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

Vivian Wilhelm [a], Alvaro Miquel [b], Luis O. Burzio [a,b,d], Mario Rosemblatt [a,d], Esteban Engel [a], Sebastián Valenzuela [a], Gustavo Parada [c], Pablo D.T. Valenzuela [a,d,e,*]

[a] *Fundación Ciencia para la Vida and Instituto Milenio de Biología Fundamental y Aplicada, Zañartu 1482, Santiago, Chile*
[b] *BiosChile IGSA, Zañartu 1482, Santiago, Chile*
[c] *Fundación Chile, Av. Parque Antonio Rabat Sur 6165, Vitacura, Santiago, Chile*
[d] *Universidad Andrés Bello, Zañartu 1482, Santiago, Chile*
[e] *Pontificia Universidad Católica de Chile, Zañartu 1482, Santiago, Chile*

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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

* Corresponding author. Tel.: +56 2 239 8969; fax: +56 2 237 2259.
E-mail address: pvalenzu@bionova.cl (P.D.T. Valenzuela).

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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