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Evaluation of three recombinant outer membrane proteins, OmpA1, Tdr, and TbpA, as potential vaccine antigens against virulent *Aeromonas hydrophila* infection in channel catfish (*Ictalurus punctatus*)

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ABSTRACT

A virulent clonal population of *Aeromonas hydrophila* (VAh) is recognized as the etiological agent in outbreaks of motile aeromonas septicemia (MAS) in catfish aquaculture in the southeastern United States since 2009. Genomic subtraction revealed three outer membrane proteins present in VAh strain ML09-119 but not in low virulence reference *A. hydrophila* strains: major outer membrane protein OmpA1, TonB-dependent receptor (Tdr), and transferrin-binding protein A (TbpA). Here, the genes encoding *ompA1, tdr,* and *tbpA* were cloned from *A. hydrophila* ML09-119 and expressed in *Escherichia coli*. The purified recombinant OmpA1, Tdr, and TbpA proteins had estimated molecular weights of 37.26, 78.55, and 41.67 kDa, respectively. Catfish fingerlings vaccinated with OmpA1, Tdr, and TbpA emulsified with non-mineral oil adjuvant were protected against subsequent VAh strain ML09-119 infection with 98.59%, 95.59%, and 47.89% relative percent survival (RPS), respectively. Furthermore, the mean liver, spleen, and anterior kidney bacterial concentrations were significantly lower in catfish vaccinated with the OmpA1 and Tdr than the sham-vaccinated control group. ELISA demonstrated that catfish immunized with OmpA1, Tdr, and TbpA produce significant antibody response by 21 days post-immunization. Therefore, OmpA1 and Tdr proteins could be used as potential candidates for vaccine development against virulent *A. hydrophila* infection. However, TbpA protein failed to provide strong protection.

1. Introduction

Since 2009, motile aeromonas septicemia (MAS) caused by a highly virulent clonal population of *Aeromonas hydrophila* (VAh) has become a major bacterial disease of catfish aquaculture in the southeastern United States. It has caused losses of about 3 million pounds of food-size fish annually [1,2]. Experimental infection indicated that VAh isolates have higher virulence for channel catfish (*Ictalurus punctatus*) compared with historical opportunistic isolates of *A. hydrophila* isolated from stressed fish [3]. Moreover, there are considerable sequence differences between VAh isolates and opportunistic strains [4,5]; these differences may account for their emergence as highly virulent strains in catfish.

Strain ML09-119 was isolated from a catfish farm in western Alabama during an epidemic outbreak of MAS [6]. Genomic subtraction based on differences between VAh (strain ML09-119) compared to opportunistic isolates revealed proteins unique to VAh strain ML09-119 and other VAh isolates [2]. Among these are three outer membrane proteins: major outer membrane protein OmpA1 (OmpA1: AHML_06755), TonB-dependent receptor (Tdr: AHML_05675), and transferrin-binding protein A (TbpA: AHML_13490).

OMPs constitute approximately 50% of the outer membrane mass, and genes encoding OMPs account for 2–3% of bacterial genomes [7,8]. Typically OMPs display β-barrel structural architecture and are involved in bacterial adaptive responses such as solute and ion uptake, iron acquisition, antimicrobial resistance, serum resistance, and bile salt resistance [9]. In pathogenic Gram negative bacteria, some OMPs contribute to adherence, colonization, and persistence in the host [10,11].

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Outer membrane protein A (OmpA) is one of the major integral proteins of the outer membrane. It contributes to maintaining integrity of the bacterial surface, serving as a receptor for phage and colicin, participating in biofilm formation, mediating F-dependent conjugation of *Escherichia coli* K1, and contributing to serum resistance [8,12,13]. In addition, OmpA is immunogenic and can elicit antibody response [14]. Recently, OmpA was shown to exist as two different allelic forms in *E. coli*, OmpA1 and OmpA2, which affects phage susceptibility [15].

TonB-dependent receptor proteins bind specific environmental substrates, and when they are bound by TonB, they transduce energy derived from the proton motive force (PMF) to allow active transport of the substrates into periplasm. Some of the substrates transported by TonB-dependent receptor proteins include iron siderophores and vitamin B_{12} [16]. Some TonB-dependent receptors are essential for virulence in pathogenic bacteria [17,18].

In Gram-negative bacteria, the transferrin receptor consists of two iron-regulated OMPs: transferrin-binding protein A (TbpA) and transferrin-binding protein B (TbpB) [19]. Tbps have been considered potential vaccine candidates since their discovery [20]. TbpA is an integral membrane protein and is a member of the family of TonB-dependent outer membrane proteins that include siderophore receptors. It serves as a channel for transport of iron across the OM [21]. TbpA has been identified in pathogenic bacteria such as *Neisseria meningitidis* [22], *Neisseria gonorrhoeae* [23], *Moraxella catarrhalis* [24], and *Haemophilus influenzae* [25].

In the current study, we undertook expression and purification of recombinant OmpA1, Tdr, and TbpA from *A. hydrophila* strain ML09-119. We also assessed the level of protection and antibody responses afforded by these three proteins against infection with *A. hydrophila* strain ML09-119 in catfish.

2. Material and methods

2.1. Ethics statement

Experimental infection of catfish was performed at Mississippi State University according to a protocol approved by the Institutional Animal Care and Use Committee (IACUC).

2.2. Bacterial cultures

Bacterial strains and plasmids used in this work are listed in Table 1. *Aeromonas hydrophila* strain ML09-119 was used as a source of genomic DNA and for experimental infections. The strain was grown on brain heart infusion (BHI) agar and broth (Difco, Sparks, MD, USA) and incubated at 37 °C. *E. coli* strain NovaBlue (Novagen, Madison, WI, USA) was used for cloning purposes. Recombinant proteins were expressed in Rosetta II (DE3) cells (EMD Millipore, San Diego, CA). All *E. coli* strains were cultured on Luria–Bertani (LB) agar or broth (Difco) supplemented with appropriate selection at 37 °C. The vector pET-28a (Novagen) was used for expression of recombinant proteins. Whenever required, isopropyl-β-D-thiogalactopyranoside (IPTG) and kanamycin (Kan: 50 μg/ml) (Sigma–Aldrich, Saint Louis, MN, USA) were added to the culture medium.

2.3. Construction of recombinant plasmids and protein expression

The DNA fragments carrying *ompA1* (AHML_06755), *tdr* (AHML_05675), and *tbpA* (AHML_13490) genes were amplified from *A. hydrophila* strain ML09-119 by PCR using the primer pairs shown in Table 2. The three amplified products were purified with the QIAquick PCR purification kit (Qiagen, Valencia, CA, USA), cut with pairs of restriction endonucleases whose recognition sequences were incorporated into the primers (Table 2), and gel purified. Each processed DNA fragment was ligated to pET28a cut with the same restriction endonucleases. Aliquots of ligated vector and insert were transformed to chemically competent NovaBlue cells, and transformants were selected on LB agar plates supplemented with Kan. Plasmid DNA was extracted from positive clones, cut with appropriate restriction endonucleases, and analyzed by electrophoresis in 1% agarose gel. Candidate plasmids with appropriate fragment patterns were sequenced using T3 and T7 terminator primers to confirm correct orientation of the insert. Three resulting recombinant plasmids (pETAhompA1, pETAhtdr, and pETAhtbpA) were introduced into *E. coli* Rosetta II (DE3) by transformation.

Expression of OmpA1, Tdr, and TbpA proteins was optimized in 25 ml cultures. Cultures of *E. coli* Rosetta II (DE3) carrying the recombinant plasmids were induced at an optical density at 600 nm (OD600) of 0.6–0.8 by adding 100 mM IPTG, and incubation was continued for 6 h. Whole cell protein samples at different time points were prepared and analyzed by electrophoresis in 12% SDS-PAGE. Non-recombinant bacteria and uninduced recombinant clone were used as negative controls.

2.4. Purification of recombinant OmpA1, Tdr, and TbpA proteins

The three recombinant proteins OmpA1, Tdr, and TbpA contained six histidine tags (His6) and were purified by His-Bind (Novagen) resin column according to the manufacturer's protocols. The recombinant OmpA1 protein was extracted following a method described previously [26] with minor modifications. Briefly, recombinant clones were grown in 500 ml of LB broth and induced by IPTG for 6 h. Bacteria were then harvested by centrifugation (14,000 rpm for 20 min at 4 °C), and the pellet was lysed using 50 mM Tris–HCl (pH 8.0), 10 mM EDTA, and 10 mg/ml lysozyme, followed by sonication (4 cycles, 10s) on ice. The sonicated suspension was centrifuged, and the pellet was washed with 0.2 M sodium phosphate buffer (pH 7.3), 1 mM EDTA, 50 mM NaCl, 5% glycerol, and 1 M urea, followed by washing with homogenization buffer (50 mM Tris–HCl (pH 8.0), 100 mM NaCl, 0.5% TritonX-100, 0.1% sodium-azide). The pellet was solubilized in 6 M guanidinium chloride, 10 mM Tris–HCl (pH 8.0), 500 mM NaCl for 1 h at 4 °C followed by centrifugation. The clarified supernatant was loaded onto a His-Bind column prepacked with Ni^{2+}-charged resin that had been preequilibrated with 10 ml of binding buffer. Non-specific proteins were removed by applying binding buffer followed by wash buffer (6 M urea, 500 mM NaCl, 20 mM imidazole, and 20 mM Tris-HCl [pH 7.9]). Recombinant OmpA1 protein was then eluted with 6 M urea, 1 M imidazole, 250 mM NaCl, 10 mM Tris-HCl. Purity of OmpA1 protein was determined by 12% SDS-PAGE analysis. Protein yield was determined on a spectrophotometer at 280 nm.

For purification of Tdr recombinant protein, expression was induced as described above by addition of IPTG at OD600 = 0.6. The bacterial pellet was collected by centrifugation, suspended in 100 mM sodium phosphate at pH 7.9, and gently sonicated. Cleared supernatant was loaded on equilibrated resin, and Tdr recombinant protein was eluted from the resin column with elution buffer and subjected to SDS-PAGE to confirm purity.

To purify recombinant TbpA, 500 ml of induced bacteria culture was harvested by centrifugation, and the pellets were lysed using BugBuster protein extraction reagent (Novagen) with gentle sonication followed by centrifugation. The soluble fraction was mixed with binding buffer and bound to a packed resin column. After elution using elution buffer, fractions of TbpA recombinant protein were analyzed by SDS-PAGE.