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Protective efficacy of recombinant hemolysin co-regulated protein (Hcp) of *Aeromonas hydrophila* in common carp (*Cyprinus carpio*)

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ABSTRACT

Motile aeromonad septicemia (MAS) caused by *Aeromonas hydrophila* is one of the common bacterial causes of fish mortalities. Prophylactic vaccination against this and other diseases is essential for continued growth of aquaculture. The type VI secretion system (T6SS) plays a crucial role in the virulence of *A. hydrophila*. The hemolysin co-regulated protein (Hcp) is an integral component of the T6SS apparatus and is considered a hallmark of T6SS function. Here, the T6SS effector Hcp was expressed and characterized, and its immunogenicity and protective efficacy were evaluated in common carp (*Cyprinus carpio*). Hcp secretion was found to be strongly induced by low temperature in *A. hydrophila*. Immunoblot analysis demonstrated that Hcp is conserved among *A. hydrophila* strains of different origins. The vaccination with recombinant Hcp resulted in an increased survival (46.67%) in common carp during a 10-day challenge time compared to non-vaccinated fish (7.14%). The vaccinated fish also showed the significantly increased levels of IgM antibody in serum and cytokines such as inerleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) in kidney, spleen and gills. The recombinant Hcp shows promise as a vaccine candidate against *A. hydrophila*.

1. Introduction

Aeromonas hydrophila is a Gram-negative bacterium widely present in freshwater habitats and causes infections in humans and animals such as amphibians and fish [1–3]. *A. hydrophila* is associated with many fish diseases like hemorrhagic septicemia and dropsy, and leads to significant economic losses worldwide. It has been accepted that vaccination is an effective method to protect fish from the infectious bacterial diseases [4]. Several studies have demonstrated that different types of vaccines such as heat-killed cells, heat or formalin-inactivated bacterial extracts and live cells of *A. hydrophila* stimulate an effective response in fish that protects against the bacterial infection [5–8]. However, it should be noted that such vaccines are not always effective, especially when the expected immune response is directed against specific antigens. And they contain complex mixtures and undefined molecules that have been evidenced to interact synergistically or antagonistically and that can stimulate, cross-react with, inhibit or even suppress the immune response to specific antigens [9]. Recently, the development of the recombinant subunit vaccine has caught a lot of attention. This vaccine contains fragments of pathogenic microorganisms, which are highly purified and immunogenic antigens. This ensures that the antigen has a well-defined composition. Also, vaccination with a protein present in a range of serotypes would overcome some of the limitations of antigenic diversity in *A. hydrophila* strains. Some previous studies targeted subunit vaccine candidates against *A. hydrophila* have mainly paid attention to the outer membrane proteins (OMPs) [10–12]. However, in the case of *A. hydrophila*, vaccination with extracellular secreted proteins may be particularly important, because its pathogenicity appears to be closely related to the production of extracellular products, which are lost, partially, in conventional bacterin preparations.

Bacterial pathogenicity critically relies on various secretion systems to deliver toxic molecules from the cytoplasm to the outer space [13]. Lately, a novel secretion system named the type VI secretion system (T6SS) was reported for several pathogens [14–17] and characterized as the most common secretion system of Gram-negative bacteria [18]. Two typical proteins of T6SS, hemolysin co-regulated protein (Hcp) and valine–glycine repeat protein G (VgrG) have been proved to function not only as structural elements of the T6SS device but also as effector proteins [17,19,20]. Furthermore, many Gram-negative pathogens have been found to

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secrete these two conserved effector proteins to the outer space through T6SS [16,17,21–23]. Of late, however, Hcp which used to be a static tubule was proven to be a chaperone and receptor of type VI secretion substrates in *Pseudomonas aeruginosa* [24]. T6SS components have been reported to concern with virulence-related mechanism of various bacteria. During *Vibrio cholerae* infection, T6SS genes were proved to be essential for toxicity in *Dictyostelium amoebae* and mammalian J774 macrophages [17]. In association with *P. aeruginosa*, Hcp1 was detected in cystic fibrosis sufferers [16]. Burtnick et al. [25] found that the recombinant Hcp2 protein provided mice with good protection (80%) against *Burkholderia pseudomallei* challenge. The above reports led us to speculate that Hcp may be a suitable vaccine candidate to prevent *A. hydrophila* infection.

The previous study from our group showed that Hcp could be recognized by immunized rabbit sera on 2-D immunoblots that were performed to evaluate the extracellular proteins of *A. hydrophila* [26]. In the present study, we demonstrated that there was a functionally active T6SS in *A. hydrophila* and investigated the immunogenicity and protective efficacy of the T6SS effector Hcp in common carp (*Cyprinus carpio*).

2. Materials and methods

2.1. Ethics statement

Animal experiments were conducted according to the Animal Welfare Council of China with approval for the experimental protocols from the Animal Ethics Committee of Nanjing Agricultural University.

2.2. Bacterial strains, plasmids and growth conditions

A total of 24 *A. hydrophila* isolates were used in the present study. *A. hydrophila* J-1 [26] and NJ-35 [27] were responsible for Aeromonad septicemia in Jiangsu Province of China in 1989 and 2010, respectively. The environmental isolate *A. hydrophila* ATCC 7966 is the type strain for this species. The whole genome sequences of strains NJ-35 (accession number CP006870.1), J-1 (CP006883.1) and ATCC 7966 (CP000462.1) have been deposited in GenBank.

The other *A. hydrophila* strains used were obtained from five different areas in China. All strains were isolated from aquatic animals of the following species: Common carp (*C. carpio*) (n = 15), Crucian carp (*Carassius carassius*) (n = 2), Silver carp (*Hypophthalmichthys molitrix*) (n = 2), Soft-shell turtle (*Trionyx Sinensis*) (n = 1), Eel (*Monopterus albus*) (n = 1).

All plasmids and *Escherichia coli* were obtained from TaKaRa (Dalian, China). *A. hydrophila* and *E. coli* used in this study were cultured in Luria–Bertani (LB) medium at 28 °C and 37 °C, respectively.

2.3. Animals

New Zealand white rabbits weighing about 1.5 kg were supplied by Jiangsu Academy of Agricultural Sciences. Common carp weighing about 10 g were obtained from the Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences in China, and maintained at 28 °C with ideal conditions of feeding, aeration and water exchange.

2.4. Expression and purification of the recombinant Hcp (rHcp) and polyclonal antibody preparation

The primer sets were designed based on the sequence of the *hcp* gene of *A. hydrophila* ATCC 7966 available in GenBank (accession no. CP000462.1). The primer sequences hcp-F (5′-GGAATTCATGCCAACTCCATGTTATATCAG-3′) and hcp-R (5′-CCGCTCGAGTTAGGCCTCGATCGGC-3′), contained the *Eco*R I and *Xho* I restriction enzyme sites (underlined), respectively. Cloning of *hcp* gene from *A. hydrophila* NJ-35 was conducted as described previously [2].

Hcp proteins were expressed in *E. coli* BL21 harboring the recombinant plasmid pET28a-*hcp*. Purification of the recombinant Hcp (rHcp) was achieved using HisTrap™ HP (GE Healthcare, USA). The purified protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to verify the identity. The Bradford Protein Quant Kit (Tiangen, China) was used to determine the protein concentration and the samples were stored at −20 °C. Approximately 10 μg of purified rHcp was subjected to SDS-PAGE, and transferred to PVDF membranes. Western blot analysis was conducted with convalescent serum from fish challenged with *A. hydrophila* NJ-35 as the primary antibody.

Rabbits were immunized subcutaneously with one milliliter (1 ml) of purified rHcp (0.5 mg/ml) emulsified with an ISA 206 adjuvant (SEPPIC, France) at a ratio of 1:1 on days 0, 14 and 28. Sera were obtained prior to injection and 7 days after the final immunization. ELISA titers of sera to rHcp were determined as described by Ni et al. [26].

2.5. PCR detection and western blot analysis

PCR was performed with the pair of primers, *hcp*-F' (5′-ATTCCGTCGGCAACATCTTC-3′) and *hcp*-R' (5′-GGATCAGTTGGGTGAAG TCAGAC-3′), to determine the distribution of *hcp* gene in *A. hydrophila* strains. PCR conditions included an initial denaturation at 95 °C for 5 min followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 57 °C for 30 s and extension at 72 °C for 25 s and a final extension at 72 °C for 10 min. Genomic DNA of ATCC 7966 and sterile deionized water were used as the templates for positive and negative controls, respectively.

Western blot was performed to explore the levels of Hcp protein expression and secretion in *A. hydrophila* strains of different origins as described elsewhere [28,29]. Briefly, all *A. hydrophila* strains were grown to OD_{600} ~ 2.0 in LB medium. One-millilliter bacterial cultures were collected and centrifuged at 10, 000 × *g* for 5 min. The cell pellets were re-suspended in 160 μl 1 × phosphate-buffered saline (PBS) and 40 μl 5 × protein sample buffer. The supernatant was filtered using a 0.22-μm membrane filter and mixed with 5 × protein sample buffer. After boiled for 10 min, equal volumes of whole-cell and supernatant samples from the strains were used for SDS-PAGE immunoblot analysis. Anti-Hcp polyclonal antiserum (prepared in our laboratory using the recombinant Hcp from this study) or anti-OmpA polyclonal antiserum [30] was used as the primary antibody and HRP-conjugated goat anti-rabbit IgG was used as the second antibody. The blots were then developed using the DAB kit.

2.6. LD_{50} determination in fish

Fish used in this study were maintained and cared for following established protocols (Pearl River Fishery Research Institute, Chinese Academy of Fishery Science). Fish challenge experiment with *A. hydrophila* NJ-35 was conducted as previously described for the zebrafish model [31]. Overnight cultures of *A. hydrophila* NJ-35 were harvested at late-log phase by centrifugation and washed twice in PBS (pH 7.4). Common carp were anesthetized by immersion with 100 mg/l tricaine methanesulfonate (MS-222) (Hangzhou Animal Medicine Factory, China). Eight groups of 10 fish were intraperitoneally (i.p.) injected with 0.1 ml of 10-fold serially diluted suspensions of bacteria (10^2–10^9 colony forming units