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A vaccine against the salmonid pathogen *Piscirickettsia salmonis* based on recombinant proteins

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Abstract

We report here the protective effect against piscirickettsiosis elicited in fish by a mixture of recombinant proteins. A comparative genomics strategy was used on a genomic library of *Piscirickettsia salmonis* in order to select optimal candidates for a recombinant subunit vaccine to protect fish from rickettsial septicaemia (SRS). Based on this information, 15 *P. salmonis* ORFs encoding heat shock proteins, virulence factors, membrane bound and other surface exposed antigens, were isolated and expressed. Seven of the most promising antigens were formulated in three mixtures (V1–V3) containing two or three recombinant proteins each and injected into salmon to test their protective efficacy. Two of the three formulations (V1, V2) elicited a strong protective response in a challenge against the pathogen, which was coincident with the humoral response against the corresponding recombinant proteins present in each formulation. V1, formulated with recombinant chaperonines Hsp60, Hsp70 and flagellar protein FlgG of *P. salmonis* achieved the highest level of protection with a relative percent survival (RPS) of 95%.

Keywords: *Piscirickettsia salmonis*; Recombinant proteins; Subunit vaccine; Recombinant antigens

1. Introduction

Piscirickettsia salmonis is the etiological agent of the salmonid rickettsial septicaemia (SRS) or piscirickettsiosis. This bacterium, isolated in 1989 from a moribund coho salmon from a saltwater net pen site in the south of Chile, was the first Rickettsia-like organism recognized as a fish pathogen [1]. Since then, the disease has also been reported to affect Atlantic salmon, the main salmonid species cultured in Chile, as well as rainbow trout and other farmed salmon species. Outbreaks of SRS have also emerged among farm-raised salmon in Canada, Norway and Ireland, however, mortalities have not been as high as those in Chile [2]. The pathogen has also been isolated from sea bass in California and *Piscirickettsia*-like organisms have been identified in Hawaiian tilapia and several other fish species [3], indicating that the disease is not only confined to salmonids.

The pathogen is a gram-negative, obligate intracellular bacterium. It is pleiomorphic, predominantly coccoid in shape and ranging in diameter from 0.5 to 1.5 μm. Molecular phylogenetic analysis based on sequencing of the 16S rRNA gene placed *P. salmonis* in a new family of *Piscirickettsiae* within the class of γ-proteobacteria, most closely related to *Coxiella*, *Francisella* and *Legionella* [4]. *P. salmonis* produces a systemic infection in fish targeting predominantly the kidney, liver, spleen, intestine, brain, ovary and gills. Fish begin to die 6–12 weeks after their transfer to seawater net pens in fall and spring. The Chilean aquaculture industry attributes annual losses of US$ 150 million to SRS [5], having

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an important effect on the economy of a country positioned as the second largest exporter of salmon and trout after Norway.

Although *P. salmonis* is sensitive in vitro to many antibiotics commonly used to control other infectious diseases in fish, infected salmonids respond poorly to this treatment, due perhaps to an insufficient concentration of antibiotics within the host cell to kill the pathogen [2]. The lack of effective treatments to control piscirickettsiosis has emphasized the need to develop techniques for disease prevention. Management of the disease is based on several husbandry practices including the application of immunostimulants of unproven efficacy and the control of vertical transmission by an expensive selection procedure during reproduction. Although vaccines made of inactivated bacteria have been successfully used to control certain bacterial disease in fish [6], preparations based on *P. salmonis* bacterins have not yielded significant protection against SRS [5,7]. This might be related to the loss of important surface antigens during both, culture of the pathogen in animal cell lines, as well as in the inactivation process. A recombinant subunit vaccine is an interesting alternative. Since its first application [8], recombinant DNA technology has been considered as a valuable technology for development of vaccines against many human and animal pathogens, including *Rickettsiae* [9], a class of intracellular bacteria related to *Piscirickettsia*. In addition, the potential use of recombinant vaccines in aquaculture has been discussed extensively [10,11]. Recently a recombinant vaccine has been introduced into the market. This product is based on the 17 kDa OspA outer surface lipoprotein from *P. salmonis* fused in tandem to T cell epitopes from tetanus toxin and measles virus. This preparation attained an 83% RPS when tested in coho salmon [7]. However, there is a need for further improvement specially regarding the creation of multivalency as a mean to insure wider protection against emerging isolates.

The present work describes the use of a predictive genomics strategy to select as vaccine targets *P. salmonis* proteins previously identified as virulence factors and protective antigens in other microorganisms. We postulate that the presence of various recombinant antigens in a treatment might improve the efficacy of the vaccine. Moreover, the inclusion of antigens conserved through species could have a cross-protective effect among different bacterial pathogens. Our efforts have been directed to express recombinant heat shock proteins and surface antigens of *P. salmonis* as antigens for an effective vaccine. We report here the protective effect against piscirickettsiosis elicited in fish by a mixture of recombinant proteins.

2. Materials and methods

2.1. Cell culture

The Chinook salmon embryo cell line CHSE-214 (ATCC 1681) was cultured in complete MEM (Gibco BRL) supplemented with non-essential amino acids, glutamine and 5% fetal bovine serum (GIBCO BRL), in T175 flasks at 16 °C.

2.2. Bacterial strains and plasmids

Escherichia coli strains NovaBlue and BL21(DE3), used for cloning and expression, respectively, were obtained from Novagen. *P. salmonis* Bios-007 was isolated in 1995 from the liver of a sick fish obtained at the location of Calbuco, in the South of Chile. To grow *P. salmonis*, frozen inoculates of about 1×10^8 bacteria/mL, were brought to room temperature, added to flasks containing confluent CHSE-214 cells and incubated overnight at 16 °C. The medium was then replaced by fresh complete MEM supplemented with non-essential amino acids, glutamine and FBS 5% and cultured for 10–14 days at 16 °C. Periodic checks of the degree of cytolysis were performed. Cultures were considered ready for harvesting when nearly 100% of the cells were lysed. Cells adhered to the flask walls were scraped, centrifuged twice at $150 \times g$ at 10 °C and the second supernatant saved as the semipurified fraction of *P. salmonis*. Further purification was performed according to Jamett et al. [12].

The plasmids pET32a (Novagen) and pGEMT (Promega) were propagated in NovaBlue cells in medium LB with 100 μg/mL ampicillin at 37 °C. *E. coli* BL21(DE3) cells transformed by pET32a were grown in LB with 100 μg/mL ampicillin.

2.3. Cloning of P. salmonis antigen coding regions

Genomic DNA was extracted from *P. salmonis* as described previously [13]. Predicted coding regions of selected antigens of *P. salmonis* were isolated by PCR amplification using specific primers (Table 1) based on the sequence information from the *P. salmonis* genomic library obtained in our laboratory. Amplified products were purified using a kit from Qiagen, ligated to pGEMT and used to transform NovaBlue competent cells. Positive clones were selected by blue/white screening using lacZ α-complementation.

2.4. DNA analysis and sequencing

Plasmid DNA was purified using a kit from Qiagen. DNA samples and restriction endonuclease digests were analyzed by electrophoresis in agarose gels. The pGEMT constructs were sequenced with the Big Dye Terminator Cycle Sequencing V.2.0 kit (Applied Biosystem Inc.) based on the procedure of Sanger et al. [14] using a 310 Genetic Analyzer (Applied Biosystem Inc.).

2.5. Production of recombinant proteins in E. coli

The coding regions of the selected genes were amplified by PCR using specific primers with restriction endonuclease sites at their 5′ ends. The amplified coding regions were

Table 1
Oligonucleotides used as primers

Gene	Forward primer	Reverse primer
Hsp10	5′-ggcgaattcatgaaaatccgtccattacat-3′	5′-cggctcgaggaattaatcttcaacgactgc-3′
Hsp16	5′-cgcgatatcatgagtcactttaatttatccc-3′	5′-gccctcgagctatgccatttttttatctacta-3′
Hsp60	5′-gacggatccggagatataagaatgtcagca-3′	5′-tatgaattcttaaccgcccatgccacccat-3′
Hsp70	5′-tatgaattcatggctgaaattattggtattg-3′	5′-gtactcgagctaaacttcttcaaactcagcatc-3′
MltB	5′-gacgaattcatgagacgatcttattggcta-3′	5′-gacctcgagtattttaagagccttttgagtg-3′
Slt70	5′-caggaattcgacataatgccatactacactt-3′	5′-cagctcgagttaaacacgcctaattccagcatt-3′
TbpB	5′-cttggatccatgaaacttaccataggcttgattgg-3′	5′-cttaagcttctcactttaattgcagcagc-3′
31 kDa protein	5′-caggaattcgttatggcagcaccacat-3′	5′-gctctcgagatgccttagtttaaccccgg-3′
VacB	5′-ggaagatatcatggtaaaaaagaagacaacaag-3′	5′-ggttggatcctaagctcttttgaatgtttcattt-3′
Omp27 (kDa)	5′-cagggatccgccatgagaagcaaacaccc-3′	5′-caggaattcatggggtgagtttcttgtg-3′
Mp13 (kDa)	5′-ctcggatccctaattatgcagttttctcgtg-3′	5′-gacgaattcccaagtattattgtatcagtagt-3′
FlgF	5′-gcagga tccgtgatcatggaccatggaatt-3′	5′-tgcgaattcttaaatttgcataatgcgtaccg-3′
FlgG	5′-cagggatccaggattatgattccagcattat -3′	5′-ctggaatcctagttatatcgtctgatttaagaa-3′
FlgH	5′-gacggatccaatattaagatgaggagtttatgg-3′	5′-ctggaattcctagaatggccatatcacact-3′
FlaA	5′-cagggatccatggaaggaagagggcgtactga-3′	5′-cacgaattcctagataagtgatagtacggc-3′

cloned into the expression vector pET32a in frame with the thioredoxin gene of *E. coli* and a histidine-tag domain. *E. coli* BL21(DE3) competent cells were transformed with the constructs and expression of recombinants was induced by incubation in LB-ampicillin supplemented with 1 mM IPTG. Recombinant proteins used for immune analysis were purified by a Ni-agarose column (Qiagen). For vaccine formulations, recombinant proteins were used as semipurified preparations either as inclusion bodies or soluble protein fractions obtained by centrifugation of sonicated bacteria at 20,000 × *g* for 15 min. Protein concentration was measured using the Micro BCA kit (Pierce). Protein analysis was performed in PAGE-SDS gels according to Laemmli [15].

2.6. Monoclonal antibody production

Two-month-old female BALB/c mice were injected intraperitoneally at 3 weeks intervals with three doses of 50 μg of purified recombinant proteins diluted in PBS and emulsified with Freund adjuvant. Ten days after the last injection, the animals were bled from the tail to obtain serum. The humoral response against the recombinant proteins was determined by an ELISA test [12]. To produce hybridoma, spleen cells from the immunized mice were isolated and fused with NS0/2 mouse myeloma cells [16].

2.7. Determination of the lethal dose 50 (LD50)

Two hundred and fifty fish (*Salmo salar*) with an average weight of 18 g were tagged and kept 2 weeks in fresh water under controlled conditions to recover before being challenged. The fish were randomly distributed in 10 groups of 20 fish. Fish from each group were injected intraperitoneally with a 200 μL suspension of increasing doses of *P. salmonis* from 1×10^2 to 1×10^8 bacteria titered as described previously [17]. Doses were prepared by serial dilutions of semipurified *P. salmonis* corresponding to the eleventh passage of isolate Bios-007. A control group, injected with the saline solution used to resuspend the pathogen, was also included. The injected fish of each group were distributed in two tanks of 1000 L (10 fish of each dose per tank) and maintained at 13 °C under controlled conditions of oxygenation, feeding and water flow until mortalities ended. Cumulative mortalities at each dose were plotted and the LD50 was determined [18]. SRS was confirmed by histopathological analysis of dead fish.

2.8. Recombinant vaccine trial

Three experimental formulations containing two or three recombinant proteins were prepared. Each mixture was emulsified with one volume of incomplete Freund adjuvant to obtain a 1:1 oil in water preparation with a final concentration of 50 μg/mL of each protein. *Salmo salar* with an average weight of 18 g were tagged for group identification. Three groups of 104 fish each were injected intraperitoneally with 0.2 mL of each vaccine preparation that contained 10 μg of each recombinant protein. The vaccinated fish were randomly distributed in eight tanks with 13 fish of each group per tank. Control fish injected with saline and incomplete adjuvant were also included in the same tanks. Salmon were held at 13 °C under controlled conditions of oxygenation, feeding and water flow for 7 weeks post-vaccination (624 degree days).

For challenge, control and vaccinated fish were injected intraperitoneally with 0.2 mL of *P. salmonis*. The fish from four tanks (208 fish) were injected with a dose of *P. salmonis* equivalent to 2 × LD50 and an identical number of fish from other four tanks were injected with a dose of bacteria equivalent to 8 × LD50. Fish of each tank injected with 8 × LD50 were transferred to each tank of fish injected with 2 × LD50 in order to have four replicas (26 fish of each group per tank) each under the same feeding and environmental conditions.

2.9. Calculation of the relative percent survival (RPS)

The protection elicited by the vaccine formulations was determined by comparing the cumulative mortality of treated and control groups. The RPS, was calculated according to the equation: RPS = [1 − (% mortality of test group/% mortality of control group)] × 100 [19].

2.10. Western blot analysis

Recombinant proteins were separated by PAGE-SDS gel electrophoresis and transferred to nitrocellulose. Total protein (30–40 μg) obtained from *P. salmonis* were analyzed with a proper dilution of the monoclonal antibodies and developed with an anti-mouse IgG conjugated with alkaline phosphatase. The monoclonal antibodies used against FlgG, FlgF, FlaA, Omp27, Hsp60, Hsp70, VacB, 31 kDa protein and TbpB were 4H8/G8, 7H11/H9, 5A4/G12, 4G1/00, 5EIO/G7, 3D5/A11, 5F7/E2, 5611/H11 and 7E1/D6, respectively. To analyze the immune response of vaccinated salmon, nitrocellulose membranes containing 1 μg of the corresponding recombinant protein were incubated with a 1:200 dilution of salmon serum. Blots were then incubated with an anti-salmon IgM monoclonal antibody and developed with an anti-mouse IgG conjugated with alkaline phosphatase.

3. Results

3.1. Identification of P. salmonis proteins as potential vaccine candidates

About 80% of the genome of *P. salmonis* has been sequenced in our laboratory. Approximately 20,000 individual sequences were obtained in both directions and assembled in 2143 contigs from a random library of genomic fragments. Although the genomic information is not complete, analysis of the contig sequences by comparison with other bacterial genomes has permitted the prediction of nearly 1500 genes of which 90% could be assigned to a known function.

Vaccine candidate were selected by searching for *P. salmonis* genes that encode proteins with sequence similarity to virulence factors involved in host-pathogen interactions or immunoreactive antigens that are secreted or located at the surface of other known pathogens. More than 40 genes were selected by these criteria, of which 15 were selected for further analysis (Table 2). These include the virulence factor VacB [20]; structural components of a putative flagellar structure such as FlgG, FlgH, FlgF and FlaA [21,22]; members of the heat shock family Hsp60, Hsp70, Hsp10 and Hsp16, which are known to be strong immunogenic determinants [23]; the membrane proteins Mp13 [24], Omp27 [25], 31 kDa [26], the transferrin binding protein TbpB and the membrane lytic transglycosylase MltB [27] and its soluble counterpart Slt70 [28].

3.2. Cloning and expression of recombinant P. salmonis proteins

In order to isolate the complete coding region of the 15 selected genes by PCR, specific primers were designed based on the genomic sequences. Some open reading frames (ORFs) were completely contained within a single contig and they were easily isolated and sequenced. Other ORFs were contained in two or more contigs and were isolated by PCR with primers designed according to the sequence flanking the missing regions. The conserved organization of some genes in clusters was particularly useful to isolate genes whose sequences were only partially represented in a single contig. Using these strategies, the 15 chosen ORFs were isolated, cloned into pGEMT and their sequences confirmed. The coding regions of each gene were then subcloned in frame with the *E. coli* thioredoxin (*Trx*) coding region present in the prokaryotic expression vector pET32a. Fusion proteins were

Table 2
Selected vaccine candidates

Gene	Protein	Basis for selection
Hsp10	Heat shock protein	Cellular and humoral response [23]
Hsp16	Heat shock protein	Cellular and humoral response [23]
Hsp60	Heat shock protein	Cellular and humoral response [23]
Hsp70	Heat shock protein	Cellular and humoral response [23]
MltB	Periplasmic membrane lytic transglycosylase anchored to the outer membrane	Strong cellular and humoral response in *N. meningitides* [27]
Slt70	Periplasmic soluble lytic transglycosylase	Highly expressed in the periplasm [28]
TbpB	Transferrin binding protein localized in the outer membrane	Strong cellular and humoral response in *N. meningitides* [27]
31 kDa protein	Outer membrane protein	Immunogenic protein in *B. abortus* [26]
VacB	Cytoplasmic virulence factor B	Virulence factor [20]
Omp27 kDa	Outer membrane protein	Extracellular antigen [25]
Mp13 kDa	Membrane protein	Defense against *F. turalensis* [24]
FlgF	Rod structure of flagellar basal body	Flagellum is highly immunogenic [21,22]
FlgG	Rod structure of flagellar basal body	Flagellum is highly immunogenic [21,22]
FlgH	L ring of flagellar basal body	Flagellum is highly immunogenic [21,22]
FlaA	C-terminus of flagellin, subunit of the extracellular flagellar filament	Flagellum is highly immunogenic [21,22]

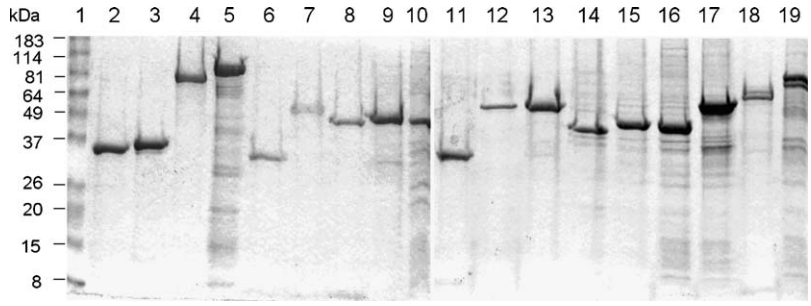

Fig. 1. SDS-PAGE analysis of purified *P. salmonis* recombinant proteins expressed in *E. coli*: (1) molecular weight markers; (2) Trx-Hsp10; (3) Trx-Hsp16; (4) Trx-Hsp60; (5) Trx-Hsp70; (6) Trx-Omp-C; (7) Trx-FlaA-C; (8) Trx-FlgF; (9) Trx-FlgG; (10) Trx-FlgH; (11) Trx-Mp13; (12) Trx-31 kDa protein; (13) Trx-TbpB-N; (14) Trx-MltB-N; (15) Trx-MltB-C; (16) Trx-Slt70-N; (17) Trx-Slt70-C; (18) Trx-VacB-N; (19) Trx-VacB-C. N and C refer to amino and carboxyl domains, respectively.

expressed in BL21(DE3) cultures under the control of T7Lac promoter upon induction with IPTG. The results are shown in Fig. 1. Most recombinant proteins were expressed as inclusion bodies with the exception of Trx-Hsp10, Trx-Hsp16 and Trx-Hsp70, which were soluble [29,30]. The ORFs of TbpB, VacB, Omp27, Slt70, FlaA and MltB were expressed either partially or in two halves [31].

3.3. Expression of the selected proteins in P. salmonis

It was of interest to study if the antigen candidates are expressed by the pathogen during infection. Therefore, extracts of *P. salmonis* growing in CHSE-214 cells were analyzed by Western blot for the presence of the native proteins. Specific monoclonal antibodies were used to detect the presence of the selected proteins in these extracts. As seen in Fig. 2, strong signals corresponding to Hsp60 and Hsp70 were found in extracts of *P. salmonis*. Less abundant, but clearly detected, are the proteins 31 KDa, TbpB and VacB. Although flagellar proteins FlgF, FlgG, flagellin and Omp27 were shown to be highly immunogenic in mice, monoclonal antibodies obtained against these recombinant proteins did not detect their presence in the bacterial extracts (results not shown). The level of expression of Slt70, Hsp10, Hsp16, FlgH, Mp13 and MltB in *P. salmonis* extracts was not analyzed due to the lack of monoclonal antibodies. Sera of mice immunized with these proteins were not used to analyze expression in bacterial extracts since they also presented reactivity against the thioredoxin moiety of the fusion proteins.

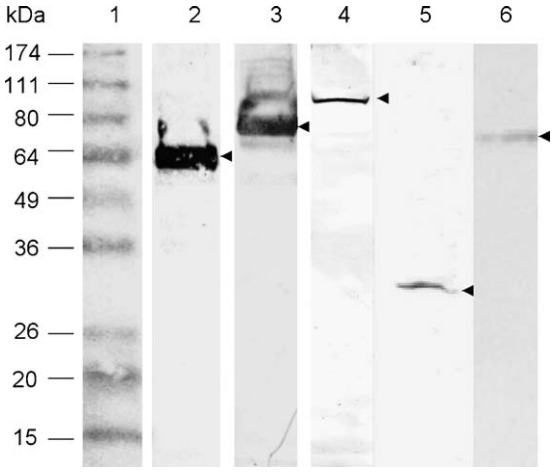

Fig. 2. Western blot analysis of antigens present in *P. salmonis*. Gel was loaded with 30–40 μg of total *P. salmonis* protein and the blotted membranes were analyzed with a 1:200 dilution of each monoclonal antibody: (1) molecular weight markers; (2–6) monoclonal antibody against Hsp60, Hsp70, VacB, 31 kDa protein and TbpB, respectively.

3.4. Recombinant vaccine trial

To measure the protective effect elicited by the recombinant proteins, formulations containing two or three of the most promising candidate proteins were prepared to be tested in a challenge with *P. salmonis*. Analysis of these mixtures by gel electrophoresis is shown in Fig. 3. The first group

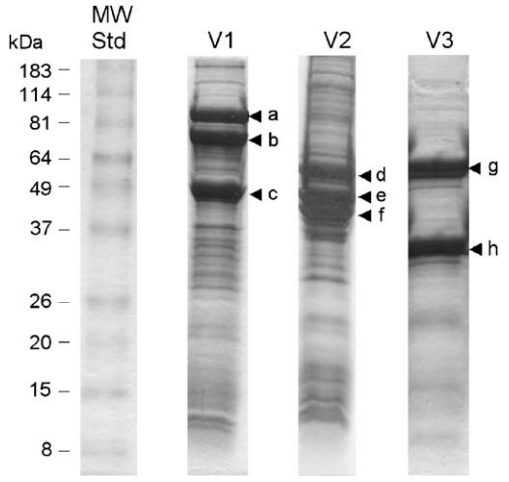

Fig. 3. SDS-PAGE analysis of recombinant vaccine formulations. Oil in water formulations containing 10 μg of each recombinant protein were used to immunize fish. MW Std: molecular weight markers; V1: formulation containing partially purified Trx-Hsp70 (a), Trx-Hsp60 (b) and Trx-FlgG (c). V2: formulation containing partially purified Trx-TbpB-N (d), Trx-MltB-C (e), Trx-MltB-N (f). V3: formulation containing partially purified Trx-FlaA-C (g) and Trx-Omp-C (h).

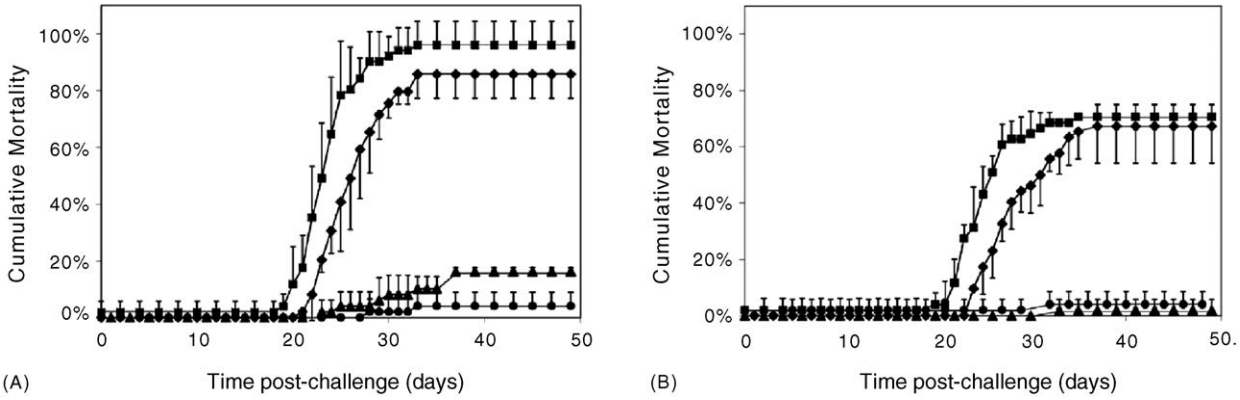

Fig. 4. Protection of recombinant vaccine against SRS in Atlantic salmon. (A) Cumulative mortality of control and immunized fish challenged with a dose of 8 × LD50 of *P. salmonis*. (B) Cumulative mortality of control and immunized fish challenged with a dose of 2 × LD50 of *P. salmonis*. (■) Control fish immunized with adjuvant; (●) fish immunized with formulation V1; (▲) fish immunized with formulation V2; (♦) fish immunized with formulation V3. The curves represent the average cumulative mortalities of four tanks of fish and the standard deviation of each value is indicated as a bar.

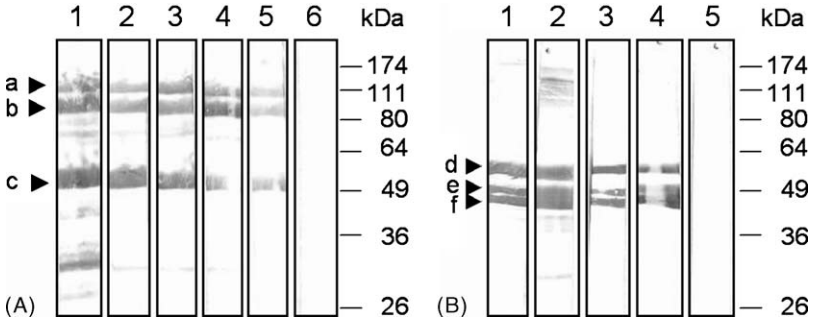

Fig. 5. Western blot analysis of serum obtained from salmon immunized with recombinant formulations V1 and V2. (A) Blots containing purified Trx-Hsp70 (a), Trx-Hsp60 (b) and Trx-FlgG (c) tested with serum of fish immunized with V1 formulation (lanes 1–5) and serum of non-immunized fish (lane 6). (B) Blots containing purified Trx-TbpB-N (d), Trx-MltB-C (e) and Trx-MltB-N (f) tested with serum of fish immunized with V2 formulation (lanes 1–4) and serum of non-immunized fish (lane 5).

(V1) is composed of Hsp60, Hsp70 and FlgG. The second group (V2) is composed of the amino terminal half of TbpB and the carboxyl and amino terminal halves of MltB. The third group (V3) is composed of the recombinant carboxyl portions of Omp27 and FlaA. Oil in water emulsions of the three recombinant preparations were injected intraperitoneally into Atlantic salmon of approximately 18 g. A saline-adjuvant control was also included in the trial.

The result of the challenge experiment with a dose equivalent to 8 × LD50 of *P. salmonis* is shown in Fig. 4A. As expected, an increasing mortality was observed in the control group. This effect began at day 2l post-challenge and reached a cumulative mortality of 96% at day 49 post-challenge. In the fish vaccinated with V3, the formulation elicited only a mild protection, with a RPS of 10.4%. Fish vaccinated with V2 exhibited a significant protective response with a RPS of 84.4%. The highest protective response was achieved with V1, reaching a RPS of 95.8%. These results represent the average mortality of each group of fish in the four tanks, which behaved as consistent replica of the trial with minor deviations (Fig. 4). As expected, the fish challenged with a *P. salmonis* dose equivalent to 2 × LD50 achieved lower cumulative mortalities (Fig. 4B), and the protective response of the V1 group (RPS 94.4%) was similar and consistent with the observed protection against the 8 × LD50 dose of *P. salmonis*.

3.5. Immune response of salmonids against recombinant proteins

Sera obtained at the end of the trial from four or five surviving fish of each of the V1 and V2 vaccinated fish was used to analyze the immune response against the recombinant component of each formulation. Serum obtained from a non-vaccinated and non-challenged fish was used as a negative control. A measurable immunoreaction against the recombinant proteins of V1 and V2 was detected by Western blot (Fig. 5), which is coincident with the ELISA immune reaction previously reported [30,31]. The specificity of the immune reaction elicited by the sera from V1 vaccinated fish was confirmed by the positive reaction against Hsp60, Hsp70 and FlgG previously excised of their thioredoxin fusion peptide (results not shown). Interestingly, the humoral response of V1-vaccinated salmon against recombinant proteins Hsp60, Hsp70 and FlgG was still high 8 months post-vaccination

(2800 degree days) as indicated by the immune reaction of their sera in a Western blot (data not shown).

4. Discussion

Prevention strategies must consider the need for a good cellular immune response to protect against the intracellular pathogen *P. salmonis*. In this respect, the most promising strategies are DNA vaccines or recombinant protein vaccines. In a first attempt using DNA vaccination against *P. salmonis*, our laboratory utilized the expression library technology to study the protection of coho salmon to the infection with *P. salmonis* with very limited success [32]. In the present work, we have concentrated our efforts on the recombinant protein subunit approach. In the case of bacterial pathogens, the selection of key proteins is essential for this strategy to succeed. We focused on molecules previously identified as virulence factors and protective antigens in other bacteria and associated with the bacterial surface. The available partial sequence of the *P. salmonis* genome permitted us to isolate and express genes to be tested as recombinant protein vaccines. Of the fifteen recombinant proteins, seven of them included in this trial were selected according to the protection conferred against other pathogens in a variety of organisms [22,23,25,27], their location in the bacterial cell and the immune response elicited in mice. The level of expression of these proteins in bacteria was also a factor considered. Two of the formulations (V1 and V2) elicited a strong protection against *P. salmonis*.

In our trials, the highest protective response (95% RPS) against *P. salmonis* was elicited by formulation V1, which contains the FlgG subunit of the basal flagellar structure and chaperonins Hsp60 and Hsp70. This result suggests that the use of more than one recombinant antigen might potentiate the immune response. However, given the varying degrees of protection achieved with the different preparations, the proper selection of antigens appears as crucial for successful protection. The efficacy of formulation V1 was confirmed by three independent challenges of Atlantic salmon, eliciting relative percent survivals of 94%, 88% and 91% (data not shown). This high level of protection in Atlantic salmon is particularly important since this is the main salmon species farmed in Chile. This level of protection was also confirmed in coho salmon by an independent research effort performed by investigators from the company that licensed this technology, who obtained an RPS of 94.5% when vaccinated coho salmon were challenged with *P. salmonis* after 1000 degree days (personal communication).

Protein FlgG is a structural component of the flagella basal body [33]; a structure very similar to the needle complex of the system III involved in the secretion of virulence factors and is present in flagellated and nonflagellated pathogens. The components of the flagellar basal body are synthesized by the *flg* operon. Recently it has been reported that they play an important role in the virulence and adhesion of the pathogen *Vibrio vulnificus* [34]. The expression of FlgG in *P. salmonis* has been indirectly proven by the specific immune reaction against FlgG of a serum from a rabbit immunized with *P. salmonis*, and by the detection of transcripts encoding this protein [Wilhelm et al., manuscript in preparation]. The efficacy of vaccine V1 is also in agreement with the protective effect elicited by Hsp60 and Hsp70 in other animal models [23]. The strong immune reaction observed with our monoclonal antibodies against Hsp60 and Hsp70 in extracts from *P. salmonis* can be correlated with the abundance of these proteins during infection in other intracellular pathogens [35]. Additionally, Hsp60 and Hsp70 have been localized on the periplasm as well as on the bacterial surface or as extracellular secreted proteins during host infection [36–38]. The surface location of the Hsp proteins as well as other properties of these molecules have been suggested to play a significant role in mediating attachment, invasion of host cells and immune modulatory activities [39,40] that could explain the strong protection elicited when the Hsp are included in vaccines. It has been proposed that the high conservation of Hsp among various microbial pathogens generates an immunologic memory for Hsp cross-reactive determinants during life due to frequent restimulation by subsequent encounters with microbes [23]. Consistent with this notion, the use of Hsp in a vaccine has the advantage of eliciting an immune response to conserved determinants shared by different bacteria, preventing colonization of the host by other microbial pathogens. Although a concern could exist regarding the use of these conserved proteins in vaccines due to the risk of an autoimmune response, the observed cross recognition of host Hsp by reactive T cells has been proposed to play a role in autoimmune processes only during chronic inflammation of the host [23]. In fact, we detected no cross-reaction with Hsp of the salmon embryonic CHSE-214 cell line with the antibodies against Hsp from *P. salmonis* (not shown). Similarly, antibodies against bacterial Hsp60 raised in mice cross-react with Hsp60 homologues of other prokaryotes, but not with the murine Hsp homologues [23].

The protection elicited against *P. salmonis* by V1 is slightly better than the protection of coho salmon reported previously for a recombinant vaccine composed of the 17 kDa antigen OspA [7]. In that report, an 83% efficacy was obtained when a quimeric protein of OspA and two T cell epitopes (TCE's) from tetanus toxin and measles virus was used, while recombinant OspA alone had a protective effect with a RPS of 30.2%. The increased efficacy of the OspA recombinant vaccine conferred by these TCE's, demonstrates for the first time the immunostimulatory effect of mammalian TCE's on the salmon immune system. One of the advantages of including Hsp in the vaccine described in this paper is the natural adjuvant effect of Hsp60 by mediating a Th1 type immune response [40] and thus favouring a cellular immune response without the need for additional TCE's. Moreover, the continuous stimulation of the immune response by cross-reactive determinants among the conserved bacterial Hsp may be an additional benefit of this vaccine because it might generate a

more lasting immunologic memory. Although long-term protection was not measured in adult fish in this study, reactive antibodies against the recombinant proteins present in formulation V1 were detected in sera of fish tested at 8 months post-vaccination. Additional analysis of cellular immune response should be considered to further explain the protective effect of the vaccine against this intracellular pathogen, which will be the focus of future research. In this regard, a protective role of LPS and some *E. coli* antigenic proteins present in these partially purified recombinant mixtures should not be ruled out. Indeed, an efficacious induction of cellular immunity has been demonstrated in the differentiation of helper T cells to a Th1 phenotype by *E. coli* LPS in vivo [41]. This evidence and the demonstration that salmon macrophages infected with *P. salmonis* express immune genes corresponding to those expressed by LPS stimulated human macrophages [42] strongly argues for a positive protective effect due to the inclusion of *E. coli* LPS in the recombinant vaccine reported here.

Formulation V2, which consists of the membrane transglycosylase and transferrin binding protein B accomplished a strong protection eliciting 85% RPS. The protective potential of these two proteins is coincident with their immunoreactivity in *Salmo salar* [31] and their capacity to induce a bactericidal activity [27,43]. Although only TbpB expression was analyzed, both proteins are significant determinants of virulence and are highly expressed by bacteria during infection [44,45]. An increase in the expression of bacterial transferrin receptors is thought to play a critical role in the infection process allowing the bacterium to compete effectively with its host for limiting iron. In addition, it has recently been shown an increased synthesis of transferrin in salmon macrophages infected with *P. salmonis*. This phenomenon has been proposed as an alternative mechanism of defence against intracellular pathogens [42]. *P. salmonis* may respond by an increased synthesis of transferrin receptors to overcome the lack of iron. These data suggest that this family of receptors is one of the most promising vaccine candidates against many pathogens [27,43,46].

Although the *E. coli* thioredoxin fusion peptide may have caused an immune reaction in mice and salmon, this protein is apparently not involved in the protection elicited by the formulations V1 and V2, because formulation V3 (that had a minor protective effect against the pathogen) also contained thioredoxin as a fusion peptide to the FlaA or Omp27 proteins. OmpA and FlaA were chosen as antigens in formulation V3 due to their high immunogenicity in mice (not shown). The lack of protection of formulation V3 in fish could be explained by the fact that only partial fragments of protein Omp27 and flagellin A were included in this mixture. Also, neither of these two proteins has been detected in *P. salmonis* with the monoclonal antibodies developed here. Our failure to detect expression of these proteins could be due to the culture conditions that may affect the expression of these antigens in vitro. In this regard, it is interesting to comment that although the pathogen has been considered non-motile, more than 37 flagellar genes exist in our genomic data [Wilhelm et al., manuscript in preparation]. Detection of transcripts encoding for some of the subunits of the flagellar structure suggests the possibility that flagella may be synthesized under certain conditions. This may influence transmission from one fish to another during the short extra-cellular stage of *P. salmonis* in seawater.

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