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Cloning and expression of the coding regions of the heat shock proteins HSP10 and HSP16 from *Piscirickettsia salmonis*

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ABSTRACT

The genes encoding the heat shock proteins HSP10 and HSP16 of the salmon pathogen *Piscirickettsia salmonis* have been isolated and sequenced. The HSP10 coding sequence is located in an open reading frame of 291 base pairs encoding 96 aminoacids. The HSP16 coding region was isolated as a 471 base pair fragment encoding a protein of 156 aminoacids. The deduced aminoacid sequences of both proteins show a significant homology to the respective protein from other prokaryotic organisms.
Both proteins were expressed in *E. coli* as fusion proteins with thioredoxin and purified by chromatography on Ni-column. A rabbit serum against *P. salmonis* total proteins reacts with the recombinant HSP10 and HSP16 proteins. Similar reactivity was determined by ELISA using serum from salmon infected with *P. salmonis*. The possibility of formulating a vaccine containing these two proteins is discussed.

INTRODUCTION

Piscirickettsia salmonis is an obligate intracellular pathogen that causes the salmonid rickettsial septicemia (SRS) (Branson and Nieto Díaz-Muñoz, 1991; Lannan and Fryer, 1993; Fryer and Mauel, 1997). This disease causes high mortality in salmonids impacting heavily the salmoniculture industry in Chile, where this bacterium was originally isolated. *P. salmonis* has also been identified in Norway (House et al., 1999), Scotland and Canada (Brocklebank et al., 1993; Jones et al., 1998). Until now prospects for the control of SRS caused by *P. salmonis* remain bleak. No antibiotic or chemotherapeutic agent has proven to be consistently effective and despite many attempts, development of an effective vaccine has not been achieved (Smith et al., 1997).

In our laboratory we have started the isolation and study of *P. salmonis* genes with potential application in the development of a vaccine to prevent SRS (Valenzuela et al., 2001). We have initially concentrated our efforts in the pathogen heat shock protein (HSP) genes (Soza et.al. 2001). It is known that pathogen-derived HSPs 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, 1999a and 1999b).

We report here the cloning and expression of the *P. salmonis* HSP10 and HSP16 genes. In addition, we show that the recombinant proteins immunoreact with a rabbit anti-*P. salmonis* serum and with sera obtained from salmon infected with this pathogen.

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MATERIALS 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 T175 flasks at 16°C with 60 ml of MEM (Gibco BRL) supplemented with nonessential aminoacids, glutamine and 5% fetal bovine serum (Gibco BRL).

2. Bacterial strains and plasmids

E. coli NovaBlue and Origami B (DE3) pLys-S strains, used for cloning and expression respectively, were obtained from Novagen.

P. salmonis inoculates of LF-89 (ATCC VR 1361) each containing about 1 x 10^8 bacteria/ml were brought to room temperature, added to flasks containing salmon CHSE-214 cells and incubated overnight at 16ºC. Then the medium was replaced by 50 ml of fresh MEM supplemented with non-essential aminoacids, 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 more than 90% of the cells show cytopathic effect. Cells adhered to the flask walls were scraped, centrifuged twice at 200 x g at 10°C, the second supernatant being the semi purified fraction of *P. salmonis*. Further purification was performed according to Jamett *et al.* (2001).

The plasmids pET32a (Novagen) and pGEMT (Promega) were propagated in NovaBlue cells in medium LB with 100 µg/ml ampicillin at 37°C. Origami B (DE3) pLys-S cells transformed by pET32a were grown in LB with 100 µg/ml of ampicillin and 34 µg/ml of chloramphenicol, as described before (Tischler et al., 2003).

3. Cloning of the HSP10 and HSP16 coding regions

Genomic DNA was extracted from the purified fraction of *P. salmonis* by the method of Binder (1995) as described previously (Wilhelm et al., 2003). Coding regions of HSP10 and HSP16 were cloned by PCR using specific primers based on information obtained from a genomic library preliminary draft recently sequenced in our laboratory and sequences of corresponding genes of phylogenetically related microorganisms.

Amplified products were purified using a commercial kit (Qiagen) and ligated to vector pGEM-T using T4 DNA ligase. NovaBlue *E. coli* competent cells were transformed with the recombinant vector. Positive clones were selected by blue/white screening using α-complementation of lacZ.

4. DNA sequencing

The HSP10 and HSP16 coding segments were sequenced by PCR according to Sanger (1977) using M13 primers. For sequencing, the Big Dye Terminator Cycle Sequencing kit (Applied Biosystem) was employed. Products were analyzed using capillary electrophoresis in an ABI Prism 310 Genetic Analyzer (Applied Biosystems Inc.).

5. Protein expression in E. coli

The HSP10 and HSP16 genes were amplified using PCR with specific primers containing restriction sites at the 5' ends for cloning into vector pET32a. Recombinant vectors propagated in NovaBlue *E. coli* cells were used to transform Origami B (DE3) pLys-S competent cells. Expression of recombinants was induced by culture in LB with IPTG 1 mM at 37°C. Recombinant proteins were purified by a Ni-agarose column (Qiagen) under native conditions as indicated by the manufacturer. The solution used for column washing was 50 mM Tris-HCl (pH 8.0), 0,3 M NaCl, 60 mM imidazole. For protein elution, the concentration of imidazole was raised to 1 M. Protein concentration was

measured using Micro BCA kit (Pierce). Protein analysis was performed in PAGE-SDS gels according to Laemmli (1970).

6. Western blot analysis

Purified recombinant HSP10 and HSP16 were separated by PAGE-SDS gel electrophoresis and transferred to nitrocellulose (Towbin et al., 1979). The membranes were incubated with a given dilution of a rabbit serum against *P. salmonis* total proteins, and developed with an anti-rabbit IgG conjugated to alkaline phosphatase.

7. ELISA with salmon serum

ELISA assays were carried out as described earlier (Aguayo et al., 2002), using recombinant HSP10 and HSP16 as antigens and dilutions from salmon serum which survived a challenge with *P. salmonis.*

RESULTS AND DISCUSSION

1. Heat-shock proteins and bacterial infectious

Bacterial HSP are abundantly produced during the course of most bacterial infectious and have been recognized for years as immunodominant antigens of many microbial pathogens (Zügel and Kaufman, 1999b). Several studies have shown that HSP are located in the periplasm and on the bacterial surface and have been linked to bacterial pathogenesis playing a significant role in mediating attachment to and invasion of the host cell (Garduño et al., 1998; Hoffman and Garduño, 1999).

We hypothesize that *P. salmonis* HSPs may play also an important role in the attachment and invasion of the salmon mucosal tissue by the pathogen and thus they might be considered as bonafide antigens in the development of experimental vaccines against SRS.

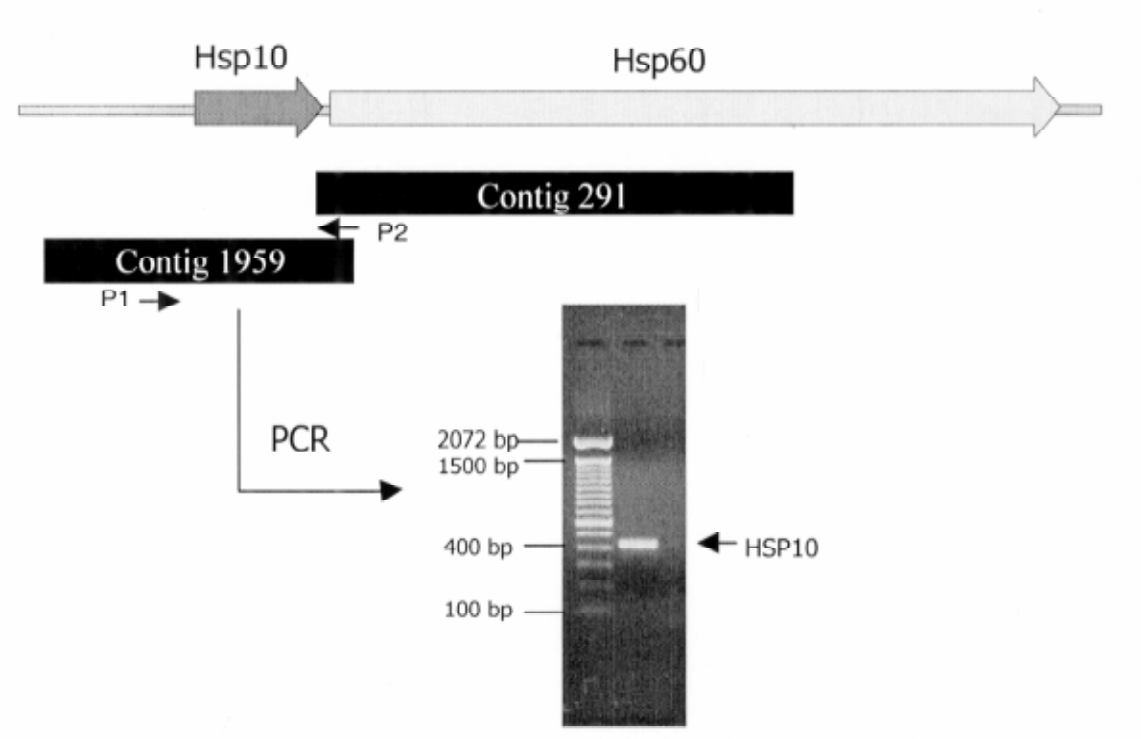

Figure 1. Isolation of the *P. salmonis* HSP10 coding region by PCR from total *P. salmonis* DNA.

2. Isolation of HSP10 and HSP16 coding regions of P. salmonis

Nearly 95% of the genome of *P. salmonis* has been sequenced in our laboratory (unpublished results). From the sequences of the contigs, we identified about 1,500 genes that are being arranged in a circular genome of about $2x10^6$ base pairs. Genes corresponding to heat shock proteins HSP10 and HSP16 were found among them. Figure 1 shows that the region coding for HSP10 is found in contig 1959 (702 base pairs). This contig has an overlap sequence of twelve nucleotides with contig 291, which encodes the amino terminal region of HSP60 (Wilhelm et al., in preparation). These two genes are found adjacent in the genome of phylogenetically related organisms, such as *Pseudoalteromonas haloplankitis* as shown in Figure 1. The short overlapping sequence in the contigs is insufficient to conclude that genes HSP10 and HSP60 are contiguous in *P. salmonis* genome. Nonetheless a 400bp fragment obtained by PCR with primers P1 and P2, which were designed based on the sequence of contigs 1959 and 291 respectively (Table 1) and flank the overlap region of both contigs, confirms the colocalization of HSP10 and HSP60 and permits the isolation of the complete open reading frame coding HSP10. Figure 2 shows that the region coding for HSP16 is found in contig 1168 (of 1,782 base pairs). Based on the sequence of this contig, we designed primers P3 and P4 (Table 1) which codify nucleotides –11 to +7 and nucleotides 150 to 170 of the 3' end of gene for HSP16 respectively. Using these primers, a fragment of 481 base pairs was amplified by PCR from *P. salmonis* genomic DNA.

3. Sequence analysis of the P. salmonis *HSP10 and HSP16 proteins*

The 400 base pairs fragment encoding HSP10 was purified and cloned into vector pGEM-T. *E. coli* clones containing the recombinant vector were identified by digestion with restriction enzymes. Positive clones were sequenced using M13 primers. Results indicate that the isolated gene codifies for a 96-amino acid protein with an approximate molecular weight of 10,560. The derived amino acid sequence of this protein shows a high degree of conservation with other microorganisms (Figure 3).

The sequence of the HSP16 coding region was obtained from the 481 base pairs fragment mentioned above. This fragment was purified and cloned into vector pGEM-T (Promega). *E. coli* clones containing the recombinant vector were identified by digestion with restriction enzymes PstI and MluI whose restriction sites were incorporated into the primers used. Positive clones were sequenced using M13 primers (forward and reverse) and the sequence of an ORF of 471 base pairs was obtained. The isolated gene codifies for a 156 amino acid protein with an estimated molecular weight of 17,160. The derived amino acid sequence of this protein shows a high degree of conservation amongst other microorganisms (Figure 4).

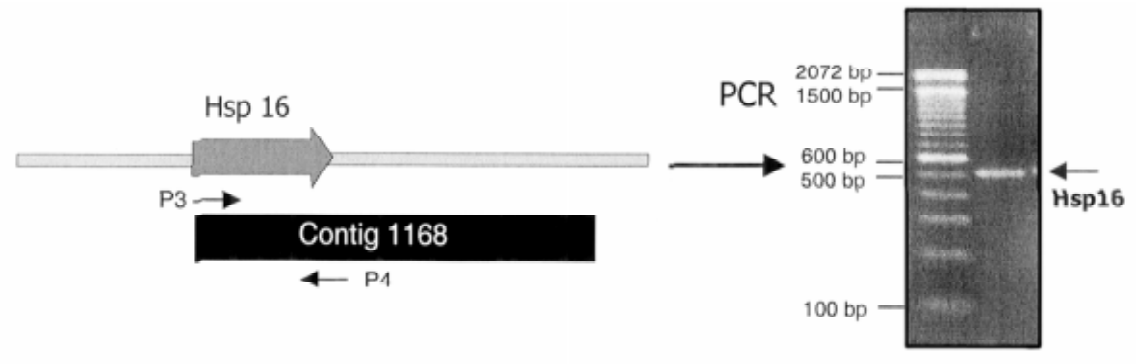

Figure 2. Isolation of the *P. salmonis* HSP16 coding region by PCR from total *P. salmonis* DNA.

TABLE I

Oligonucleotide sequence of the primers used for PCR

P1	:	5'-CAGTCCAATTAATCAAGGCGA-3'
P2	:	5'-GCTGACATTCTTATATCTCCA-3'
P3	:	5'-CCGCTGCAGGAGTAATTCATATGAGTCA-3'
P4	:	5'-CCCACGCGTCTATGCCATTTTTTTATCTAC-3'
P5	:	5'-GGCGAATTCATGAAAATCCGTCCATTACAT-3'
P6	:	5'-CGGCTCGAGGAATTAATCTTCAACGACTGC-3'
P7	:	5'-CGCGATATCATGAGTCACTTTAATTTATCCC-3'
P8	:	5'-GCCCTCGAGCTATGCCATTTTTTTATCTACTA-3'

4. Synthesis and characterization of recombinant P. salmonis *HSP10 and HSP16*

In order to characterize the HSP10 and HSP16 from *P. salmonis* and to study their immunogenic properties we proceeded to express in *E. coli* the corresponding recombinant proteins using expression vector pET32a. Using this vector, the genes are expressed as fusion proteins with *E. coli* thioredoxin, which allows an efficient expression of cloned genes.

For this, the HSP10 coding region was amplified from *P. salmonis* DNA with primers P5 and P6 (Table 1), which contain restriction sites for EcoRI and XhoI respectively, and cloned into vector pGEM-T. The gene was then isolated by digestion with EcoRI and XhoI and cloned into pET32a. Plasmid DNA isolated from a pET32a-HSP10 positive clone was used to transform *E. coli* Origami cells in order to express the fusion protein. After induction with IPTG, the recombinant protein obtained was analyzed in a 15% PAGE-SDS gel. We detected a strong induction of a protein of the expected size found in the soluble fraction of the bacterial extract even in the first hour of induction (Figure 5A). The protein was purified using a nickel-agarose affinity column (Figure 5B).

To express the recombinant protein Trx-HSP16, the HSP16 coding region was amplified from *P. salmonis* DNA with primers P7 and P8 (Table I), complementary to the 5' and 3' ends of the gene. They contain the restriction sites for EcoRV and XhoI required for cloning in the expression vector pET32a. To express the fusion protein, plasmid DNA from a positive pET32a-HSP16 was used to transform *E. coli* specifically designed to respond to promoter T7 with IPTG. After induction with IPTG, the recombinant protein obtained was analyzed in a 15% PAGE-SDS gel. We detected an induction in the

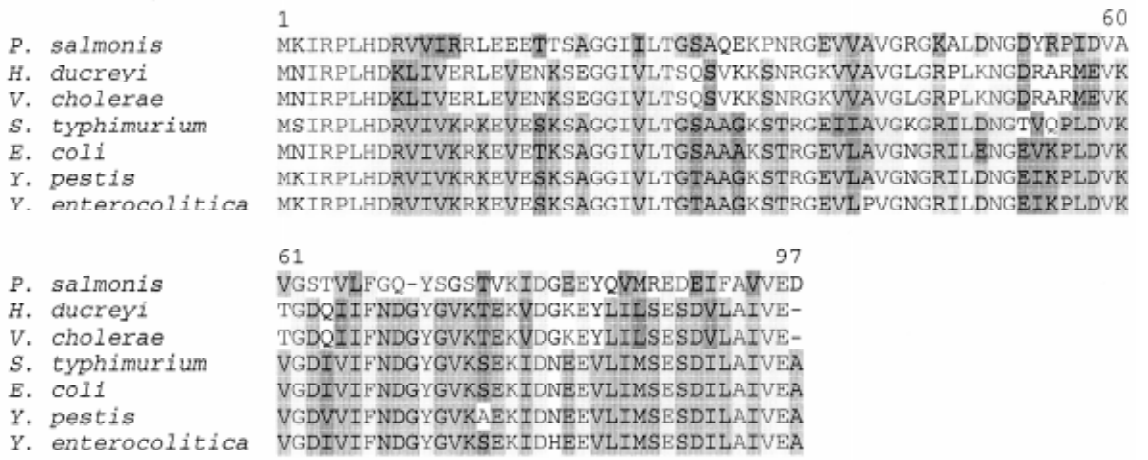

Figure 3. Protein sequence comparison of *P. salmonis* HSP10 with the corresponding proteins from other bacteria.

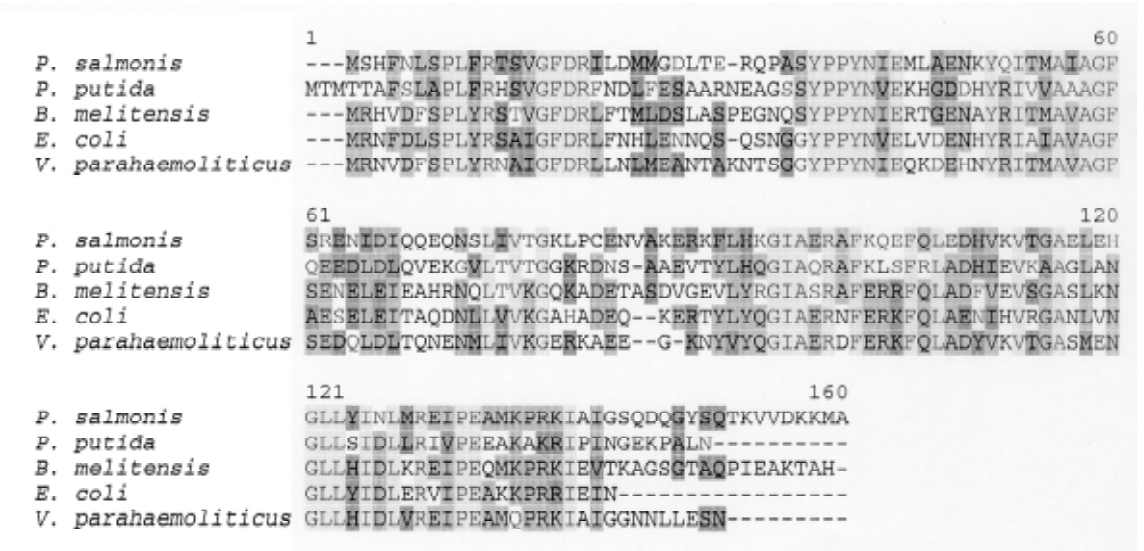

Figure 4. Protein sequence comparison of *P. salmonis* HSP16 with the corresponding proteins from other bacteria.

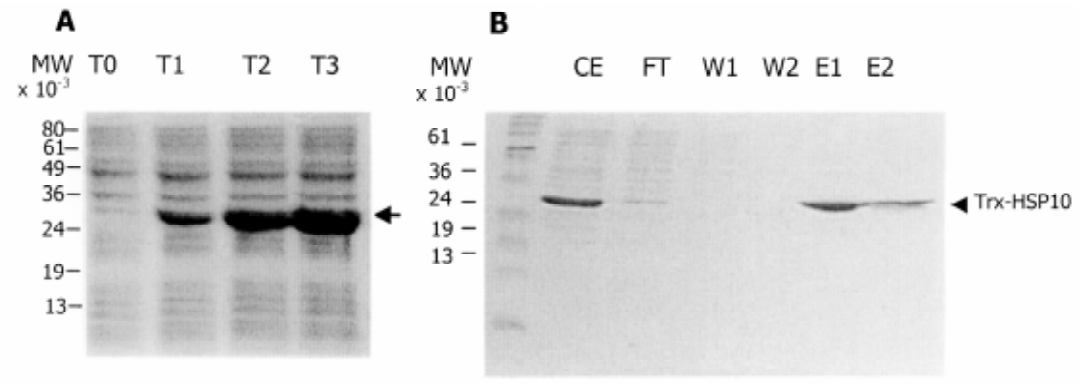

Figure 5. Expression in *E. coli* and purification of *P. salmonis* HSP10 fusion protein.
A. Gel electrophoresis showing the expression of Trx-HSP10 in *E. coli*. T0: *E. coli* extract before induction with IPTG; T1, T2, T3: *E. coli* extract after induction with IPTG for 1, 2 and 3 hours respectively.
B. Gel electrophoresis showing the purification of recombinant Trx-HSP10 by Ni-agarose column. CE: crude extract; FT: flow-trough; W1 and W2: washings; E1 and E2: protein elution with imidazole.

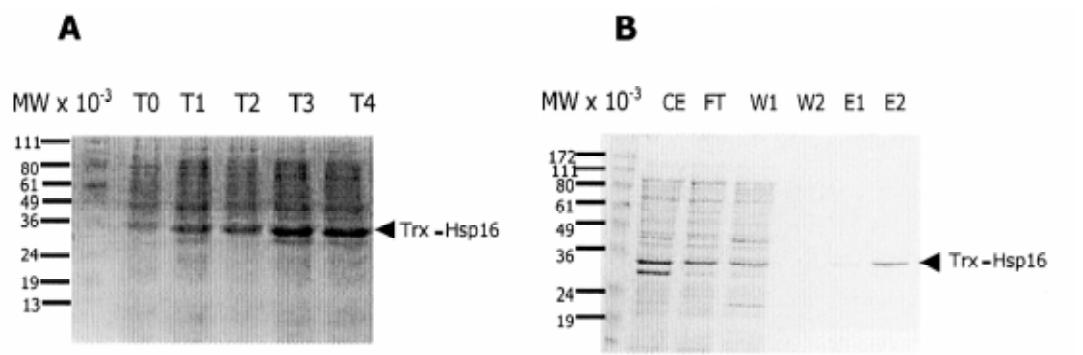

Figure 6. Expression in *E. coli* and purification of *P. salmonis* HSP16 fusion protein.
A. Gel electrophoresis showing the expression of Trx-HSP16 in *E. coli*. T0: *E. coli* extract before induction with IPTG; T1, T2, T3 and T4: *E. coli* extract after induction with IPTG for 1, 2, 3 and 4 hours respectively.
B. Gel electrophoresis showing the purification of recombinant Trx-HSP16 by Ni-agarose column. CE: crude extract; FT: flow-trough; W1 and W2: washings; E1 and E2: protein elution with imidazole.

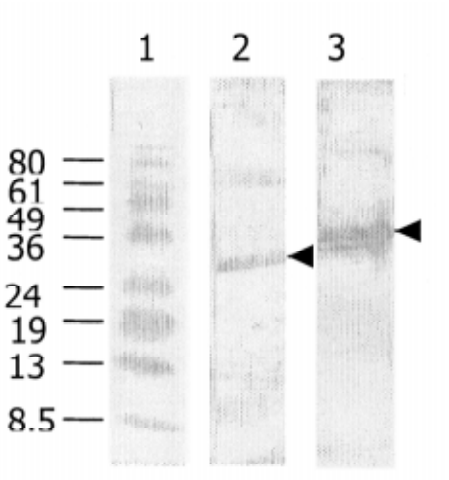

Figure 7. Western blot analysis of the reaction of recombinant HSP10 and HSP16 with anti *P. salmonis* serum.
Lane 1: molecular weight standards; lane 2: Trx-HSP10 and lane 3: Trx-HSP16.

soluble fraction of proteins even in the first hour of induction (Figure 6A). The protein was purified by affinity chromatography, using a nickel-agarose resin. The resin links to a histidine tag present in the fusion protein (Figure 6B).

5. Immunogenic properties of the P. salmonis *HSP10 and HSP16*

Immunogenic reactivity of recombinant proteins HSP10 and HSP16 was studied by the Western Blot technique using a mouse policlonal serum against an extract of *P. salmonis* obtained from a cell culture. Figure 7 shows that both recombinant proteins are recognized by the antiserum. This suggests that native HSP10 and HSP16 are expressed during the intracellular life cycle of *P. salmonis* and the immune response elicited by these native proteins also recognize the recombinant proteins. We also found that serum from coho salmon that have survived the infections by *P. salmonis* reacts with the recombinant proteins HSP10 and HSP16 in an ELISA assay (Figure 8). These results suggest that the HSP10 and HSP16 of *P. salmonis* are highly immunogenic to salmon and thus they are bonafide antigens to be included in a experimental vaccine to prevent SRS.

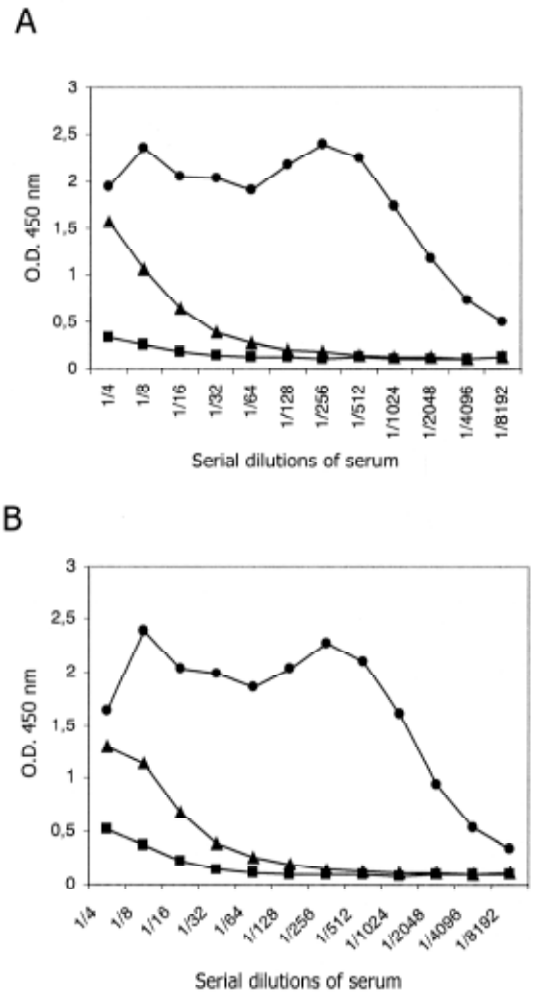

Figure 8. ELISA assay showing the reactivity of salmon serum that have survived the infections with *P. salmonis* with the recombinant HSP10 and HSP16.
A. Reactivity with HSP10. B. Reactivity with HSP16. ●:serum from salmon infected with approximately 2.500 bacteria per ml; ▲: serum from salmon infected with approximately 100 bacteria per ml; ■: serum from control non-infected salmon.

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