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Research paper

Identification and expression profiles of multiple genes in Nile tilapia in response to bacterial infections

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ABSTRACT

To understand the molecular mechanisms involved in response of Nile tilapia (*Oreochromis niloticus*) to bacterial infection, suppression subtractive cDNA hybridization technique was used to identify upregulated genes in the posterior kidney of Nile tilapia at 6 h post infection with *Aeromonas hydrophila*. A total of 31 unique expressed sequence tags (ESTs) were identified from 192 clones of the subtractive cDNA library. Quantitative PCR revealed that nine of the 31 ESTs were significantly ($p < 0.05$) upregulated in Nile tilapia at 6 h post infection with *A. hydrophila* at an injection dose of 10^5 CFU per fish (~20% mortality). Of the nine upregulated genes, four were also significantly ($p < 0.05$) induced in Nile tilapia at 6 h post infection with *A. hydrophila* at an injection dose of 10^6 CFU per fish (~60% mortality). Of the four genes induced by *A. hydrophila* at both injection doses, three were also significantly ($p < 0.05$) upregulated in Nile tilapia at 6 h post infection with *Streptococcus iniae* at doses of 10^6 and at 10^5 CFU per fish (~70% and ~30% mortality, respectively). The three genes induced by both bacteria included EST 2A05 (similar to adenylate kinase domain containing protein 1), EST 2G11 (unknown protein, shared similarity with *Salmo salar* IgH locus B genomic sequence with *e* value of 0.02), and EST 2H04 (unknown protein). Significant upregulation of these genes in Nile tilapia following bacterial infections suggested that they might play important roles in host response to infections of *A. hydrophila* and *S. iniae*.
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1. Introduction

Aeromonas hydrophila, a Gram-negative motile bacillus widely distributed in aquatic environments, is a causative agent of motile aeromonad septicaemia (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-Bañuelos 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* could also become a primary pathogen, causing 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 West Alabama, an 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

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AL09-71, 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). However, it is currently unknown whether *A. hydrophila* AL09-71 is also highly virulent to Nile tilapia (*Oreochromis niloticus*), an intensively cultured food fish that is economically important to many countries. Furthermore, it is currently unclear how Nile tilapia would respond to *A. hydrophila* AL09-71 infection at the molecular level. Time course studies (1, 2, 4, 6, 12, 24, 36, and 48 h) in fish under normal and acutely infected conditions have revealed that the transcriptional levels of all five toll-like receptors (TLR2, TLR3, TLR5, TLR20a, and TLR21) were significantly induced at 6 h post infection of *Edwardsiella ictaluri*. 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 4–8 h post challenge compared to that at 2 h post challenge (Chu and Lu, 2008). Therefore, we chose 6 h post infection as the time point between 4 and 8 h post infection in this study to understand host response at the molecular level. The objectives of this study are: (1) to determine whether *A. hydrophila* AL09-71 is highly virulent to Nile tilapia; (2) to identify upregulated genes in the posterior kidney of Nile tilapia at 6 h post infection with *A. hydrophila* AL09-71; and (3) to determine whether the upregulation of identified gene(s) is also induced by *Streptococcus iniae* infection.

2. Materials and methods

2.1. Bacteria source and growth conditions

The AL09-71 isolate of *A. hydrophila* was obtained from diseased channel catfish in 2009 from West Alabama. The AL98-C1B isolate of *A. hydrophila* was isolated from diseased channel catfish in July of 1998 and stored in tryptic soy broth (TSB) (Difco, Sparks, MD) containing 5% glycerol at −80 °C. The *S. iniae* ISET0901 isolate, originally isolated from diseased Nile tilapia in Israel in 2005 and stored at −80 °C, was used to determine whether gene upregulation in Nile tilapia was specific to *A. hydrophila* infection. The two *A. hydrophila* isolates have been confirmed to be *A. hydrophila* through molecular identification (Pridgeon and Klesius, 2011a). All isolates were cultured on tryptic soy agar (TSA) plates according to published procedures (Panangala et al., 2007). The *S. iniae* isolate was re-isolated from Nile tilapia after passing it three times in fish and confirmed as *S. iniae* by API 20 Strep test (BioMerieux, Durham, NC) and gas chromatography analysis of fatty acid methyl ester using MIDI microbial identification system (Shoemaker et al., 2005).

2.2. Experimental fish

Nile tilapia (8 ± 2 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 h light:dark period was maintained and supplemental aeration was supplied by an air stone. 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.

2.3. Virulence of bacterial isolate to Nile tilapia

All bacterial isolates were grown in TSB at 28 °C for 18–24 h. The concentration of colony forming units per milliliter (CFU/mL) used in this study was determined through serial dilutions as described previously (Pridgeon and Klesius, 2011a). Briefly, an optical density (OD) of 1.0 of the bacterial cultures was measured at 540 nm using Thermospectronic spectrophotomer (Fisher Scientific, Pittsburgh, PA). Serial dilutions (in triplicates) of each *A. hydrophila* isolate were prepared in TSB immediately and 100 μL of serially diluted *A. hydrophila* were immediately plated onto TSA plates. After incubating the plates for 24 h at 28 °C, the number of colonies were counted and the average number of CFU/mL at $OD_{540\ nm}$ = 1 was calculated for the two isolates of *A. hydrophila*. At least three replicates were performed on three different days to determine the average CFU/mL for both *A. hydrophila*. After exposing fish to *A. hydrophila* at different doses, mortality was recorded daily for 7 and 14 days post exposure, respectively. The presence or absence of *A. hydrophila* in dead fish was determined by culturing posterior kidney samples on blood agar plates followed by gas chromatographic analysis of fatty acid methyl ester MIDI microbial identification system (Shoemaker et al., 2005).

2.4. Bacterial load determination, total RNA extraction, and cDNA synthesis

To determine the amount of *A. hydrophila* in the posterior kidney (the dark linear organ which runs the entire length of the peritoneal cavity) of Nile tilapia at 6 h post injection with *A. hydrophila* AL09-71, posterior kidney samples were collected from three fish per treatment group. As controls, posterior kidney fish samples at 6 h post injection of TSB were also collected. Tissues were weighed, grounded, and serially diluted in TSB containing 0.1% Triton-X 100. One hundred microliters of serially diluted sample was plated onto TSA plates. After 24 h incubation at 28 °C, the number of CFU/mg was calculated for control fish or Nile tilapia that was injected with *A. hydrophila* AL09-71. Total RNA was isolated from Nile tilapia posterior kidney samples (five fish per treatment) at 6 h post injection with TSB control or bacteria 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 AMV reverse tran-