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ARTICLE

# Production and immune response of recombinant Hsp60 and Hsp70 from the salmon pathogen *Piscirickettsia salmonis*

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Dirección para Correspondencia

## ABSTRACT

We have isolated and sequenced the genes encoding the heat shock proteins 60 (Hsp60) and 70 (Hsp70) of the salmon pathogen *Piscirickettsia salmonis*. The sequence analysis revealed the expected two open reading frames that encode proteins with calculated molecular weights of 60,060 and 70,400. The proteins exhibit a 70-80% homology with other known prokaryotic Hsp60 and Hsp70 sequences. The coding regions have been expressed in *E. coli* as thioredoxin fusion proteins. Both recombinant proteins were shown to elicit a humoral response when injected intraperitoneally in Atlantic salmon and also conferred protection to fish challenged with *P. salmonis*. The present data will facilitate further studies on the involvement of heat shock proteins in protective immunity of fish to infection by *P. salmonis* and their potential use in recombinants vaccines against this intracellular pathogen.

**Key terms:** *Piscirickettsia salmonis*, Hsp60, Hsp70, *groEL*, *dnaK*, immune response

## INTRODUCCION

The obligate intracellular bacterium *Piscirickettsia salmonis* is the etiological agent of salmonid rickettsial septicemia (SRS), a disease responsible for extensive mortality in the Chilean salmon industry (Branson and Nieto, 1991; Fryer et al., 1990; Lannan and Fryer, 1993; Fryer and Mauel, 1997). This pathogen has also been identified in the Northern hemisphere (House et al., 1999; Brocklebank et al., 1993; Jones et al., 1998).

Recently, SRS has been partially controlled through the use of antibiotics, although they are not fully effective and have problems derived from their toxicity and generation of resistance. Vaccines have proven to work well in preventing infection. However, no effective vaccines are currently commercially available against *P. salmonis*. This pathogen grows very slowly in cell lines; it takes 15 to 20 days to develop a full cytopathic effect, making it difficult to process large quantities of cells and to separate them from host material.

In our search for potential vaccine candidates we have started the identification and study of *P. salmonis* genes coding for proteins with potential application in the production of an effective vaccine to control SRS. In addition to the membrane bound transglycosylase and transferrin binding protein (Wilhelm et al., 2004), we have directed our efforts toward the *P. salmonis* heat shock protein genes. It is well known that pathogen-derived Hsp are targets used by the host immune response to control infection. The Hsp serve as important antigens inducing very strong cellular and humoral immune responses (Kaufman, 1990, 1991; Kaufman et al., 1990). Immune responses to Hsp have been observed in infectious diseases caused by bacteria, protozoa, fungi and nematodes (Zügel and Kaufman, 1999).

We report here on the isolation, sequencing, and expression of the *P. salmonis* Hsp60 (*groEL*) and Hsp70 (*dnaK*) genes and the ability of the recombinant proteins in raising an antibody response in salmon.

## MATERIAL AND METHODS

*1. Cell cultures.*

Inoculates of the Chinook salmon embryo cell line CHSE-214 (ATCC 1681) maintained in liquid nitrogen were thawed, pelleted, resuspended and cultured for 7 days or until confluence in 60 ml of 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. Bacterial strains and plasmids.*

*E. coli strains* NovaBlue and BL21 (DE3), used for cloning and expression respectively, were obtained from Novagen.

Inoculates of *P. salmonis* LF-89 (ATCC VR1361), each containing approximately 1 x $10^8$ bacteria/ml, were brought to room temperature, added to flasks containing salmon cells, and incubated overnight at 16 C. The medium was then replaced by 50 ml of 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 or propagation when

almost 100% of the cells were cytopathic. Cells adhering to the flask walls were scraped, centrifuged twice at 150 xg, and the second supernatant was collected as the semi-purified fraction of *P. salmonis.* Further purification was performed according to Miquel et al., (2003).

The plasmids pET32a (Novagen), pET21a and pGEMT (Promega) were propagated in NovaBlue cells in medium Luria Broth (LB) with 100 µg/µl ampicillin at 37°C. *E. coli* BL21(DE3) cells transformed by pET32a or pET21a were grown in LB with 100 µg/ml ampicillin.

*3. Cloning of the Hsp60 and Hsp70 coding regions.*

Genomic DNA was extracted from the purified fraction of *P. salmonis* using Binder's procedure (1995) as described previously (Wilhelm et al., 2003). Coding regions of Hsp60 and Hsp70 were isolated by PCR amplification using specific primers based on information deduced from the sequence of a genomic library obtained in our laboratory (Valenzuela et al., unpublished results). The sequence of the primers is shown in Table I.

Amplified products were purified employing the Qiaquick PCR purification kit (Qiagen), ligated to pGEMT at 14°C in the presence of T4 DNA ligase and used to transform NovaBlue competent cells. Positive clones were selected by blue/white screening using lacZ α-complementation.

TABLE I

Oligonucleotides used as primers

| Primer | Sequence |
|---|---|
| p1 | 5'-atggagatataagaatg-3' |
| p2 | 5'-ccgcccatgccacccat-3' |
| p3 | 5'-atggctgaaattattggtat-3' |
| p4 | 5'-aacttcttcaaactcagcatc-3' |
| p5 | 5'-gacggatccggagatataagaatgtcagca-3' |
| p6 | 5'-tatgaattcttaaccgcccatgccacccat-3' |
| p7 | 5'-tatgaattcatggctgaaattattggtattg-3' |
| p8 | 5'-gtactcgagctaaacttcttcaaactcagcatc-3' |

*4. DNA sequencing.*

The Hsp60 and Hsp70 coding sequences were obtained employing Sanger's dideoxy procedure (1977) using M13 primers. Products were separated by capillary

electrophoresis in an ABI 310 Genetic Analyzer (Applied Biosystem Inc.). The *P. salmonis* Hsp60 and Hsp70 coding sequences are registered in the Gene Bank data base under accession numbers AY686756 and AY686757 respectively.

*5. Protein production in* E. coli*.*

The Hsp60 and Hsp70 coding regions previously cloned in pGEM-T were sub-cloned in pET32a or pET21a. The plasmids of the recombinant clones were used to transform *E. coli* BL21(DE3) competent cells. Expression of recombinants was induced by incubation in LB with 1 mM IPTG. Recombinant proteins were purified by a Ni-agarose column (Qiagen). Protein concentration was measured using the Micro BCA kit (Pierce). Protein analysis was performed in PAGE-SDS gels according to Laemmli (1970).

*6. Monoclonal antibody production.*

Two-month-old female BALB/c mice were injected intraperitoneally three times at 3-week intervals with 50 µg of recombinant proteins Hsp60 or Hsp70 diluted in PBS and emulsified with Freund adjuvant. Ten days after the final injection, the animals were bled from the tail to obtain serum. The humoral response against the recombinant proteins was determined by an ELISA according to Jamett et al., (2001). To produce hybridoma, spleen cells from the immunized mice with the highest titer against the recombinant proteins were isolated and fused with NS0/2 mouse myeloma cells according to the general procedure used by Köhler and Milstein (1975) with minor modifications as described previously (Jamett et al., 2001).

*7. Immunization of salmon.*

A group of 104 *Salmo salar* with an average weight of 18 grams were tagged for group identification and injected intraperitoneally with 200 µl of an oil-water emulsion containing 10 µg of each recombinant Hsp60 and Hsp70. The fish were maintained at 13°C under controlled conditions of oxygenation, feeding, and water flow in 4 tanks. Non-immunized control fish were maintained in the same tanks. Serum from some of the treated and control fish was obtained 3 months after vaccination, and the immune response against the recombinant proteins was analyzed by ELISA.

*8. Challenge.*

After 625 degree days, a tank containing 13 vaccinated fish, 13 adjuvant injected fish, and 13 control non-vaccinated fish was challenged with a dose of *P. salmonis* equivalent to 2XLD50. Mortality of fish was registered periodically and dead fish were analyzed to confirm that SRS was the cause of mortality. LD50 was previously determined by analyzing cumulative mortality of intraperitonally injected fish with serial dilution of *P. salmonis*. The effectiveness of the vaccine was determined by the relative percent survival (RPS), which was calculated as follows: RPS = [1- (% mortality of test group ÷ % mortality of control group)] x 100%.

*9. Western blot analysis.*

Recombinant Hsp60 and Hsp70 or whole-cell protein obtained from *P. salmonis* were separated by PAGE-SDS gel electrophoresis, transferred to nitrocellulose (Towbin et al., 1979) and analyzed with a dilution of anti-*P. salmonis* rabbit polyclonal serum,

specific monoclonal antibodies or with a 1:200 dilution of serum obtained from immunized salmon. The blots were developed with an anti-mouse IgG conjugated to alkaline phosphatase, or an anti-rabbit IgG conjugated to alkaline phosphatase. Incubation with a monoclonal antibody against salmon IgM was included in the analysis of salmon serum.

*10. ELISA.*

ELISA assays were carried out according to Aguayo et al. (2002) with minor modifications, using recombinant Hsp60 and Hsp70 as antigens and dilutions of serum from immunized salmon. Incubation with an anti-salmon IgM monoclonal was included before developing the reaction with anti-mouse IgG conjugated to alkaline phosphatase.

## RESULTS AND DISCUSSION

*1. Isolation of Hsp60 and Hsp70 coding regions of* P. salmonis.

Our laboratory has sequenced approximately 90% of the *P. salmonis* genome. The sequences of the contigs has allowed us to identify about 1,500 genes that are being arranged in a circular genome of about $2x10^6$ base pairs. Genes corresponding to heat shock proteins Hsp60 and Hsp70 were identified by comparison to sequences of genomes of phylogenetically related organisms such as *Pseudoalteromonas haloplankitis* for Hsp60 and *Legionella pneumophila* for Hsp70. Figure 1 shows that the region coding for Hsp60 is found in contigs 291 (1,072 base pairs) and 750 (566 base pairs). Contig 291 includes from the 5' end up to nucleotide 1004 and contig 750 comprises the coding region for the carboxyl end of the protein, from nucleotide 1070 to nucleotide 1,636.

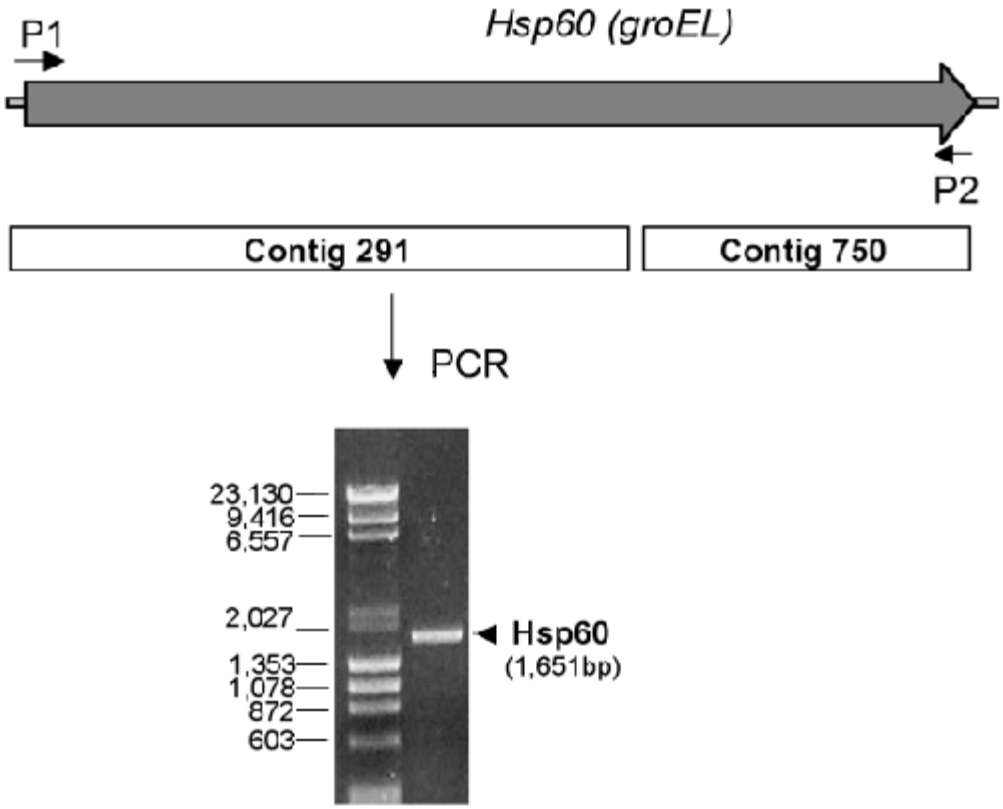


**Figure 1.** Isolation of the *P. salmonis* Hsp60 coding region by PCR from genomic *P. salmonis* DNA. The DNA MW ladder is a mixture of Hind III-digested Lambda DNA and Hae III digested ØX174 DNA.

This was confirmed by amplifying a fragment of 1,651 base pairs (Fig. 1) with primers p1 and p2 (Table I). This fragment was cloned into pGEM-T and sequenced. An open reading frame of 1,638 base pairs codifies a protein of 546 amino acids (Fig. 2) corresponding to a protein of 60,060 daltons. A high degree of homology was found when comparing this protein with Hsp60 of other microorganisms (Fig. 2). Comparative analysis of Hsp60 of *P. salmonis* with that of phylogenetically-related microorganisms shows that the first
lacks a sequence of about 5 amino acids in the carboxyl end, due probably to the incomplete data available of the genomic library.

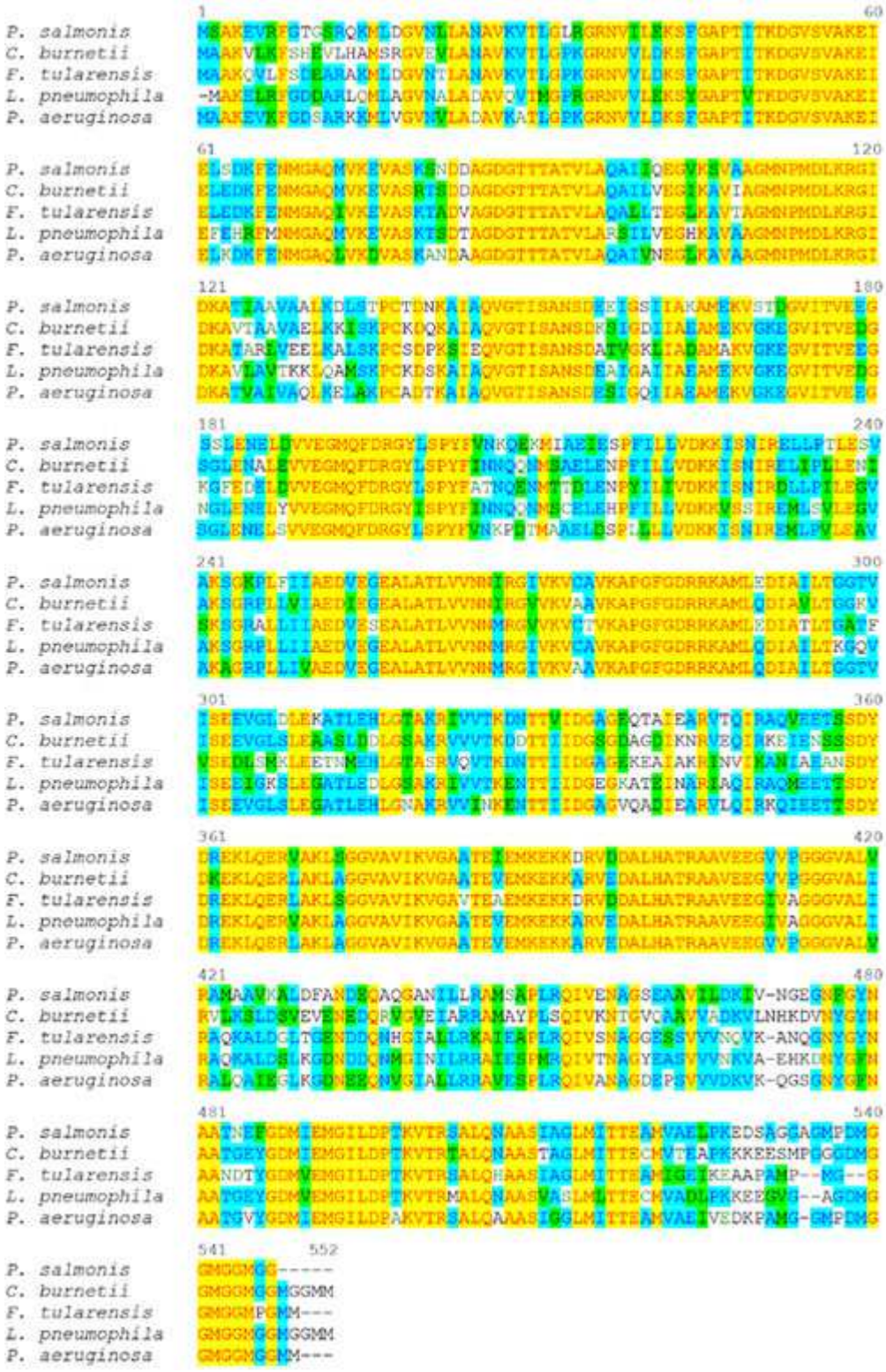


**Figure 2.** Protein sequence comparison of *P. salmonis* Hsp60 with the corresponding proteins from other bacteria. Multi-alignment was developed by the Vector NTI Suite 6 program.

Approximately 70% of the region coding for Hsp70 was found in contigs 2130 (595 base pairs), 1687 (619 base pairs) and 1748 (581 base pairs) based on an alignment with *Legionella pneumophila* (Fig. 3). The Hsp70 gene was amplified using specific primers designed based on the 5' and 3' end sequences of the gene. Primer p3 (Table I) contains the sequence of the first 20 nucleotides of the gene found in contig 2130. The 3' end was not found in the genomic sequence; as a consequence, primer p4 (Table I) was designed using a consensus sequence based on a highly conserved domain in phylogenetically-related species (see Fig. 4). PCR amplification resulted in a fragment of 1920 base pairs, which corresponds to the size of this gene in other microorganisms (Fig. 3). The sequence of the 1920 base pairs fragment codifies a 70,400 dalton protein of 640 amino acids (Fig. 4). The protein shows a high degree of homology with Hsp70 of other microorganisms.

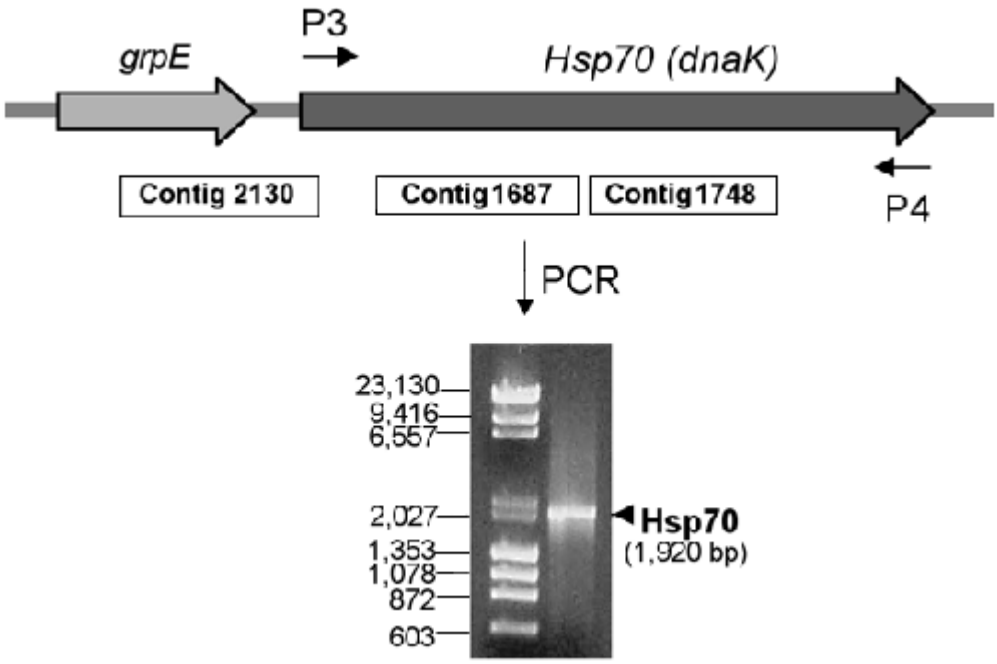


**Figure 3.** Isolation of the *P. salmonis* Hsp70 coding region by PCR from genomic *P. salmonis* DNA. The DNA MW ladder is a mixture of Hind III digested Lambda DNA and Hae III digested ØX174 DNA.

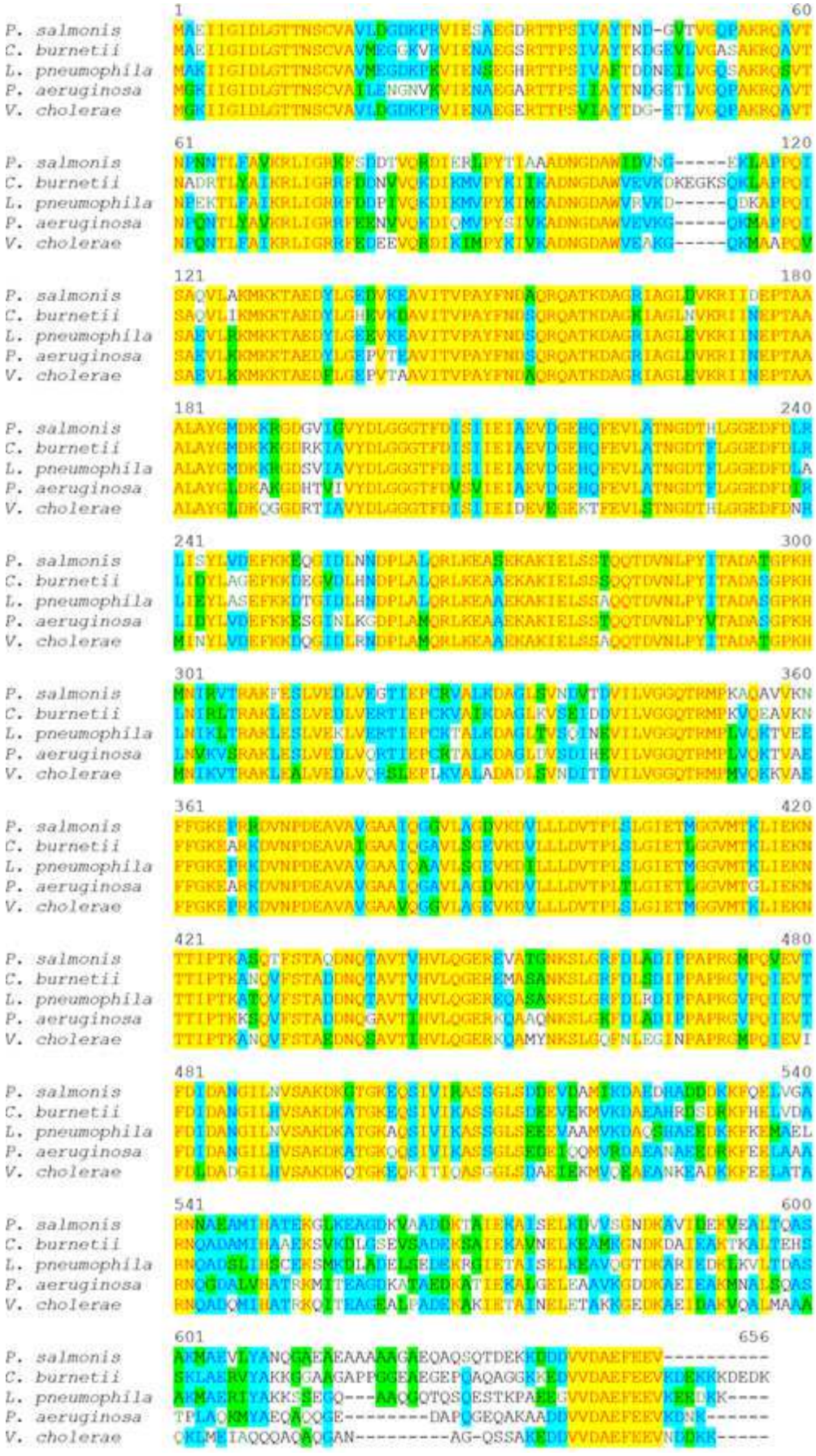
1 60
P. salmonis MAEIIGIDLGTTNSCVAVLDGDKPRVIESAEGDRTTPSIVAYTND-GVTVGQPAKRQAVT
C. burnetii MAEIIGIDLGTTNSCVAVMEGGKVRVIENAEGSRTTPSIVAYTKDGEVLVGASAKRQAVT
L. pneumophila MAKIIGIDLGTTNSCVAVMEGDKPKVIENSEGHRTTPSIVAFTDDNEILVGQSAKRQSVT
P. aeruginosa MGKIIGIDLGTTNSCVAILEGNGNVKVIENAEGARTTPSIIAYTNDGETLVGQPAKRQAVT
V. cholerae MGKIIGIDLGTTNSCVAVLDGDKPRVIENAEGERTTPSVIAYTDG-ETLVGQPAKRQAVT
61 120
P. salmonis NPNNTLFAVKRLIGRKFSDDTVQRDIERLPYTIAAADNGDAWLDVNG-----EKLAPPQI
C. burnetii NADRTLYAIKRLIGRRFDDNVVQKDIKMVPYKIIKADNGDAWVEVKDKEGKSQKLAPPQI
L. pneumophila NPEKTLFAIKRLIGRRFDDPIVQKDIKMVPYKIMKADNGDAWVEVKD-----QDKAPPQI
P. aeruginosa NPQNTLYAVKRLIGRRFEENVVQKDIQMVPYSIVKADNGDAWVEVKG-----QKMAPPQI
V. cholerae NPQNTLFAIKRLIGRRFEDEEVQRDIKIMPYKIVKADNGDAWVEAKG-----QKMAAPQV
121 180
P. salmonis SAQVLAKMKKTAEDYLGEDVKEAVITVPAYFNDAQRQATKDAGRIAGLDVKRIIDEPTAA
C. burnetii SAQVLIKMKKTAEDYLGHEVKDAVITVPAYFNDSQRQATKDAGKIAGLNVKRIINEPTAA
L. pneumophila SAEVLRKMKKTAEDYLGEEVKEAVITVPAYFNDSQRQATKDAGRIAGLEVKRIINEPTAA
P. aeruginosa SAEVLKKMKKTAEDYLGEPVTEAVITVPAYFNDSQRQATKDAGRIAGLDVKRIINEPTAA
V. cholerae SAEVLKKMKKTAEDFLGEPVTAAVITVPAYFNDAQRQATKDAGRIAGLEVKRIINEPTAA
181 240
P. salmonis ALAYGMDKKRGDGVIGVYDLGGGTFDISIIEIAEVDGEHQFEVLATNGDTHLGGEDFDLR
C. burnetii ALAYGMDKKRGDRKIAVYDLGGGTFDISIIEIAEVDGEHQFEVLATNGDTFLGGEDFDLR
L. pneumophila ALAYGMDKKRGDSVIAVYDLGGGTFDISIIEIAEVDGEHQFEVLATNGDTFLGGEDFDLA
P. aeruginosa ALAYGLDKAKGDHTVIVYDLGGGTFDVSVIEIAEVDGEHQFEVLATNGDTELGGEDFDIR
V. cholerae ALAYGLDKQGGDRTIAVYDLGGGTFDISIIEIDEVEGEKTFEVLSTNGDTHLGGEDFDNR
241 300
P. salmonis LISYLVDEFKKEQGIDLNNDPLALQRLKEAAEKAKIELSSTQQTDVNLPYITADATGPKH
C. burnetii LIDYLAGEFKKDEGVDLHNDPLALQRLKEAAEKAKIELSSSQQTDVNLPYITADASGPKH
L. pneumophila LIEYLASEFFKDTGIDLHNDPLALQRLKEAAEKAKIELSSAQQTDVNLPYITADASGPKH
P. aeruginosa LIDYLVDEFKKESGINLKGDPLAMQRLKEAAEKAKIELSSTQQTDVNLPYVTADASGPKH
V. cholerae MINYLVDEFKKDQGIDLRNDPLAMQRLKEAAEKAKIELSSAQQTDVNLPYITADATGPKH
301 360
P. salmonis MNIRVTRAKFESLVEDLVERTIEPCRVALKDAGLSVNDVTDVILVGGQTRMPKAQAVVKN
C. burnetii LNIRLTRAKLESLVEDLVERTIEPCKVALKDAGLKVSEIDDVILVGGQTRMPKVQEAVKN
L. pneumophila LNIKLTRAKLESLVEKLVERTIEPCKTALKDAGLTVSQINEVILVGGQTRMPLVQKTVEE
P. aeruginosa LNVKVSRAKLESLVEDLVQRTIEPCRTALKDAGLDVSDIHEVILVGGQTRMPLVQKTVAE
V. cholerae MNIKVTRAKLEALVEDLVQRSLEPLKVALADADLSVNDITDVILVGGQTRMPMVQKKVAE
361 420
P. salmonis FFGKEPRKDVNPDEAVAVGAAIQGGVLAGDVKDVLLLDVTPLSLGIETMGGVMTKLIEKN
C. burnetii FFGKEARKDVNPDEAVAIGAAIQGAVLSGEVKDVLLLDVTPLSLGIETLGGVMTKLIEKN
L. pneumophila FFGKEPRKDVNPDEAVAVGAAIQAAVLSGEVKDILLLDVTPLSLGIETMGGVMTKLIEKN
P. aeruginosa FFGKEARKDVNPDEAVAVGAAIQGAVLAGDVKDVLLLDVTPLTLGIETLGGVMTGLIEKN
V. cholerae FFGKEPRKDVNPDEAVAVGAAVQGGVLAGEVKDVLLLDVTPLSLGIETMGGVMTKLIEKN
421 480
P. salmonis TTIPTKASQTFSTAQDNQTAVTVHVLQGEREVATGNKSLGRFDLAGIPPAPRGMPQVEVT
C. burnetii TTIPTKANQVFSTADDNQTAVTVHVLQGEREMASANKSLGRFDLSDIPPAPRGVPQIEVT
L. pneumophila TTIPTKATQVFSTADDNQTAVTVHVLQGEREQASANKSLGRFDLRDIPPAPKGVPQIEVT
P. aeruginosa TTIPTKKSQVFSTADDNQGAVTIHVLQGERKQAAQNKSLGKFDLADIPPAPRGVPQIEVT
V. cholerae TTIPTKANQVFSTAEDNQSAVTIHVLQGERKQAMYNKSLGQFNLEGINPAPRGMPQIEVI
481 540
P. salmonis FDIDANGILNVSAKDKGTGKEQSIVIRASSGLSDDEVDAMIKDAEDHADDDKKFQELVGA
C. burnetii FDIDANGILHVSAKDKATGKEQSIVIKASSGLSDEEVEKMVKDAEAHRDSDRKFHELVDA
L. pneumophila FDIDANGILNVSAKDKATGKAQSIVIKASSGLSEEEVAAMVKDAQSHAEEDKKFKEMAEL
P. aeruginosa FDIDANGILHVSAKDKATGKQQSIVIKASSGLSEDETQQMVRDAEANAEADRKFEELAAA
V. cholerae FDIDADGILHVSAKDKQTGKEQKITIQASGGLSDAEIEKMVQEAEANKEADKKFEELATA
541 600
P. salmonis RNNAEAMIHATEKGLKEAGDKVAADDKTAIEKAISELKDVLSGNDKAVIDEKVEALTQAS
C. burnetii RNQADAMIHAAEKSVKDLGSEVSADEKSAIEKAVNELKEAMKGNDKDAIEAKTKALTEHS
L. pneumophila RNQADSLIHSCEKSMKDLADELSEDEKRQIETAISELKEAVQGTDKARIEDKLKVLTDAS
P. aeruginosa RNQGDALVHATRKMTEAGDKATAEDKATIEKAIGELEAAVKGDDKAEIEAKMNALSQAS
V. cholerae RNQADQMIHATRKQTEAGEALPADEKAKIETAINELETAKKGEDKAEIDAKVQALMAAA
601 656
P. salmonis AKMAEVLYANQGAEAEAAAAAGAEQAQSQTDEKKDDVVDAEFEEV----------
C. burnetii SKLAERVYAKKGAAGAPPGGEAEGEPQAQAGGKKEDVVDAEFEEVKDEKKKDEDK
L. pneumophila AKMAERIYAKKSEGQ---AAQGQTQSQESTKPAEEGVVDAEFEEVKEELKK----
P. aeruginosa TPLAQKMYAEQAQQGE---------DAPQGEQAKAADDVVDAEFEEVKDNK------
V. cholerae QKLMEIAQQQAQAQGAN---------AG-QSSAKEDDVVDAEFEEVNDDKK-----

**Figure 4.** Protein sequence comparison of *P. salmonis* Hsp70 with the corresponding proteins from other bacteria. Multi-alignment was developed by the Vector NTI Suite 6 program.

*2. Synthesis and characterization of* P. salmonis *Hsp60 and Hsp70.*

In order to characterize Hsp60 and Hsp70 from *P. salmonis* and study their immunogenic properties we proceeded to express in *E. coli* the corresponding recombinant proteins using vector pET32a, in which the cloned genes are expressed as a fusion protein with *E. coli* thioredoxin. For this purpose, the Hsp60 coding region was amplified with primers complementary to the ends of the gene (primers 5 and 6 in Table I) containing the restriction sites BamHI and EcoRI. Primer p6 also contained an extra nucleotide to complete the glycine codon inferred from the consensus sequence for Hsp60 (Fig. 2) and a stop codon. The amplified fragment that codifies an Hsp60 protein 5 amino acids shorter at the carboxyl end in comparison with its homologue in phylogenetically-related species (Fig. 2) was cloned into pGEM-T and digested with EcoRI and BamH I. The fragment released was ligated to vector pET32a, which was previously digested with the same restriction enzymes. Positive clones were identified by enzymatic digestion. BL21(DE3) cells were transformed with the recombinant vector pET32a-Hsp60 to express the recombinant protein. After a 3-hour induction the recombinant protein was analyzed in a 12.5%PAGE-SDS gel. The protein of the expected MW (80 KDa) is found in both, the insoluble and the soluble fractions (Fig. 5). When necessary, the insoluble fraction was solubilized in 4M urea and the recombinant Hsp60 purified in an affinity column with Ni-agarose resin under denaturating conditions (Fig. 5).

The procedure followed for the gene encoding Hsp70 is in essence the same one used for Hsp60. The Hsp70 coding region was amplified from the genome of *P. salmonis* with primers 7 and 8 (Table I),complementary to the ends of the gene and containing the restriction sites EcoRI and XhoI respectively and a stop codon. The amplified fragment was cloned into pGEM-T and later subcloned in pET32a. *E. coli* BL21 (DE3) cells transformed with pET32a-Hsp70 were induced to express the recombinant protein for 3 hours, and the recombinant protein was analyzed in a 12.5 PAGE-SDS gel. The protein is primarily found in the soluble protein fraction. The recombinant protein was purified using a nickel-agarose resin to approximately 85% of purity (Fig. 5).

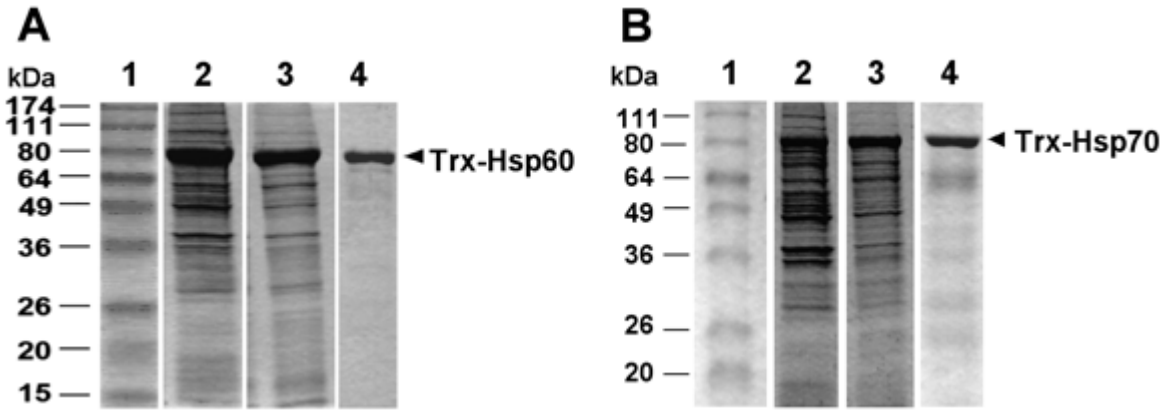


**Figure 5.** SDS-PAGE analysis of the expression in *E. coli* and purification of *P. salmonis* Hsp60 and Hsp70 fusion proteins.

A: Lane 1 : Molecular weight markers. Lane 2: 10 µg of insoluble protein fraction of recombinant BL21(DE3)/ pET32-Hsp60 cells after 3 hours of induction with IPTG. Lane 3: 10 µg of soluble protein fractions of recombinant BL21(DE3)/pET32-Hsp60 cells after 3 hours of induction with IPTG. Lane 4: Purified Trx-Hsp60 recombinant protein after Ni-agarosa purification.

B: Lane 1 : Molecular weight markers. Lane 2: 10 µg of insoluble protein fraction of recombinant BL21(DE3)/pET32-Hsp70 cells after 3 hours of induction with IPTG. Lane 3: 10 µg of soluble protein fraction of recombinant BL21(DE3)/pET32-Hsp70 cells after 3 hours of induction with IPTG. Lane 4: Purified Trx-Hsp70 recombinant protein after Ni-agarosa purification.

*3. Detection of Hsp60 and Hsp70 in P. salmonis cultured in cell lines.*

A good vaccine candidate is expected to be expressed during host infection by the pathogen. In an attempt to determine whether the genes coding for Hsp60 and Hsp70 were expressed during the replication of *P. salmonis* inside the host cells, CHSE cells infected with *P. salmonis* were cultured until a 90% cytopathic effect was achieved; the bacteria was then harvested as described in Methods. Whole-cell *P. salmonis* protein was analyzed by Western blot with monoclonal antibodies produced in mice against the recombinant proteins Trx-Hsp60 and Trx-Hsp70. The specificity of the monoclonal antibodies for Hsp60 and Hsp70 was previously confirmed by ELISA and Western blot against each recombinant protein respectively (data not shown). These antibodies recognized the recombinants excised from the thioredoxin fusion peptide with enterokinase, confirming their specific interaction with the target. The Western blot in Figure 6 shows the presence of Hsp60 and Hsp70 in *P. salmonis* cultured in the CHSE cell line. The detected proteins migrate according to the expected size of the native proteins. The mouse antibodies elicited by the recombinant proteins appear specific for the *P. salmonis* heat shock proteins and has no cross reactivity with the eukaryotic counterparts since the monoclonal antibodies do not react with proteins of non-infected CHSE cells (data not shown).

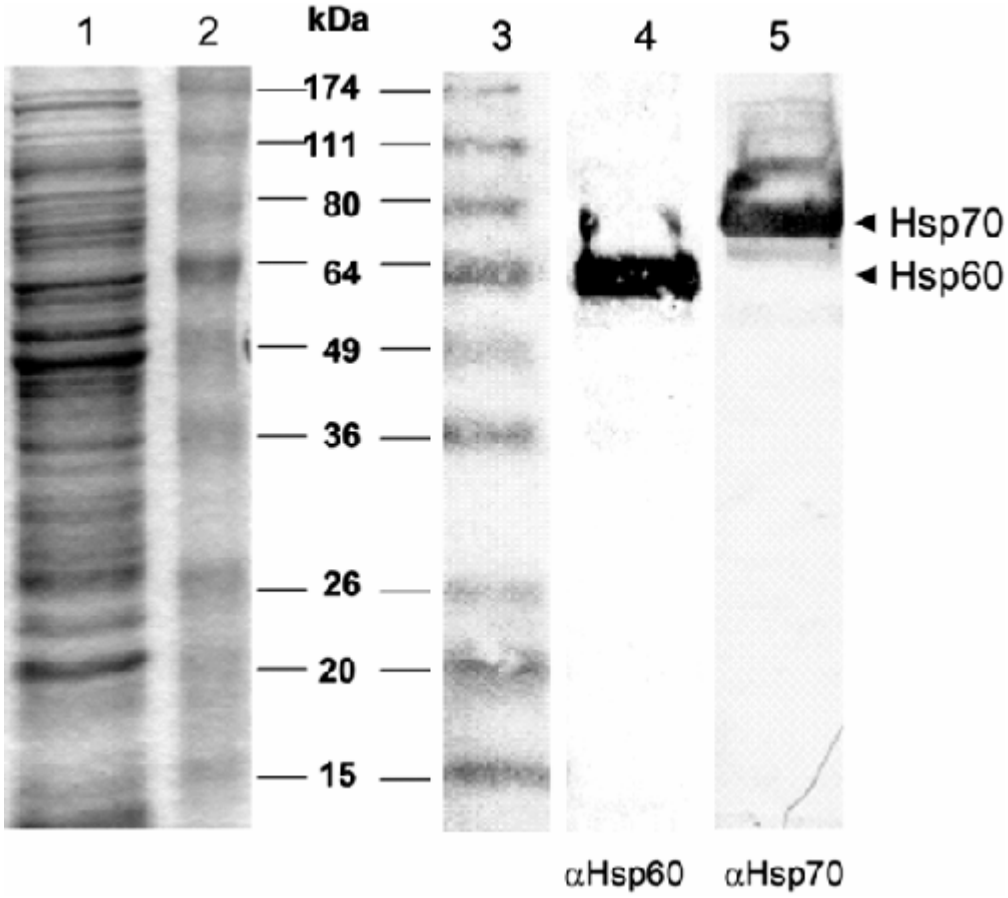


**Figure 6.** Western blot of Hsp60 and Hsp70 present in *P. salmonis.*

Gel was loaded with 30 µg of whole-cell *P. salmonis* protein and the blotted membrane was analyzed with a 1:200 dilution of each monoclonal antibody. 1: Coomasie blue staining of *P. salmonis* lysate. 2 and 3: Molecular weight markers. 4: Membrane analyzed with a monoclonal antibody against Hsp60. 5: Membrane analyzed with a monoclonal antibody against Hsp70.

The high expression of Hsp60 and Hsp70 detected in *P. salmonis* is coincident with the strong production of these proteins described for other intracellular bacteria during infection (Fernández et al, 1997). In contrast to their cytoplasmic location in most bacteria and eukaryotes, Hsp60 and Hsp70 have been detected in pathogenic bacteria in the periplasm, on bacterial surfaces, and sometimes as extracellular secreted proteins during host infection (Garduño et al., 1998; Scorpio et al., 1994; Gillis et al., 1985). No leader sequences or other recognizable motif that suggest a secretory role are present in the chaperonin proteins, raising the possibility of a unique specific transport mechanism for Hsp in these pathogens. The surface location of Hsp has been linked to a significant role in mediating attachment and invasion of host cells as well as immune modulation activities (Ensgraber and Loos, 1992; Huesca et al., 1996; Retzlaff et al., 1994; Hoffman and Garduño, 1999).

*4. Immunogenic properties of the P. salmonis Hsp60 and Hsp70.*

Immunogenic reactivity of *P. salmonis* Hsp60 and Hsp70 was studied by the Western Blot technique using a polyclonal rabbit serum developed against an extract of *P. salmonis* obtained from a cell culture. Figure 7 shows that both recombinant proteins are recognized by the antiserum against *P. salmonis*. This result confirms that native Hsp60 and Hsp70 expressed during the intracellular life cycle of *P. salmonis* are immunogenic in rabbits. We also found that serum from Atlantic salmon that had been immunized three months earlier with a formulation containing the two recombinant Hsp reacted with the fusion Trx-Hsp60 and Trx-Hsp70. This was analyzed by ELISA (Fig. 8) and Western blot (Fig. 9A). To confirm that fish serum recognized epitopes of the Hsp and was not merely reacting against the thioredoxin fusion protein, the serum was also tested by Western blot against recombinant Hsp60 and Hsp70 not fused to thioredoxin obtained by expression with vector pET21a (Fig. 9B and 9C). The recognition of both recombinants confirmed that *P. salmonis* Hsp60 and Hsp70 are immunogenic in salmon and thus are bonafide antigens to be included in an experimental vaccine to study their ability to protect against *P. salmonis* infection.

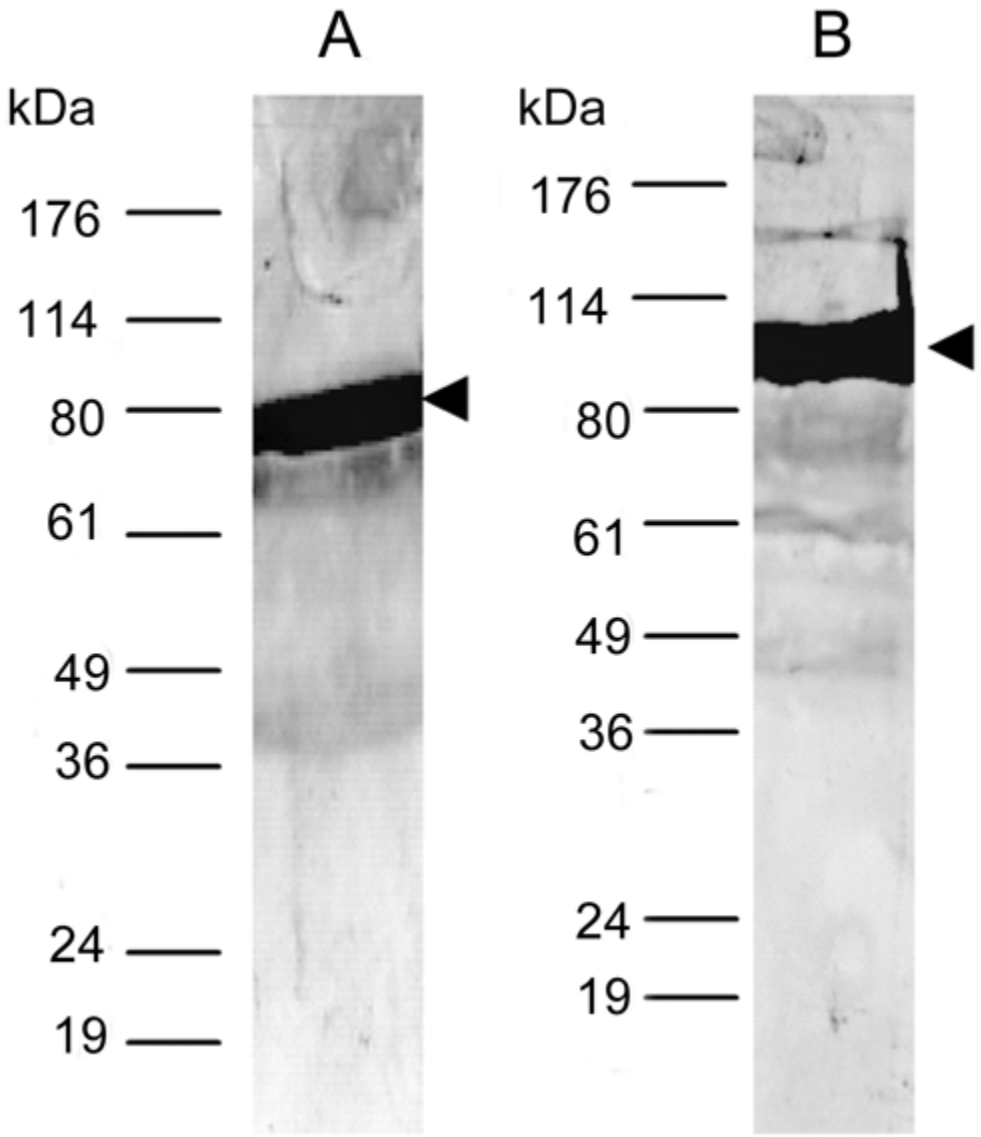


**Figure 7.** Western blot analysis of recombinant Hsp60 and Hsp70 with

anti *P. salmonis* serum.
A: Trx-Hsp60 tested with a 1:1000 dilution of *P. salmonis* polyclonal serum.
B: Trx-Hsp70 tested with a 1:1000 dilution of *P. salmonis* polyclonal serum.

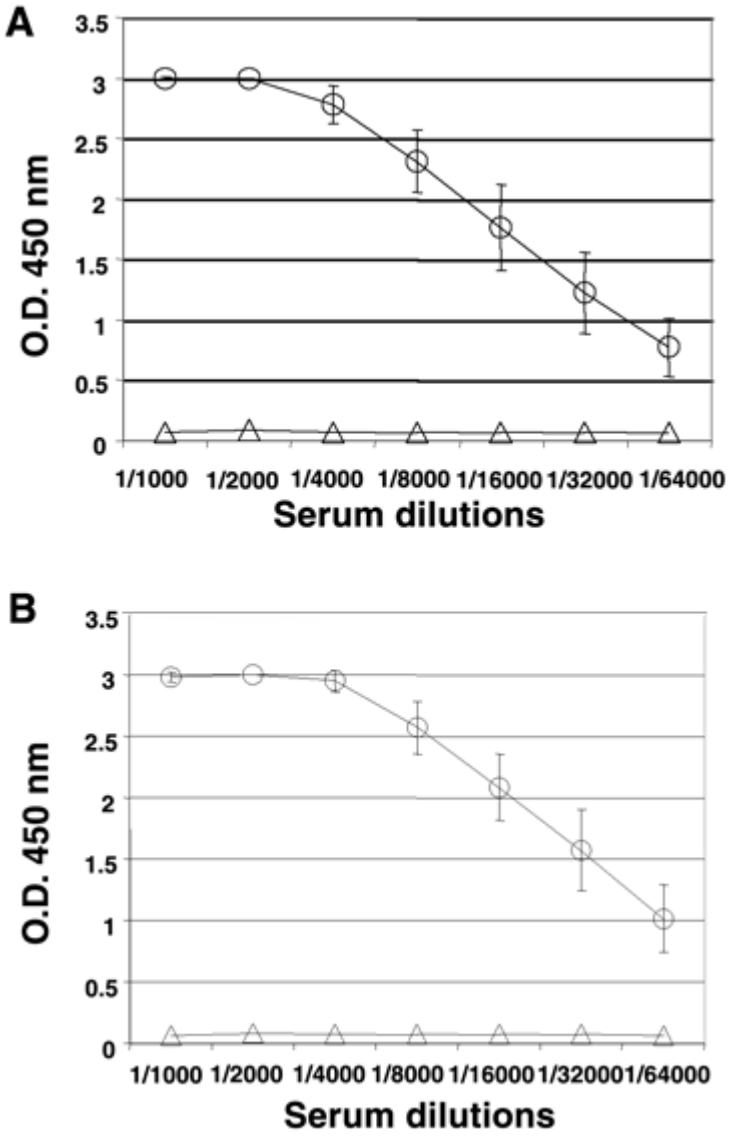


**Figure 8.** Measurement of antibodies to Trx-Hsp60 and Trx-Hsp70 in serum of salmon immunized with both recombinant proteins.
A: ELISA analysis of antibodies against Hsp60 in immunized (O) and control fish (Δ).
B: ELISA analysis of antibodies against Hsp70 in immunized (O) and control fish (Δ).

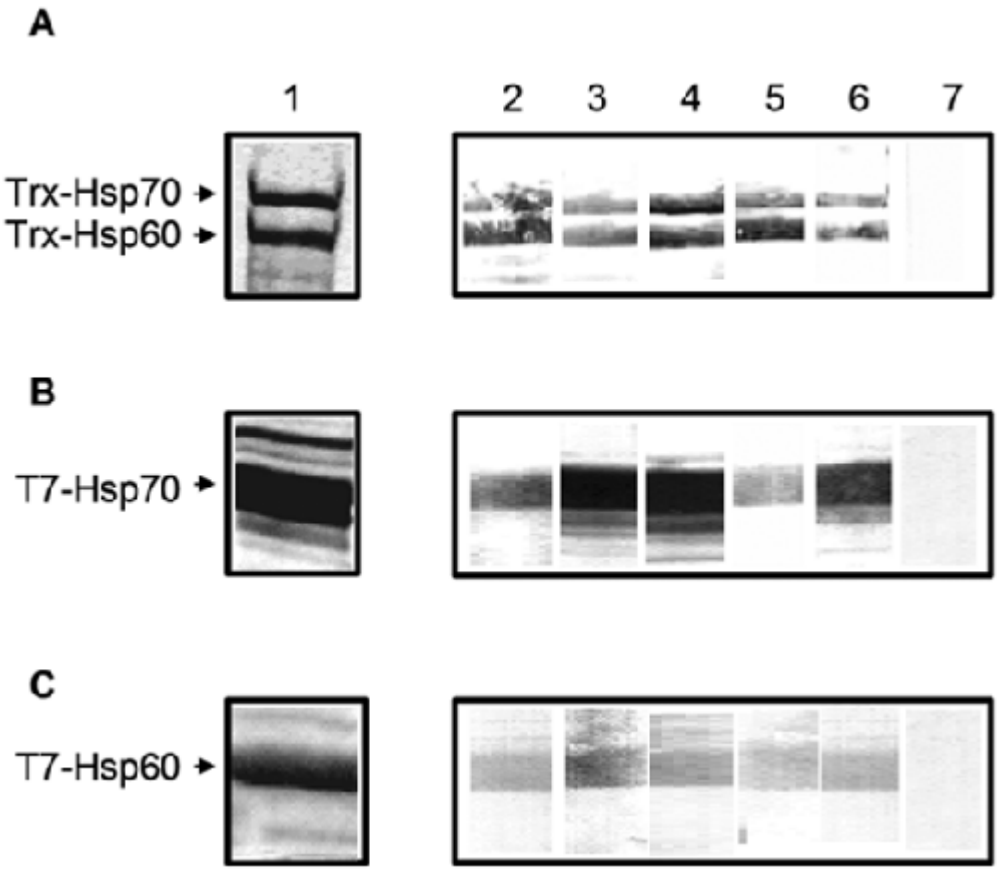


**Figure 9.** Western blot analysis of serum obtained from salmon immunized with recombinant Hsp60 and Hsp70.
A: Lane 1 : Electrophoretic pattern of a mixture of purified Trx-Hsp60 and Trx-Hsp70; Lanes 2-6: blots tested with serum of immunized fish; Lane 7: blot tested with serum of non-immunized fish.
B: Lane 1 : Electrophoretic pattern of whole-cell extract containing recombinant T7-Hsp70; Lanes 2-6: blots tested with serum of immunized fish; Lane 7: blot tested with serum of non-immunized fish.
C: Lane 1 : Electrophoretic pattern of whole-cell extract containing recombinant T7-Hsp60; Lanes 2-6: blots tested with serum of immunized fish; Lane 7: blot tested with serum of non-immunized fish.

*5. Protection against* P. salmonis *in vaccinated Atlantic salmon.*

One tank containing vaccinated and control non-injected fish was challenged with *P.salmonis* 625 degree days after immunization with the formulation that contained recombinant Hsp60 and Hsp70. A group of adjuvantinjected fish was also included in the trial as control. At day 21 after *P. salmonis* infection, control fish started to die and cumulative mortality increased dramatically during the next 20 days. In contrast, a reduced number of vaccinated fish died during this period, achieving a relative percent survival (RPS) of 89.6% at the end of the challenge (Table II). The strong protective response elicited by the recombinant formulation contrast with the weak protection that has been described for bacterins against *P. salmonis* (Smith et al., 1997) and is coincident with the protective effect elicited by Hsp60 and Hsp70 in other animal models (Ferrero et al., 1995; Gómez et al., 1995). In conclusion our data strongly

supports the use of Hsp60 and Hsp70 as antigens in a recombinant vaccine against the intracellular pathogen *Piscirickettsia salmonis.*

TABLE II

Protective response of immunized salmon to *P. salmonis*

| Group | Challenge | | | |
|---|---|---|---|---|
| | 30 days | | 50 days | |
| | Mortality | RPS | Mortality | RPS |
| V1 | 8% | 82.6 | 8% | 89.6 |
| V0 | 58% | -26 | 69% | 10.4 |
| Control | 46% | | 77% | |

| | | |
|---|---|---|
| RPS | = | Relative percent survival |
| Control | = | Non-injected fish |
| V0 | = | Adjuvant-injected fish |
| V1 | = | Fish injected with recombinant proteins |

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