Fish and Shellfish Immunology 81 (2018) 37–48

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Nile tilapia fry fed on antimicrobial peptide Epinecidin-1-expressing *Artemia* cyst exhibit enhanced immunity against acute bacterial infection

Chen-Hung Ting, Yi-Chun Chen, Jyh-Yih Chen*

Marine Research Station, Institute of Cellular and Organismic Biology, Academia Sinica, 23-10, Dahuen Road, Jiaushi, Ilan, 262, Taiwan

ARTICLE INFO

Keywords:
Tol2-transposon
Microinjection
Epinecidin-1
Antimicrobial peptide
Immunity

ABSTRACT

Artemia are often used as a live feed for fry in aquaculture. We have previously demonstrated that supplementing adult zebrafish feed with *Artemia*, which express an *Epinephelus coioides*-derived antimicrobial peptide, Epinecidin-1 (Epi-1), protects against bacterial infection. Thus, *Artemia* may serve as a bioreactor for producing biofunctional molecules. However, the application of *Epi-1* transgenic *Artemia* in larval aquaculture of commercial fish species has not been investigated. Here we used a *Tol2*-transposon system to generate stable *Epi-1* expressing *Artemia*. Nile tilapia (*Oreochromis niloticus*) fry were then fed with decapsulated transgenic cysts and acutely challenged with Gram-positive *Streptococcus iniae* or Gram-negative *Vibrio vulnificus* (204). Survival analysis revealed that tilapia fry fed with *Epi-1* transgenic cysts were resistant to acute bacterial infection. Immune-related gene expression profiling showed that *S. iniae* and *V. vulnificus* inoculations produced distinct immunomodulatory effects in the tilapia fry. Upon *S. iniae* infection, tilapia fry fed on control diet exhibited an immune response dominated by *Tlr-7/MyD88*, wherein *Tnf-α*, *Il-8* and *Cxcl-10* expression were all induced; conversely, the tilapia fry fed with *Epi-1* transgenic cysts showed a *Tlr-2/Tlr-5*-dominant immune response, marked by the induction of *Il-1β*, *Il-8* and *Il-12* expression. However, after *V. vulnificus* (204) infection control fry exhibited a *Tlr-2/MyD88/Traf-6*-dominant response with activation of *Tnf-α* and *Il-8* expression; meanwhile tilapia fry fed on *Epi-1* transgenic cyst showed a dominant *Tlr-2/Tlr-5*-mediated immune response, including induction of *Il-1β*, *Il-8*, *Il-12*, and *Cxcl-10* expression. These findings suggest that feeding larval fish fry with *Epi-1* transgenic *Artemia* cysts confers enhanced immunity toward bacterial challenge. *Epi-1* transgenic cysts should therefore be considered as a potential functional feed for larval aquaculture.

1. Introduction

Nile tilapia (*Oreochromis niloticus*) are mainly freshwater fish that are commonly farmed in Taiwan and other Asian countries due to their hardiness, fast growth and environmental adaptability. Despite their hardiness, tilapia and other fish species are susceptible to massive mortality caused by streptococcosis, which poses a major problem in tilapia farming [1]. Outbreaks of streptococcal infections can be acute or chronic, resulting in mass deaths over the course of a few days or extending over several weeks. *Streptococcus iniae* and *Streptococcus agalactiae* are the dominant species of gram-positive pathogenic bacteria isolated from infected fishes [2–5] and have been classified as emerging zoonotic pathogens, which cause invasive infection in humans [6–8]. In addition to *Streptococcus* sp., Gram-negative *Vibrio vulnificus* (204) infection of cultured tilapia in freshwater and low-salinity environments is frequently reported and may cause severe wound infections and life-threatening septicemia [9–11]. In order to combat bacterial infections, traditional antibiotics have been widely applied as prophylactics in aquaculture; however, this strategy is only effective at the primary phase of infection. Other strategies for protecting fish from bacterial infection are mainly based on health management, such as keeping fish in ideal conditions, providing a nutritious diet or minimizing environmental stresses that may weaken the immune system.

Artemia sp., also known as brine shrimp, are primitive arthropods that comprise a group of 15 species around the world [12]. *Artemia* are distributed throughout coastal salinas as well as in salt lakes, and their cyst can tolerate arid conditions, high salinity, and extremely low dissolved oxygen environments [13–15]. *Artemia* can reproduce through either oviparous or ovoviviparous means, depending on environmental cues that include food availability, light, salinity, and oxygen availability [16,17]. Under optimal conditions, fertilized eggs develop into nauplius larvae in an ovoviviparous process; conversely, in suboptimal conditions, the eggs develop into encysted gastrula embryos (cysts), which suspend development by entering diapause (oviparity) [18]. *Artemia* sp. is widely used as a feed for marine larval animals [19] due to the high nutritional value of cysts and freshly hatched nauplii

* Corresponding author.
E-mail address: zoocjy@gate.sinica.edu.tw (J.-Y. Chen).

https://doi.org/10.1016/j.fsi.2018.07.008
Received 2 February 2018; Received in revised form 25 June 2018; Accepted 4 July 2018
Available online 05 July 2018
1050-4648/

[19–23]. The cysts and nauplii of some *Artemia* species, particularly *Artemia franciscana* and *Artemia sinica*, are enriched with highly unsaturated fatty acids (HUFAs), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are known to enhance the survival, growth rate, and growth potential of fry [19,24,25]. In addition, *Artemia* have been shown to serve as an ideal bioreactor for the production of exogenous protein supplements [26,27]. For example, zebrafish larvae fed on transgenic *Artemia*, which expressed growth hormone from yellowfin porgy (*Acanthopagrus latus*), exhibited enhanced body length gain rates [26]. Additionally, our group previously demonstrated that after being fed with Epinecidin-1 (Epi-1)-expressing transgenic *Artemia*-supplemented fodder, adult zebrafish showed enhanced survival and immune modulatory activity upon *V. vulnificus* (204) infection. The data further suggested that the transgenic *Artemia* had both bacteriostatic and bactericidal activities [27]. Epi-1 was identified from grouper (*Epinephelus coioides*) [28] and represents an evolutionarily conserved component of the innate immune system, which functions to protect against a broad range of pathogens [27–35]. *Epi-1*-transgenic zebrafish were shown to be protected from *V. vulnificus* (204) and *Streptococcus agalactiae* infection through regulation of immune-related genes, including interleukin (*Il*)-associated genes and toll-like receptor (*Tlr*)-associated genes, such as nuclear factor (NF)-κB, *MyD-88*, and tumor necrosis factors (*Tnfs*) [33]. In addition, Epi-1 administration enhanced the survival rate of *V. vulnificus* (204)-infected zebrafish, implying that Epi-1 may be used to combat fish bacterial infections [32]. Proteomics and bioinformatics analyses showed that Epi-1-mediated antibacterial action in zebrafish was linked to cytoskeletal regulation, implying a possible antimicrobial peptide (AMP)-induced host-defense mechanism [36]. Moreover, microarray studies showed that Epi-1 modulates Nervous necrosis virus (NNV)-induced host signaling mechanisms in medaka (*Oryzias latipes*), including B cell/T cell activation, adipocytokine signaling, mast cell activation, thereby preventing viral multiplication in host organisms [37]. We and others have shown that several recombinant AMPs may have beneficial effects in animals when used as feed additives [27,38–41]. Thus, AMPs may provide effective alternatives to traditional antibiotics with high potential for application in the aquaculture feed industry.

Farmed fish are commonly fed with antibiotic feed supplements to protect against disease. However, antibiotic residue may represent an environmental stress for fish and also may create antibiotic-resistant bacteria that can damage human health on a global scale. Therefore, it is particularly important to develop alternative strategies to reduce antibiotic use, while still preventing disease in farmed fish. The goal of this study was to develop and evaluate a bioactive *Artemia* cyst as a functional feed for tilapia fry that can protect from bacterial infections of *S. iniae* and *V. vulnificus* (204), which were isolated from an aquaculture farm in Taiwan [5,42]. To this end, an *Artemia* strain that stably produces Epi-1 AMP was created and used as a supplement in feed for tilapia fry. Furthermore, the survival rate and immune responses of the tilapia fry that were fed with the *Epi-1* transgenic (TG) cysts were evaluated after acute bacterial infection.

2. Materials and methods

2.1. Bacterial strains and aquaculture

All fish care and handing procedures were performed in accordance with Academia Sinica guidelines. Experiments using fish were performed according to “The Ethical Guideline for Using Vertebrates as Experimental Animals in Taiwan” approved by the “Ethical Committee for Using Vertebrates as Experimental Animals” of Academia Sinica (Protocol ID: 13-12-609). *S. iniae* and *V. vulnificus* (204) were purchased from the Bioresource Collection and Research Center (BCRC) and were cultured as recommended. For assays after bacterial challenge, *S. iniae* were grown for 24 h in Brian Heart Infusion broth at 28 °C and *V. vulnificus* (204) were grown for 12 h in Tryptic Soy Broth at 28 °C. Bacterial growth was monitored by measuring the optical density (OD) at 650 nm. Late-logarithmic-phase cultures, which corresponded to 0.7–0.8 OD for both bacteria, were harvested for use as bacterial challenges. *Artemia* cyst was purchased from INVE AQUACULATRE, Inc. and cultured as previously described [27]. Tilapia (*Oreochromis niloticus*) were purchased from a private company in Changhua County (Taiwan) and were acclimated in a fiber-reinforced plastic tank at 28 °C. Once female fish were observed to be carrying fertilized eggs in the mouth, the fish were cultured separately for 1 week to allow the egg hatching and growth within the mouth. Fry were then removed and cultured to about 0.8 cm in length. Cultured fry were grouped for twice daily feeding with either decapsulated wild-type (TAIKONG Corp.) or TG *Artemia* eggs for 14 consecutive days.

2.2. Constructs, mRNA preparation, and microinjection

To generate the pTLR-CMV-epi-1-dsRed2 (CMV-Epi-1) vector (transposon construct), the Mylz2 promoter of the pTLR-Mylz2-Epi-1-dsRed2 vector [33] was replaced with the CMV promoter. First, the pEGFP-C3 vector (Clontech) was digested with AseI and blunted by DNA polymerase I; the CMV promoter was then excised by the restriction enzyme, NheI. Next, the Mylz2-Epi-1 was linearized by SalI and blunted by DNA polymerase I; the Mylz2 promoter was then removed by NheI. The backbone DNA was then ligated with the CMV promoter using T4 DNA ligase. The transposase vector, pKJ-Tol2, was used as previously described [33]. For *in vitro* transcription, the pKJ-Tol2 vector was linearized by the restriction enzyme, XbaI, and *Tol2* transposase mRNA was synthesized using the mMESSAGE mMACHINE T7 kit (Ambion® | Thermo Fisher Scientific) in accordance with the standard protocol. The mRNAs were purified by the MEGAclear™ Transcription clean up kit (Ambion® | Thermo Fisher Scientific). For microinjection, *Artemia* cysts were decapsulated as previously described. Decapsulated cysts were soaked in distilled water and shaken for 30 min. Cysts were then placed on a 1.5% agarose gel and dried. Each *Artemia* cyst was co-injected at 12–25 psi with 1–2 nL of a mixture that contained *Tol2* transposase mRNA (258.2 μg/μL), plasmid DNA (258.2 μg/μL), and RNaseOUT™ Recombinant Ribonuclease Inhibitor (Thermo Fisher Scientific). TG *Artemia* cysts were then aerated in seawater for 16 h.

2.3. Genotyping, semi-quantitative PCR, and breeding strategy

For genotyping, genomic DNA (gDNA) was sampled from the 6th abdominal segment of 14-day-old *Artemia* and dissolved into 20 μL dilution buffer containing 0.5 μL DNA Release additive (Thermo Fisher Scientific). Mixtures were incubated at room temperature (RT) for 10 min and then digestion was stopped by incubating for 2.5 min at 98 °C. The gDNA was then used for PCR analysis with Phire Tissue Direct PCR Master Mix (Thermo Fisher Scientific). Specific primer pairs (*Epi-1* forward1 and *Epi-1* backward1; *MT-Nd4* forward and *MT-Nd4* backward) (Table 1) were designed to recognize transgenic *Epi-1* and the internal control, *MT-Nd4* (encoding NADH: Ubiquinone Oxidoreductase Core Subunit 4). The PCR program began with a 98 °C denaturation for 5 min, followed by 40 cycles of 95 °C/5 s, 60 °C/5 s and 72 °C/20 s, followed by 72 °C/60 s for the final extension step. To obtain *Artemia* with germ-line transmission of the *Epi-1* gene, organisms from the F0 generation were mated with wild-type *Artemia* to generate F1 generations. Individual organisms from each generation were genotyped and inbred to produce offspring until the F14 generation. To determine the copy number of the *Epi-1* transgene, the *Dmrt* gene, which encodes Double sex and Mab-3-related transcription factor, was used as a homozygous control (copy number = 2). *Dmrt* and *Epi-1* were amplified using specific primer pairs: *Dmrt* forward and *Dmrt* backward; CMV-*Epi-1*-forward2 and Epi-backward2 (Table 1). *MT-Nd4* amplification was used to control for equal amounts of template in each reaction. The PCR reaction was run for 30, 35 and 40 cycles to assay the linear