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Identification of antigens for the development of a subunit vaccine against *Photobacterium damselae* ssp. *piscicida*

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ABSTRACT

Photobacterium damselae ssp. *piscicida* (*Ph.d.p.*), the causative agent of photobacteriosis, is among the most important pathogens affecting finfish aquaculture globally. With the emergence of recombinant technology, subunit vaccines have been actively pursued, but mostly for viral diseases. Bacterial subunit vaccines are more difficult to develop since the bacterial genome is more complex, with numerous candidate antigens, leading to a lengthy and laborious screening process. Immunoproteomics, using western blotting on protein analyzed with 2DE and LC-MS/MS to isolate immune-reactive proteins and acquire amino acid sequences, followed by recombinant technology to clone the candidate gene, identified eight candidate antigens from *Ph.d.p.*, which have been cloned and expressed in *Escherichia coli* BL21(DE3). These proteins were purified and used as antigens in an efficacy trial. Three, rHSP60, rENOLASE, and rGAPDH proteins, elicited higher specific antibody titers and stronger protective immunity than the other five and an inactivated *Ph.d.p.* whole bacterial vaccine. These three antigens may be candidates for the development of a subunit vaccine against *Ph.d.p.*

1. Introduction

Cobia (*Rachycentron canadum* L.) is a promising species for aquaculture, the husbandry for which was developed in Taiwan [1]. However, cobia is subject to photobacteriosis-related mortalities due to infection with *Photobacterium damselae* ssp. *piscicida* (*Ph.d.p.*) (formerly *Pasteurella piscicida*) [2]. Laboratory inactivated whole bacteria vaccines have been developed for *Ph.d.p.* [3], but their protective efficacy is not optimal [3,4]. Improvements have been made by adding an extra-cellular lipopolysaccharide (LPS) component [5] and an extracellular product (ECP) [6].

Development of subunit vaccines began with the emergence of genetic engineering technology with cloning and expression of candidate antigens in single cell expression hosts such as yeasts and *Escherichia coli*. Several effective subunit vaccine products have been developed and applied for disease prevention [7–10]. Identification and screening of each potential antigen from bacterial pathogens is tedious and labor-intensive. Reverse vaccinology [11–13] offers a novel method for rapid identification of immune-reactive proteins and has been applied to research and development of vaccines. It has been used for the identification of antigenic proteins such as those of *Helicobacter pylori* [14,15], *Chlamydia trachomatis* [16], *Edwardsiella tarda* [17,18], *Edwardsiella ictaluri* [19], *Shigella flexneri* [20], *Borrelia garinii* [21], and *Nisseria meningitides* [22].

The aim of this study was to screen, clone and isolate immune-reactive antigens from *Ph.d.p.* expressed in *E. coli*. Several antigens were purified, and the immune response and protective efficacy in cobia evaluated.

2. Materials and methods

2.1. Experimental fish

As there are no specific pathogen free cobia available, morphologically healthy and antibody negative 80-day post-hatching cobia, approximately 5–8 g, were obtained from a hatchery in Pingtung, Taiwan. Fish were held in 300 l fiberglass reinforced plastic tanks with a recirculation system of sand-filtered, UV-treated, aerated sea water at 25–28 °C. They were fed daily with commercial feed (Seabass feed, Uni-President, Taiwan). Fish

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health and quality were monitored for 14 days by observing swimming behavior and appearance. Risk of *Ph.d.p.* infection was checked with microbiological tests of internal organs and PCR [23]. ELISA was performed on blood samples to ensure that the level of *Ph.d.p.* antibody titer remained near background values prior to vaccination. The fry were 15–20 g when used.

2.2. Bacteria culture

A *Ph.d.p.* (PFPP-02) strain isolated from a photobacteriosis outbreak in farmed cobia in Penghu, Taiwan [23] was used for this study. The strain was grown in brain/heart infusion broth (BHIB) (Difco, Detroit, MI, USA) with 3% NaCl, with agitation (rpm 200) for 24 h at 25 °C. After the density reached $OD_{600nm} = 1$, bacteria were aliquoted into vials containing BHIB with 30% glycerol and stored at −80 °C. Before each use, a fresh culture was started from a frozen sample.

2.3. Collection of antiserum

Anti-*Ph.d.p.* antibody was prepared from blood of 30 cobia surviving a natural outbreak of photobacteriosis in Penghu, Taiwan, titered following the method of Lin [24], and used as a control serum antibody and for 2DE immunoblotting.

2.4. Preparation of total proteins (TPs)

Frozen *Ph.d.p.* cells were refreshed in BHIB with 3% NaCl at 25 °C for 3 h, inoculated into fresh medium at a dilution of 1:100, and incubated at 25 °C overnight to $OD_{600nm} = 1$ (log phase). Total protein preparation followed the protein extraction method in the instruction manual (GE healthcare, USA). After centrifugation, the cell pellet was re-suspended in distilled sea water twice. The cell pellets were disrupted by lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT) and sonication. Cell debris was removed by centrifugation at 13,000 × g for 10 min., precipitated by TCA-acetone solution (13.3%, w/v) with 20 mM DTT at -20 °C for 2 h, and washed in 80% acetone. After preparation, the pellet was air dried by vacuum concentration (Maxi Dry-Lyo, Heto Lab Equipment, Denmark) to remove residual acetone and stored at −70 °C until use. The concentration of TP in the final preparation was determined by bicinchoninic acid kit (BCA protein assay kit) (Pierce, USA).

2.5. Preparation of extracellular products (ECPs)

Extracellular product preparation followed Liu's protocol [2] with *Ph.d.p.* bacterial cells grown as described. After harvest, the cells were re-suspended in 5 ml PBS buffer, and sprayed onto autoclave-sterile cellophane overlaying a BHIB with 3% NaCl agar plate (25 × 20 cm), and incubated at 25 °C for 24 h. Cells were scraped from the surface of the cellophane and re-suspending in 10 ml PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 7.4) followed by centrifugation at 10,000 × g at 4 °C for 30 min. The supernatant was passed through 0.22 μm filters (Millipore, Bedfore, MA, USA) to remove cell debris, air-dried as before, and stored in 1 ml aliquots at −20 °C until use. The concentration of ECP in the final preparation was determined by BCA protein assay.

2.6. Isolation of outer membrane proteins (OMPs)

Outer membrane proteins were prepared using a modification of the sodium lauryl-sarcosinate method [19]. Overnight cultures were harvested and repeatedly washed by centrifugation with autoclaved sterile seawater. The pellet was re-suspended in three volumes (v/w) of French press buffer (50 mM Tris–HCl, 1 mM EDTA, pH 7.4) and subsequently disrupted by French press (10,000 psi, Taitek-Sci, Taipei, Taiwan). The cellular debris and unbroken cells were removed by centrifugation twice at 10, 000 × g for 10 min at 4 °C. The supernatant was collected and centrifuged at 41,000 × g for 2 h at 4 °C. The pellet fraction was re-suspended in 2% (w/v) sodium lauryl sarcosine (Sarkosyl reagent, Sigma, USA) and incubated 20 min at 25 °C to solubilize the inner membrane. The OMP fraction was pelleted after centrifugation at 100,000 × g for 40 min at 4 °C, re-suspended in the French press buffer, air dried under vacuum, and stored at −70 °C until use. The concentration of OMP in the final preparation was determined by BCA protein assay.

2.7. Two dimensional electrophoresis (2DE)

The 2DE of proteins followed the method of Fichmann [25]. The first dimension of isoelectric focusing used the Ettan IPGphor II Isoelectric Focusing System (GE healthcare, USA). Five hundred μg of ECP, OMP, and TP of each fraction were rehydrated in buffer (7 M urea, 2 M thiourea, 2% CHAPS, 8.7% glycerol, and 0.002% bromophenol blue), mixed with IEF solution (9.8 M urea, 1.6% ampholine, pH 4–7 and 0.4% ampholine pH 3–10, 2% Triton X-100, 100 mM DTT, 50 mM Tris–HCl), and separately loaded onto a 13 cm Immobline™ dry strip with pH 4–7 gradient (GE healthcare, USA) in a strip holder. Rehydration was run at 20 °C. for 13 h at 30 V. Isoelectric focusing was run at 20 °C, at the following voltage gradient: 1 h at 500 V, 1 h at 1000 V, 1 h at 5000 V, 1 h at 5000–8000 V on a linear gradient and 3 h at 8000 V. After isoelectric focusing, the strips were treated with equilibration buffer (6 M urea, 2% SDS, 50 mM Tris, 30% glycerol, 0.002% bromophenol blue). The second dimension electrophoresis was performed in a Hoefer SE 600 Ruby electrophoresis system (GE healthcare, USA) using 10~20% gradient gels. Electrophoresis was performed at 15 mA for 15 min for pre-electrophoresis and then at 30 mA for 6 h. After electrophoresis, protein samples were stained with Brilliant Blue R-250 (Merck, Germany) and scanned and analyzed in a high-resolution scanner (ImageScanner, GE healthcare, USA).

2.8. Immunoblotting

After electrophoresis, the gels were transferred to a polyvinylidene difluoride membrane (PVDF) (PerkinElmer, USA) by the electro-transfer method, using Tris–glycine buffer (2.5 mM Tris, 9.2 mM glycine, pH 8.3) containing 20% methanol under a constant current of 400 mA at 4 °C for 8 h. using a Hoefer power supply (EPS2A200, Hoefer, USA). The PVDF membrane was soaked with blocking solution (5% skimmed milk in PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 7.4) for 1 h, and incubated with cobia anti-*Ph.d.p.* serum at 1:500 dilution as the primary antibody. Rabbit anti-cobia Ig antibody, prepared in our laboratory following the method of Watts [26], was used at 1:1000 dilution as the secondary antibody, and a commercial goat anti-rabbit IgG alkaline phosphatase conjugated antibody (Bethyl Laboratories, USA) at 1:1000 dilution was employed as the tertiary antibody. The membranes were developed colorimetrically with Nitro-Blue Tetrazolium Chloride (NBT, Amresco, USA) and 5-Bromo-4-Chloro-3′-Indolyphosphate *p*-Toluidine salt (BCIP, Amresco, USA).

2.9. LC-MS/MS protein identification

Isolated protein spots excised from the gels were subjected to LC-MS/MS analysis, and peptide mass fingerprints were predicted from the NCBI database using Mascot Daemon software (Matrix Science, UK).