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Outer membrane protein FrpA, the siderophore piscibactin receptor of *Photobacterium damselae* subsp. *piscicida*, as a subunit vaccine against photobacteriosis in sole (*Solea senegalensis*)

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ABSTRACT

Photobacteriosis caused by *Photobacterium damselae* subsp. *piscicida* (*Pdp*) remains one of the main infectious diseases affecting cultured fish in Mediterranean countries. Diverse vaccine formulations based in the use of inactivated bacterial cells have been used with unsatisfactory results, especially in newly cultured species like sole (*Solea senegalensis*). In this work, we describe the use of the outer membrane receptor (FrpA) of the siderophore piscibactin produced by *Pdp* as a novel subunit vaccine against photobacteriosis. FrpA has been cloned and expressed in *Escherichia coli* under an arabinose-inducible promoter. A recombinant protein (rFrpA) containing the *pelB* localization signal and a His tag was constructed to obtain a pure native form of the protein from *E. coli* outer membranes. The immunogenicity of rFrpA, and its protective effect against photobacteriosis, was tested by i.p. injection of 30 μg of the protein, mixed with Freund's adjuvant, in sole fingerlings with two immunizations separated by 30 days. Results showed that using either pure rFrpA or whole cells as immobilized antigens in ELISA assays, rFrpA induces the production of specific antibodies in sole. An experimental infection using fish vaccinated with rFrpA or formalin-killed whole cells of *Pdp* showed that both groups were protected against *Pdp* infection at similar levels, with no significant differences, reaching RPS values of 73% and 79%, respectively. Thus, FrpA constitutes a promising antigen candidate for the development of novel more effective vaccines against fish photobacteriosis.

1. Introduction

Photobacterium damselae subsp. *piscicida* (hereafter *Pdp*) is a γ-proteobacterium member of *Vibrionaceae* that was identified aswh the causative agent of a fish disease known as photobacteriosis (formerly pasteurellosis or pseudotuberculosis). This disease causes large economic losses in marine aquaculture worldwide due to its broad host range, affecting more than 20 economically relevant fish species, and wide geographical distribution [1–3]. Since 1990 it has been the major pathological problem in culture of sea bream and sea bass in European Mediterranean countries [2,4], and it is affecting now to other emerging high value cultured species such as sole (*Solea senegalensis* and *S. solea*) [5].

Several virulence factors have been described in this bacterium such as polysaccharide capsular material [6], extracellular products with phospholipase, cytotoxic and hemolytic activities [7–9], or the synthesis of the plasmid-encoded exotoxin AIP56, that displays apoptogenic activity against fish macrophages and neutrophils [10], and that it is secreted through a type II secretion system [11]. Several other putative virulence factors were suggested after the analysis of the complete genome of two pathogenic European strains [12]. A key virulence factor present in *Pdp* is the ability to acquire iron from the host by production of siderophores [13,14]. These are low molecular weight compounds with the ability to chelate and solubilize Fe^{3+} [15]. Siderophore-Fe^{3+} complexes are transported into the bacterial cell by outer membrane receptors and specific transporters [16]. *Pdp* synthetizes a specific

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siderophore known as piscibactin for which the chemical structure and biosynthetic pathway were previously reported [17]. Previous studies allowed also the identification and characterization of the *Pdp* genes encoding the synthesis of piscibactin and its cognate outer membrane receptor, FrpA [18]. These genes are harbored in a pathogenicity island which is part of the 69 kb plasmid pPHDP70 that plays a key role in the virulence of *Pdp* [14].

Antibiotics were the first treatment of photobacteriosis but after few years *Pdp* acquired resistance due to the appearance of plasmid- or chromosome-encoded resistance to several antimicrobials [19–21]. In addition, antibiotics used in aquaculture and their degradation products may cause negative effects on human and animal health and on the natural environment [22,23]. Thus, vaccines should be the most effective way to control photobacteriosis and several vaccine formulations have been reported [3,24]. Bacterins which contain heat or formalin killed bacteria are the most common type of vaccines used for prevention of photobacteriosis. In order to improve protection, bacterins have been enriched with extracellular products (ECP), outer membrane proteins (OMP) or lipopolysaccharides (LPS). However, the conferred protective immunity of bacterins is unsatisfactory, with a Relative Percentage Survival (RPS) nearby 60% [25]. In addition, currently used vaccines have been developed for use in sea bass and sea bream, but there is not a specific vaccine registered for use in other species such as sole, for which current vaccines are not as effective as for other fish species [5].

Progress in molecular biology technologies has allowed the development of novel vaccine formulations. Recently, promising novel vaccine preparations against *Pdp* and other fish pathogens have been reported, including purified subunits (proteins or glycoproteins) of the pathogen, which are cloned and purified from *E. coli* cultures, in combination with adjuvants or nano and micro vector systems [26–30]. Bacterial outer membrane proteins related to iron acquisition have been proposed as efficient antigens to formulate subunit vaccines against several bacterial pathogens [31–34]. In this study, the outer membrane protein receptor of the siderophore piscibactin, FrpA, was cloned, expressed and purified from *E. coli* cultures and its effectiveness as vaccine against *Pdp* in sole (*Solea senegalensis*) was evaluated.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Pdp strain DI21 was previously isolated from gilthead sea bream [4]. *Pdp* was grown in Tryptic Soy Agar (TSA) or Tryptic Soy Broth (TSB) supplemented with 1% NaCl (TSA-1 and TSB-1, respectively) at 25 °C. *E. coli* strains were grown in LB-Broth high salt (Sigma-Aldrich) with 200 μg/mL ampicillin and 50 μg/mL chloramphenicol at 28 °C. All strains were preserved in vials of LB with 20% glycerol at −80 °C.

2.2. Plasmid construction

An expression plasmid harboring *frpA* gene with *pelB* sequence [35] and 10 Histine residues in the *N*-terminal was prepared as follows. The gene encoding FrpA from *Pdp* DI21 (protein accession No. AKQ52529.1, coded by KP100338.1:10003...11985) was PCR amplified using high-fidelity Phusion polymerase (Thermo Scientific) and the chimeric primers shown in Table 1. FrpA-containing pBAD derivatives were made by overlap extension PCR [36].

Two steps of overlap extension PCR were required for obtaining the final expression plasmid pBAD-pelB-10XHis-FrpA. This plasmid contains the *pelB* localization signal, a 10-His tag (10XHis) and a localization-signal-free FrpA peptide. In the first step, primers Fwd-FrpA-pBAD and Rev-FrpA-pBAD were used to yield a pBAD derivative carrying the whole *frpA* ORF (pBAD-FrpA) under the control of the pBAD promoter. In a second step, primers Fwd-pBAD-pelB and pelB-His-FrpA-Rev were used to remove the FrpA localization signal and to introduce the pelB-10XHis peptide sequence (as determined by SignaIP 4.1 Server) [37]. The resulting constructs were verified by sequencing and then transformed into chemically competent *E. coli* TG1 cells (*F'[traD36 lacIq Δ(lacZ) M15 proA + B+] glnV (supE) thi-1 Δ(mcrB-hsdSM)5 (rK-mK- McrB-) thi Δ(lac-proAB)*) or BL21 CodonPlus (Agilent Technologies) cells.

2.3. Expression and purification of recombinant FrpA

Recombinant FrpA expression was induced in *E. coli* BL21 CodonPlus (Agilent Technologies) by addition of 0.2% L-arabinose (Sigma-Aldrich). The cells were incubated at 28 °C with shaking for 38 h. After induction, the cells were harvested by centrifugation at 3,000 *g* for 10 min at 4 °C. Bacterial pellets were resuspended in 10 mM Tris-HCl pH 8.0, 50 mM NaCl, lysozyme (5 mg per gram of cells) (Alfa Aesar), DNase RQ1 RNase-Free (9 μL per gram of cells) (Promega) and protease inhibitor (Sigma-Aldrich) at the concentration recommended by the manufacturer. Then, the bacterial suspension was lysed by sonication. After low-speed centrifugation at 2,400 g for 10 min at 4 °C to remove cell debris, whole membranes were sedimented at 33,000 g for 1 h at 4 °C. The inner membranes were solubilized in the presence of 1.5% (w/v) sodium N-lauryl-sarcosine (Sigma-Aldrich) for 1 h at room temperature and then, the FrpA-containing outer membranes were pelleted by centrifugation (33,000 g for 1 h at 4 °C) and finally, solubilized overnight in 5% Elugent (v/v) (Calbiochem) at 4 °C.

Residual membrane fragments and inclusion bodies were removed by centrifugation (33,000 g for 1 h at 4 °C). The resulting supernatant was mixed with equilibration buffer (10 mM Tris-HCl pH 8.0, 50 mM NaCl, 0.25% elugent and 10 mM imidazole) and applied to a pre-equilibrated, 1-mL Ni-NTA resin (Thermo Scientific). FrpA was eluted with 500 mM imidazole buffer. After buffer exchange by diafiltration with phosphate-buffered saline buffer supplemented with 0.25% Elugent (PBS-Elugent buffer), the final concentration of FrpA was 2.55 mg/mL by using a Qubit fluorometric quantitation system (Thermo Scientific). The purity of the protein was monitored by SDS-PAGE electrophoresis after Coomassie blue staining.

2.4. Structural analysis

2.4.1. Trypsin digestion

FrpA (10 μL of a 2.55 mg/mL solution in PBS-Elugent buffer) was denatured by addition of 0.1% SDS and incubation at 100 °C for 10 min. Denatured FrpA, together with an equivalent volume of untreated sample, were digested with 4 μg/mL of trypsin (Sigma-Aldrich) for

Table 1
Primers used for preparation of pBAD derivatives for FrpA expression.

Primer	Sequence 5'→3'[a]
Fwd-FrpA-pBAD	*CCCGTTTTTTTGGGCTAACAGGAGGAATTAACC***ATGTACAGGAACAGTTTCTCGCTCTCTCC**
Rev-FrpA-pBAD	*CGAATTCCAATGACAACTCCGTCTTCC***TTACCACTCTAGCTTCATATTCACACCC**
Fwd-pBAD-pelB	*GGGCTAACAGGAGGAATTAACCATG*AAATACCTGCTGCCGACCGCTGCTGCTGGTCTGCTGCTCCTCGCTGCCCAGCCGGCGATGGCC
pelB-His-FrpA-Rev	**CCGTAACTTCTGGATTATGTTCATC**ATGATGATGATGGTGGTGGTGATGGTGATGGG CCATCGCCGGCTGGGCAGC

[a] *frpA* sequence is shown in bold letters; pBAD sequence shown in italics; pelB sequence is underlined; His-tag sequence shown in roman letters.

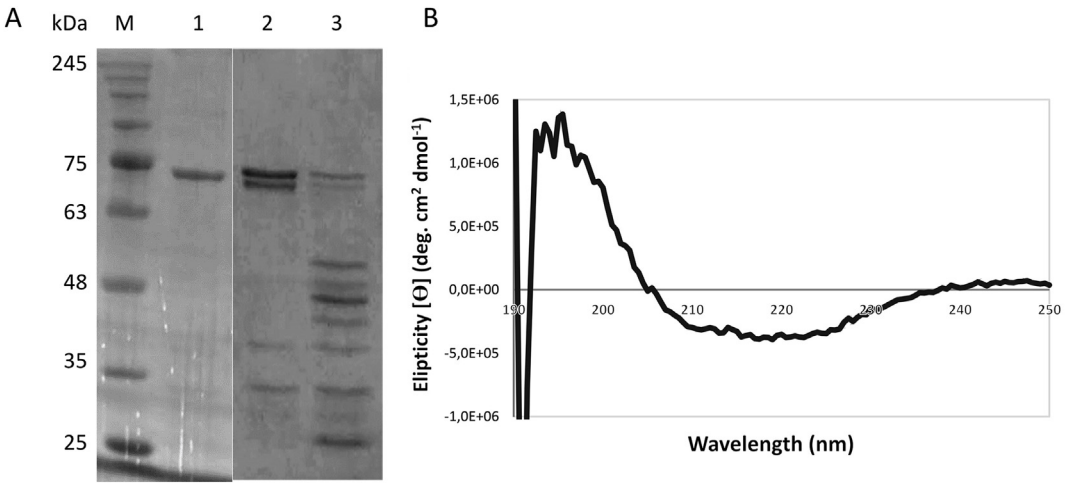

Fig. 1. A) SDS-PAGE of FrpA. M) Molecular mass marker. 1) rFrpA after nickel-affinity column. 2) Tryptic fragments of native rFrpA. 3) Tryptic fragments of rFrpA denatured by boiling with 0.1% SDS. B) CD spectrum of native rFrpA.

15 min at room temperature. Digestion was stopped by addition of protease inhibitor (Sigma-Aldrich). The samples were analyzed by SDS-PAGE.

2.4.2. Mass spectrometry analysis

After SDS-PAGE the protein band with the expected molecular weight (71.92 kDa) was excised (see Fig. 1A, lane 1). The sample was in-solution reduced, alkylated, and digested with trypsin. Briefly, the sample was reduced with dithiothreitol (DTT) and subsequently alkylated with iodoacetamide. Then, the sample was digested with 12.5 ng/L sequencing grade trypsin (Roche Applied Science) for 16 h at 37 °C. After digestion, the supernatant was collected, and 1 μL was spotted onto a MALDI target plate (384-spot OptiTOF plates, Sciex) and allowed to air dry at room temperature. Subsequently 0.5 μL of a 3 mg/mL solution of α-cyano-4-hydroxy-*trans*-cinnamic acid matrix in 0.1% trifluoroacetic acid and 50% acetonitrile was added to the dried peptide digest spots and again allowed to air dry. The sample was analyzed using the MALDI-TOF/TOF mass spectrometer 4800 Proteomics Analyzer (ABSciex, Framingham, MA) and 4000 Series ExplorerTM software (ABSciex). MALDI-TOF spectra were acquired in reflector positive ion mode using 1000 laser shots per spectrum. Data Explorer version 4.2 (ABSciex) was used for spectra analyses and generating peak picking lists. All mass spectra were internally calibrated using autoproteolytic trypsin fragments and externally calibrated using a standard peptide mixture (ABSciex). TOF/TOF fragmentation spectra were acquired by selecting the 10 most abundant ions of each MALDI-TOF peptide mass map (excluding trypsin autolytic peptides and other known background ions) and averaging 2000 laser shots per fragmentation spectrum. The parameters used to analyze the data were a signal to noise threshold of 20, a minimum area of 100, and a resolution higher than 10,000 with a mass accuracy of 20 ppm.

2.4.3. Database search

Raw data were analyzed using Protein Pilot 4.0 software (ABSciex). Protein Pilot search parameters were as follows: Cys-alkylation: iodoacetamide; digestion: trypsin; ID focus: biological modifications; database: last SwissProt release; species filtering: none; search effort: thorough ID and detection protein threshold unused ProtScore (Conf) > 1.3 (95%). The scoring model was defined by the Paragon algorithm.

2.4.4. Circular dichroism (CD) spectroscopy

The CD spectrum of FrpA in PBS-Elugent buffer (0.16 mg/mL) was measured using a CD spectro-polarimeter (J-815; Jasco Inc.) with a 1-mm-path-length cuvette (Hellma Analytics) at 20 °C. The wavelength range scanned was 190–250 nm. The data were processed using Spectra Manager software (version 1.54.03 Jasco Inc.).

2.5. Fish immunization

Sole (*Solea senegalensis*) fingerlings with an average weight of 10 g, obtained from a fish farm in Northwestern Spain, were acclimatized in tanks with filtered seawater at 18 °C and were fed daily with a commercial pellet diet. Fish health was tested out during 15 days by observing behaviour, appetite and appearance. Prior to experiments, the fish were randomly distributed into five groups of 50 individuals in tanks of 50 L. Each group of fish received a different treatment by injecting intraperitoneally 100 μL per fish of: 1) 30 μg rFrpA diluted in PBS with 0.25% Elugent mixed in a 1:1 ratio with Freund's complete adjuvant (Sigma-Aldrich); 2) a formalin-killed bacterial suspension made from *Pdp* strain DI21 (bacterin), adjusting the final concentration to OD_{600} = 1 (equivalent to 1.3×10^9 CFU/mL) with PBS; 3) PBS with 0.25% Elugent mixed in a 1:1 ratio with Freund's complete adjuvant; 4) PBS with 0.25% Elugent. A fifth group of non-vaccinated fish was used as control. A booster vaccination was done for all groups 30 days after the initial immunization using the same dosage and treatment with the exception of the adjuvant used that was Freund's incomplete (Sigma-Aldrich).

2.6. Challenge

Challenge experiments were carried out 30 days after the second vaccination. Fish were injected intraperitoneally with 10^6 CFU per fish of *Pdp* DI21 suspended in PBS. Mortality was recorded daily for 15 days and expressed as cumulative mortality. The cause of death was verified by re-isolation of the bacteria from internal organs. The relative percentage of survival (RPS) was determined as follows: RPS = [1 - (% mortality in vaccinated groups/% mortality in control group)] x 100. Kaplan-Meier and log-rank tests (SPSS for Windows, version 20) were used to evaluate differences in survival rates among groups over time.

2.7. Determination of antibody levels

The antibody levels in fish sera were determined in three different periods during the experiment: 1) before immunization, 2) one month after the first immunization and 3) one month after the second immunization. Blood samples from 5 randomly chosen fish per group were obtained by caudal vein puncture, samples were allowed to clot for 2 h at 4 °C and centrifuged. Serum was collected, diluted 1:1 (v/v) in glycerol and stored at −20 °C until use. To determine specific anti-*Pdp*

antibodies content in sera, indirect ELISA was carried out using anti-Gourami (*Osphronemus goramy*) IgM monoclonal antibody (AQUATIC Diagnostics Ltd) according to the manufacturer protocol. Monoclonal antibodies from sole were not commercially available when the experiments were done. Formalin-inactivated *Pdp* cells (100 μL per well containing 10^8 cells) or purified rFrpA (10 ng per well) were used as coating antigens on a 96-well micro-titer plate. Wells were blocked with non-fat dry milk in TBS (50 mM Tris, 0.15 M NaCl; pH 7.4). After incubation for 30 min at 25 °C with the serum to be tested (50 μL of a dilution 1:100 in PBS per well), bound *Solea senegalensis* immunoglobulins were detected by reaction with the anti-Gourami IgM monoclonal antibody. Subsequently, bound antibodies were detected with horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad) using TMB Peroxidase EIA Substrate Kit (Bio-Rad). Finally, absorbance at 450 nm was measured in a micro-titer plate reader (iMark, BioRad). Determination of antibody content was conducted by triplicate. The results were compared by analysis of variance (ANOVA) followed by Duncan's test. Differences were considered significant at $p < 0.05$.

2.8. Ethical statements

All experiments involving fish were conducted in strict accordance with the guidelines established by the European Union (2010/63/UE) and the Spanish legislation (RD 53/2013) for the use of laboratory animals. All animals were anesthetized before i.p. injection and survivors were euthanized after finishing the experiments. All procedures were authorized by the Bioethics Committee of the University of Santiago de Compostela.

3. Results

3.1. Heterologous expression, purification and characterization of FrpA

To isolate FrpA from outer membranes of the heterologous bacterium *E. coli*, we followed a method based on that of Yue et al. [35]. Briefly, we constructed the recombinant antigen as a fusion protein containing the *pelB* localization sequence, followed by a 10XHis tag, and a localization-signal-less FrpA peptide. The construct was cloned into the L-arabinose-inducible plasmid pBAD, overexpressed in *E. coli* BL21 CodonPlus cells and the native recombinant protein was purified from outer membranes as described in Materials and Methods. SDS-PAGE showed a single band with a mobility that agreed with the molecular weight predicted for recombinant 10XHis-FrpA (here after called rFrpA; Fig. 1A). Peptide mass fingerprinting was used to confirm that the band indeed contained a protein with the FrpA sequence.

The conformation of rFrpA was investigated by trypsin digestion and CD spectroscopy. Trypsin digestion has been often used to distinguish between folded and unfolded proteins [38,39]. Comparison of untreated and SDS-denatured rFrpA on SDS-PAGE gels, following trypsin digestion, reveals that denatured rFrpA is completely digested whereas rFrpA native resists the tryptic attack (Fig. 1A), in agreement with the idea that our purification protocol yields folded rFrpA. These results were further confirmed by CD spectrum analysis showing a clear minimum around 219 nm that is consistent with the presence of abundant β-sheet structure (Fig. 1B) [38,40]. In summary, our results show that, as expected, outer-membrane-isolated rFrpA is folded into its native conformation.

3.2. Presence of FrpA-specific antibodies in fish serum after immunnization with either Pdp bacterin or rFrpA

All groups of fish were given a first immunization followed by a booster vaccination after 30 days (both by i.p. injection) and the antibody titers were measured by ELISA in the sera obtained on days 1, 30 and 60 using whole *Pdp* cells or purified FrpA as antigens (Fig. 2).

After 30 days post-vaccination, the maximum levels of anti-*Pdp* antibodies where observed in the serum of fish immunized with *Pdp* DI21 bacterin (Fig. 2A). High levels of antibodies against whole *Pdp* cells were observed. Conversely, a weak anti-*Pdp* antibody levels (ca. 14% of the levels obtained against whole cells) were detected in the serum of fish vaccinated with rFrpA. Unexpectedly, these sera showed lower levels of antibodies anti-*Pdp* than the adjuvant control, suggesting that rFrpA induce the production of low levels of anti-*Pdp* antibodies. Interestingly, antibody levels using *Pdp* bacterin as antigen reach almost the maximum after 30 days and the booster dose seems not increase the antibody levels significantly. However, the antibody levels using rFrpA as antigen kept increasing after the second immunization and after 60 days were almost twice of those obtained after 30 days. This could suggest a last longer protection using rFrpA as antigen.

When we measured antibody levels against rFrpA, we obtained similar antibody levels in sera from fish vaccinated either with the *Pdp* bacterin or with rFrpA (Fig. 2B), with very low antibody levels in serum from fish vaccinated only with adjuvant. These findings clearly indicate that FrpA is an immunogenic protein for sole that could be used as antigen in vaccine formulations against *Pdp*.

3.3. Protection against photobacteriosis in fish immunized with rFrpA

In order to elucidate if the use of rFrpA as subunit antigen conferred a significant protection against *Pdp*, one month after booster vaccination (day 60 after initial immunization) a lethal dose of *Pdp* was i.p. injected to each fish. As expected, fish of the control group immunized with PBS began to die at 4 days post infection and mortality achieved 97% on day 8 (Fig. 3). The RPS of the adjuvant control group was 39.1%. Interestingly, while the RPS of the group treated with inactivated whole *Pdp* cells (bacterin) was 79.3%, the RPS of the group immunized with rFrpA reached 72.9% and no significant differences were found between both survival curves. All died fish showed typical symptons of photobacteriosis and only *Pdp* was re-isolated from internal organs. From these results, we can conclude that rFrpA, although inducing lower levels of antibodies than the bacterin, gives similar protection against photobacteriosis in sole. Thus, it could be possible to use rFrpA alone to protect fish against the infection by *Pdp*.

4. Discussion

Bacterins which contain formalin killed bacteria are the most common type of vaccines used in the treatment of photobacteriosis. In order to improve their protection, bacterins are enriched with extracellular products (ECP), outer membrane proteins (OMP) or lipopolysaccharides (LPS). However the conferred protective immunity against photobacteriosis in several species is limited [25]. The rearing of sole (*Solea senegalensis*) is a growing aquaculture industry in several European countries, but it is limited by its sensitivity to photobacteriosis, for which a sole-specific vaccine is not yet available. Some *Pdp* bacterins are currently being used to prevent photobacteriosis, but they do not always achieve satisfactory protection levels [24]. For this reason, many efforts were done in the last years to develop new vaccines against *Pdp*. They included works focused on the identification of immunogenic proteins that could be used as antigens in subunit vaccines [26–28,41,42] and the development of a DNA vaccine [43].

Subunit vaccines are considered a much safer alternative than traditional bacterins since they allow a rapid production of focused vaccines based on a single antigen [44], avoiding possible problems related to bacterins toxicity. Bacterins are usually made from cells cultured in rich media [45] that largely differ from conditions that bacteria encounter within the host. Thus, many antigens present in bacterial cells during infection are likely not expressed when cultured in laboratory media [42]. Furthermore, some cell components like extracellular toxins can have a toxic effect on fish [7,10]. Other disadvantages for killed vaccines are the possible presence of immunosuppressive

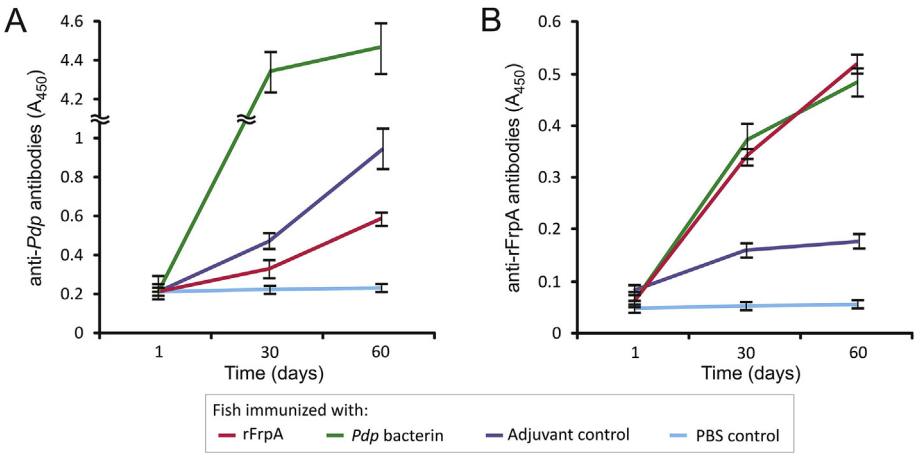

Fig. 2. Antibody levels in fish sera, determined by ELISA, against whole cells of *Pdp* (A) or against rFrpA (B) measured at 30 and 60 days post-immunization with rFrpA plus Freund's adjuvant, *Pdp* bacterin, and controls (see Material and Methods).

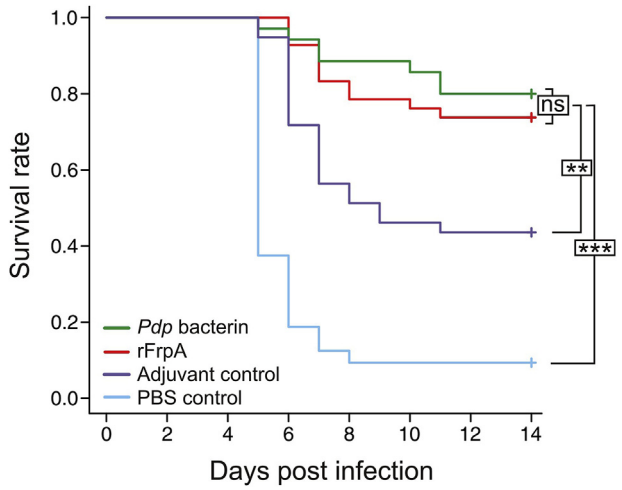

Fig. 3. Challenge trials results expressed as survival percentages after the inoculation of fish with live *Pdp* at 10^6 UFC per fish. Asterisks denote statistically significant differences among treatments: *, $p < 0.05$**, $p < 0.01$; ***, $p < 0.001$; ns, no statistically significant differences.

antigens, toxic reactions caused by immune-enhancing adjuvants, reduced immunogenicity due to denaturation of proteins and systemic reactions [46–48]. Residual formaldehyde used for bacterins preparation can produce also toxic effects in fish. All these problems could be avoided or greatly reduced, using as antigens, instead of whole inactivated cells, components from the bacterial cells which are expressed during infection in fish.

Protein antigens are usually efficient activators of adaptative immune response since they can be processed by either macrophages or B-lymphocytes and presented to a T cell, which signals B cell differentiation [49]. Thus, the first step for development of protein-based vaccines is the accurate selection of appropriate protein candidates. In this regard, iron uptake systems from *Pdp*, including heme uptake components and piscibactin synthesis enzymes, are expressed *in vivo* during infection in fish [42,50,51]. Besides, outer membrane proteins from *Pdp* were reported to be good candidates for use as antigens in vaccine preparations [52]. Thus, in this work we evaluated the use of recombinant FrpA, a *Pdp* outer membrane protein that acts as receptor for the siderophore piscibactin, as possible antigen to use in vaccine formulations against photobacteriosis in sole (*Solea senegalensis*).

FrpA belongs to a group of bacterial outer membrane receptors involved in the uptake of siderophores. All these proteins display a 22-stranded β-barrel structure with a globular plug domain within the barrel [16,53]. Since only FrpA in its native form was expected to display all the epitopes normally present on the outer membrane of *Pdp*, we reasoned that the immunogenic potential of native FrpA preparations would be largely superior to those consisting of non-native protein. Thus, we made every effort to isolate native FrpA. Sorting of β-barrel proteins to the outer membranes of Gram-negative bacteria and their insertion on the lipidic bilayer are highly conserved processes that are essential for the delivery of correctly folded and functional conformers of these proteins [54–56]. Integration of β-barrel proteins in the bacterial outer membranes requires the β-barrel assembly machinery (BAM), which in *E. coli* consists of BamA and four lipoprotein subunits, BamB, BamC, BamD and BamE [57]. Such mechanistic conservation enables the heterologous expression of β-barrel membrane proteins in their native conformation in Gram-negative model systems such as *E. coli*. Yue et al. [35] demonstrated that overexpressed recombinant β-barrel proteins could be correctly targeted to *E. coli* outer membranes by using the *pelB* targeting sequence. On the other hand, it has been shown that siderophore receptors isolated from outer membranes in the presence of certain detergents retain their function, thus arguing for the maintenance of native conformation under these conditions [58]. Thus, isolation of recombinant β-barrel proteins from *E. coli* outer membranes, is usually taken as an indicator of their correct folding into the native state. In fact, we have been able to crystallize and solve the structure of rFrpA, which shows the conserved fold of siderophore receptors (manuscript in preparation). This method, which allows the expression and isolation of native forms of outer membrane receptors, could be easily adapted to produce any such type of proteins from other Gram-negative pathogens.

After vaccination of fish with inactivated whole *Pdp* cells (bacterin), the existence of anti-FrpA antibodies in fish serum could be demonstrated. This result clearly suggest that the FrpA protein located at the surface of *Pdp* cells is immunogenic. Interestingly, fish immunized with rFrpA showed a significant immunity response that was equivalent to that observed in fish immunized with *Pdp* bacterin. Therefore, both antigens (either bacterin or rFrpA) resulted in production of specific anti-FrpA antibodies in fish sera. Thus, our results showed that the immunogenic potential of native rFrpA preparations is similar to that induced by FrpA located on the surface of *Pdp* cells, which make us hypothesized that rFrpA could be used as a subunit antigen to induce protection against photobacteriosis.

Challenge trials showed that fish groups vaccinated with bacterin or rFrpA were protected against photobacteriosis with similar protection levels, showing survival rates (RPS) of ca. 79% and 73% respectively. This result suggests that FrpA is an immunogenic protein that is produced by *Pdp* during host infection, and that FrpA represents a promising antigen for the prevention of photobacteriosis. The observation

that with lower levels of antibodies than using the *Pdp* bacterin (Fig. 2A), the degree of protection is similar (Fig. 3), suggests that antibodies raised against whole cells are mainly non-protective antibodies [59].

It is known that the correct selection of an appropriate adjuvant is a crucial step to enhance the immunogenicity of protein-based subunit vaccines [60,61]. Additionally, it was reported that the combined utilization of more than one protein as antigen could significantly enhance protection against *Pdp* infections [28]. Thus, further studies must be carried out to study the immunogenicity and protection of alternative vaccine formulations using rFrpA mixed with other adjuvants or using rFrpA combined with other potential antigens.

In conclusion, this work shows that FrpA, the outer membrane receptor involved in piscibactin uptake in *Pdp*, represents a promising antigen for the development of novel subunit vaccines for prevention of photobacteriosis in sole. Further work, currently under way, includes testing diverse vaccine formulations that contain rFrpA in their composition, which could allow to increase the protection levels achieved and to explore the vaccine's commercial use.

Declaration of competing interest

The use of FrpA as part of a vaccine formulation against photobacteriosis in sole is protected under a patent issued by the Spanish Office for Patents and Trademarks (OEPM), patent number ES2549702B1 (W02017009511A1).

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