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Quantitative proteomic analysis of iron-regulated outer membrane proteins in *Aeromonas hydrophila* as potential vaccine candidates

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ABSTRACT

The iron-regulated outer membrane protein (OMP) of *Aeromonas hydrophila* is an effective vaccine candidate, but its intrinsic functional components are largely unknown. In this study, we compared the differentially expressed sarcosine-insoluble fractions of *A. hydrophila* in iron-limited and normal medium using tandem mass tag labeling-based quantitative proteomics, and identified 91 upregulated proteins including 21 OMPs and 83 downregulated proteins including 10 OMPs. Subsequent bioinformatics analysis showed that iron chelate transport-related proteins were enriched in increasing abundance, whereas oxidoreductase activity and translation-related proteins were significantly enriched in decreasing abundance. The proteomics results were further validated in selected altered proteins by Western blotting. Finally, the vaccine efficacy of five iron-related recombinant OMPs (A0KGW8, A0KFG8, A0KQ46, A0KIU8, and A0KQZ1) that were increased abundance in iron-limited medium, were evaluated when challenged with virulent *A. hydrophila* against zebrafish, suggesting that these proteins had highly efficient immunoprotectivity. Our results indicate that quantitative proteomics combined with evaluation of vaccine efficacy is an effective strategy for screening novel recombinant antigens for vaccine development.

1. Introduction

Aeromonas hydrophila is a type of pathogen that can cause serious diseases such as motile aeromonad septicemia and multifocal *Aeromonas* osteomyelitis, and is connected with momentous sources of infections linked to natural disasters including hurricanes, tsunamis, and earthquakes. Furthermore, *A. hydrophila* has been associated with emerging or new illnesses such as near-drowning events, prostatitis, and hemolytic-uremic syndrome, and can cause heavy economic losses worldwide [1,2]. Large amounts of antibiotics are used in aquaculture each year to cure fish diseases including *A. hydrophila* infection, but they cause environmental disruption and emergence of bacterial antibiotic resistance [3]. Thus, an alternative reagent or method is urgently needed to reduce the use of antibiotics. Vaccines are a common and powerful method for curing bacterial infection. In addition to attenuated strains, immunogenic antigens including outer membrane proteins (OMPs), lipopolysaccharide, and S-layer and extracellular secreted proteins are potential vaccine candidates [4–7]. Of these recombinant subunit vaccine candidates, the protective efficacy of OMPs has been particularly well explored because of their high antigenicity and the fact that they stimulate specific immune responses [8]. For example, Li et al. [9] compared and identified two OMPs (VP1061 and VP2850) in *Vibrio parahaemolyticus* that could potentially be used as broad cross-protective vaccines with the immunoproteomics method. For *A. hydrophila*, recombinant Omp48 (LamB), Omp38, OmpG, ompTS, OmpF, OmpC, Aha1 and OmpW were reported to induce protective immune responses as well [9–13].

However, to date, only a few OMPs have been found to correlate with the immunoprotective efficacy of at least 50 OMPs in

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A. hydrophila, according to records in the UniPort protein database (http://www.uniprot.org/uniprot/). Meanwhile, the immunogenicity and immunoprotectivity of many OMPs remain largely unknown, most likely for two reasons. First, most OMPs are major outer membrane components that are generally immunogenic antigens, which are easily identified using immunoblotting or immunoproteomics strategies. However, a high abundance of these major proteins can suppress the immunoblot or mass spectrometry (MS) signal. Second, current *in vitro* bacterial cultivation for the discovery of recombinant protein vaccines is mainly conducted in enriched nutrient medium such as Luria–Bertani (LB) broth, which is significantly different from the *in vivo* environment in which free iron concentrations are low as a mechanism of defense [13,14]. Thus, strategies for simulating the iron-limited *in vivo* environment for vaccine development have garnered much attention. Several studies have reported that attenuated bacterial strains and OMP fractions cultured in iron-limited conditions enhance vaccine efficacy in *Flavobacterium psychrophilum* and *A. salmonicida* [15,16]. However, there has been little focus on the components of these iron-regulated OMP fractions, and the evaluation of their immunogenicity and immunoprotectivity is lacking, especially for *A. hydrophila*.

In this study, we compared the differential expression of OMP fractions of *A. hydrophila* in iron-limited and normal medium using tandem mass tag labeling (TMT)-based quantitative proteomics. Subsequent bioinformatics analysis showed that several iron-regulated OMPs were significantly increased in the iron-limited condition. Immunoblot analysis and virulence challenge experiments showed that some of the recombinant proteins had high vaccine efficacy, making them potential candidates for vaccine development against virulent *A. hydrophila* infection. These results may provide a novel strategy for the discovery of recombinant protein vaccine candidates.

2. Material and methods

2.1. Bacterial strains and sample preparation

The bacterial strain *A. hydrophila* ATCC 7966 was kept in our laboratory. *A. hydrophila* LP-1 is a virulent strain isolated from diseased grass carp, which was kindly provided by Dr. Pang from Guangdong Ocean University (Zhanjiang, China). One colony of *A. hydrophila* ATCC 7966 was incubated in 5 mL LB medium overnight, and then diluted in 100 mL LB at a 1:100 ratio with or without 200 μM 2,2'-dipyridyl (DIP) until the OD_{600} reached 1.0. The OMP fractions were prepared using a previously described sarcosine-insoluble method [17]. Briefly, after washing twice with saline, the cell pellets were lysed by sonication in 50 mM Tris-HCl and centrifuged to remove cell debris. The supernatants were ultra-centrifuged at 100,000 × g for 1 h at 4 °C using the Optima LE-80 K ultracentrifuge (Beckman, Palo Alto, CA, USA). The pellets were incubated in 2% (w/v) sodium lauryl sarcosinate (Sigma Aldrich, St. Louis, MO, USA) at room temperature for 30 min and then ultra-centrifuged again under the same conditions. The pellets were dissolved in 8 M urea for subsequent experiments.

2.2. Digestion and TMT labeling

The isolated proteins were digested in solution as previously described [18]. Briefly, 100 μg samples were diluted to 1 M urea in 0.5 M triethylammonium bicarbonate buffer (TEAB) and digested in trypsin at a 1:50 ratio after being reduced and alkylated by 10 mM dithiothreitol and 25 mM iodoacetamide. Then, the peptides were labeled using TMT Isobaric and Isotopic Mass Tagging Kits (Thermo Fisher, San Jose, CA, USA). Samples were independently prepared twice as biological replicates. *A. hydrophila* with and without DIP treatment were labeled as 126 and 128, respectively, and their corresponding biological replicates were labeled as 127 and 129, respectively. The labeled peptides were pooled and desalted with the Strata X C18 desalting column (Phenomenex, Torrance, CA, USA) according to the manufacturer's protocol, and then dried using a CentriVap concentrator (Labconco Inc., Kansas City, MO, USA) for subsequent MS.

2.3. Quantitative analysis by liquid chromatography tandem-mass spectrometry

Labeled peptides were re-suspended in 0.1% formic acid with 2% acetonitrile, and then analyzed on a Q-Exactive mass spectrometer (Thermo Fisher) at the same settings as previously described [19]. Each sample was analyzed in triplicate. The raw data (.raw format) was changed to. mgf format with Proteome Discoverer 1.3 software, and used to search the MASCOT database (version 2.3.0, on-site license, Matrix Science Framingham, MA, USA) for peptide identification and quantitation against *A. hydrophila* ATCC 7966. A 1% false discovery rate was selected as the cut-off for peptide identification. Meanwhile, the average TMT reporter ion ratio (DIP treatment vs. control) was ±1.2 fold in abundance, and peptides with p-values ≤0.05 in both biological replicates were considered significantly altered. The average of the protein ratios in the replicates was selected for further bioinformatics analysis.

2.4. Bioinformatics analysis

The biological functions and related processes of the altered proteins were classified according to their Gene ontology (GO) categories and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways using DAVID online software (http://david.abcc.ncifcrf.gov/) [20]. The quantifiable proteins were further divided into the following four quantitative categories according to their ratios: Q1, ratio range from minimum to 0.667, Q2, 0.667 to 0.83, Q3, 1.2 to 1.5, and Q4, 1.5 to maximum. Then the proteins from the four categories were plotted for GO and KEGG enrichment-based cluster analysis. Prediction of subcellular location prediction was performed using the Gneg-mPLoc computer program (version 2.0, http://www.csbio.sjtu.edu.cn/bioinf/Gneg-multi/#) [21]. Prediction of protein–protein interaction (PPI) was done using the STRING database (version 10.0) and visualized using Cytoscape software (version 3.4.0) [22,23].

2.5. Purification of recombinant proteins

The genes of interest *AHA_0972* (protein name is A0KGW8), *AHA_4275* (A0KQZ1), *AHA_3963* (A0KQ46), *AHA_0461* (A0KFG8) and *AHA_1663* (A0KIU8) were cloned into the pET-32a plasmid and overexpressed in *Escherichia coli* BL21 using the primers listed in Supplementary Table 2. The recombinant proteins were purified by Ni-NTA affinity chromatography as previously described [24]. Briefly, *E. coli* BL21 strains containing the recombinant plasmids were incubated in 5 mL LB medium overnight at 37 °C 200 rpm, diluted to 1:100 (v:v) in fresh LB medium with 100 μg/mL ampicillin, and shaken continuously. When the OD_{600} was 0.6, 1 mM IPTG was added to induce protein expression followed by incubation for 7 h at 20 °C. The cultures were centrifuged at 10,000 × g for 10 min at 4 °C and washed three times with phosphate-buffered saline (PBS). The bacterial pellets were re-suspended in binding buffer (25 mM $Na_2HPO_4 \cdot 12H_2O$, 10 mM $NaH_2PO_4 \cdot 2H_2O$, 500 mM NaCl, 5 mM imidazole), and then lysed by sonication for 30 min in an ice bath. After centrifugation, the supernatants containing fusion proteins were loaded onto a Ni-NTA resin column. Recombinant