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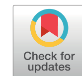
The protective efficacy of four iron-related recombinant proteins and their single-walled carbon nanotube encapsulated counterparts against *Aeromonas hydrophila* infection in zebrafish



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ABSTRACT

Iron-related proteins play important roles in iron homeostasis, and they may be potential vaccine candidates against pathogenic *Aeromonas hydrophila*. In addition, the encapsulation of antigens in single-walled carbon nanotubes (SWCNTs) has recently been shown to effectively stimulate the host immune response. To investigate the immune response of zebrafish to iron-related proteins and SWCNT-encapsulated proteins, we overexpressed and purified four iron-related recombinant proteins (P55870, A0KGK5, A0KPP0, and A0KIY3) from *A. hydrophila*. We then vaccinated zebrafish with these proteins and their SWCNT-encapsulated counterparts via both intraperitoneal injection and bath immunization. The target proteins evoked an immune response in zebrafish after intraperitoneal injection, and SWCNT-encapsulation significantly increased the immune response after bath immunization. When challenged with virulent *A. hydrophila*, zebrafish administered 5 μg intraperitoneal injections of SWCNT-P55870, A0KGK5, A0KPP0, or A0KIY3 had remarkably high relative percent survivals (RPSs) (50%, 55.6%, 66.7%, and 94.44% respectively). The RPSs of zebrafish vaccinated via immunization bath with 40 mg/L SWCNT-encapsulated counterparts were also high (52.94%, 55.56%, 61.11%, and 86.11%, respectively). These results indicated that zebrafish vaccinated with P55870, A0KGK5, SWCNT-P55870, and SWCNT-A0KGK5 were partially protected, while A0KPP0 and A0KIY3 were promising vaccine candidates against pathogenic *A. hydrophila* infection.

1. Introduction

Aeromonas hydrophila is a well-known opportunistic pathogen that infects many species of fish, including *Ictalurus punctatus*, *Oncorhynchus mykiss,* and *Pelteobagrus fulvidraco*. This bacterium causes symptoms of motile *Aeromonas* septicemia, including hemorrhagic septicemia, orbital cellulitis, and ocular rupture [1,2]. Every year, *A. hydrophila* infections cause severe economic losses for the aquaculture industry [3]. Therefore, there is an urgent need to identify effective immunoprotective vaccine candidates to control the spread of this disease. To date, several types of vaccines have been developed against *A. hydrophila,* including DNA, LPS, attenuated strain, and recombinant proteins [4–7]. The recombinant outer membrane proteins (OMPs) of *A. hydrophila*, including OmpA1, Tdr, LamB, Omp48, OmpF, Aha1, and OmpW, may be effective protective antigens [8–13]. Moreover, a new reported that the perturbation of iron scavenging dynamics by adding iron chelator in epidemic *A. hydrophila* cultures enhanced the virulence, which indicates some iron-related bacterial fractions may be potential vaccine candidates [14]. Moreover, we previously tested the effects of an iron-limited growing environment on *A. hydrophila* protein expression by comparing the altered proteomic profiles of OMPs and extracellular proteins of *A. hydrophila* growing in normal medium to those of *A. hydrophila* growing in medium supplemented with 2,2-dipyridyl (DIP), which mimicked the iron-limited environment *in vitro* [15]. We identified 21 proteins that were more abundant during iron starvation than in the iron-rich medium. A subsequent immunoprotective evaluation suggested that at least five upregulated iron chelate transport-related proteins might be potential recombinant vaccine candidates. However, the protective effects of other iron-related proteins, especially in uncharacterized OMPs, are still largely unknown.

In addition to the protective antigens, adjuvant is an important vaccine component that stimulates the immune system of host, promoting antigen uptake, and increasing protection duration [16]. The ideal fish adjuvant should be effective, nontoxic, and biodegradable

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[17]. Several adjuvants are currently used in fish vaccines: mineral oil, Freund, Montanide ISA711, and ISA763A, but additional effective adjuvants, without severe side effects, are still needed [18].

Nanoparticles are biocompatible and biodegradable, and have been proposed as potent adjuvants in clinical medicine [19]. Recently, it was shown that when the polylactide-*co*-glycolide (PLGA) or polylactide (PLA) nanoparticle encapsulated *A. hydrophila* OMPs, the strength and duration of the immune response was increased [20]. Functionalized single-walled carbon nanotubes (SWCNTs) enhance the immunoprotective effect of DNA, acting as a delivery vehicle for recombinant proteins targeting viral and bacterial pathogens in fish such as the common carp, zebrafish, and grass carp, and are low- or nontoxic [21–23]. Therefore, the use of nanoparticles like SWCNTs to encapsulate recombinant antigens are considered promising for antibacterial or antiviral vaccine candidates relevant to fish aquaculture [22]. As a fish model, zebrafish were well suited for both developmental and genetic analysis, also the well-developed immune system and its available genome information made zebrafish a good model for immunity research [24–27].

Here, we tested the immune response of zebrafish to four *A. hydrophila* proteins: three iron-related outer membrane proteins (A0KGK5, A0KPP0, and A0KIY3) and one extracellular protein (P55870), as well as the protective efficacy of these proteins. We also prepared SWCNT-recombinant protein vaccines and compared the immunoprotective effects of these vaccines against pathogenic *A. hydrophila* in zebrafish when administered via traditional intraperitoneal injection and via bath immunization.

2. Methods and materials

2.1. Fish and bacterial strains

Zebrafish with the length of 3.0 ± 1 cm and weight of 0.3 ± 0.1 g were purchased from the Fuzhou Flower, Bird, and Fish Market (Fuzhou, China). Zebrafish were randomly divided into several aquaria, each with aerated water maintained at 25 °C. Water was changed and fish were fed once per day. Fish were acclimatized to laboratory conditions for one week prior to experimentation. One strain of *A. hydrophila* used in this study, *A. hydrophila* strain ATCC 7966, was kept at −80 °C. The other strain of *A. hydrophila, A. hydrophila* LP-2, which is a virulent strain isolated from diseased silver carp, was donated by Dr. Pang, Guangdong Ocean University (Zhanjiang, China). Both bacteria were inoculated as needed into liquid LB medium and incubated overnight at 30 °C with shaking.

2.2. Expression and purification of the recombinant proteins

Gene-specific primers were designed based on the *A. hydrophila* ATCC 7966 genome (Table 1). PCR products were ligated with the cloning vector pET-32a and transformed into *Escherichia coli* BL21. The recombinant plasmids were incubated overnight in 5 mL LB medium at 37 °C with shaking at 200 rpm, and then transferred to 200 mL fresh LB medium, supplemented 1:100 (v/v) with 100 μg/mL ampicillin (Solarbio, Beijing, China). When the OD600 reached 0.3–0.6, the strains were induced with 1 mM IPTG for 6–8 h at 18 °C. After washing three times with phosphate-buffered saline (PBS), bacterial pellets were resuspended in binding buffer (25 mM $Na_2HPO_4 \cdot 12H_2O$, 10 mM $NaH_2PO_4 \cdot 2H_2O$, 500 mM NaCl, and 5 mM imidazole), and then sonicated on ice for 30 min to disrupt cell membranes. The supernatants were loaded onto a Ni-NTA resin column (GE Healthcare Bio-Science, Sweden) and purified as has been previously described [28]. Briefly, after washing with 30 mL binding buffer and 10 mL washing buffer (25 mM $Na_2HPO_4 \cdot 12H_2O$, 10 mM $NaH_2PO_4 \cdot 2H_2O$, 500 mM NaCl, and 20 mM imidazole), samples were eluted with 5 mL elution buffer (25 mM $Na_2HPO_4 \cdot 12H_2O$, 10 mM $NaH_2PO_4 \cdot 2H_2O$, 500 mM NaCl, and 300 mM imidazole). Recombinant protein concentrations were measured with the Bradford assay, and stored at −20 °C.

2.3. Western blotting

The purified recombinant proteins were used to immunize rabbits to produce specific polyclonal antibodies and Western blotting was performed to confirmed the expression of interested proteins as previously described [28]. The preparing of mouse anti-live or -inactivated bacteria sera was the same as previously described except for the pathogenic bacteria was *A. hydrophila* [29]. For Western blotting, identical volumes of protein, some treated with 200 μM of the iron chelator DIP, were separated with 12% SDS-PAGE. After separation, protein samples were transferred to polyvinylidene fluoride (PVDF) membranes with the *Trans*-Blot Turbo Transfer System (Bio-Rad, Hercules, CA) at 1.3 A for 30 min. After washing with PBST, PVDF membranes were blocked in 5% (w/v) skim milk and incubated at room temperature for 1 h. Next, primary antibodies were dissolved in PBS buffer with 5% (w/v) skim milk at 1:5000 (v/v), and incubated with the PVDF membranes at 4 °C for 16 h. The membranes were washed three times with PBST and incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody, diluted 1:5000 in PBST at room temperature for 1 h. After washing five times with PBST, the membranes were exposed using a Clarity Western ECL Substrate Kit (Bio-Rad, Hercules, CA) and viewed with the ChemiDoc MP imaging system (Bio-Rad, Hercules, CA) to detect immune-stained proteins [30,31]. Finally, PVDF membranes were stained with Coomassie R-350 as a loading control.

2.4. Preparation of SWCNT-recombinant protein vaccine candidates

Oxidized single-walled carbon nanotubes (o-SWCNTs) were purchased from Chengdu Organic Chemicals Co., Ltd. (Chengdu, China). SWCNT-recombinant proteins were constructed from the recombinant proteins of interest (A0KGK5, P55870, A0KIY3, and A0KPP0) by covalently bonding the recombinant proteins on the o-SWCNTs via a diimide-activated amidation process, as previously described [32]. Briefly, about 200 mg o-SWCNTs were mixed with 100 mL 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer solution for 30 min. We then added 1.0 g 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and 1.4 g *N*-hydroxysuccinimide to the buffer solution, and sonicated the mixture for 2 h. After washing with 0.1 M MES buffer,

Table 1
Primers sequences used for gene cloning.

Gene	Primer sequences (5′→3′)	Restriction sites
AHA_0854	F: CCGGAATTCATGAAAAAGACAGCATTGACCATGG	*Eco*RI
	R: CCCAAGCTTTTAGAAGTTGTACTGCAGGGCCACG	*Hin*dIII
AHA_1512	F: CCGGAATTCATGAAAAACAAAAAACCACGCAAAT	*Eco*RI
	R: CCCAAGCTTTCAGTGACTGGCCGGTGGCCCGAA	*Hin*dIII
AHA_1698	F: CCGGAATTCATGGGTCTTTATATCAATACGAACG	*Eco*RI
	R: CCCAAGCTTTCAGCCCTGCAGCAGGGAGAGTGC	*Hin*dIII
AHA_3793	F: CGGGAATTCATGAATAAAACACTGATTACCTTGC	*Eco*RI
	R: CCCAAGCTTTCACTGCTGAACTTCCGAGATCCCT	*Hin*dIII

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