Fish and Shellfish Immunology xxx (2018) xxx-xxx

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com

Short communication

Detection of specific immune response in sole (*Solea senegalensis*) against *Photobacterium damselae* subsp. *piscicida* antigens

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ARTICLE INFO

Keywords:
Solea senegalensis
Photobacterium damselae subsp. *piscicida*
Immunization
Antibody
ELISA
2D-PAGE
Western blot
Antigen identification

ABSTRACT

The pathogenic bacteria *Photobacterium damselae* subsp. *piscicida* affects the development of *Solea senegalensis* culture. Vaccines made with inactivated cells have produced a relative protection against the sickness, however the administration of subcellular and purified antigens as vaccine could increase the effectiveness of the immune response.

Thus, the aim of this work was the determination of antigens of *P. damselae* subsp. *piscicida* involved in the specific immune response of *S. senegalensis*.

Fish were immunized by intraperitoneal injection (i.p.) with inactivated extracellular polymeric substances (ECP) and whole cells of *P. damselae* subsp. *piscicida*, and Freund's incomplete adjuvant. Two months later fish were boosted with the same antigens. Serum from fish was collected to determine by ELISA the title of antibodies against subcellular fractions of bacteria (ECP, capsule, outer membrane proteins, O antigen and formalized whole cells). Significant differences were found between control and immunized fish, but differences between first immunization and booster were only found for O antigen and capsule.

Western blots derived from 2D-PAGE of ECP and Outer Membrane Proteins (OMP), using sole immunized serum, detected two high reactive antigens from ECP. Proteins were identified, by mass spectrometry, as ATP-dependent metalloprotease and Telurite resistance proteins. In the case of OMP, three antigenic proteins were detected and identified as Nrfa Y218f, Anti-oxidant AhpC/TSA, and a protein domain DNA binding heat shock related.

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Senegalense sole (*Solea senegalensis*) has a high potential for diversification of aquaculture. However, its production is limited by the emergence of pasteurellosis outbreaks caused by the pathogenic bacteria *Photobacterium damselae* subsp *piscicida*.

Several studies have reported the protection against *P. damselae* subsp. *piscicida* by different designs of vaccines in cultured fish [1–5]. Vaccines are usually composed of inactivated whole cells (bacterin), containing a wide pool of antigens. However, not all the antigens protect against pathogens. Different studies showed that vaccines consisting of immunogenic fractions can induce higher protection than inactivated whole cell bacteria in fish [6,7]. Therefore, the detection of immunogenic bacterial antigens is vital to design an antigen-based vaccine. Extracellular substances (ECP) and outer membrane have demonstrated a high specific immune response in immunized fish [8,9] and could be good candidates to select immunogenic proteins.

The aim of this work is the identification of subcellular components of *Photobacterium damselae* subsp. *piscicida* involved in the immunogenic response of Senegalese sole (*Solea senegalensis*).

1. Fish

Solea senegalensis specimens, ranging from 50 to 100 g, were obtained from fish farms in the south of Spain (Cádiz and Motril) and maintained in aerated marine water at 17–22 °C. Feeding was to 2% body weight daily with commercial feed. All the experiments were performed using 6–8 fish as replicates.

2. Bacteria strain

Photobacterium damselae subsp. *piscicida* Lg41/01, a virulent strain isolated from a sick sole (LD50 2.8×10^4 cfu/g), was cultured in tryp-

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https://doi.org/10.1016/j.fsi.2018.12.050
Received 29 July 2018; Received in revised form 18 December 2018; Accepted 23 December 2018
Available online xxx
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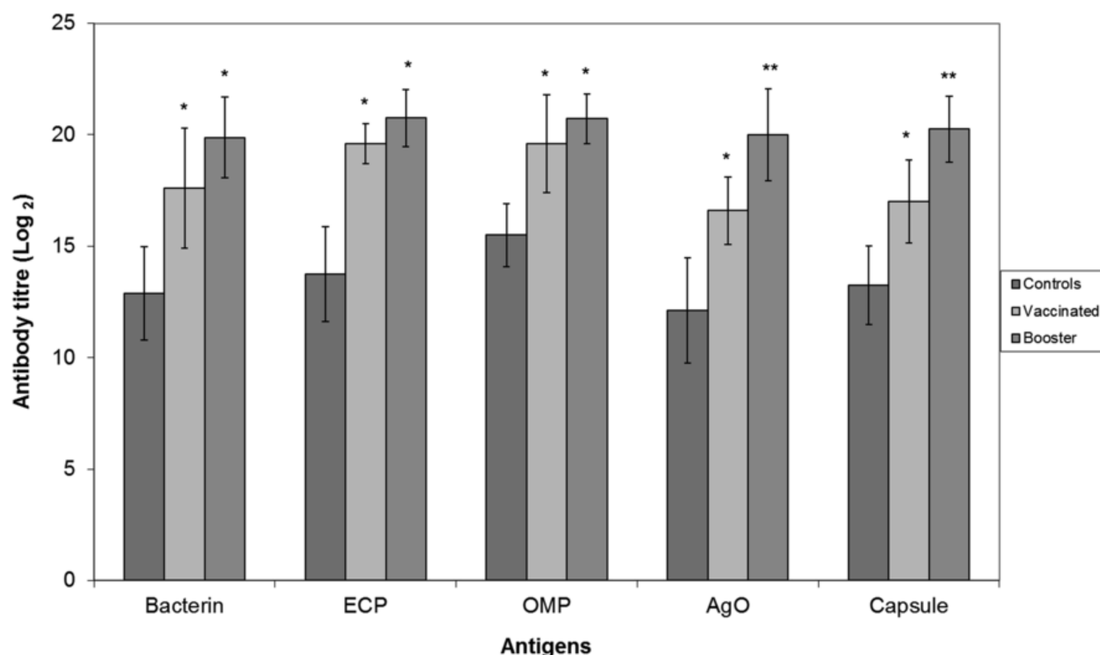

Fig. 1. Specific antibody titre for Lg41/01 antigens in serum of sole immunized with *P. damselae* subsp. *piscicida* whole cells and ECP by i.p. injection using bacterine, extracellular products (ECP), outer membrane proteins (OM), somatic antigen (AgO) and capsule as antigens. The Y axe shows the values of the limit dilution of sera expressed in log_2. *Significant differences to the control, **significant differences to the control and once vaccinated fish ($p < 0.05$).

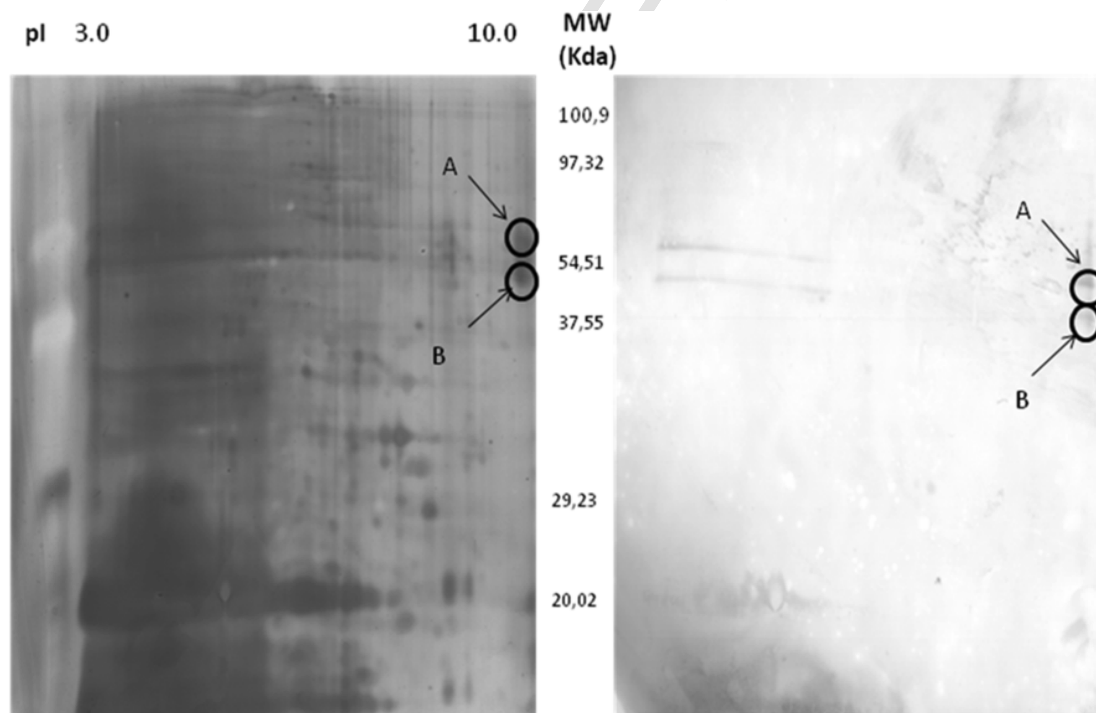

Fig. 2. ECP spots from 2D-PAGE (a) and Western blotting (b) using sole immunized serum against *P. damselae* subsp. *piscicida* whole cells and ECPs. A: metalloprotease ATP-dependent; B: tellurite resistance protein.

ticase soy broth and trypticase soy agar (Oxoid) supplemented with NaCl at 2% final concentration (TSBs and TSAs respectively).

3. Fish immunization to obtain polyclonal antiserum

Fish were immunized with inactivated whole cells (bacterin) and ECP of Lg41/01 strain according Arijo et al. [2]. The bacterin was obtained scraping the whole cells into phosphate-buffered saline (PBS) to obtain a suspension of 10^{10} cells/mL. Formalin to 1% (v/v) final concentration was added with incubation overnight at 4 °C to inactivate the cells. The suspensions were washed thrice in PBS, and then resuspended in PBS to 10^9 cells/mL. The extracellular polymeric substances (ECP) of *P. damselae* subsp. *piscicida* were obtained using the cellophane technique described by Liu [10]. The ECP were inactivated by the addition of formalin to a final concentration of 1% (v/v), and then heating at 100 °C for 30 min.

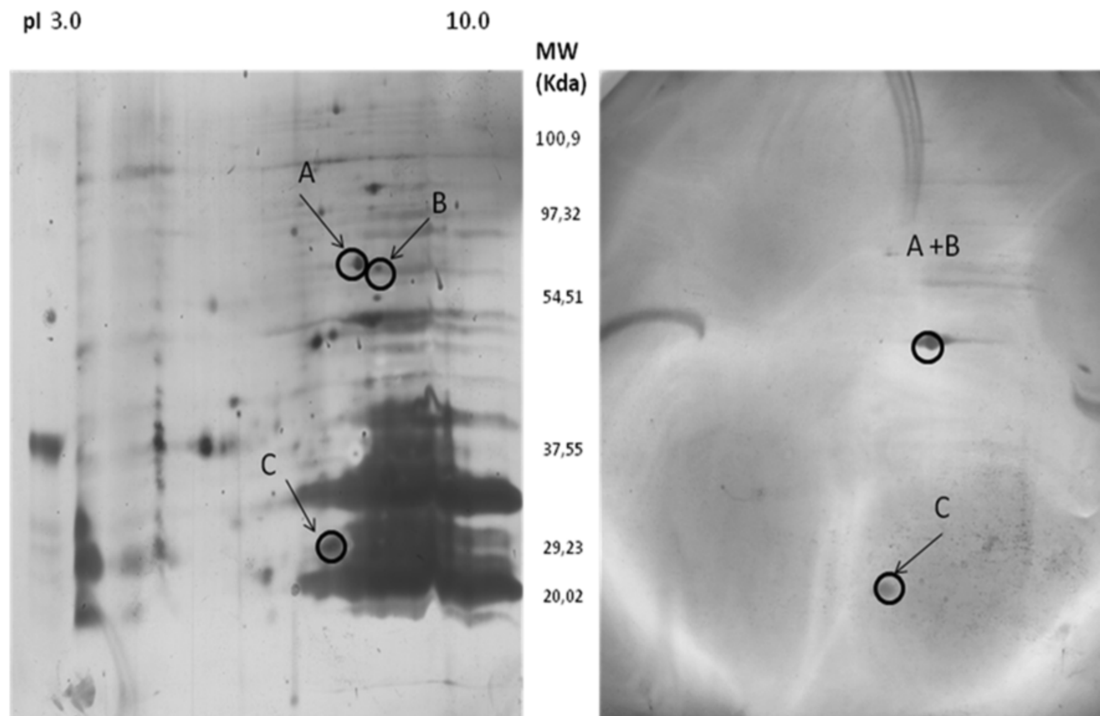

Fig. 3. OMP spots from 2D-PAGE (a) and Western blotting (b) using sole immunized serum against *P. damselae* subsp. *piscicida* whole cells and ECPs. A: Nrfa; B: AhpC/TSA; C: protein with DNA binding heat shock domain.

Table 1
ECP and OMP spots isolated from *P. damselae* subsp. *piscicida* by 2D-PAGE and identified with confidence by MASCOT (confidence interval > 75%). MW: molecular weight; PI: isoelectric point; and Accession number according the National Center for Biotechnology (http://www.ncbi.nlm.nih.gov/).

Sample	Protein	Specie	Protein score	Confidence interval	Protein MW (Da)	PI	Accession number
ECP A	ATP-dependent metalloprotease protein	*Serratia proteamaculans*	78	*99,768*	*70577,4*	*5,95*	gi|157368730
ECP B	Telurite resistance protein	*Bacillus cereus*	81	*99,881*	*41656,6*	*5,37*	gi|54298038
OMP A	ChainA, Nrfa Y218f protein (nitrite reductase)	*Wolinella succinogenes*	94	*99,994*	*55771,8*	*7,56*	gi|168988896
OMP B	Anti-oxidant Ahp/CTSA family protein	*Colwellia psychrerythraea, Vibrio cholerae, Photobacterium sp…*	159	*100*	*22146,8*	*4,69*	gi|71282444
OMP C	cold-shock DNA-binding domain-containing protein	*Shewanella loihica*	136	*100*	*7432,7,4*	*5,09*	gi|127512097

Suspension of inactivated whole cells was supplemented with 10% of inactivated ECP (800 μg proteins/ml). Then, sole specimens were inoculated with 0.1 mL of inactivated whole cell-ECP preparation in 0.1 mL of Freund's incomplete adjuvant (FIA; Sigma-Aldrich) by i.p. injection. Two months later, the fish were re-inoculated with the same antigens and adjuvant. Two months after the first immunization and one month after the second immunization, groups of fish were anaesthetized and blood collected by venepuncture, allowed to clot to obtain sole antiserum to Lg41.01 and stored at −80 °C until its use.

4. Detection of specific antibody titre of serum

Indirect ELISAs, following the method described by Arijo et al. [6], were carried out to test the antibody titre in sole serum. The following bacterial antigens were used in ELISAs: inactivated whole cells (bacterin); ECP (isolated as before); bacterial outer membrane proteins (OMP), purified according to the method of Lambert [11]; O-antigens (Ag-O), obtained following the technique described by Edwards and Ewing [12]; and capsule, isolated using the technique described by Bonet et al. [13].

Briefly, bottomed microtitre plates were coated with inactivated whole cells, ECP, OMP, Ag-O and capsule. Antigen-coated wells were sequentially incubated with serial dilutions of fish serum, polyclonal rabbit antibody anti-sole immunoglobulin (produced by the University of Malaga) (dilution 1:1000), horseradish peroxidase-conjugated antibody goat anti-rabbit immunoglobulin (Sigma) (1:5000), and developing solution (o-phenylendiamine dihydrochloride, Sigmafast OPD; Sigma-Aldrich). The reaction was stopped by 3 M H_2SO_4. Absorbance values were determined using a microplate reader (Thermo Electron Corporation) at 490 nm. The limit dilution method was used for the determination of the specific antibody titre (absorbance of the highest serum dilution ≥average of blanks absorbance + two SD) [14]. Results were compared using a Student's T-test, with significant differences at $p < 0.05$.

A significant increase of specific antibody level was detected in immunized fish for all the *P. damselae* subsp. *piscicida* subcellular compounds tested regard to unvaccinated fish (Fig. 1). Similar results for *P. damselae* subsp. *piscicida* vaccines have been reported for gilthead seabream (*Sparus aurata*) [8]. However, the booster did not significantly increase the antibody titre, comparing with one immunized fish,

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except for somatic antigen and capsule fractions in which boosters increased the titre obtained respect to the first immunization (Fig. 1). This result may also be influenced by the use of Freund's incomplete adjuvant [15]. In this case, for OMP and ECP, a booster could be unnecessary.

There were no significant differences in antibody titres between the different subunits, so we can consider that any of them is optimal for vaccine development.

5. Identification of immunogenic proteins

ECP and OMP were selected for the identification of immunogenic proteins in base of that a high level of antibody titre is obtained with only one immunization. The analysis was carried out using two-dimensional PAGE [16]. Samples were added to 11 cm immobilized pH gradient strips (IPG, pH gradient 3–10, ReadyStrip IPG strips, Bio-Rad). Proteins in IPG strips were electrofocused using a Protean Isoelectrofocusing (IEF) Cell, (BioRad).The IPG strips were placed on top of 10% acrylamide gel subjected to immunoblot analysis. Second dimension separations were performed on an Omnipage platform (Cleaver Scientific). Proteins were separated according their molecular weight at 250 V. Proteins in the gels were fixed with 50% methanol/10% acetic acid. The gels were stained by silver stain (Proteo Silver Plus, Sigma) and were scanned at 700 ppi (GS-800, BioRad).

Western Blot analysis was carried out according to the method described by Arijo et al. [6]. After electrophoresis, gels were transferred into 0.45 μm pore size nitrocellulose membranes (Sigma) in a semi-dry transblotter (BioRad). Then, the membranes were incubated in Senegalese sole sera immunized against *P. damselae* subsp. *piscicida* (dilution 1:400). Sera collected from control fish were used as negative control. The membranes were sequentially incubated with rabbit anti-Senegalese sole Ig (1:1000), goat anti-rabbit Ig G horseradish peroxidase-conjugate (Sigma) diluted 1:5000, and developer 3,3′,5,5′-Tetramethylbenzidine (TMB; Sigma). The 2-D image of the gel from each sample was compared with the Western Blot, selecting the spots that reacted with the sole antiserum.

The protein identification was carried out by mass spectrometry MALDI/TOF-TOF (Matrix Assisted Laser Desorption/Time of Flight, 4700 Proteomics Analyzer, Applied Biosystems), obtaining a mass spectrum (MS) or "Footprint Protein" [17]. A database search was then performed, combining the results of the fingerprint peptide (MS) with a fragmentation (MS/MS) of peptides from each spot according to the MS spectrum, using MASCOT as the search method.

ECP showed two spots corresponding to immunogenic proteins with high reactivity close to 50 kDa (Fig. 2), while 3 OMP were highly reactive proteins from 50 kDa to 20 kDa (Fig. 3). These proteins had a Mascot Score greater than 75, representing a significant identification ($p < 0.05$) and upper confidence interval of 99.7% (Table 1). Western Blot analysis using control sera did not show any reactive protein.

The two ECP identified have both a potential role in virulence and antimicrobial resistance (Table 1): ECP-A was identified as a metalloprotease ATP-dependent. The ATP-dependent proteases break transmembrane proteins and activate virulence factors [18,19]. Also, several metalloproteases have been identified as virulence factor secreted by *P. damselae* subsp. *piscicida* and it is abundantly [20,21]. ECP-B was a protein that confers resistance to tellurite. Despite being a rare element in the environment, the tellurite resistance gene operon (ter) is found in many pathogenic species. Although the ter operon has been associated in tellurite resistance and pathogenicity, its mode of action still remains unclear. Ter proteins could acts against host defenses produced by the cells of the immune system by employing mechanisms equivalent to tellurite reduction [22].

Regarding the immunogenic OMP (Table 1), OMP-A was identified as a protein Nrfa, a cytochrome c nitrite reductase. Nitrite reductase proteins diminish the impact of nitric oxide generated by the immune response [23]. The OMP-B was homologous to a protein antioxidant AhpC/TSA (peroxiredoxin). Peroxiredoxin family proteins protect the pathogen against reactive oxygen species [24]. The Ahpc genes increase their transcription when *P. damselae* subsp. *piscicida* infect *S. senegalensis* [25]. On the other hand the OMP-C was homologous to a protein domain DNA binding heat shock related. There is very little information available about the immune importance of OMP-C, a protein that protects in the low-temperature [26].

In conclusion, *P. damselae* subsp. *piscicida* subcellular components produce a specific immune response in farmed soles even with one immunization. At least two ECP proteins and three OMP are good candidates to design vaccines. However, more investigations must be developed to demonstrate their ability to protect the host against this pathogen.

Acknowledgements

This work was supported by the Ministry of Science of Spain (*Plan Nacional de Investigación*, AGL2010-17789) and the European Regional Development Fund. We also wish to thank the collaboration of Mr. Victor Fernandez, for the supply and maintenance of fish used in the experiments, and the Instituto Español de Oceanografía (IEO) of Vigo and Santander, for supplying fish serum. English language assistance provided by Mrs. Marie-Louise Smets.

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