhitov and Janeway, 1997; Dixon and Stet, 2001; Medzhitov, 2007; Alvarez-Pellitero, 2008; Magnadóttir, 2010).

The immune response mechanisms against *A. hydrophila* have been studied in several fish species (Sahu et al., 2007; Rodríguez et al., 2008; Poobalane et al., 2010; Reyes et al., 2010; Harikrishnan et al., 2010; Mu et al., 2010; Pridgeon et al., 2011; Reyes-Becerril et al., 2011). The objectives of this study are to: 1) identify up-regulated genes in channel catfish after secondary infection compared with primary infection; and 2) determine the transcriptional profiles of genes identified in response to primary and secondary infections at different time points.

Materials and methods

Bacteria source and growth conditions

The AL09-71 strain of *A. hydrophila* was obtained from diseased channel catfish in 2009 from West Alabama and has been confirmed to be *A. hydrophila* through biochemical and molecular identification (Pridgeon and Klesius, 2011a). The isolate was grown in tryptic soy broth (TSB) (Fisher Scientific, Pittsburgh, PA) for 18 h at 28 ℃ before challenge.

Experimental fish

Channel catfish (21.2±3.3 g) were obtained from stocks maintained at USDA-ARS, Aquatic Animal Health Research Laboratory (Auburn, AL, USA). All fish were maintained in dechlorinated water in 340 L tanks. Prior to experiments, fish were acclimated in flowthrough 57 L aquaria supplied with ~2 L h^{-1} dechlorinated water for 14 days. Experimental fish were confirmed to be not infected by bacteria by culturing posterior kidney tissues from representative groups of fish on tryptic soy agar plates. A 12:12 h light:dark period was maintained and supplemental aeration was supplied by air stones. Mean dissolved oxygen was ~5.6 mg L^{-1} at water temperature ~27 ℃, with pH ~7.1 and hardness ~100 mg L^{-1}. Fish were fed ~3% body weight daily with commercial dry fish food.

Sample collection from primary and secondary infected fish

The infection dose of *A. hydrophila* AL09-71 was $1x10^5$ colony forming unit per fish (CFU/fish) based on previous challenge experiment result (LD_{50}=$1x10^5$ CFU/fish). For the primary challenge experiment, five hundred channel catfish were infected by intra-peritoneal (ip.) injection ($1x10^6$ CFU/fish in 100 µl TSB). And, two hundred and fifty non-infected control fish were injected with 100µl sterile TSB. At different time points (0 h, 3 h, 6 h, 12 h, 24 h, 2 days, and 7 days post infection), anterior kidney samples from 15 infected fish at each time point were collected and each five were pooled together. Anterior kidney samples from 15 fish intraperitoneally (IP) injected with TSB at each time point were also collected and each five

were pooled together as control. In the primary challenge experiment the infected fish received an ip. injection and were observed for 28 days. In the secondary challenge experiment, the surviving fish from the primary challenge received an additional injection of ($1x10^6$ CFU/fish in 100 µl TSB) bacteria 28 days after the primary infection. The non-infected control fish were also reinjected with sterile TBS at day 28 days post-primary injection. In secondary challenge experiment, anterior kidney samples were collected from infected fish and non-infected fish as the same as we did in primary challenge experiment at each time point. All anterior kidney tissues were flash frozen on dry ice during collection followed by storage at -80 ℃ until RNA extraction.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from anterior kidney tissues using TRIzol Reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. All RNAs were quantified on a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Rockland, DE) and analyzed on 1% agarose gel by electrophoresis to confirm the integrity. For each pooled sample, 2 mg of total RNA was used to synthesize the first strand cDNAs. The first strand cDNAs used for quantitative PCR were synthesized by using the cloned AMV first strand cDNA synthesis kit (Invitrogen, Carlsbad, CA) following the protocol.

Construction of subtractive cDNA library

Our previous experiment results have shown that some immune related genes up-regulated at 6h post-infection (Mu et al., 2011). Therefore, in this study, we chose 6 h post infection as the time point to identify up-regulated genes after secondary infection compared with primary infection. For subtractive library construction, total RNAs were extracted from pooled anterior kidney samples of five fish either after primary infection or secondary infection

with *A. hydrophila* AL09-71 at the 6 h post infection. cDNAs were then synthesized using PCR-select cDNA Subtraction Kit (Clontech, Palo Alto, CA). Two-step subtractive hybridizations were performed according to procedures described previously (Mu et al., 2011). cDNAs from secondary infection experiment were used as tester and cDNAs from primary infection experiment were used as driver. According to the manufacturer's instructions, cDNAs from both tester and driver were digested by RsaI at 37 ℃ overnight. The digested tester cDNAs were purified, subdivided equally and ligated with two different adaptors (adaptor 1 and adaptor 2R supplied by the kit) respectively. Two hybridizations were performed. In the first, an excess of driver was added to each adaptor-ligated tester sample followed by 98 ℃ for 1.5 min, 63 ℃ for 6 h. In the second hybridization, the denatured driver was added into two primary hybridization samples which were not denatured followed by 63 ℃ overnight. Finally, the ratio of driver DNA:tester DNA sample was 50:1. After filling in the adapter ends with DNA polymerase, the entire population of molecules was then subjected to PCR to amplify the tester-specific sequences as described in the manual. The secondary PCR amplification product was cloned into pGEM-T Easy vector (Promega, Madison, WI, USA) following manufacturer's instructions and transformed into one Shot® TOP10 chemically competent E. coli (Invitrogen, Carlsbad, CA) according to the manual. Transformed cells were then plated on Luria–Bertani (LB) plates containing ampicillin (100 μg/ml) and X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) (40 μg/ml).

Plasmid DNA isolation and sequencing

From the library, a total of 96 colonies were subsequently picked to grow overnight in LB broth in the presence of ampicillin (100 ug/ml) at 37 ℃ and 235 rpm in Innova^{TM} 4000 Incubator Shaker (New Brunswick Scientific, Edison, NJ). Overnight cultures were then sent to USDA-

ARS Mid South Genomic Laboratories in Stoneville, MS for plasmid DNA extraction and DNA sequencing with an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA). Vector and adaptor sequences were then manually trimmed. Trimmed sequences were analyzed using the national Center for Biotechnology Information (NCBI) BLAST program to search for sequence homologies.

Primer design and quantitative PCR

Sequencing results of different clones were used to design gene-specific primers by using Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Quantitative PCR (QPCR) was performed using Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). For each cDNA sample, channel catfish 18S ribosomal RNA primers were included as an internal control to normalize the variation in cDNA amount as published previously (Pridgeon et al., 2010a). All QPCR was performed using Platinum® SYBR® Green qPCR SuperMix-UDG with ROX (Invitrogen, Carlsbad, CA) in a total volume of 12.5 ul. The QPCR mixture consisted of 1 µl of cDNA (input RNA of 10 ng), 0.5 µl of 5 µM gene-specific forward primer, 0.5 µl of 5 uM gene-specific reverse primer and 10.5 µl of 1xSYBR Green SuperMix. The QPCR thermal cycling parameters were 50 ℃ for 2 min, 95 ℃ for 10 min followed by 40 cycles of 95 ℃ for 15 s and 60 ℃ for 1 min. All QPCR was run in duplicate for each cDNA sample and three pooled cDNA samples were analyzed by QPCR. The fluorescence intensities of the control and treatment products for each gene, as measured by cycle threshold (C_t) values, were compared and converted to fold differences by the relative quantification method (Pfaffl, 2001) using the Relative Expression Software Tool 384 v. 1 (REST) and assuming 100% efficiencies. Expression differences between control and treatment groups were assessed for statistical significance using a randomization test in the REST software. The mRNA

expression levels of all genes were normalized to the levels of 18S ribosomal RNA gene in the same sample. Expression levels of 18S were constant between all samples (<0.30 change in C_t). Each primer set amplified a single product as indicated by a single peak present for each gene during melting curve analysis.

Data analysis

The relative transcriptional levels of different genes were 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: $\Delta C_t = C_t$ (sample) − C_t (calibrator). The relative expression level of a specific gene in control fish or in infected fish were compared to that of average control fish by the formula $2^{-\Delta\Delta Ct}$ where $\Delta\Delta C_t = \Delta C_t$ (infected) − ΔC_t (control) as described previously (Pridgeon et al., 2010a). The relative expression data of a specific gene in control or infected fish were examined by unpaired t-test using SigmaStat statistical analysis software (Systat Software, San Jose, CA) and the differences were considered significant when the P value was less than 0.05.

Results

Characteristics of the subtractive cDNA library

A total of 96 clones were obtained from the subtractive library using the samples from secondary infected fish as tester and samples from primary infected fish as driver. All 96 clones were subjected to sequencing. Of the 96 clones, 94 contained inserts. The Blastx and Blastn results revealed that a total of 28 unique expressed sequence tags (ESTs) (30%) were obtained from 94 sequences (Table1). The insert sizes of the 28 unique ESTs ranged in size from 124 bp to 1054bp. The average insert size was 452 bp (Table 1). According to the Blastx and Blastn results, 26 (93%) of 28 unique ESTs were found had an E-value lower than 10^{-20}(Table 1). 18 of

the 28 sequences shared high homologies with channel catfish. Six shared homology with zebrafish (*Danio rerio*), the other four ESTs shared with other four organisms.

Expression of the 28 ESTs at 6 h post-infection

For relative QPCR experiments, 28 pairs ESTs-specific primers for the 28 ESTs were designed in order to determine whether the expression levels of the 28 ESTs isolated from the subtractive library were up-regulated in secondary infected channel catfish comparing with that in primary infected fish (Table 2). QPCR results revealed that 8 ESTs were significantly ($P<0.05$) induced in secondary infected fish compared to that in primary infected fish at 6 hpi (Figure 1). In the 8 upregulated ESTs, two (*Ictalurus punctatus* strain Auburn XbaI element 5 and *Ictalurus punctatus* strain Stuttgart XbaI element 7) in secondary infection were up-regulated greater than 3-fold compared to primary infection (Figure 1). The other 6 were up-regulated around 2-fold including TLR20-1 and TLR20-2 genes, inward rectifier potassium channel 13, unknown gene on immunoglobulin heavy chain locus, prolactin, NADH dehydrogenase subunit 2, reverse transcriptase-like protein (Figure 1).

Expression kinetics of the 8 genes at different time points

To determine whether the expression levels of these 8 ESTs in secondary infection were also up-regulated at the other time points compared to that in primary infection, the relative QPCR analysis were also conducted on anterior kidney cDNA samples from other time points using uninfected fish (IP injected with TSB) as control. The expression kinetics of the 8 ESTs identified by SSH is summarized in Fig. 2. All ESTs have higher expression level in primary infection than that in secondary infection at 3 hpi, 12 hpi, 24 hpi, 2 dpi and 7dpi except 6 hpi. All ESTs' expression level were significantly ($P<0.05$) increased and rapidly reached the first and highest peak at 3hpi. Of 8 ESTs, transcriptional levels of the two XbaI elements were

significantly increased over 100 folds in both primary and secondary infections at 3 hpi compared to un-infected controls (Figure 2). Following the primary infection, these two ESTs were induced all most 300 folds at 3 hpi. After 3 hpi, expressions of all 8 ESTs in both primary and secondary infections dropped rapidly. The transcriptional levels of six ESTs in primary experiment reached another peak at 24 hpi, but another two genes (prolactin and NADH dehydrogenase subunit 2) reached the second peak earlier at 12 hpi. The second peaks were much lower than the first peak. With the advancement of time, the transcriptional levels of 8 ESTs in primary infection almostly dropped to the same level as uninfected controls at 2 dpi. But, at 7 dpi, the third significantly up-regulation was detected in 4 genes including: TLR20-1 and TLR20-2 genes, unknown gene on the immunoglobulin heavy chain, prolactin, NADH dehydrogenase subunit 2. For the transcripts of 8 ESTs in secondary infection, there was no peak after 3 hpi. Only slightly up-regulations following secondary infection after 3 hpi were detected at 24 hpi (inward rectifier potassium channel 1 and reverse transcriptase-like protein) and at 7 dpi (immunoglobulin heavy chain gene locus, prolactin and NADH dehydrogenase subunit 2).

Discussion

Using SSH technique, 28 unique ESTs were identified from a total of 96 clones, only 8 were confirmed to be significantly up-regulated following secondary infection compared with those following primary infection at 6 hpi. This result may suggest that these 8 ESTs might play a more important role in the adaptive immune response against *A. hydrophila* infection than the innate immune response. However, the expression kinetics results showed that, at the other time points (at 3 hpi, 12 hpi, 24 hpi, 2 dpi and 7dpi), these 8 ESTs have higher expression levels following primary infection compared with that following secondary infection. Our results also revealed that the transcriptional levels of all 8 ESTs had 3 peaks after primary infection, while

only one peak at 3 hpi was detected following secondary infection. Similarly, the low transcriptional levels of immune genes following the secondary infection were also found in rainbow trout (*Oncorhynchus mykiss*) infected with *Yersinia ruckeri* (Raida and Buchmann, 2008).

EST A08 shew a higher homology (E-value 1.00E-143, identity 95%) with channel catfish TLR20-1 and TLR20-2 genes complete cds submitted by Quiniou. (2010). This EST was significantly induced up to 60-fold following primary infection and up to almost 30-fold following secondary infection at 3 hpi. Our results suggest it may play an important role in the innate and adaptive immune response against *A. hydrophila* infection. The dynamic expression levels of TLR20-1 and TLR20-2 genes were first reported. In addition, TLR20a, which was specific in teleost, were identified in zebrafish (*Danio rerio*) (Meijer et al., 2004) and channel catfish (Baoprasertkul et al., 2007). Our previous results showed that expression level of TLR20a was significantly induced by *Edwardsiella ictaluri* infection at 6 hpi in channel catfish and the transcriptional level was higher than those of TLR2, TLR3 or TLR21 (Pridgeon et al., 2010a). However, EST A08 did not show any homology with TLR20a in channel catfish.

Of all 8 ESTs, EST A10 and B04 were up-regulated the most after primary infection (up to 300-fold) and secondary infection (up to 200-fold). Both of them shared higher identities with XbaI element. EST A10 shared 99% identity with channel catfish strain Auburn XbaI element 5, complete sequence. EST B04 shared 96% identity with channel catfish strain Stuttgart XbaI element 7. In channel catfish, the XbaI elements were firstly characterized as a kind of A/T-rich tandem repetitive DNA sequence with four copies of the ATTA repeat and eight copies of $(A)_{3–6}$ GT/TG motifs (Liu et al., 1998). They may be useful for the identification of strains by polymerase chain reaction analysis based on their polymorphism (Liu et al., 1998). Though it is

unknown whether RNA transcripts of these tandem repetitive DNA sequence are produced merely by "accident" due to failure of transcription termination or whether their production serves some biological function during the developmental stage at which they are synthesized, substantial information has been gathered for repetitive sequences in eukaryotic DNA concerning their structures and their associations with protein encoding sequences both in DNA and RNA transcripts (Jelinek and Schmid, 1982). Some repetitive DNA sequence can be transcribed to nuclear RNA and form RNA-RNA duplexes which might regulate the production of messenger RNA of some genes (Davidson and Britten, 1979). Therefore, the higher transcription levels of these two XbaI elements suggest that they may play an important role in the immune response through regulate the production of messenger RNA of some immune genes.

EST C11 was characterized to share 99% identity with protaclin (PRL) in channel catfish. As we know, PRL, a kind of peptide hormone, shares many properties with cytokines such as homologous receptor structure, similar signal transduction pathway, and immunomodulatory action (Kooijman et al., 1996; Yu-Lee, 2002). PRL is also known to enhance immune functions in fish as in mammals (Balm, 1997; Clark, 1997; Harris et al., 2000; Olavarr á et al., 2010). In innate immune response, PRL can enhance the mitotic activity of leukocytes of the chum salmon (*Oncorhynchus keta*) (Sakai et al., 1996) and stimulate the phagocytic activity of fish leukocytes. These results indicate that there is a kind of cross-talk between the PRL and the TLR-induced pathway for activation of leukocytes (Balm, 1997; Clark, 1997; Harris et al., 2000). PRL is also known to be necessary to maintain circulating levels of IgM in the rainbow trout (*O. mykiss*) (Yada et al., 1999). Our results revealed that PRL was rapidly induced up to almost 60-fold after primary infection and over 20-fold after secondary infection at 3 hpi. The expression pattern is

similar to those of TLR20-1 and TLR20-2 genes and unknown gene on the immunoglobulin heavy chain locus. Our results suggest that PRL may has a positive relation with TLR on the activation of leukocytes and PRL may also play an important role in maintaining circulating levels of IgM in channel catfish. Moreover, to the best of our knowledge, PLR was first found to be expressed in the anterior kidney of channel catfish.

EST C09 was identified to show lower e-value (6.00E-102) and share 89% with unknown gene on the immunoglobulin (Ig) heavy (H) chain gene locus of channel catfish. As reported, the catfish Ig heavy chain locus is a translocon-type locus with three Igδ genes linked to an Igμ gene or pseudogene, which is estimated to contain approximately 200 variable region genes representing 13 families as well as at least three diversity and 11 joining genes (Bengtén et al., 2006). The unknown gene was induced rapidly to over 25 folds following primary infection and almost 10 folds following secondary infection at 3 hpi. However, the expression level dropped also more rapidly after primary infection compared that after secondary infection, which leaded to a relative higher expression level at 6 hpi after secondary infection.

EST C12 was up-regulated greater than 10-fold following primary infection and 6-fold after secondary infection at 3 hpi respectively. EST C12 shared 94% identity with NADH dehydrogenase subunit 2 of channel catfish at protein level. The NADH dehydrogenase complex, also called NADH coenzyme Q oxidoreductase or Complex I, is an enzyme located in the inner mitochondrial membrane that catalyzes the transfer of electrons from NADH to coenzyme Q. Since energy production is more efficient with the NADH dehydrogenase pathway than with the succinate ubiquinone reductase pathway (the second complex of the electron chain which uses FADH2) (Marchand et al., 2006), the up-regulation of this gene might indicate an optimization of energy production in the immune response. In addition, NADH dehydrogenase subunit 3 and

NADH dehydrogenase 1 alpha subcomplex 4 were induced by modified live *Edwardsiella ictaluri* vaccination in the anterior kidney of channel catfish (Pridgeon et al., 2010b). Further more, in Atlantic salmon (*Salmo salar*), NADH dehydrogenase chain 1 were also found to be up-regulated in anterior kidney and liver of fish infected by *A. salmonicida* (Tsoi et al., 2004). Taken together, our results suggest that NADH dehydrogenase complex may play an important role in the immune response against infection of *A. hydrophila* through increasing the energy production, which may be used to support the immune activity and increasing respiration of host after infection.

EST C04 was up-regulated greater than 50-fold in primary infection and 20-fold in secondary infection at 3 hpi respectively. EST C04 shared 74% with inward rectifier potassium channel (Kir) 13 of zebra fish at protein level (E-value=3.00E-104). Earlier studies have shown that macrophages express two types of K^+ channels, inwardly rectifying K^+ channels and voltage-gated K^+ channels (Mackenzie et al., 2003; Vicente et al., 2003). These earlier research have also revealed that the potassium channels activity can affect the macrophage antimicrobial functions. It has been found that the activation of potassium channels is an early step in the transmembrane signal transduction in macrophage and the perturbation of potassium channels would significantly impair antimicrobial functions of macrophages in goldfish (*Carassius auratus*) (Stafford et al., 2002). A recent study has found that increase in the activity of macrophage K^+ channels is necessary but not sufficient to induce macrophage to release IL-1β (Olavarr á et al., 2010). Therefore, the significant up-regulation of Kir 13 may induce the expression level of IL-1β, which, in turn, activates the functions of macrophages.

EST E05 was up-regulated greater than 50-fold after primary infection and 5-fold after secondary infection at 3 hpi respectively. EST E05 shared 45% with reverse transcriptase-like

protein of *Takifugu rubripesat* (E-value= 1.00E-21). This reverse transcriptase-like protein has also been found to be up-reglated in spleen of mandarin fish *Siniperca chuatsi* infected with infectious spleen and kidney necrosis virus at 4 dpi (He et al., 2006). However, the reverse transcriptase-like protein was found to be down-regulated after vaccination with an attenuated *E. ictaluri* at 2 dpi (Pridgeon et al., 2012).

In conclusion, 8 ESTs were identified and confirmed to significantly ($P < 0.05$) induced by secondary infection compared to those induced by primary infection in anterior kidney at 6 h post challenge. All 8 ESTs were significantly induced at 3 hpi by both primary and secondary infections, suggesting their important roles in earlier immune response. For most of ESTs, other significant up-regulations were also detected out later at 12 hpi, 24 hpi or 7dpi after primary infection, which suggests that these ESTs might take part in the development of adaptive immunity or other functions. However, after 3 hpi, only a few up-regulations were detected to be induced by secondary infection.The expression levels of 8 ESTs following secondary infection are much lower than that following primary infection at most time points except 6 hpi. Raida and Buchmann (2008) reported that one explanation of the lower expression levels induced by secondary infection is that the pathogen is killed very fast in re-injected fish, whereby the associated expression of genes is kept at a minimum. However, the slight drop of all 8 ESTs at 6 hpi after secondary infection makes them have higher expression level compared to those after primary infection.

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Table 1. List of genes isolated from the secondary infected versus primary infected catfish subtractive cDNA library.

Clone no.	Accessetion no.	Homology gene name or protein name	Organism	E-value	Identies	Insert size
A01	GU329038	Voucher INHS 47559 12S ribosomal RNA gene, partial sequence; mitochondrial	Ictalurus punctatus	7.00E-52	100%	498
A03	GQ465835	Clone BS2 18S ribosomal RNA gene, partial sequence	Ictalurus punctatus	0	100%	424
A04	XM_002663870	C-Jun-amino-terminal kinase-interacting protein 4-like (LOC100333761), mRNA	Danio rerio	9.00E-25	75%	813
A05	JC1348	Hypothetical 18K protein - goldfish mitochondrion	Carassius auratus	2.00E-29	54%	812
A07	AF482987	Ictalurus punctatus mitochondrion, complete genome	Ictalurus punctatus	0	99%	389
A08	HQ677723	Ictalurus punctatus TLR20-1 and TLR20-2 genes, complete cds	Ictalurus punctatus	1.00E-143	95%	400
A09	GU385707	voucher OP-10-CG-NBFGR-LKO 28S ribosomal RNA gene, partial sequence	Ompok pabda	0	100%	549
A10	AF112197	Ictalurus punctatus strain Auburn XbaI element 5, complete sequence	Ictalurus punctatus	2.00E-89	99%	192
B03	AY458870	12S ribosomal RNA gene, partial sequence	Ictalurus punctatus	8.00E-123	100%	428
B04	AF112199	Ictalurus punctatus strain Stuttgart XbaI element 7, complete sequence	Ictalurus punctatus	4.00E-83	96%	625
B06	AF112195	Ictalurus punctatus strain Kansas XbaI element 3, complete sequence	Ictalurus punctatus	3.00E-40	96%	184
B10	NP_612135	NADH dehydrogenase subunit 5	Ictalurus punctatus	1.00E-131	97%	1054
B11	GQ465835	Ictalurus punctatus clone BS2 18S ribosomal RNA gene, partial sequence	Ictalurus punctatus	5.00E-145	99%	293
C02	XR_117934	PREDICTED: Danio rerio hypothetical LOC100537045 , miscRNA	Danio rerio	2.00E-65	99%	146
C04	NP_001039014	Inward rectifier potassium channel 13	Danio rerio	3.00E-104	74%	735
C09	DQ400445	Immunoglobulin heavy chain gene locus, partial sequence and unknown gene	Ictalurus punctatus	6.00E-102	89%	308
C10	AF401559	Ribosomal protein L7 mRNA, partial cds	Ictalurus punctatus	1.00E-110	100%	316
C11	AF267990	Prolactin gene, complete cds	Ictalurus punctatus	8.00E-145	99%	296
C12	NP_612126	NADH dehydrogenase subunit 2	Ictalurus punctatus	1.00E-07	94%	157

Table 1. Continue

Clone no.	Accessetion no.	Homology gene name or protein name	Organism	E-value	Identies	Insert size
D01	NP_612128	Cytochrome c oxidase subunit II	Ictalurus punctatus	7.00E-42	96%	543
D12	CU861890	DNA sequence from clone CH1073-21K6 in linkage group 20	Danio rerio	3.00E-18	81%	596
E05	AAD19348	Reverse transcriptase-like protein	Takifugu rubripes	1.00E-21	45%	816
E06	NP_001038715	Rho GTPase-activating protein 10	Danio rerio	7.00E-42	88%	273
E07	NP_001187074	40S ribosomal protein S9	Ictalurus punctatus	5.00E-79	100%	373
F01	NM_001200275	Leukocyte DNA binding receptor (LOC100305038), mRNA, complete cds	Ictalurus punctatus	7.00E-29	94%	438
F08	AY049812	Internal transcribed spacer 2	Callorhinchus milii	6.00E-93	96%	218
G08	NM_131310	Heat shock protein 90	Danio rerio	0	86%	624
H04	JN872359	Isolate IP-P-03 control region, complete sequence; mitochondrial	Ictalurus punctatus	0	99%	481

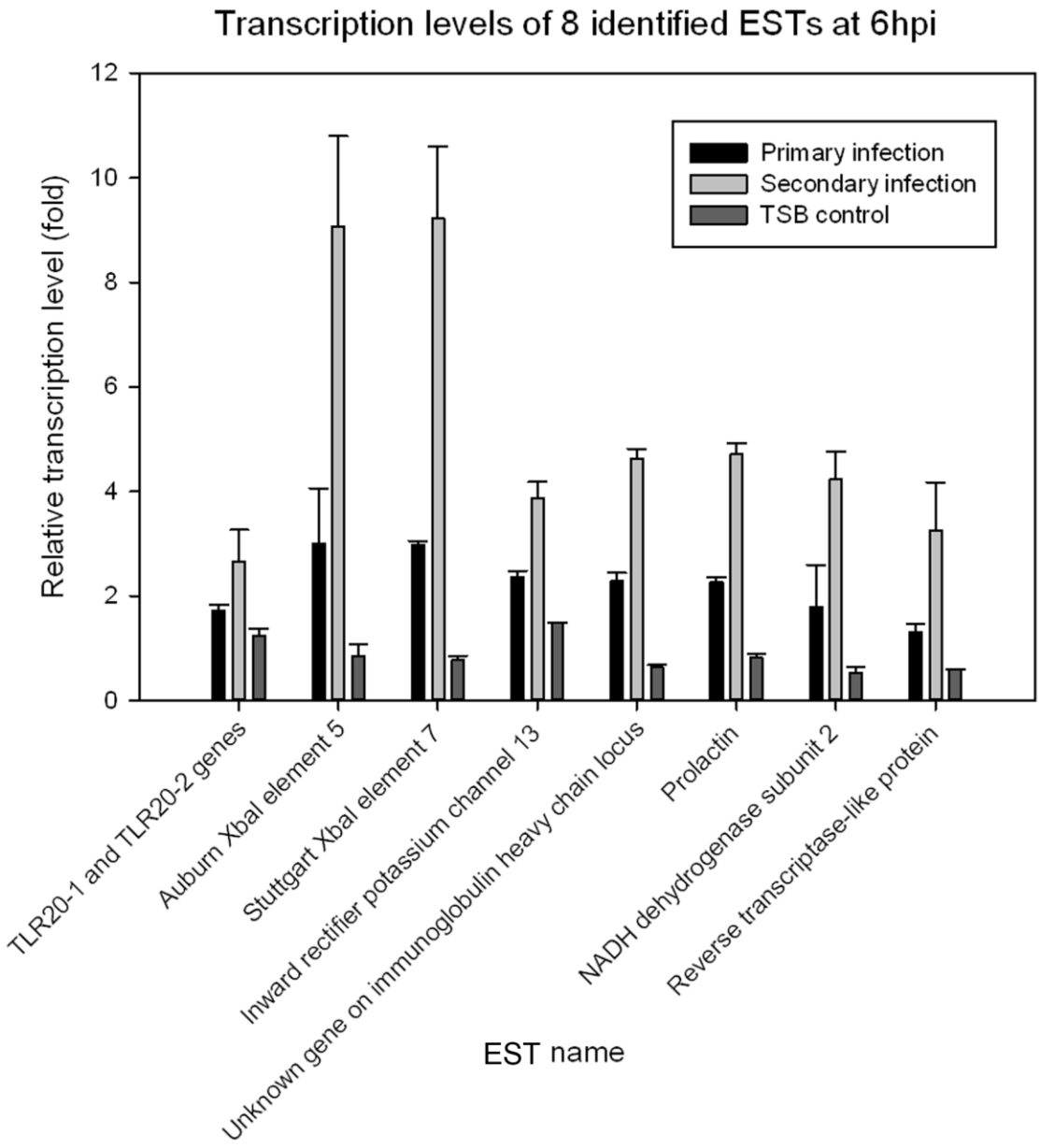
Table 2. Gene-specific primers used in qPCR.

No.	Clone no.	Forward primer	Reverse primer
1	A01	CCCACCTAGAGGAGCCTGTT	GGCGGGATAAACAAGAAGTG
2	A03	CGGCGTCCAACTTCTTAGAG	ATCAACGCGAGCTTATGACC
3	A04	TGACAAGCCAACAGCAGACT	TTAACCATGACCTGCCACAC
4	A05	CCTCGCCTGTTTACCAAAAA	ACAGTTAAGCCCTCGTTCCA
5	A07	CCTCGCCTGTTTACCAAAAA	ACAGTTAAGCCCTCGTTCCA
6	A08	CATCCAACCTGATGGAGCTT	GCACCTTTGGCACCAATTAC
7	A09	TGGGTTTTAAGCAGGAGGTG	ACGCTTGGTGAATTCTGCTT
8	A10	TCGCATTCATCCTGTGTCTC	GAGTAGGTCGGGCAACTGAT
9	B03	AGGACACAACCTCACCAGGA	TTGTAGTCTGCCGGAGCTTT
10	B04	TTGTGCTCTTTATCCGCTCA	TTGGACTACGTTGTGCACTTG
11	B06	TGTTTCTGACCCAAAATATTACGA	TTTTTCAACAACTTTTGCATTTT
12	B10	ACGTCTAGCCTGAGGAAGCA	GGAAAAGTTGTGGGGAGTCA
13	B11	TTTGATCGCTCCACACGTTA	CGGCTCGAGGTTATCAAGAG
14	C02	CCAGAGGAAACTCTGGTGGA	TCGGAGGGAACCAGCTACTA
15	C04	ACCTTGTCCCCTCTTCCTGT	GCTGCCATATTGGATCTCGT
16	C09	TCGAGTCAGTGCAAAAACCTT	TGAGCATCTTGCAGAACCAG
17	C10	CGAAAGCCTCCTGAAGAAGA	GTAGATCAGGTTCCGGGTGA
18	C11	AGAGCGAGCTCCTGTCTCTG	GTCCTGCAGCTCTCTGGTCT
19	C12	ACCATCCTATTTGCCAGCAC	GGGGCTAGTCCTACTTTTAATGC
20	D01	GTCCCAGGACGACTAAACCA	CAAAGTGCTCTAGGGGAACG
21	D12	GGATCCTGAAAAAGGACTAGAGC	TCTTCAGAACCTCCGTCTGC
22	E05	GCTACACGCTCCACTCAAAA	TGGTTTGACGCCATTTACAA
23	E06	GCCATCATGGACCTCAAGTT	GCGTGCCTGCTTCTTAGACT
24	E07	CGTCGTCCCTTTGAGAAGTC	CTTCATCCAGCACACCAATG
25	F01	ATGCTCCCTGGGATAGGC	TCCATCCATCTTCTACTACTTACTCC
26	F08	GCCGAAACGATCTCAACCTA	GTAACCCGGCTTTCGGTTC
27	G08	TCATTCCCAACAAGCATGAA	AGAAACCCACACCAAACTGC
28	H04	GAGGGTCACACAACTTGCAC	TGTTTGTTGATTGCCTGCAT

Figure legends

Figure 1. Effect of *Aeromonas hydrophila* treatment on the transcriptional levels of the 8 ESTs at 6 h post-infection including primary, secondary infections and TSB control. Data are presented as means ±S.D. from three replicates.

Figure 2. Effect of *Aeromonas hydrophila* treatment on expression kinetics of the 8 genes at different time points post infection including primary, secondary infections and TSB control. Data are presented as means ±S.D. from three replicates.


Transcription levels of 8 identified ESTs at 6hpi
Relative transcription level (fold)
0
2
4
6
8
10
12
Primary infection
Secondary infection
TSB control
TLR20-1 and TLR20-2 genes
Auburn XbaI element 5
Stuttgart XbaI element 7
Inward rectifier potassium channel 13
Unknown gene on immunoglobulin heavy chain locus
Prolactin
NADH dehydrogenase subunit 2
Reverse transcriptase-like protein
EST name

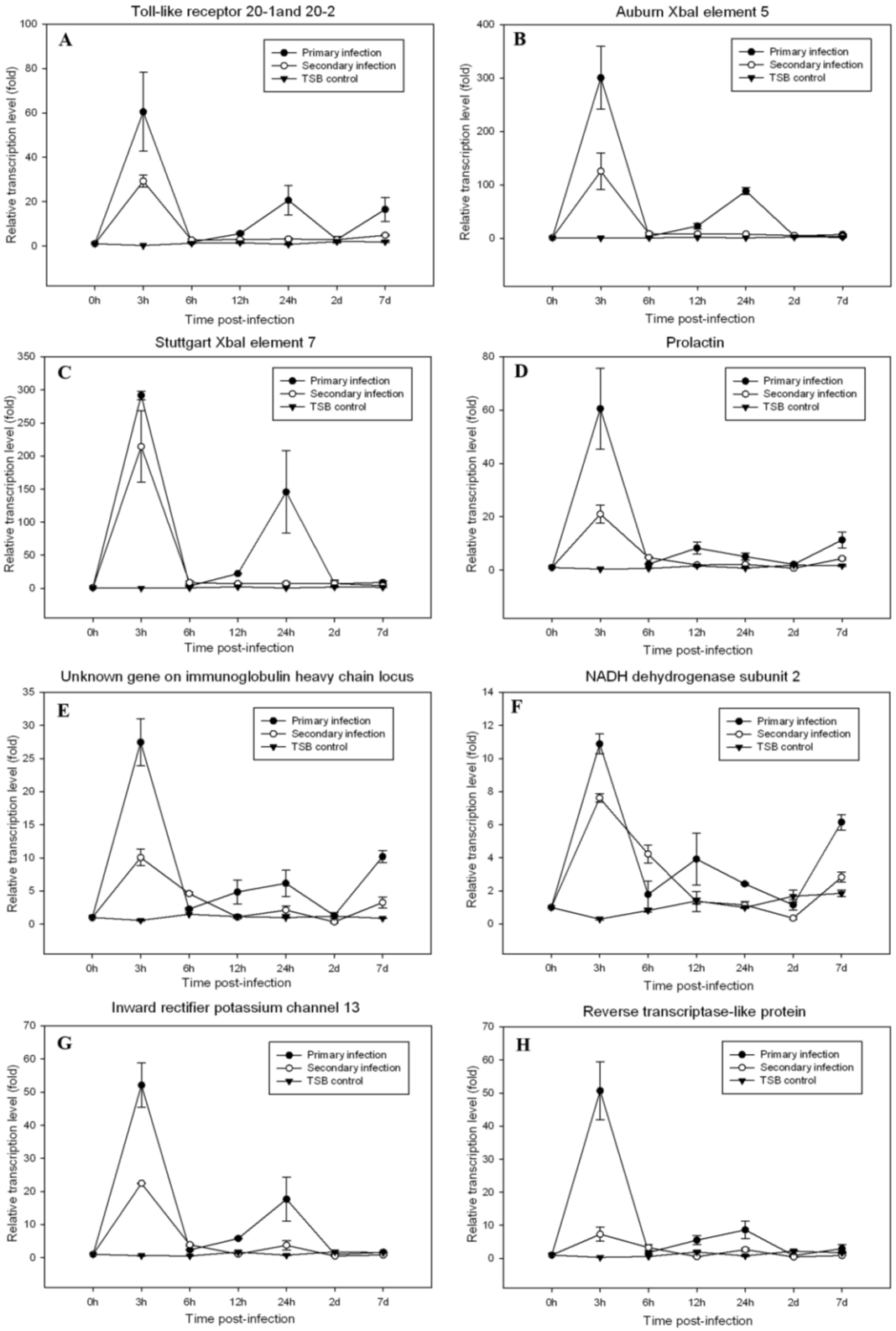

Toll-like receptor 20-1and 20-2
A
Auburn Xbal element 5
B
Stuttgart Xbal element 7
C
Prolactin
D
Unknown gene on immunoglobulin heavy chain locus
E
NADH dehydrogenase subunit 2
F
Inward rectifier potassium channel 13
G
Reverse transcriptase-like protein
H
Primary infection
Secondary infection
TSB control
Relative transcription level (fold)
Time post-infection
0h
3h
6h
12h
24h
2d
7d

Figure 2

V. TRANSCRIPTIONAL PROFILES OF MULTIPLE KNOWN GENES IN CHANNEL CATFISH AFTER SECONDARY EXPOSURE TO *AEROMONAS HYDROPHILA* COMPARED TO PRIMARY EXPOSURE

Introduction

Aeromonas hydrophila, a Gram-negative motile bacillus widely distributed in aquatic environments, is a causative agent of motile aeromonad septicemia (MAS) (Harikrishnan et al., 2003). Although usually considered as a secondary pathogen associated with disease outbreaks, *A. hydrophila* could also become a primary pathogen, causing outbreaks in fish farms with high mortality rates (Thore and Roberts, 1972; Nielsen et al., 2001; Fang et al., 2004). In West Alabama, MAS disease outbreaks caused by *A. hydrophila* in 2009 alone led to an estimated loss of more than 3 million pounds of food size channel catfish (Pridgeon and Klesius, 2011a). Virulence studies have revealed that AL09-71, a 2009 West Alabama strain of *A. hydrophila*, is highly virulent to channel catfish (*Ictalurus punctatus*), killing fish within 24h post exposure (Pridgeon and Klesius, 2011b). In 2010, *Aeromonas* disease outbreaks were common (Hemstreet, 2010).

To prevent future disease outbreaks caused by the highly virulent West Alabama 2009 isolate, an attenuated vaccine specifically targeting *A. hydrophila* AL09-71 was developed through selection for resistance to both novobiocin and rifampicin (named AL09-71 N+R, Pridgeon and Klesius, 2011c). Recently, transcriptions of some genes encoding both innate and adaptive immune parameters in the anterior kidney following vaccination with AL09-71 N+R

and infection with AL09-71 have been described. Knowledge about the immune defence of channel catfish against *A. hydrophila* is important in terms of control and prevention. Previously we reported that the transcriptional levels of 43 channel catfish genes were induced by vaccination with attenuated *Edwardsiella ictaluri* vaccine (Pridgeon et al., 2010b). Similarly, the transcriptional levels of 28 genes have been reported to be up-regulated by attenuated *Flavobacterium columnare* vaccine in channel catfish (Pridgeon and Klesius, 2010). In addition, the transcriptional levels of five toll-like receptors (TLRs) have been reported to be induced by acute infection with *E. ictaluri* (Pridgeon et al., 2010a). The bacterial infections can aslo induce the expression levels of tumor necrosis factor (Zhang and Wang, 1998), interleukin-1β (Zhang et al., 1998; Mu et al., 2011), interleukin-10 (Zhang et al., 1998; Mu et al., 2011), chemokine CXCL10 (Baoprasertkul et al., 2004; Mu et al., 2011) in channel catfish according to the ealier reports. Furthermore, 8 antimicrobial peptides (AMPs) genes (NK-lysin type 1, NK-lysin type 2, NK-lysin type3, bactericidal permeability-increasing protein, cathepsin D, liver-expressed AMP 2, hepcidin and transferrin) were up-regulated in the anterior kidney of channel catfish in response to the infection of *A. hydrophila* or *E. ictaluri* (Liu et al., 2010; Mu et al., 2011; Pridgeon et al., 2012). Recently, Mu et al. (2011) have reported the expression kinetics of six channel catfish genes, which were identified by SSH, in response to the primary exposure to *A. hydrophila* vaccine. Although those genes are up-regulated by infection after primary exposure to either vaccine or infection, little is known whether they might be up-regulated by secondary exposure. Therefore, the objective of this study is to analyze the transcription levels of these genes in channel catfish after secondary exposure compared to primary exposure.

In fish, the anterior kidney is the central organ, which plays the main function in immune defence. This organ works as the principal immune organ (Tort et al., 2003) responsible for

phagocytosis (Danneving et al., 1994), antigen processing (Brattgjerd et al., 1996; Kaattari et al., 1985), formation of IgM and immune memory through melanomacrophagic centres (Herraez et al., 1986; Tsujii et al., 1990). Due to the central role of the anterior kidney in immune response, our study focus on analyzing the expression kinetics of genes encoding toll-like receptors (TLRs), antimicrobial peptides (AMPs), cytokines, lysozyme C and other reported genes in the anterior kidney. At the same time, the present study also analyzed the activity of lysozyme in serum following primary and secondary exposures.

In the present study, we analyzed the transcriptional profiles of 22 genes, selected from 94 reported immune related genes, at different time points and compared their transcriptional patterns after primary and secondary infections.

Materials and methods

Bacteria source and growth conditions

The AL09-71 isolate of *A. hydrophila* was obtained from diseased channel catfish in 2009 from West Alabama and has been confirmed to be *A. hydrophila* through biochemical and molecular identification (Pridgeon and Klesius, 2011a). The isolate was grown in tryptic soy broth (TSB) (Fisher Scientific, Pittsburgh, PA) for 18 h at 28 ℃.

Experimental fish

Channel catfish (21.2 ± 3.3 g) were obtained from stocks maintained at USDA-ARS, Aquatic Animal Health Research Laboratory (Auburn, AL, USA). All fish were maintained in dechlorinated water in 340 L tanks. Prior to experiments, fish were acclimated in flow through 57 L aquaria supplied with ~2 L h^{-1} dechlorinated water for 14 days. Experimental fish were confirmed to be not infected by bacteria by culturing posterior kidney tissues from representative groups of fish on tryptic soy agar plates. A12:12 h light:dark period was maintained and

supplemental aeration was supplied by air stones. Mean dissolved oxygen was ~5.6 mg L^{-1} at water temperature ~27 ℃, with pH ~7.1 and hardness ~100 mg L^{-1}. Fish were fed ~3% body weight daily with commercial dry fish food.

Sample collection from primary and secondary infected fish

The infection dose of *A. hydrophila* AL09-71 was $1x10^5$ colony forming unit per fish (CFU/fish) based on previous challenge experiment result ($LD_{50}=1x10^5$ CFU/fish). For the primary challenge experiment, five hundred channel catfish were infected by intraperitoneal (ip.) injection ($1x10^6$ CFU/fish in 100 µl TSB). And, two hundred and fifty non-infected control fish were injected with 100 µl sterile TSB. At different time points (0 h, 3 h, 6 h, 12 h, 24 h, 2 days, and 7 days post infection), anterior kidney samples from 15 infected fish at each time point were collected and each five were pooled together. Anterior kidney samples from 15 fish ip. injected with TSB at each time point were also collected and each five were pooled together as control. In the primary challenge experiment the infected fish received an ip. injection and were observed for 28 days. In the secondary challenge experiment the surviving fish from the primary challenge received an additional injection of ($1x10^6$ CFU/fish in 100 µl TSB) bacteria 28 days after the primary infection. The non-infected control fish were also reinjected with sterile TBS at day 28 days post-primary injection. In secondary challenge experiment, anterior kidney samples were collected from infected fish and non-infected fish as the same as we did in primary challenge experiment at each time point. All anterior kidney tissues were flash frozen on dry ice during collection followed by storage at -80 ℃ until RNA extraction.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from anterior kidney tissues using TRIzol Reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. All RNAs were quantified on a Nanodrop

ND-1000 spectrophotometer (Nanodrop Technologies, Rockland, DE) and analyzed on 1% agarose gel by electrophoresis to confirm the integrity. For each pooled sample, 2 mg of total RNA was used to synthesize the first strand cDNAs. The first strand cDNAs used for quantitative PCR were synthesized by using the cloned AMV first strand cDNA synthesis kit (Invitrogen, Carlsbad, CA) following the protocol.

Genes reported in literatures screened in this study and primer design

Primers of 94 reported genes were purchased from SigmaeAldrich (St. Louis, MO) and used in this study to determine which genes were up-regulated by secondary infection. Of 94 reported genes, 22 genes were determined to be up-regulated at 3hpi following secondary infection. For each cDNA sample, channel catfish 18S ribosomal RNA primers were included as an internal control to normalize the variation in cDNA amount as published previously (Pridgeon et al., 2010a). Primers used for amplification of 22 genes listed in Table 1. The transcriptional levels of five toll-like receptors (TLRs) genes (TLR2, TLR3, TLR5, TLR20a and TLR21) have been reported to be up-regulated by acute infection with *E. ictaluri* and *A. hydrophila* (Pridgeon et al., 2010a; Pridgeon et al., 2010b; Mu et al., 2011). The expression levels of 8 antimicrobial peptides (AMPs) genes (NK-lysin type 1, NK-lysin type 2, NK-lysin type3, bactericidal permeability-increasing protein, cathepsin D, liver-expressed AMP 2, hepcidin and transferrin) were studied in the anterior kidney of channel catfish in response to the infection of *A. hydrophila* and *E. ictaluri* (Liu et al., 2010; Mu et al., 2011; Pridgeon et al., 2012). Furthermore, the expression of four cytokine genes, interleukin-1 beta (Zhang et al., 1998; Mu et al., 2011), interleukin-10 (Zhang et al., 1998; Mu et al., 2011), chemokine CXCL10 (Baoprasertkul et al., 2004; Mu et al., 2011), tumor necrosis factor alpha, alpha-induced protein 2 (TNFAIP2) (Pridgeon et al., 2010b), and some other reported genes such as lysozyme C (Mu et al., 2011);

cadherin EGF LAG seven-pass G-type receptor 1 (CELSR1) (Mu et al., 2011), lymphokine-activated killer T-cell-originated protein kinase-like (TOPK) (Mu et al., 2011), protein-arginine deiminasae type II-like (PADII) (Pridgeon et al., 2010b), very large inducible GTPase 1 (VLIG) (Pridgeon et al., 2010b) were investigated in this study. Sequencing results of different clones were used to design gene-specific primers by using Primer3 program (http://frodo.wi.mit.edu/cgibin/primer3/-primer3_www.cgi). Primers for these total 22 selected genes were purchased from SigmaeAldrich (St. Louis, MO) and used in this study to determine which genes were up-regulated by the A. hydrophila AL09-71.

Quantitative PCR

All QPCR was performed using Platinum® SYBR® Green qPCR SuperMix-UDG with ROX (Invitrogen, Carlsbad, CA) in a total volume of 12.5 µl. The QPCR mixture consisted of 1 µl of cDNA (input RNA of 10 ng), 0.5 µl of 5 µM gene-specific forward primer, 0.5 µl of 5 µM gene-specific reverse primer and 10.5 µl of 1xSYBR Green SuperMix. The QPCR thermal cycling parameters were 50 ℃ for 2 min, 95 ℃ for 10 min followed by 40 cycles of 95 ℃ for 15 s and 60 ℃ for 1 min. All QPCR was run in duplicate for each cDNA sample and three pooled cDNA samples were analyzed by QPCR. The fluorescence intensities of the control and treatment products for each gene, as measured by cycle threshold (C_t) values, were compared and converted to fold differences by the relative quantification method (Pfaffl, 2001) using the Relative Expression Software Tool 384 v. 1 (REST) and assuming 100% efficiencies. Expression differences between control and treatment groups were assessed for statistical significance using a randomization test in the REST software. The mRNA expression levels of all genes were normalized to the levels of 18S ribosomal RNA gene in the same sample. Expression levels of

18S were constant between all samples (<0.30 change in C_t). Each primer set amplified a single product as indicated by a single peak present for each gene during melting curve analysis.

Lysozyme assay

Plasma lysozyme activity was measured spectrophotometrically according to the method as described by Sankaran and Gurnani (1972). The lysozyme substrate was a 0.025% (w/v) suspension of Micrococcus lysodeikticus (Sigma) was prepared freshly with sodium phosphate buffer (0.04 M, pH 6.0). Serum (20 µl/well in duplicate) from 5 fish/group was placed in a microtiter plate and 250 µl of bacterial cell suspension was added to each well. Hen egg white lysozyme was used as an external standard. The initial and final (25 min incubation at 30 ℃) absorbances of the samples were measured at 450 nm. The rate of reduction in absorbance of samples was converted to lysozyme concentration (µg/ml) using a standard curve. The results were expressed as µg/ml equivalent of hen egg white enzyme activity.

Data analysis

The relative transcriptional levels of different genes were 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: $\Delta C_t = C_t$ (sample) − C_t (calibrator). The relative expression level of a specific gene in control fish or in infected fish were compared to that of average control fish by the formula $2^{-\Delta\Delta Ct}$ where $\Delta\Delta C_t = \Delta C_t$ (infected) − ΔC_t (control) as described previously (Pridgeon et al., 2010a). The relative expression data of a specific gene and lysozyme activity in control or infected fish were examined by unpaired t-test using SigmaStat statistical analysis software (Systat Software, San Jose, CA) and the differences were considered significant when the P value was less than 0.05.

Results

Expression kinetics of five TLRs in the anterior kidney after infection

Using quantitative PCR (QPCR), the relative transcriptional levels of five TLRs (TLR2, TLR3, TLR5, TLR20 and TLR21) were studied in the anterior kidney of channel catfish under TSB control, primary infection and secondary infection at different time points (3 hpi, 6 hpi, 12 hpi, 24 hpi, 2 dpi and 7 dpi). The results of time course studies revealed that all these 5 genes have a similar transcriptional pattern following primary and secondary infection (Fig. 1). All five TLRs were significantly ($P < 0.05$) up-regulated at 3 hpi, declined to the control level at 6 hpi and significantly ($P < 0.05$) induced again at 7 dpi following both primary and secondary infection (Fig. 1). At 12 hpi, most genes remained lower expression level like that at 6 hpi (Fig. 1B, C, D, E) except TLR2 which was significantly induced in primary infection (Fig. 1A). The expression levels of TLR2, TLR5 and TLR20 were stable from 12 hpi to 2 dpi (Fig. 1A, C, D). But, TLR3 and TLR21were rapidly induced from 12 hpi to 24 hpi (Fig. 1B, E) after primary infection. From 24 hpi to 2 dpi, the expression level of TLR21 remained stable (Fig. 1E), whereas the expression level of TLR3 dropt rapidly after primary infection (Fig. 1B). At 3 hpi and 24 hpi, the expression levels of TLR3, TLR20 and TLR21 in the primary experiment were significantly ($P < 0.05$) higher than those in the secondary experiment (Fig. 1B, D, E). And, at 7 dpi, the expressions of all 5 genes under primary infection were significantly ($P < 0.05$) induced much more highly than those under secondary infection (Fig. 1). However, at 6 hpi, the expression levels of TLR2, TLR3 and TLR5 under secondary infection were significantly ($P < 0.05$) higher than those under primary infection (Fig. 1A, B, C). Of the five TLRs genes, TLR5 was induced the most by the infection at 3 hpi, while induced slightly at 7 dpi as compared to the other 4 TLRs (Fig. 1C).

Expression kinetics of eight AMPs in the anterior kidney after infection

The expression kinetics of the eight reported AMPs genes were summarized in Fig. 2. The transcriptional patterns of NK-lysin type 1 (NKL-1), NK-lysin type 2 (NKL-2), NK-lysin type 3 (NKL-3), Bactericidal permeability-increasing protein (BPI), hepcidin and transferrin were found to be similar under both primary and secondary infection (Fig. 2. A, B, C, E, G, H). Under primary infection, the transcriptional profiles of these genes showed 3 peaks at 3 hpi (except NKL-2, transferrin (first peak at 6 hpi)), 24 hpi (except BPI and transferrin) and 7 dpi respectively (the highest peak) respectively (Fig. 2. A, B, C, E, G, H). But, under secondary infection, only tow peaks were found in most genes except hepcidin, which still has three peaks like under primary infection, among the six genes with similar transcriptional pattern (Fig. 2.A, B, C, E, G, H). It was notable that the transcriptional levels of NKL-2, BPI and transferrin after secondary infection were significantly higher than that after primary infection at 6 hpi (Fig. 2.B, E, H). Of the eight AMPs, hepcidin was upreglated most up to 3800-fold and 1500-fold under primary and secondary infection respectively at 7 dpi (Fig. 2.G). As shown in Fig. 2.H, transferrin was significantly induced (around 40-fold) at 6 hpi, and, thereafter, was up-regulated again (around 500-fold) at 7 dpi by both primary and secondary infections. Interestingly, at these two time points, the expressional levels of tranferrin were significantly ($P < 0.05$) higher under secondary infection as compared to that under primary infection. All three types NKL genes were induced over 25-fold at 7 dpi (Fig. 2.A, B, C). The other two genes (cathepsin D and liver-expressed AMP 2) have lower expression levels compared with the other six genes described above (Fig. 2.D, F). Remarkably, the expression levels of NKL-1 and NKL-2 after secondary exposure were more significantly ($P < 0.05$) higher than those after primary exposure at 7 dpi (Fig. 2.A, B).

Expression kinetics of four cytokines in the anterior kidney after infection

The expression kinetics of four cytokine genes encoding the interleukin-1 beta (IL-1β), interleukin-10 (IL-10), chemokine CXCL10 (IP10) and tumor necrosis factor alpha, alpha-induced protein 2 (TNFAIP2) were determined in the anterior kidney of channel catfish. Both primary and secondary infections significantly ($P < 0.05$) induced all four cytokine at 3 hpi. However, expression levels (around 20-fold) under primary infection were much higher than those under secondary infection (around 10-fold) (Fig. 3.). Another two expression peaks (around 20-fold) of IL-1β were detected at 24 hpi and 7 dpi respectively following primary infection (Fig. 3.A). While, the expression level of IL-10 touched the peak (60-fold) at 12 hpi following primary infection (Fig. 3.B). The transcriptional patterns of IP10 and TNFAIP2 were similar after primary infection with highest peak (around 40-fold) at 24 hpi (Fig. 3. C, D). The only difference happened at 7 dpi, where the expression of IP10 was up-regulated to more than 20-fold, while the expression of TNFAIP2 was stable from 2 dpi to 7 dpi under primary infection (Fig. 3. C, D). The expression patterns of all four cytokine genes were similar following secondary infection with significant up-regulations at early stage of infection (3 hpi and 6 hpi), but lower than those of primary infection (Fig. 3. A, B, C, D).

Expression kinetics of five reported genes induced by A. hydrophila infection

As shown in Fig. 4, the expression kinetics of five reported genes were summarized, lysozyme C; cadherin EGF LAG seven-pass G-type receptor 1 (CELSR1), lymphokine-activated killer T-cell-originated protein kinase-like (TOPK), protein-arginine deiminasae type II-like (PADII), very large inducible GTPase 1 (VLIG). The transcription level of lysozyme C peaked at 24 hpi (around 16-fold) under primary infection, while, in secondary infection, it peaked ealier at 12 hpi (around 5-fold) (Fig. 4. A). CELSR1 was significantly induced over 15-fold at 3 hpi, 24

hpi and 7 dpi respectively (Fig. 4. B). The expression of TOPK peaks at 12 hpi around 8-fold, and, thereafter, it declined to lower than control at 2 dpi and 7 dpi (Fig. 4. C). The expression levels of PADII and VLIG were rapidly up-regualted at 3 hpi by primary infection (over 15-fold) and secondary infection (over 5-fold) (Fig. 4. D, E). With the advancement of time, the expressions of these two genes after secondary exposure gradually declined to normal level. However, their expressions after primary exposure were rapidly induced again (over 10-fold) at 12 hpi (Fig. 4. D, E).

Lysozyme activity

Plasma lysozyme activity was increased 3.3-fold under primary infection and 3.0-fold under secondary infection at 12 hpi as compared to the activity level under control (Fig. 5). After 12 hpi, the lysozyme activity started decreasing. At 2 dpi, the lysozyme activity after secondary infection declined to lower than control. At 7 dpi, the lysozyme activity following primary infection was also detected to be lower than control level. It was notable that the lysozyme activity after primary exposure was significantly higher than that after secondary exposure at almost all time points except 12 hpi.

Discussion

TLRs are transmembrane proteins, which are essential in recognizing the highly conserved pathogen-associated molecular patterns (PAMPs) and activating immunostimulatory and immunomodulatory cell signalling pathways, which are essential for both innate immune and adaptive immune responses (Janeway and Medzhitov, 2002). TLRs are able to elicit the inflammatory and immune responses in vertebrate (Medzhitov and Janeway, 2000). The best characterized ligand that TLRs recognize include: (1) lipoteichoic acid and lipoproteins by TLR2; (2) dsRNA by TLR3; (3) lipopolysaccharide (LPS) by TLR4; (4) bacterial flagellin by

TLR5, (5) single stranded RNA (ssRNA) by TLR7, and (6) dsDNA by TLR9 (Baoprasertkul et al., 2007b; Iwasaki and Medzhitov, 2010). Additionally, TLRs were also reported to have multi-functions and act together in pathogen recognition and signaling (Ishii et al., 2005; Baoprasertkul et al., 2007a). For example, even though TLR2 is best known as a receptor recognising conserved components of Gram-positive bacteria such as lipoteichoic acid and lipoproteins, TLR2 is also able to interact with a wide range of additional ligand types such as zymosan, derived from yeast, glycosylphosphatidylinositols (GPIs) from protozoan parasites, LPS of Gram-negative bacterium which is generally considered to be the ligand of TLR4 (Baoprasertkul et al., 2007a). The previous studies have shown that TLR2 may function together with TLR4 (Hadley et al., 2005) or independently in this role (O'Connell et al., 2006). Furthermore, TLR3 is well known to also responsible for virus detection with dsRNA as its ligand. However, the successful stimulation of TLR3 with bacterial PAMPs has also been detected in zebrafish and channel catfish after infection with the Gram-negative *E. tarda* and *E.ictulari*, respectively (Bilodeau et al., 2005; Bilodeau and Waldbieser, 2005).

Recently, Pridgeon et al. (2010a) reported that both TLR2 and TLR 3 in the anterior kidney were significantly induced by the infection of *E. ictulari* and touched peaks at 6 hpi, while, thereafter, the transcriptional levels of two genes declined. In addition, TLR2 was most significantly ($P < 0.05$) induced at 12 hpi in the kidney of Indian major carp, mrigal (*Cirrhinus mrigala*), infected by *A. hydrophila*, and, after that, the TLR2 expression level declined (Basu et al., 2012a). Similarly, in our study, TLR2 and TLR3 were also found to be up-regulated at 3hpi, suggesting that TLR2 and TLR3 play important roles in the host defense of channel catfish against infection of *A. hydrophila* at the earlier infection time course.

TLR5 in channel catfish was found to be significantly up-regulated at 4 and 6 hpi after acute infection of *E. ictaluri* (Pridgeon et al., 2010a). Recently, Mu et al. (2011) reported that, after infection of *A. hydrophila*, the expression level of TLR5 in the anterior kidney of channel catfish increased at 3 hpi and significantly peaked at 6 hpi. In addition, the expressions of TLR5 in the kidney were found to touch the peaks at 6 hpi (around 17-fold) and decline to around 3 fold at 24 hpi in Indian major carp (*Cirrhinus mrigala*) infected by *A. hydrophila* (Basu et al., 2012b). Similarly, the present study reveals that, of the five TLRs, TLR5 was up-regulated most highly (around 50-fold) at 3 hpi by both primary and secondary infections, which further confirms the essential role of TLR5 in recognizing flagellated bacteria in both innate and adaptive immunity.

The channel catfish TLR20 and TLR21 were characterized by Baoprasertkul et al. (2007b), which belong to so-called 'fish-specific' TLR family and were not identified in mammalian (Rebl et al., 2010). Although they appear to branch with the murine TLR11, 12 and 13 in phylogenetic analyses, they form distinct branches (Baoprasertkul et al., 2007b; Palti, 2011). In this study, TLR20 and TLR21 in the anterior kidney were induced significantly at 3 hpi by both primary and secondary infections. In addition, TLR20 and TLR21 in the anterior kidney were significantly up-regulated at 6 hpi in channel catfish infected by *Edwardsiella ictaluri* (Pridgeon et al., 2010a). Therefore, TLR20 and TLR21 may play important roles in channel catfish immune against bacterial infection. However, no direct evidence of ligand specificity has been shown for TLR20 and TLR21 (Palti, 2011). The function of TLR20 and TLR21 are unknown in channel catfish. Future studies investigating the ligand specificity of the TLR20 and TLR21 genes will provide a better understanding of the function of these two genes.

Interestingly, a significant up-regulation was detected out at 7 dpi in all five TLRs after both primary and secondary infections, suggesting the TLRs may have some special functions at 7 dpi in both innate and adaptive immune repons. Especially, the primary infection induced the highest expressions of TLR2, TLR3, TLR20 and TLR21 at 7 dpi, which were much higher than those induced by secondary infection. Kawai and Akira (2011) reported that TLRs play important roles in initiation of adaptive immunity, inflammation and tissue repair through activating different downstream signaling pathways. Thereafter, we speculate the up-regulation of TLRs at 7 dpi may be related to the activation of adaptive immunity, inflammation or tissue repair.

Fish secrete different kinds of antimicrobial peptides (AMPs), which are positively charged short amino-acid-chain molecules involved in host defense mechanisms. An increasing number of AMPs have been isolated from fish and with their abundance in many tissues, they may represent the most important innate defense in fish (Noga et al., 2011). In channel catfish, eight AMPs (NK-lysin type 1, NK-lysin type 2, NK-lysin type 3, bactericidal permeability-increasing protein (BPI), cathepsin D, hepcidin, liver-expressed AMP 2 (LEAP2), and transferrin) have been reported. Of the eight AMPs, hepcidin and transferrin were induced mostly by both primary and secondary infections. Recently, hepcidin has been found to act as an important iron regulator by binding to ferroportin (a key iron exporter on macrophages), thereby decreasing iron transfer into blood plasma from macrophages and inducing ferroportin-mediated endocytosis and proteolysis in mammalian (Ganz, 2011; Kroot et al., 2012; Zhu et al., 2012). Transferrin also has a critical role in iron metabolism, maintaining low levels of extracellular free iron and transporting iron to tissues as required, which also participates in a wide variety of metabolic processes, including immune regulation, antimicrobial and antioxidant activity, DNA

synthesis, cytoprotection, and electron transport (Stafford et al., 2003; Ong et al., 2006). The dramatical and significant up-regulations of hepcidin and transferrin were observed in our study, suggesting that both hepcidin and transferrin play very important roles in immune against *A. hydrophila* through decreasing the ion level in extracelluar environment. Recent studies show that the expression of hepcidin is regulated by a transferrin in the zebrafish embryo (Fraenkel et al., 2009) and transferrin is a major determinant of hepcidin expression in hypotransferrinemic mice (Bartnikas et al., 2011), suggesting that there is a kind of synergy between transferring and hepcidin. Our time course study reveals that they have a similar expression pattern following primary and secondary infections, indicating the existence of the cooperation of them in innate and adaptive immunity against *A. hydrophila* infection. Remarkably, the expression level of transferrin under secondary infection was significantly ($P < 0.05$) higher than that under primary infection at 6 hpi and 7 dpi (Fig. 2.H), which suggests that transferrin may play a more important role in adaptive immunity than that in innate immunity. In addition, the striking inductions of hepcidin were also detected at 6 hpi in *A. hydrophila* and *E. ictaluri* infected channel catfish (Mu et al., 2011; Pridgeon et al., 2012).

Three distinct NK-lysin transcripts have been cloned in channel catfish and a higher expression level was detected at 7 dpi in the anterior kidney in channel catfish infected by *E. ictaluri* (Wang et al., 2006 a, b), which is in agreement with our result in this study (Fig. 2.A, B, C). We found that NK-lysin type 1 and 2 may function heavier at 7 dpi after secondary infection, since they have higher expression levels following secondary infection (Fig. 2.A, B). Whereas, the express pattern of NK-lysin3 is different in this study, suggesting a difference in function. BPI was identified and detected to be up-regulated at 7 dpi in the anterior kidney of *E. ictaluri* infected channel catfish (Xu et al., 2005). Our study also revealed a strong expressional induce of

BPI at 7 dpi following both primary and secondary infections (Fig. 2. E). In addition, cathepsin D and LEAP2 were also found to be up-regulated by primary and secondary infections, but not as highly as the other six AMPs. Furthermore, recent study shows that five AMPs (NKL-1, NKL-3, BPI, cathepsin D and hepcidin) were significantly up-regulated within 48 hpi in the anterior kidney of channel catfish infected by *E. ictaluri* (Pridgeon et al., 2012). The higher and similar temporal expression profiles were found in NKL-1, NKL-2, NKL-3, BPI, transferrin and hepcidin, which suggest these six AMPs might cooperate and play major roles in the immune defense against *A. hydrophila*. Furthermore, NKL-2, BPI and tranferrin may play more important roles in the adaptive immunity at 6 hpi, since the expression levels of these three AMPs following secondary infection were significantly higher than in primary infection at 6 hpi.

Interleukin-1 β (IL-1β) is an important early response pro-inflammatory cytokine that mediates immune regulation in both innate and adaptive immunity (Bird et al., 2002; Huising et al., 2004; Wang et al., 2006c). However, interleukin-10 (IL-10) is regarded as an anti-inflammatory cytokine and plays a crucial role in the regulation of inflammation in mammalian by downregulating expression of other cytokines such as IL-1β, primarily at the transcriptional level (Aste-Amezaga et al., 1998). Significant up-regulation of IL-1 β has been reported in the kidney of ray-finned fish (*P. sarana*) at 1, 3, and 6 h (Das et al., 2011) and in the anterior kidney of channel catfish at 3 and 6h post *A. hydrophila* infection (Mu et al., 2011). In consistent with these reports, our results also revealed that IL-1 β was significantly up-regulated over 20-fold at 3 hpi, 6 hpi, 24 hpi and 7 dpi under primary infection, further confirming that IL-1 β plays an important role in the pro-inflammatory response to *Aeromonas* infection. Additionally, in this experiment, a clear association was observed between higher expression (around 60-fold) of IL-10 and a corresponding lower expression (around 10-fold) of IL-1 β at 12 hpi under primary

infection, indicating a suppressive role of IL-10 on the transcriptional level of the pro-inflammatory cytokine IL-10. This association suggests that IL-10 might have anti-inflammatory activity in channel catfish. Similar report can be found in Atlantic cod (*Gadus morhua*) (Seppola et al., 2008). On the other hand, the relative slightly up-regulation of IL-10 at 7 dpi and 3 hpi may reflect the processes of anterior kidney rebuilding and the limitation of potentially harmful inflammatory responses which start immediately after induction of the inflammatory reaction caused by IL-1 β, respectively. Chemokine CXCL10 was firstly identified and reported to be up-reglated at 4 hpi and peaked at 24 hpi in the anterior kidney of *E. ictaluri* infected channel catfish (Baoprasertkul et al., 2004). Recently, a significantly up-regulation was detected at 3 hpi and peaked at 12 hpi in *A. hydrophila* infected catfish (Mu et al., 2011). Similarly, in this study, we found that Chemokine CXCL10 was pronounced up-regulated at 3 hpi and peaks at 24 hpi, which suggests CXCL10 may play an important role in recruiting monocytes, neutrophils, lymphocytes and other effector cells to the inflammatory sites to eliminate *A. hdrophila* in the early stages of infection. TNFAIP2 is also a TNFa-regulated gene that is first found expressed in human endothelial cells (Sarma et al., 1992). Although the role of TNFAIP2 is still unclear, it is an important gene involved in apoptosis (Liu et al., 2011). TNFAIP2 may also play an important role in activating effector cells to eliminate *A. hdrophila* since its expression pattern is similar with that of Chemokine CXCL10.

Lysozyme C is an important molecule of innate immune system for the defense against bacterial infections. G-type and c-type lysozymes have been identified in fish and other vertebrates (Ye et al., 2010). The completed cDNA sequences of g-type and c-type lysozymes in channel catfish were reported by Chen et al. (2010). Recently, we reported that the expression of lysozyme C was significantly induced at 12 h post challenged with *A. hydrophila* in the anterior

kidney of channel catfish, and thereafter it touched the peak at 24 hpi (Mu et al., 2011). In this study, a same expression pattern of lysozyme C was shown in the primary infection (Fig. 4. A). The secondary response was significantly induced earlier at 3 hpi and reached a peak at 12 hpi (Fig. 4. A). The expression level of lysozyme C touched the peak later as compared to other up-regulated genes (at 3 hpi) in this study, which suggests it may be the down-stream gene of other genes. Lysozyme is effective when the outer cell wall of Gram-negative bacteria is disrupted due to the action of complement and other enzymes (Saurab et al., 2008). The results (Fig. 5.) of plasma lysozyme activity reflect the transcriptional profiles (Fig. 4.A) after both primary and secondary infections, suggesting that up-regulation of lysozyme genes might be responsible for the rise in plasma lysozyme activity of catfish following *A. hydrophila* infection. Furthermore, the lysozyme activity and expression level under primary infection are significantly higher than those under secondary infection except at 12 hpi. This result may indicate lysozyme is more important in innate immune defense against infection of *A. hydrophila*.

Very large inducible GTPase 1 (VLIG) can be induced by interferons (IFNs) in humans and mice and play a critical role in preventing microbial infections, while its function is not well understood (MacMicking et al., 2004; Martens and Howard, 2006; Li et al., 2009). VLIG was firstly reported to be up-regulated at 48h post vaccination in the anterior kidney of channel catfish vaccinated with attenuated *E. ictaluri* (Pridgeon et al., 2010b). Our study showed that VLIG was also significantly induced at 3 hpi, 12 hpi and 7 dpi in the anterior kidney of *A. hydrophila* infected channel catfish (Fig. 4.B). For the remaining 3 genes, cadherin EGF LAG seven-pass G-type receptor 1 (CELSR1), and lymphokine-activated killer T-cell-originated protein kinase-like (TOPK), they were all significantly induced by the infection of *A. hydrophila*. Our results indicate they may all function in the immune response against *A. hydrophila*

infection in channel catfish. For all these 5 five genes, it is notable that the expressions under secondary exposure were much lower as compared to those under primary exposure except at 6 hpi, suggesting these genes may be more important in innate immunity than that in adaptive immuntiy.

In conclusion, we determined the expression kinetics of 22 genes, selected from 94 reported immune related genes, at different time points and compared after primary and secondary infections. Of 22 genes, hepcidin and tranferrin were detected to be the most highly induced two genes. Though literatures indicate their function in immune response, further study might need to focus on their correlation and interaction based on their associated immune functions. We also found that some genes were up-regulated earlier at 3 hpi or 6 hpi compared to our previous study at 6 hpi or 12 hpi (Mu et al., 2011). This may be due to the higher challenge dose ($1x10^6$ CFU/fish) (Pridgeon et al., 2011d) and bigger fish (21.2 ± 3.3 g) used in this study. Furthermore, even though the transcriptional levels of most of 22 genes following primary infection are higher than those following secondary infection except at 6 hpi, we found several genes that had higher transcriptional levels after secondary infection such as transferrin, NKL-1, and NKL-2 except at 6 hpi. The results suggest that these 3 genes may be more important in the adaptive immune response compared with that in innate immune response. Our results also revealed that a lot of genes have a higher expression level at 6 hpi after secondary exposure than that after primary exposure such as TLR2, TLR3, TLR5, NKL-2 and so on, which is caused by the slighter drop of expression level after 3 hpi under secondary infection. Most interestingly, it was noteworthy the lower expression levels of investigated genes in re-infected channel catfish. This corresponds to the transcriptional data of the immune response in rainbow trout reacting to *Yersinia ruckeri* where, following full recovery from the primary infection, secondary infection

did not elicit higher transcription levels (Raida and Buchmann, 2008). Raida and Buchmann (2008) reported that one explanation of the lower expression levels induced by secondary infection is that the pathogen is killed very fast in re-injected fish, whereby the associated expression of genes is kept at a minimum. Most genes were significantly induced at 3 hpi by both primary and secondary infections, suggesting their important roles in earlier immune response. However, for most of these genes, significant up-regulations were also detected later at 24 hpi and 7dpi after primary infection, which suggests that these genes might take part in some other functions such as important roles in initiation of adaptive immunity, inflammation and tissue repair.

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Table 1. Gene-specific primers used in qPCR.

Gene Name	Accession no	Forward primer	Reverse Primer	Gene Source
18s rRNA	BE469353	ATGGCCGTTCTTAGTTGGTG	TAGGTAGCACACGCTGATCG	Pridgeon et al., 2010a
TLR2	DQ372072	GCGTGGTTAAGAGCGAAAAG	GGAAGGAAGTCTCGCTTGTG	Baoprasertkul et al., 2007a; Pridgeon et al., 2010a
TLR3	DQ423776	TTGCACCTGTGAGAGCATTC	AGTGCACCAGGAAGGCTAGA	Bilodeau & Waldbieser 2005; Pridgeon et al., 2010a;
TLR5	GO898818	CAGAAACAGCTTTGCACCTG	CTCATTCCCCTGTCCATCAC	Baoprasertkul et al., 2007b; Mu et al., 2011
TLR20	DQ529275	CACCTCTCTGGGACTGGTGT	GCTCATCTTTCCCGCAGTAG	Baoprasertkul et al., 2007b; Pridgeon et al., 2010a;
TLR21	DQ529276	TTCCTCTGCAGTGAGTGGTG	TGTGTCCAGAACAGCTCCTG	Baoprasertkul et al., 2007b; Pridgeon et al., 2010a;
NKL-1	AY934592	GGGCCATGAAGAAAGTGAAA	GCTTGGAACAATTCCAGCAT	Wang et al., 2006; Pridgeon et al., 2012
NKL-2	DQ153186	TGTAAGTGGGCCATGAACAA	TCCTCCACCAAGGTATCCAA	Wang et al., 2006; Pridgeon et al., 2012
NKL-3	DQ153187	GGCTGTGACAAACTCCCAGT	GGATCAATTCCCACATGTCC	Wang et al., 2006; Pridgeon et al., 2012
BPI	AY816351	TGTTGGCTTTGCTCTCCTTT	TGCCTATGGGAGACACCTTC	Xu et al., 2005; Pridgeon et al., 2012
Cathepsin D	GU588646	CTGGGAGGGAAAGTGTTCAA	GGTGTAGAAACGGCCCATAA	Cho et al., 2002; Pridgeon et al., 2012
Hepcidin	AY834209	TGCAGCTTTACCATCTGAGG	AGGTGACTCTGACGCTTCGT	Bao et al., 2005; Mu et al., 2011
LEAP2	AY845141	TTGGAAGCGCTACAAATCCT	ACCCGGAGGTTGAATAATCC	Bao et al., 2006; Pridgeon et al., 2012
Transferrin	FJ176740	AAACAAATGTGACGCATGGA	CAGATTGCACTTTCCAGCAA	Liu et al., 2010
TNFAIP2	GO898786	TCATGTATGACCCAGCCTCA	CTTGATGGGGTGCATAGACA	Pridgeon et al., 2010b
IL-1β	DQ157743	CAGTCACCTCCAGCTGTTCA	CAGAAAGTTTTCGGGAGCTG	Wang et al., 2006; Mu et al., 2011
IL-10	FD020902	TGCTGACTGTTCTGCTGCTT	AGGTGTCCAGGTCATCCTTG	Liu, 2008; Mu et al., 2011
IP10	AY335951	GCCAGGACCAGTGTAAGGAG	TTCAGATTCCGGATTCAAGC	Baoprasertkul et al., 2004; Mu et al., 2011
Lysozyme C	JK088422	TCTGGCTAACTGGGTTTGCT	TGCCCTGCTGTCTCACTATG	Mu et al., 2011
CELG	JK088421	CCGTGACGGTGTCTTCATC	GCGGTTCAGGTAGATTTGCT	Mu et al., 2011
TOPK	JK088415	CTTCTGCACGGAGACATGAA	GTGAGCCCATACGCAAAGAT	Mu et al., 2011
PADT	GO898811	TGAGACGTGCTCTTTGCTTG	TCTCACAGCTCAAGGTTCCA	Pridgeon et al., 2010b
IGTP	GO898801	TCCATGAGCACAGTGGAGAG	AAGGCTCATCTTGGGGTTTT	Pridgeon et al., 2010b

Figure legends

Figure 1. Transcription profiles of 5 TLRs in anterior kidney of channel catfish at different time points including primary, secondary infections and TSB control. Data are presented as means ±S.D. from three replicates.

Figure 2. Transcription profiles of 8 AMPs in anterior kidney of channel catfish at different time points including primary, secondary infections and TSB control. Data are presented as means ±S.D. from three replicates.

Figure 3. Transcription profiles of 4 cytokines in anterior kidney of channel catfish at different time points including primary, secondary infections and TSB control. Data are presented as means ±S.D. from three replicates.

Figure 4. Transcription profiles of 5 reported genes in anterior kidney of channel catfish at different time points including primary, secondary infections and TSB control. Data are presented as means ±S.D. from three replicates.

Figure 5. Plasma lysozyme activity in primary, secondary infected and TSB control fish. Data are presented as means ±S.D. from three replicates.

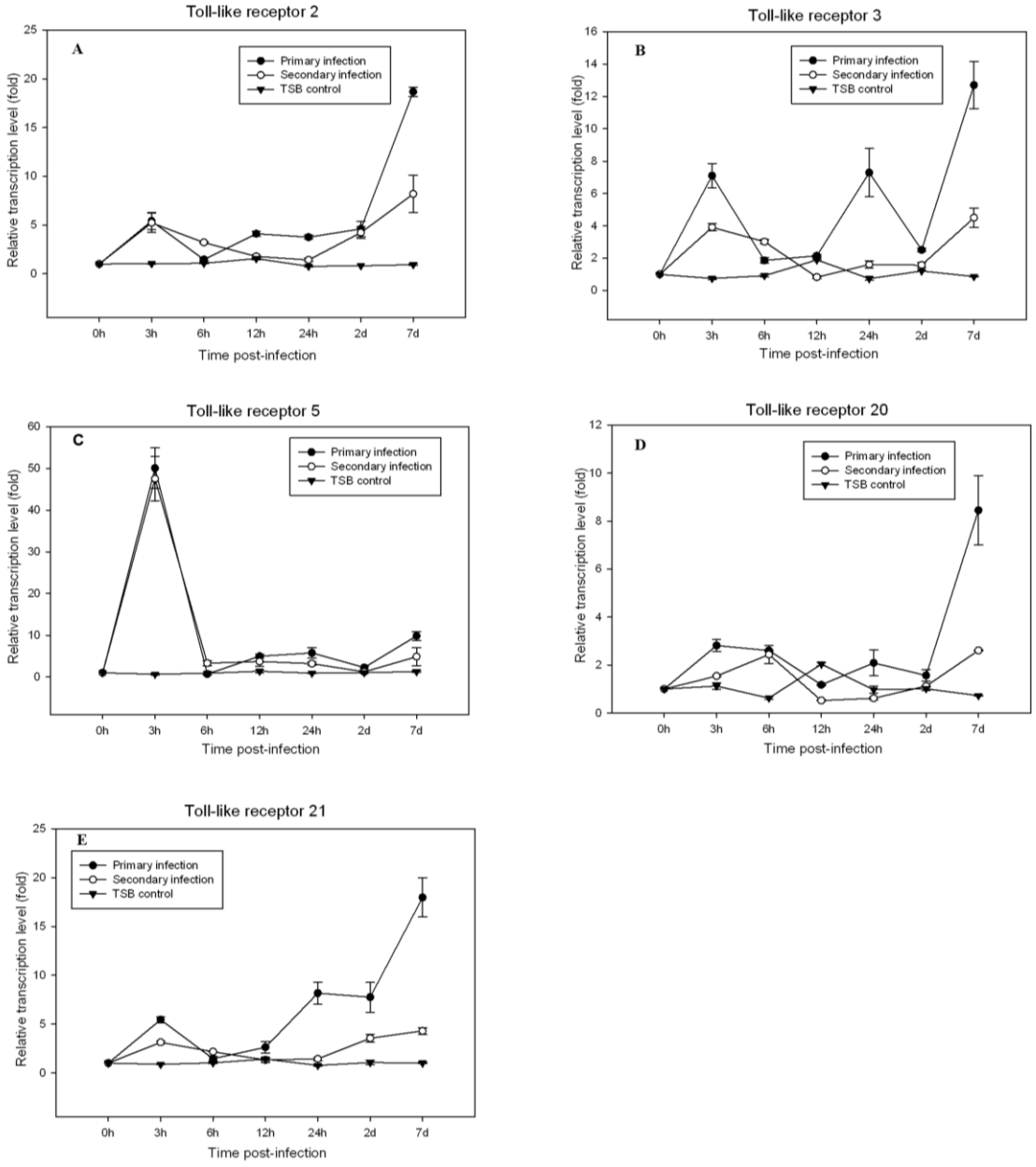
Toll-like receptor 2
A
Primary infection
Secondary infection
TSB control
Relative transcription level (fold)
Time post-infection
0h
3h
6h
12h
24h
2d
7d
Toll-like receptor 3
B
Toll-like receptor 5
C
Toll-like receptor 20
D
Toll-like receptor 21
E

Figure 1

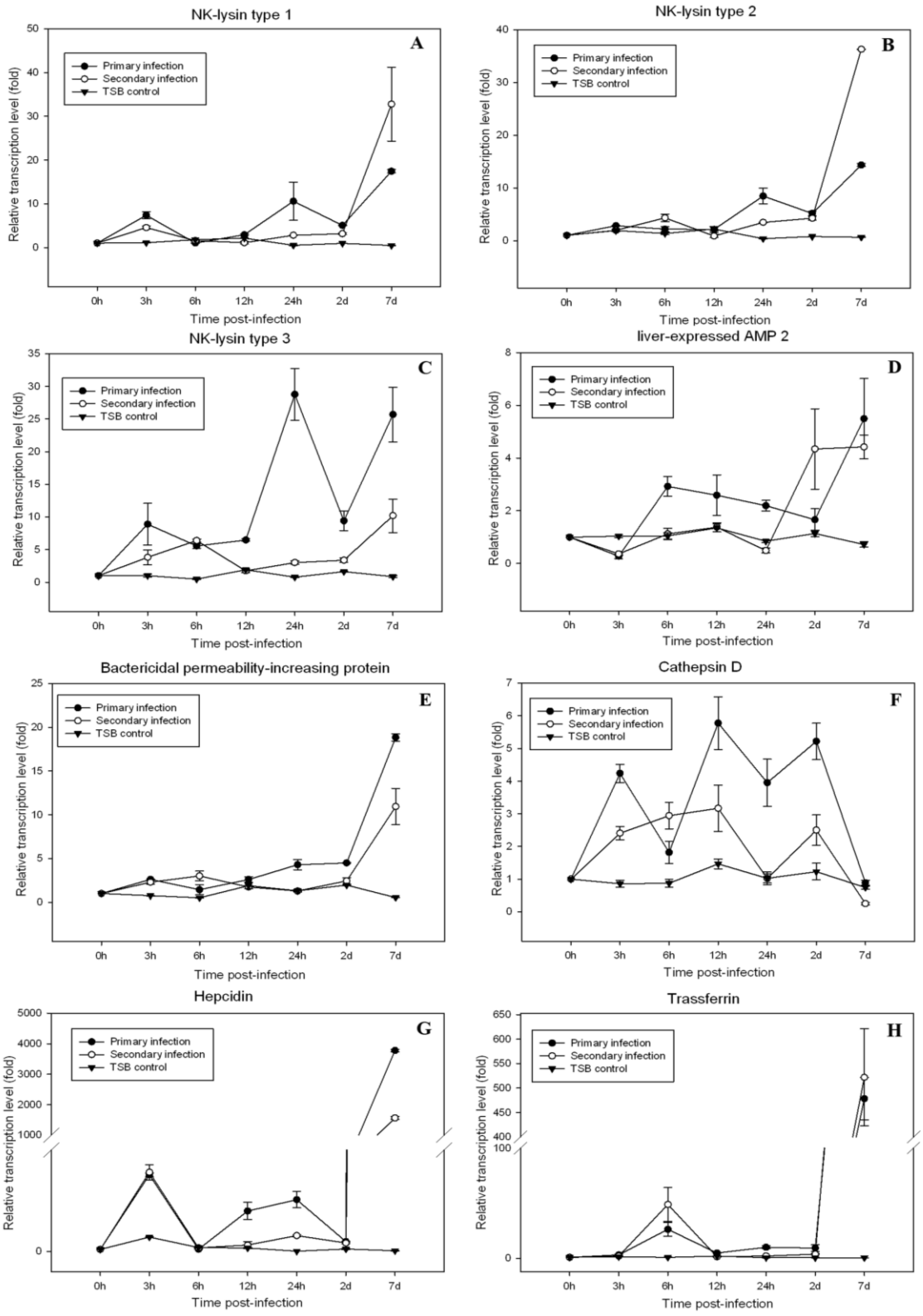
NK-lysin type 1
A
Primary infection
Secondary infection
TSB control
Relative transcription level (fold)
Time post-infection
0h
3h
6h
12h
24h
2d
7d
NK-lysin type 2
B
NK-lysin type 3
C
liver-expressed AMP 2
D
Bactericidal permeability-increasing protein
E
Cathepsin D
F
Hepcidin
G
Trassferrin
H

Figure 2

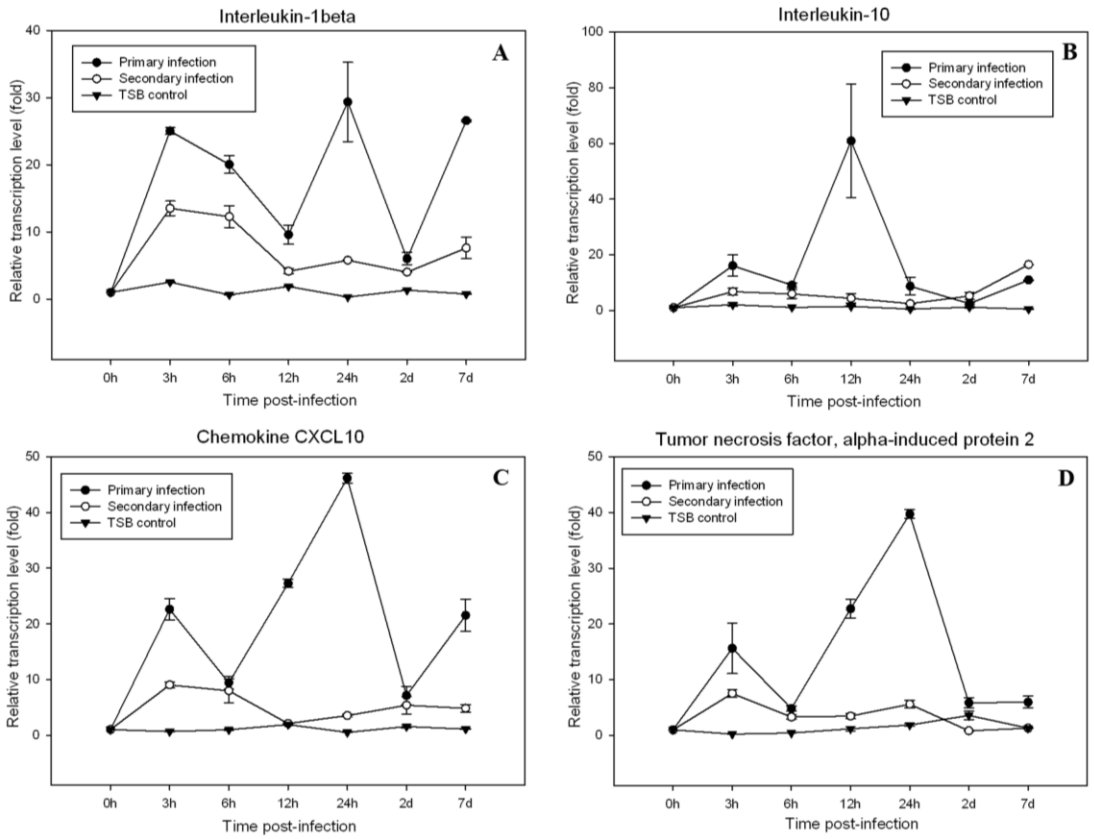
Interleukin-1beta
A
Primary infection
Secondary infection
TSB control
Relative transcription level (fold)
Time post-infection
0h
3h
6h
12h
24h
2d
7d
Interleukin-10
B
Chemokine CXCL10
C
Tumor necrosis factor, alpha-induced protein 2
D

Figure 3

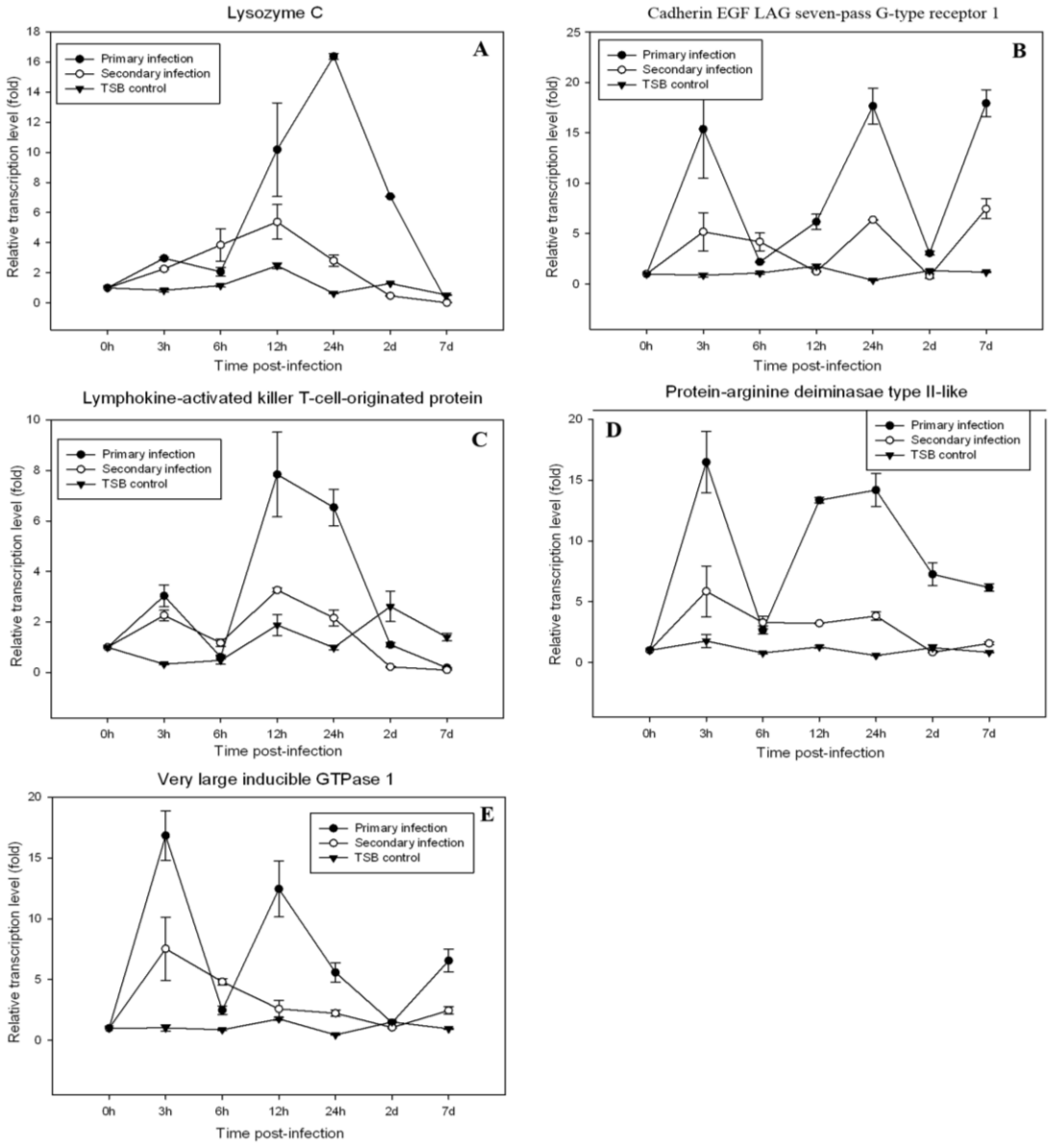
Lysozyme C
A
Primary infection
Secondary infection
TSB control
Relative transcription level (fold)
Time post-infection
0h
3h
6h
12h
24h
2d
7d
Cadherin EGF LAG seven-pass G-type receptor 1
B
Lymphokine-activated killer T-cell-originated protein
C
Protein-arginine deiminasae type II-like
D
Very large inducible GTPase 1
E

Figure 4

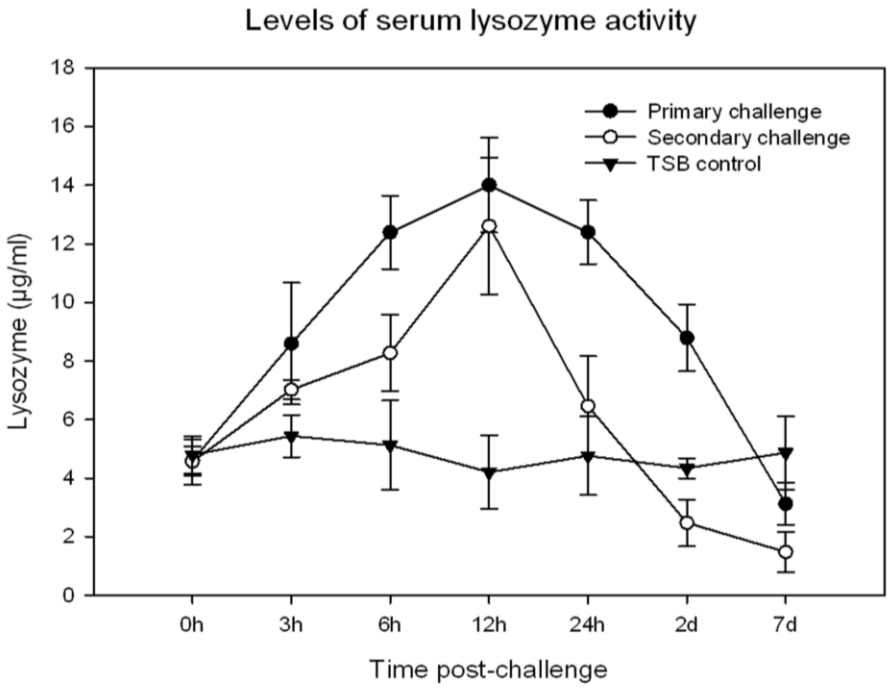
Levels of serum lysozyme activity
Primary challenge
Secondary challenge
TSB control
Lysozyme (µg/ml)
0
2
4
6
8
10
12
14
16
18
0h
3h
6h
12h
24h
2d
7d
Time post-challenge

Figure 5

VI. OVERALL RESULTS AND FUTURE DIRECTIONS

Overall results and discussion

To characterize an attenuated *Aeromonas hydrophila* strain (AL09-71 N+R) compared with its parent strain (AL09-71), the growth rate, motility, chemotactic response, invasion ability and whole cellular fatty acid profile were investigated and compared between parent (AL09-71) and mutant (AL09-71 N+R) strain. Our results indicate that the attenuated vaccine strain *A. hydrophila* AL09-71 N+R has smaller colony size and slower growth rate compared to its parent AL09-71. *In vitro* motility assay revealed that AL09-71 N+R was immotile whereas AL09-71was motile. The chemotactic response of AL09-71 N+R to channel catfish mucus was significantly lower than that of AL09-71. The ability of AL09-71 N+R to invade catfish gill cells was significantly lower than that of AL09-71. Furthermore, significantly different cellular fatty acid profiles were detected between the vaccine strain and its virulent parent strain. However, at genomic DNA level, the vaccine strain and its virulent parent strain appeared to be similar to each other.

To understand the molecular mechanisms of protection elicited by the attenuated AL09-71 N+R vaccine strain in catfish, suppression subtractive hybridization (SSH) was used to identify genes up-regulated by the vaccine. A total of 22 unique genes were identified at 12 h post vaccination. Of the 22, six were confirmed to be significantly induced by vaccination. In addition, 88 channel catfish genes that were reported to be associated with host immunity were included in the expression analysis. Of the 88 genes, 14 were found to be significantly up-

regulated by the vaccination at12 hour post infection. Expression profiles of the 20 genes at different time points showed that the pattern of gene up-regulation in vaccinated fish was similar to that in infected fish, confirming that vaccination of attenuated bacteria mimics infection by live bacteria at molecular level. However, the extent of gene induction by the infection differed from that by the vaccination. For example, at 6 hpv, IL-1β was induced less than 10 fold by the vaccination, whereas the infection resulted in more than 100 fold up-regulation. Similarly, vaccination induced hepcidin less than 50 fold at 6 hpv, whereas infection up-regulated hepcidin more than 200 fold, suggesting that their regulation might be associated with severity of the infection. Time course studies revealed that Na^+/K^+ ATPase subunit was highly and significantly up-regulated at 3 hpv or 3 hpi, indicating that it might play an early role in fish immune response to *Aeromonas* infection. After infection of *A. hydrophila*, the expression level of TLR5 increased at 3 hpi and significantly peaked at 6 hpi, suggesting that TLR5 plays an essential role in recognizing flagellated bacteria. In addition, our results also revealed that IL-1β was significantly up-regulated at 3 and 6 hpi, confirming that IL-1β plays an important role in the early immune response to *Aeromonas* infection. In addition to IL-1β, IL-10 was also significantly up-regulated at 6 and 12 hpi. Since IL-10 was not significantly up-regulated at 3 hpi, whereas IL-1β was significantly up-regulated at 3 hpi, suggesting that IL-10 is the downstream cytokine of IL-1β. From 6 hpi to 12 hpi, the induced level of IL-10 was reduced from 40 fold to 11 fold, whereas the induced level of chemokine CXCL10 was increased from 3 fold to 5 fold, suggesting that CXCL10 is a downstream chemokine followed by the release of IL-10. Moreover, our time course studies also revealed that hepcidin was significantly up-regulated at 6, 12, 24, and 48 hpi of *A. hydrophila*. In addition to hepcidin, NK-lysin-type 2 antimicrobial peptide was also significantly up-regulated at 6 and 12 hpi, further confirming that

AMPs play important roles in host defense against bacterial infections. Furthermore, in this study, lysozyme c and lysomal-associated transmembrane protein 5 were both found to be significantly up-regulated at 6, 12, and 24 hpv or hpi, further confirming the important roles they play in the immune response against bacterial infection. Taken together, our results suggest that vaccination with attenuated *A. hydrophila* mimics infection with live bacteria, inducing multiple immune genes in channel catfish.

To understand whether channel catfish response to secondary infection is similar to primary infection, SSH was used to identify genes up-regulated by secondary infection. Of the 28 unique genes identified by the library, eight were confirmed to be significantly induced by secondary infection compared to that by primary infection at 6 hpi. All 8 ESTs were confirmed significantly up-regulated at 6 hpi following secondary infection compared those following primary infection, indicating that these 8 ESTs might play a more important role in the adaptive immune response against *A. hydrophila* infection. However, the expression kinetics results showed that, at the other time points (at 3 hpi, 12 hpi, 24 hpi, 2 dpi and 7dpi), these 8 ESTs have higher expression levels following primary infection compared with that following secondary infection. The results also showed that the transcriptional levels of all 8 ESTs had 3 peaks under primary exposure, while only one peak at 3 hpi was detected out under secondary expoure. EST A08 shows a higher homology (E-value 1.00E-143, identity 95%) with channel catfish TLR20-1 and TLR20-2 genes complete cds, which was significantly induced up to 60-fold following primary infection and up to almost 30-fold following secondary infection at 3 hpi. Of all ESTs identified, EST A10 and B04 were up-regulated the most (up to 300-fold) after primary infection and (up to 200-fold) after secondary infection. Both of them shared the higher identity with XbaI element. EST A10 and B04 shared 99% identity with channel catfish strain Auburn XbaI

element 5, complete sequence and 96% identity with channel catfish strain Stuttgart XbaI element 7 respectively. The higher transcription levels of these two XbaI elements indicate they may play an important role in the immune response against the infection through regulating the expression of immune related genes. EST C11 was characterized to share 99% identity with protaclin (PRL) in channel catfish. Our results on the expression kinetics of PRL after primary and secondary infection suggests that PRL may has a positive relation with TLR on the activation of leukocytes and may also play an important role in maintaining circulating levels of IgM in channel catfish. Moreover, to the best of our knowledge, the expression of PLR was first found in the anterior kidney of channel catfish. EST C12 was up-regulated greater than 10-fold under primary exposure and 6-fold under secondary exposure at 3 hpi respectively. EST C12 shares 94% identity with NADH dehydrogenase subunit 2 of channel catfish at protein level. Our results indicate that NADH dehydrogenase complex may play an important role in the immune response against infection of *A. hydrophila* through increasing the energy production, which can be used to support the immune activity. EST C04 which shares 74% with inward rectifier potassium channel (Kir) 13 of zebra fish was up-regulated greater than 50-fold in primary infection and 20-fold in secondary infection at 3 hpi respectively, suggesting that it might play an important role in immune response through increasing the IL-1β level and activating antimicrobial functions of macrophages.

In addition to the eight genes identified by SSH, 94 genes known to be associated with host immunity were also subjected to expression analysis. Of the 94 genes, 22 were identified to be induced and differential regulated at different time points. Of the 22 genes, hepcidin and transferrin were induced most under both primary and secondary infections. Our time course study reveals that they have a similar expression pattern following primary and secondary

infections, indicating the existence of the cooperation of them in innate and adaptive immunity against *A. hydrophila* infection. Remarkably, the expression level of transferrin under secondary exposure was significantly ($P < 0.05$) higher than that under primary exposure at 6 hpi, which suggests that transferring may play a more important role in adaptive immunity than that in innate immunity. Our present study reveals that, of the five TLRs, TLR5 was up-regulated most highly (around 50-fold) at 3 hpi by primary and secondary infection, which further confirms the essential role of TLR5 in recognizing flagellated bacteria and suggests that TLR5 is not only necessary in the innate immunity but also essential in the adaptive immunity against *A. hydrophila* infection. Interestingly, a significantly up-regulation was detected at 7 dpi in all five TLRs under both primary and secondary infections, suggesting the TLRs has some special function at 7 dpi in innate and adaptive immune. All investigated eight AMPs may play important roles in the innate and adaptive immune defense against *A. hydrophila* infection. The higher and similar temporal expression profiles were found in NKL-1, NKL-2, NKL-3, BPI, transferrin and hepcidin, which suggest these six AMPs might cooperate and play major roles in the immune defense. our results also revealed that IL-1 β was significantly up-regulated over 20-fold at 3 hpi, 6 hpi, 24 hpi and 7 dpi under primary infection, further confirming that IL-1 β plays an important role in the pro-inflammatory response to *Aeromonas* infection. Additionally, in this experiment, a clear association was observed between higher expression (around 60-fold) of IL-10 and a corresponding lower expression (around 10-fold) of IL-1 β at 12 hpi under primary exposure, indicating a suppressive role for IL-10 on the transcriptional level of the pro-inflammatory cytokine IL-10. This association suggests that IL-10 might have anti-inflammatory activity in channel catfish. We also found that some genes were up-regulated earlier at 3 hpi or 6 hpi compared to our previous study at 6 hpi or 12 hpi (Mu et al., 2011). This may be due to the

use of higher challenge dose ($1x10^6$ CFU/fish) and bigger fish (21.2 ± 3.3 g) in this study. Even though the transcriptional levels of most of 22 genes following primary infection are higher than those following secondary infection, we found several genes that had higher transcriptional levels after secondary infection such as transferrin, NKL-1, and NKL-2. The results suggest that these 3 genes may be more important in adaptive immune response as compared to that in innate immune response. Taken together, we believe that the study of the expression kinetics of immune related genes following secondary infection is helpful to identify more valuable immune response related genes.

Future directions

For characterizing the attenuated *A. hydrophila* strain, more characters need to be investigated in future study such as chemotaxis assay, adhesion abilities, invasion abilities, whole protein profile and total lipopolysaccharide. Next generation sequencing technology might be used to for further investigation on the genomic difference between parent and mutant strains. The mutant on the transcriptional level also needs to be determined by SSH or next generation sequencing technology.

For the transcriptional analysis of multiple immune response genes, hepcidin and transferrin were found to be the most up-regulated two genes after infection. Though literatures indicate their function in immune response, further study might need to focus on their correlation and interaction based on their associated immune functions. For some other up-regulated EST or genes such as XbaI elements, TLR20, TLR21, NKL-1 and NKL-2, functions are still unclear in the process of immune response against bacterial infection. In future study, we might need to investigate their functions by over-express or down-express these genes in model animal through different technologies such as Targeted Mutations, RNAi, Gain-of-Function Mutations. In this

study, the transcriptional profiles of multiple genes were just analyzed until 7 days post infection. In future, more time points need to be analyzed especially after 7dpi, which will be more helpful in understanding the host immune response.

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