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ARTICLE

Isolation and expression of the genes coding for the membrane bound transglycosylase B (MltB) and the transferrin binding protein B (TbpB) of the salmon pathogen *Piscirickettsia salmonis*

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Dirección para Correspondencia

ABSTRACT

We have isolated and sequenced the genes encoding the membrane bound transglycosylase B (MltB) and the transferring binding protein B (TbpB) of the salmon pathogen *Piscirickettsia salmonis*. The results of the sequence revealed two open reading frames that encode proteins with calculated molecular weights of 38,830 and 85,140. The deduced aminoacid sequences of both proteins show a significant homology to the respective protein from phylogenetically related microorganisms. Partial sequences coding the amino and carboxyl regions of MltB and a sequence of 761 base pairs encoding the amino region of TbpB have been expressed in *E. coli*. The strong humoral response elicited by these proteins in mouse confirmed the immunogenic properties of the recombinant proteins. A similar response was elicited by both proteins when injected intraperitoneally in Atlantic salmon. The present data indicates that these proteins are good candidates to be used in formulations to study the protective immunity of salmon to infection by *P. salmonis*.

Key terms: *Piscirickettsia salmonis*, MltB, TbpB, recombinant antigens, recombinant vaccines

INTRODUCTION

Piscirickettsia salmonis is the etiological agent of salmonid rickettsial septicemia (SRS), a disease that causes high mortality in salmonids, heavily impacting the salmoniculture industry in Chile, where this bacterium was originally isolated (Branson and Nieto Díaz-Muñoz, 1991; Lannan and Fryer, 1993; Fryer and Mauel, 1997). *P. salmonis* has also been identified in Norway (House et al., 1999), Scotland and Canada (Brocklebank et al., 1993; Jones et al., 1998), although with less economical impact. To date prospects for controlling SRS caused by *P. salmonis* remain bleak. No antibiotic or chemotherapeutic agent has proven to be consistently effective, and despite many attempts, no effective vaccine is available for the control of this obligate intracellular pathogen (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 aimed to express genes encoding membrane-bound or periplasmic proteins that have previously been shown to behave as significant antigens in other microorganisms. Among these genes, an open reading frame coding for the membrane-bound transglycosylase B (MltB) and another for transferrin binding protein B (TbpB) were of particular interest because of their high immunogenical potential described in other microorganisms (Pizza et al., 2000). The transferrin binding protein is highly expressed under iron restriction condition such as during host infection where iron is not free but bound to transferrin molecules (Alexander & Ingram, 1992). Lytic transglycosylases have an important role in peptidoglycan metabolism and cell division, and are also significant determinants of pathogenicity (Höltje & Tuomanen, 1991; Adu-Bobie et al., 2004). These properties make both proteins attractive vaccine candidates against *P. salmonis.* We report here the cloning and expression of the *P. salmonis* MltB and TbpB structural genes and the immune response elicited by these recombinant proteins in salmon.

MATERIALS AND METHODS

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 nonessential aminoacids, glutamine and 5% fetal bovine serum (FBS) (GIBCO BRL) in T175 flasks at 16°C.

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 16º C. The medium was then replaced by 50 ml of fresh complete 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 nearly 100% of the cells were cytopathic. Cells that adhered to the flask walls were scraped and centrifuged twice at 150 x g at 10°C. The second supernatant contained the semi-purified fraction of *P. salmonis.* Further purification was performed according to Jamett et al., (2001).

The plasmids pET32a (Novagen) and pGEM-T (Promega) were propagated in NovaBlue cells in Luria Broth (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 as described previously (Tischler et al., 2003).

Cloning of the MltB and TbpB coding regions

Genomic DNA was extracted from the purified fraction of *P. salmonis* using the procedure of Binder (1995) as described previously (Wilhelm et al., 2003a). Coding regions of MltB and TbpB 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 using kit 28104 (Qiagen), ligated to pGEM-T, 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

p1 = 5'-atggagggtcatcaatggtg-3'
p2 = 5'-caattggttgattacctggc-3'
p3 = 5'-ccaggtaatcaaccaattgc-3'
p4 = 5'-gaacacgaataggcggcac-3'
p5 = 5'-caactcactgacctaccagg-3'
p6 = 5'-gcataaccgtgtgttcgtaag-3'
p7 = 5'-atgaaact(at)acc(at)taggctt(ag)at(ct)gg-3'
p8 = 5'-gcgctcatcgagtgaagac -3'
p9 = 5'-caaggatccatgccaaattcagggaaaacaacg-3
p10 = 5'-cttgaattcgcataaccgtgtgttcgtaac-3'
p11 = 5'-gacgaattcatgagacgatcttattggcta-3'
p12 = 5'-cagctcgagttatcactgcgccgcttataatc-3'
p13 = 5'-ctcgaattcaatgtactaacaacgttagcgt-3'
p14 = 5'-gacctcgagtattttaagagccttttgagtg-3'
p15 = 5'-cctggatccctgtctatcaatattggcggc-3'
p16 = 5'-cgtgaattcttactgtgttagtgtgcgctgtg-3'

DNA sequencing

The MltB and TbpB coding sequences were obtained by the dideoxy procedure of Sanger (1977) using M13 primers and the Big Dye Terminator Cycle Sequencing kit (Applied Biosystem). Products were separated using capillary electrophoresis in a 310 Genetic Analyzer (Applied Biosystem Inc.). The *P. salmonis* MltB and TbpB coding sequences are registered in the Gene Bank data base under accession numbers AY770981 and AY770982 respectively.

Protein production in E. coli

The MltB and TbpB coding regions were amplified by PCR with specific primers and ligated to pET32a and used to transform Novablue competent cells. The plasmids of the recombinant clones were used to transform *E. coli* BL21 (DE3) competent cells. Expression of recombinants was induced by treatment with IPTG as described previously (Wilhelm et al., 2003b). Recombinant proteins were purified by a Ni-agarose column (Qiagen) or consecutive washes with increasing concentrations of urea. Protein concentration was measured using the Micro BCA kit (Pierce). Protein analysis was performed in PAGE-SDS gels according to Laemmli (1970).

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 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 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 of Köhler and Milstein (1975), with minor modifications as described in Jamett et al., (2001).

Immunogenicity in salmon

A group of 104 Salmo salar with an average weight of 18 grams were injected intreperitoneally with 200 μl of a water in oil emulsion containing 10 μg of each recombinant MltB-N (N-terminal domain (region) of 167 aminoacids), MltB-C (C-terminal domain (region) of 207 amino acids), and TbpB-N (N-terminal domain (region) of 255 amino acids). The fish were maintained at 13º C under controlled conditions of oxygenation, feeding, and water flow. Control non-injected fish were maintained in the same tanks. Serum of 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.

ELISA of salmon serum

ELISA assays were carried out according to Aguayo et al., (2002) with minor modifications, using recombinant MltB-N, MltB-C or TbpB-N as antigens and dilutions of serum from salmon which were immunized with a formulation that contained the recombinant proteins. Incubation with a monoclonal anti-salmon IgM was included before developing the reaction with the anti-mouse IgG conjugated to alkaline phosphatase.

RESULTS AND DISCUSSION

Isolation of MltB and TbpB coding regions of P. salmonis

Approximately 90% of *P. salmonis* genome has been sequenced recently in our laboratory.

Fragments of approximately 1,000 bp were obtained from highly purified genomic bacterial DNA and cloned into the pIK96 vector. The sequencing procedure yielded approximately 20,000 readings in both directions of an average of 500 base pairs each, which were assembled into 2,143 contigs. As a result of a comparative study of the sequences present in these contigs with other bacterial genomes, more than 1,500 genes of *P.salmonis* have been identified and organized into a circular genome of approximately 2 x 10^6 base pairs. Among these genes, an open reading frame coding for the membrane-bound transglycosylase B (MltB) and another for transferrin binding protein B (TbpB) were selected because of their high immunogenical potential described in other microorganisms (Pizza et al., 2000). The transferrin binding protein B, which is highly expressed during host infection to capture the iron bound to transferrin molecules has been shown to have protective properties against different microorganisms (Webb & Cripps, 1999; McMichael 2000; West et al, 2001). Lytic transglycosylases are abundant antigens present on the bacterial wall, where they play an important role in cell structure and division, and by the release of highly bioactive cell wall subcomponents, they have a major impact on the course of infection *in vivo* (Höltje & Tuomanen, 1991; Adu-Bobie et al., 2004). These antigenic and immunogenic properties make both proteins attractive vaccine candidates against *P. salmonis.*

An open reading frame coding the first 848 bp of the MltB gene was located in contig 741 and amplified with specific primers designed based on the contig sequence (primers p1 and p2 of Table I). According to the alignments with other phylogenic related microorganisms, a 276 bp fragment coding for the carboxyl terminus of the MltB protein was missing in this sequence and not present in the other contigs obtained from the genomic library of *P. salmonis* (Fig. 1). In an effort to obtain this missing segment, a comparison of genes flanking MltB in the genomes of related bacteria was performed. Genes coding for a rare lipoprotein A (*rlpA*) and a DD-carboxypeptidase penicillin-binding protein (*dacA)* were found in several *Aeromonas* downstream of the MltB gene (Stover et al., 2000). The orf coding *dacA* was localized in contig 541 of our *P. salmonis* genome data as shown in Figure 1. Assuming that this organization was also present in *P. salmonis*, a sense primer coding the nucleotides 730-749 of MltB (primer p3) and the antisense primer coding the 5' sequence of gene *dacA* (primer p4) were used. The expected amplification of a 1,200 bp fragment confirmed the positioning of gene *dacA* downstream of the MltB gene in the *P. salmonis* genome (Fig. 1).

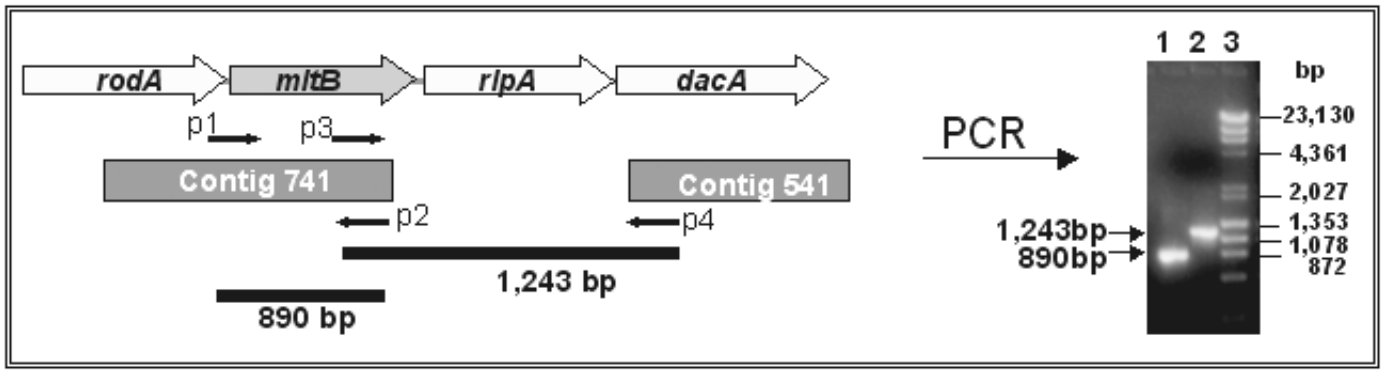


Figure 1. Isolation of the *P. salmonis* MltB coding region by PCR from total *P. salmonis* DNA.
1: PCR product from primers p1 & p2;
2: PCR products from primers p3 & p4;
3: MW standards.

The isolation of the gene coding the other outer membrane-bound protein, TpbB, was achieved

based on the information obtained by aligning the *P. salmonis* and the *Pseudomonas aeruginosa* genes. As seen in Figure 2, the partial sequences coding the amino and carboxyl regions of TbpB were located in contigs 1953 and 653 respectively. However, there is a gap of approximately 680 bp between them in the *P. salmonis* database. A sequence of 120 bp at the 5'end is also missing in the *P. salmonis* database. Primers p5 and p6 were designed according to the 5' end of contig 1953 and the 3'end sequence of the TbpB gene present in contig 653. They were used in a PCR reaction, obtaining an amplified fragment of 2,244 bp which corresponds to most of the TbpB coding sequence. Nevertheless, 120 bp were still missing at the 5' end of the gene. In order to isolate the entire coding region, a degenerate primer (primer p7 in Table I) was designed according to a highly conserved domain present at the 5'end of the gene in related microorganisms. A PCR performed with primer p7 and an internal primer approximately 219 bp downstream of the 5' end (primer p8) successfully amplified the expected 219 bp fragment containing the missing sequence.

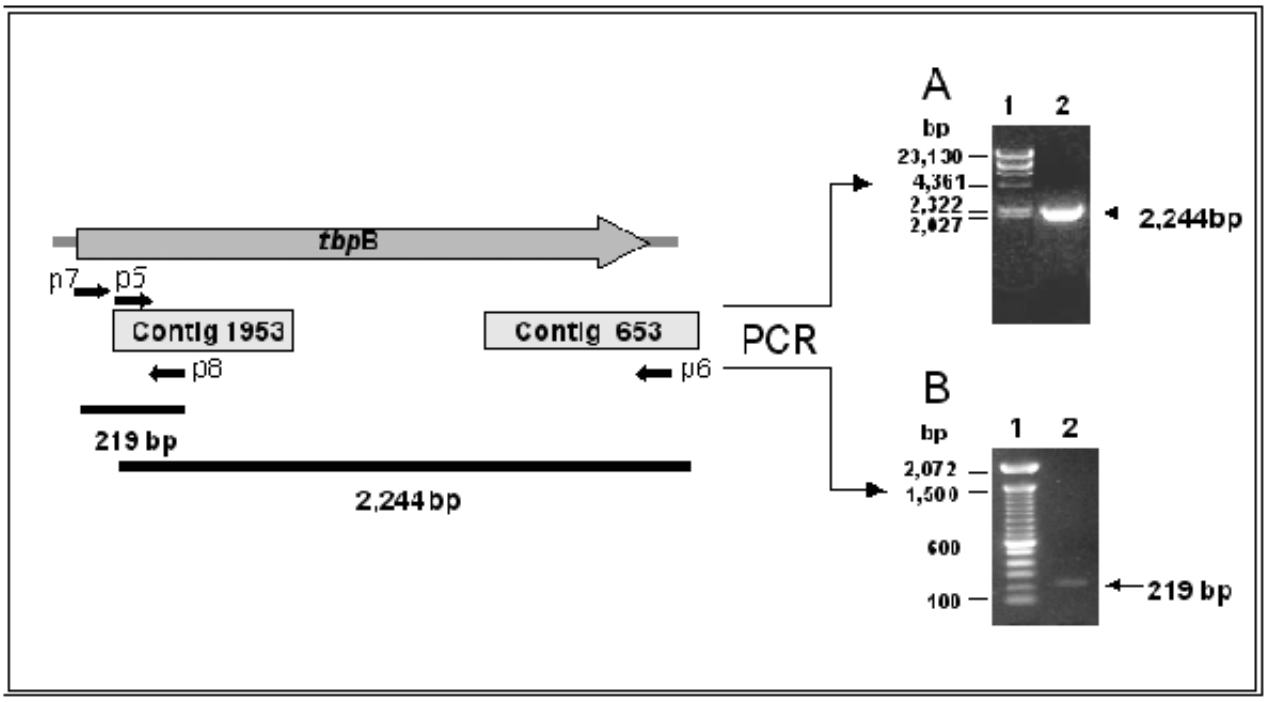


Figure 2. Isolation of the *P. salmonis* TbpB coding region by PCR from total *P. salmonis* DNA.
A: PCR product from p5 & p6; B: PCR products from p7 & p8.

Sequencing of the P. salmonis *MltB and TbpB genes.*

The fragment containing the first 848 bp of the Mlt structural gene was cloned in pGEM- T and sequenced. In parallel we also cloned the 1,200 bp fragment, which contains the last 276 bp encoding the carboxyl termini of the MltB protein. The sequence of these two amplified fragments allowed the identification of the complete *MltB* open reading frame, which is 1,062 bp long. The 353 codons of this gene predict a protein of 38,830 Daltons. As seen in the alignment (Fig. 3), *P. salmonis* MltB conserves several domains described for MltB of other γ-proteobacteria. The most important conservation is found in the region spanning from amino acids 193 to 246 that aligns

with residues 216-268 of *E. coli*. In *E. coli* this region forms the core domain described as a deep groove involved in peptidoglycan binding and is where the conserved catalytic residues Glu162, Ser216 and Asn339 are positioned. Crystallographic studies done on Slt35, which corresponds to the proteolytic product of MltB (Ehlert et al., 1995), have demonstrated the presence of an EF - hand calcium-binding fold at the core region and the aminoacids involved in this metal binding motif are also conserved in the *P. salmonis* MltB sequence (Van Asselt & Dijkstra, 1999; Van Asselt et al., 2000). The sequencing also confirmed that *rlpA* is positioned in between genes *MltB* and *dacA* in the *P. salmonis* genome, confirming the conservation of this cluster of genes along with *Pseudomonas aeruginosa* (Stover et al., 2000). The cluster which also includes *rodA* upstream of *MltB* is involved in cell shape determination and peptidoglycan metabolism (Spratt et al., 1980).

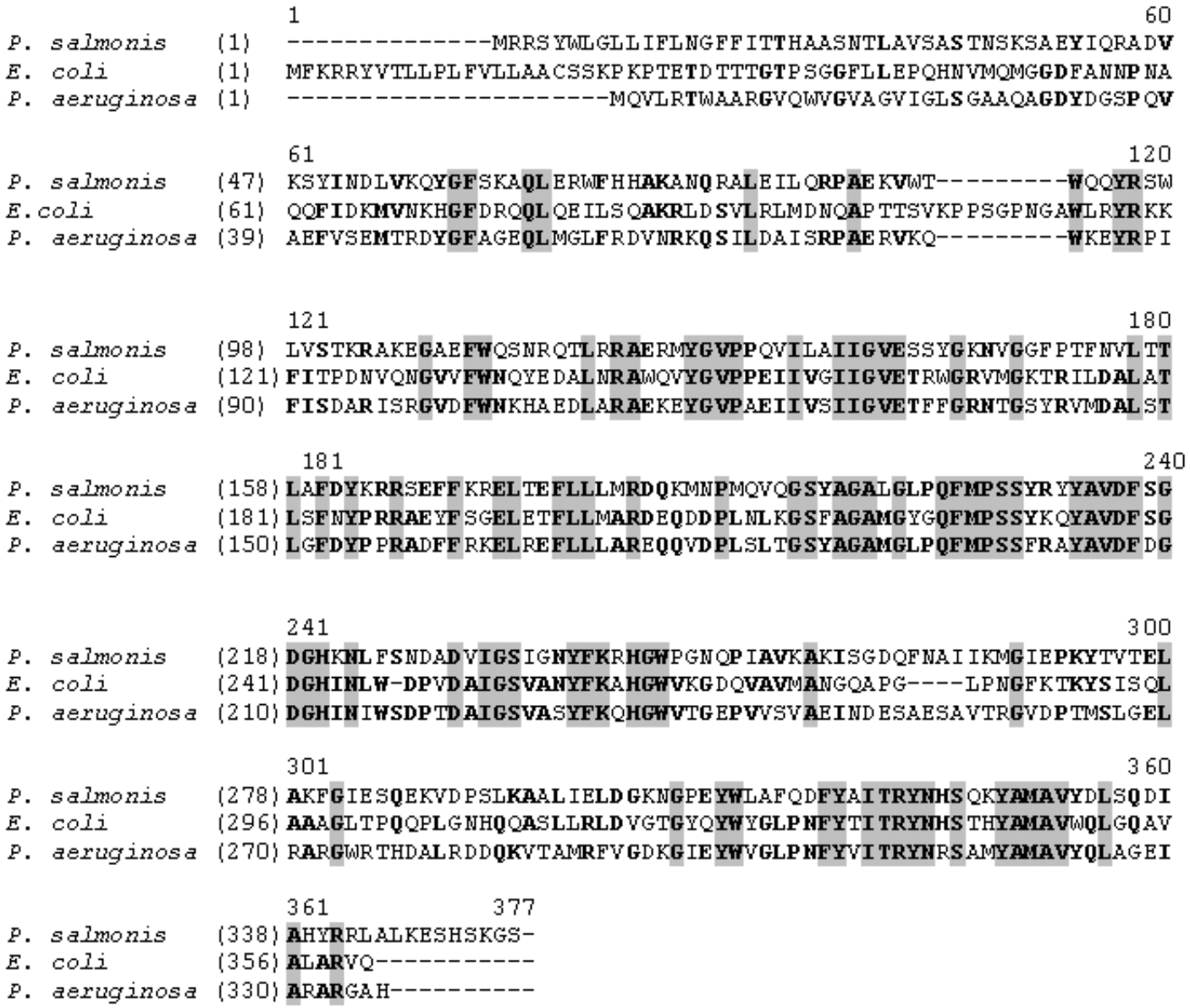
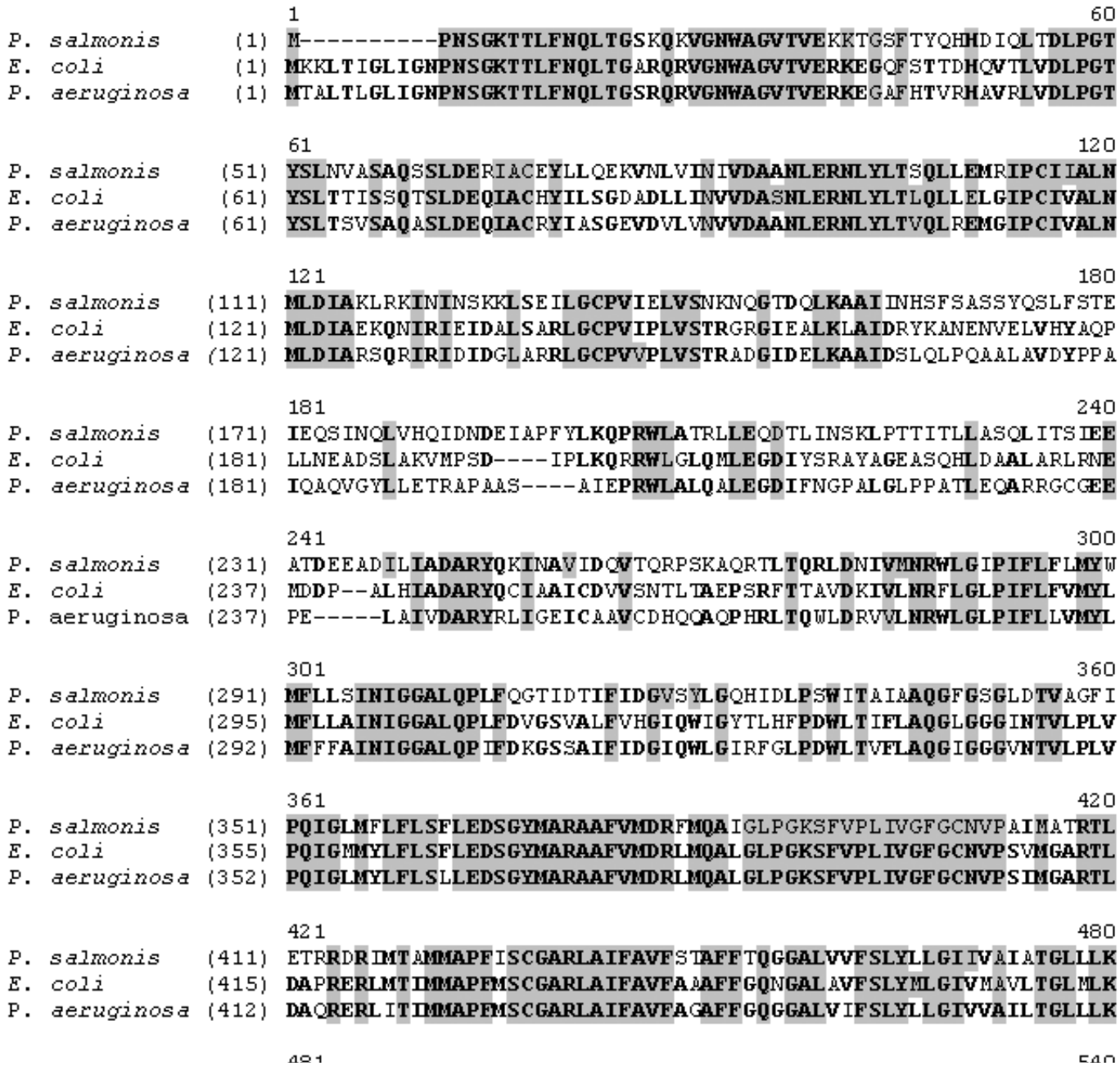


Figure 3. Protein sequence comparison of *P. salmonis* MltB with the corresponding proteins from other bacteria.

The 2,244 bp fragment, amplified with the primers designed according to the TbpB coding

sequences, was cloned in pGEM-T and sequenced. Using M13 primers and specific oligonucleotides to walk along the gene we sequenced almost the entire TbpB orf. The 120 bp of the 5' end obtained with the 5' terminal degenerated primer was also cloned and sequenced, allowing us to elucidate the missing amino terminal coding region of TbpB and to complete the sequence of the gene. It was not possible to establish the correct sequence of the first 30 bases of the *P. salmonis* TbpB gene because a degenerate primer mixture encoding this sequence based on other TBPB sequences was used to amplify the gene. Therefore, a primer located 10 codons downstream (primer p9) was used with p10 to amplified the complete coding sequence. The sequence described in Figure 5 corresponds to the segment amplified with the primers mentioned above and is named TbpBΔ30 to indicate the absence of the 10 first codons in the isolated gene. This sequence of 2,295 base pairs encodes 764 amino acids of a predicted protein of 84,040 Da (Fig. 4). The remarkable degree of conservation of the *P. salmonis* TbpB with other related microorganisms at the amino terminal region (Fig. 4) is coincident with the predicted ATP binding domain (Kammler et al., 1993) and also with potential epitopes shown by the hydrophilicity pattern of the protein.



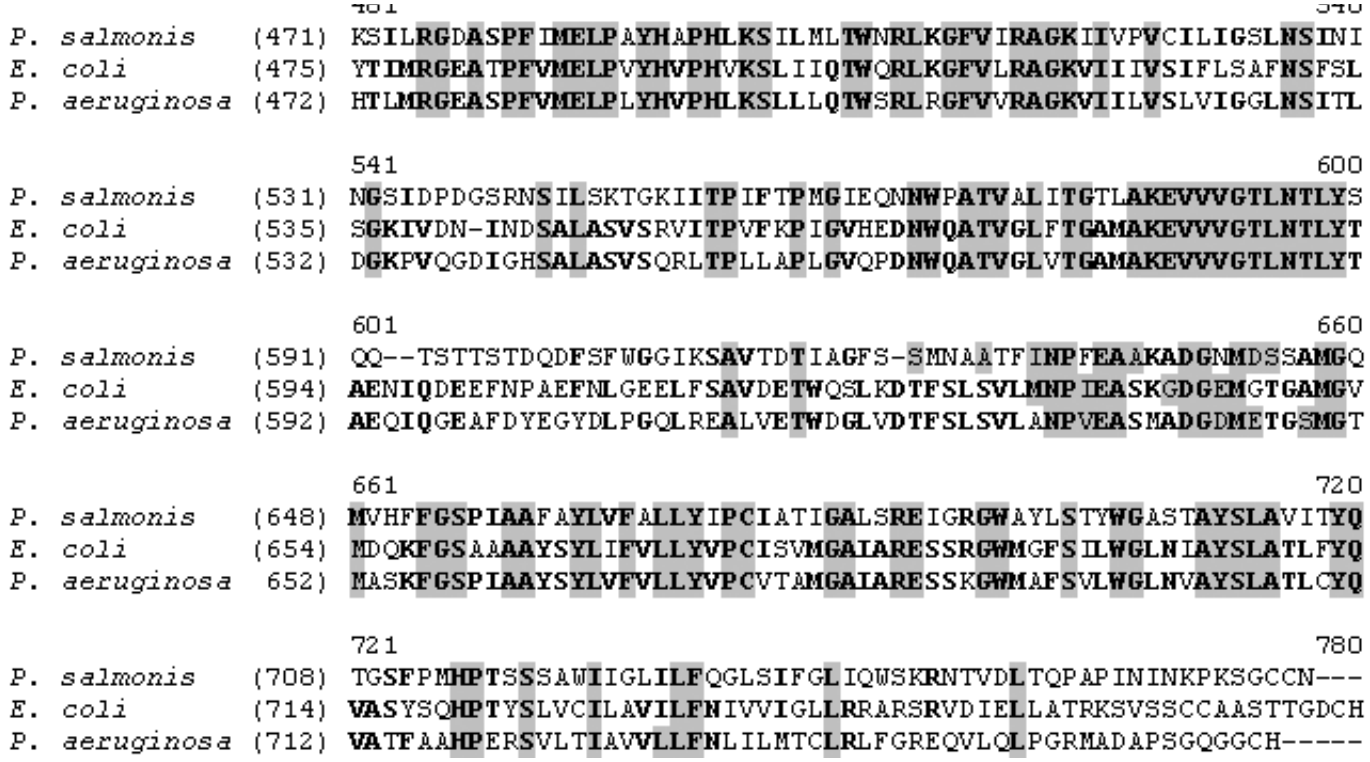


Figure 4. Protein sequence comparison of *P. salmonis* TbpB with the corresponding proteins from other bacteria.

Synthesis and characterization of recombinant MltB and TbpB of P. salmonis*.*

The entire region coding MltB was first amplified with primers that carried restriction sites in their 5' end to clone the gene in frame downstream of the thioredoxin gene (Trx) in the pET32a expression vector. The resulting pET32a_MltB was introduced in BL21 cells which failed to express the recombinant protein in the presence of IPTG. The lack of expression of MltB could be explained by an alteration of the endogenous peptidoglycan metabolism caused by the recombinant lytic enzyme adversely affecting the dynamic structure of the bacterial sacculus, as seen for other heterologous expressed peptidoglycan enzymes (Despreaux & Manning, 1993). To overcome the probable toxicity caused by the recombinant in *E. coli,* two segments coding the amino and carboxyl regions of MltB were amplified and cloned independently into the pET32a vector in frame with Trx. The predicted antigenic domains of the protein were taken into consideration in designing the internal primers in order not to loose an important antigenic locus determinant. The first half of the MltB gene was amplified with primer p11 and p12 of Table I and carries the restriction sites for EcoR I and XhoI respectively in their 5' end. The segment codifies from nucleotide 1 to 500 and is defined as MltB-N. The second half of MltB gene was amplified with primes p13 and p14, also carrying restriction sites for EcoR I and Xho I in their 5' end. This second half spans from nucleotide 457 to the stop the codon of the gene, encoding the last 207 aminoacids of the carboxyl region of the protein and is defined as MltB-C. Both amplified fragments were sequenced and cloned in pET32a. The recombinant vectors pET32a-MltB-N and pET32a-MltB-C were used to transform Bl21 cells and the proteins induced with IPTG. Figure 5A shows the induction of both halves of MltB when expressed as fusion protein with thioredoxin. The two fusion proteins Trx-MltB-N and Trx-MltB-C with expected sizes of 38,400Da and 42,800Da were detected in the insoluble protein fraction of the sonicated bacteria. Concentrations as high as 8 M urea were insufficient to solubilize the proteins and as a consequence Trx-Mlt-N and Trx-Mlt-C were partially purified by consecutive washes of the pellet with increasing

concentrations of urea and finally resuspended in 0.1% SDS (Fig. 5B).

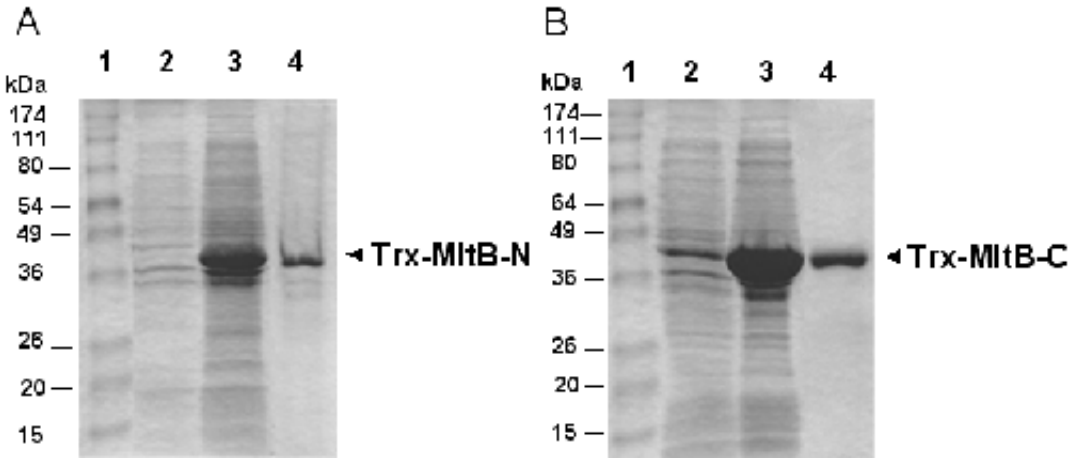


Figure 5. Expression in *E. coli* of fusion proteins encoding the amino and carboxyl region of the *P. salmonis* MltB.
A. 1: MW standards; 2: *E. coli* pellet before induction; 3: *E. coli* pellet after 3 hr. induction with IPTG; 4: partially purified recombinant Trx-MltB-N.
B. 1: MW standards; 2: *E. coli* pellet before induction; 3: *E. coli* pellet after 3 hr. induction with IPTG; 4: partially purified recombinant Trx-MltB-C.

A similar strategy was used to express TbpB. In an early attempt, the entire coding sequence was cloned in vector pET32a downstream of the Trx gene. Although the sequence of the amplified gene was correct, the expression of recombinant TbpB failed. Therefore, internal primers (p15 and p16) were designed to amplify the gene by halves as was done for MltB. Antigenic domains predicted by the database were taken into account for this purpose. Two independent fragments were amplified and cloned into pGEM-T to confirm their sequence. TbpB-N encodes the first 761bp of the amino terminal region, which includes the predicted ATP binding and GTPase activity domains (Kammler et al., 1993). TbpB-C encodes 1,410 bp spanning from nucleotide 761 to the stop codon. The presence of transferrin binding domains has been demonstrated on both halves of TbpB (Retzer et al., 1999; Sims et al., 2003; Renauld-Mongenieet al 2004). Fragments encoding TbpB-N and TbpB-C were isolated from pGEM-T and cloned in pET32a. BL21 cells transformed with pET32-TbpB-N were induced with IPTG and the soluble and insoluble protein fractions analyzed by PAGE.-SDS (Fig. 6). The fusion protein Trx_TbpB-N with the expected size of 48,000 Da was detected in the insoluble protein fraction. This protein was solubilized in 4M urea and purified using an affinity column of nickel-agarose (Fig. 6). The induction of TbpB-C was however unsuccessful, and even the partition of this sequence into two smaller fragments failed to express this region of TbpB.

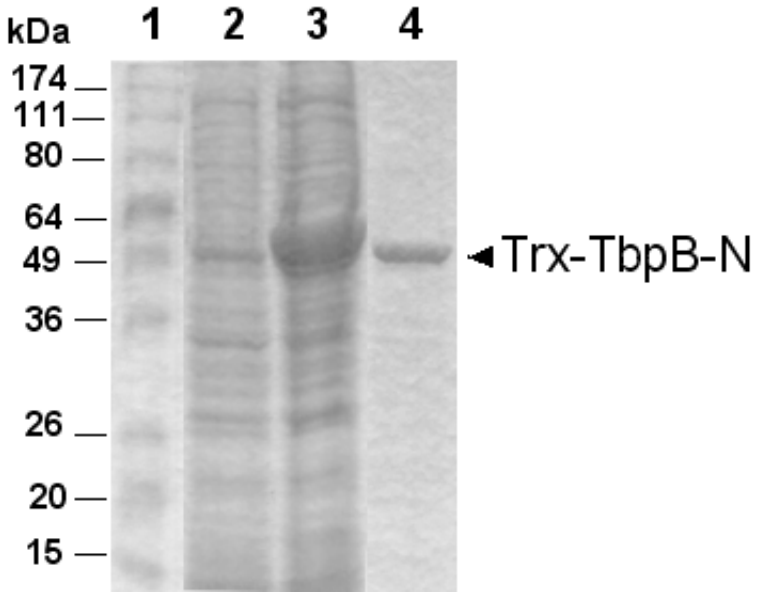
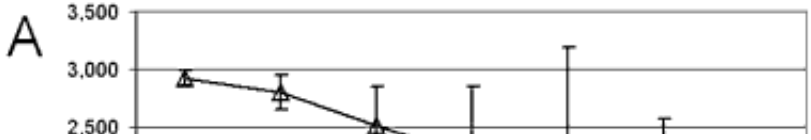


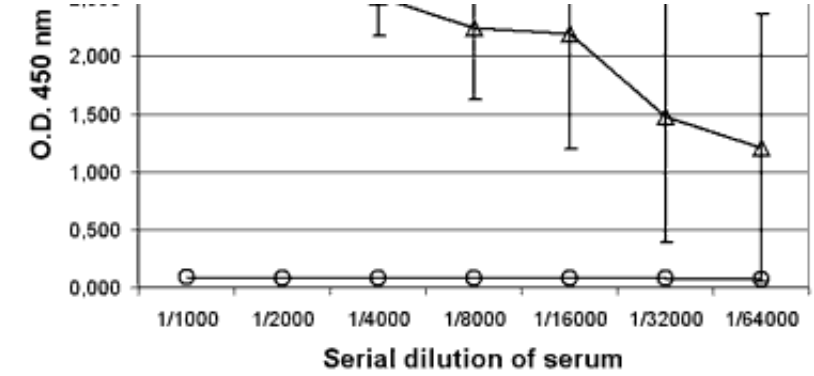
Figure 6. Expression in *E. coli* of the fusion protein encoding the amino region of the *P. salmonis* TbpB.
1: MW standards; 2: *E. coli* pellet before induction; 3: *E. coli* pellet after 3 hr. induction with IPTG; 4: partially purified recombinant Trx-TbpB-N.

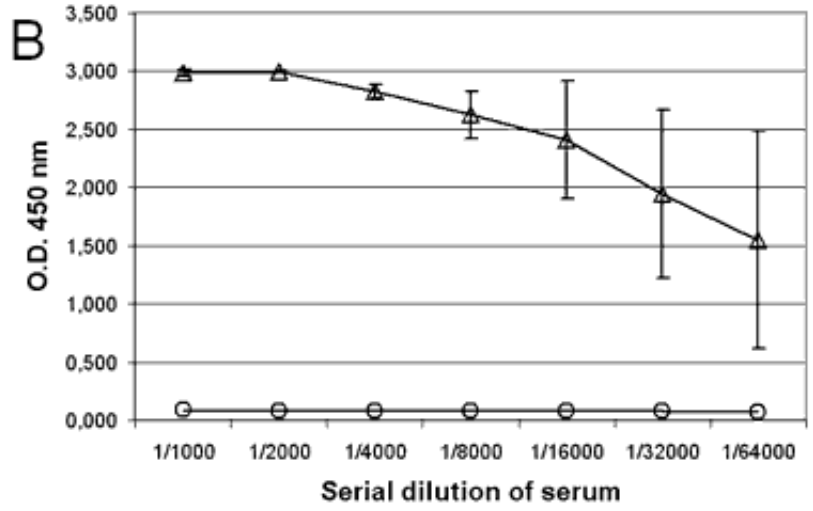
Immunogenic properties of MltB and TbpB of P. salmonis *.*

In an attempt to analyze the immunogenic properties of the recombinant proteins Mlt-N, Mlt-C and TbpB-N, BALB/c mice were vaccinated with the purified proteins. The immunogenic response was evaluated by ELISA with the corresponding recombinant proteins. The results (not shown) indicate that the three recombinant proteins elicit a strong immunoresponse. The mice that gave the highest titer were selected to produce monoclonal antibodies. Four monoclonal antibodies were obtained against Mlt-N, seven against Mlt-C and seven against TbpB-N. All the monoclonal antibodies recognized the corresponding recombinant proteins by Western blot (data not shown). The antibodies also recognize the native TBPB in protein preparations of *P. salmonis*, confirming that the gene is expressed during culture of the pathogen.

The primary purpose of this project is the development of an efficient recombinant vaccine. In this regard and due to the poor knowledge about the fish immune system, it was important to analyze whether a mixture with these recombinant proteins could elicit an immune response when injected into fish. We found that serum from Atlantic salmon that have been immunized three months prior with a formulation that contained the three recombinant Trx-Mlt-N, Trx-Mlt-C and Trx-TbpB-N reacted with the respective recombinant. This was analyzed by ELISA (Fig. 7). This strong immune response in salmon is coincident with the bactericidal immune response elicited by these two proteins in other microorganism (Webb & Cripps, 1999; McMichael 2000; Pizza et al., 2000; West et al, 2001) and supports the use of MltB and TbpB as bonafide antigens in experimental vaccines against *P. salmonis* infection.







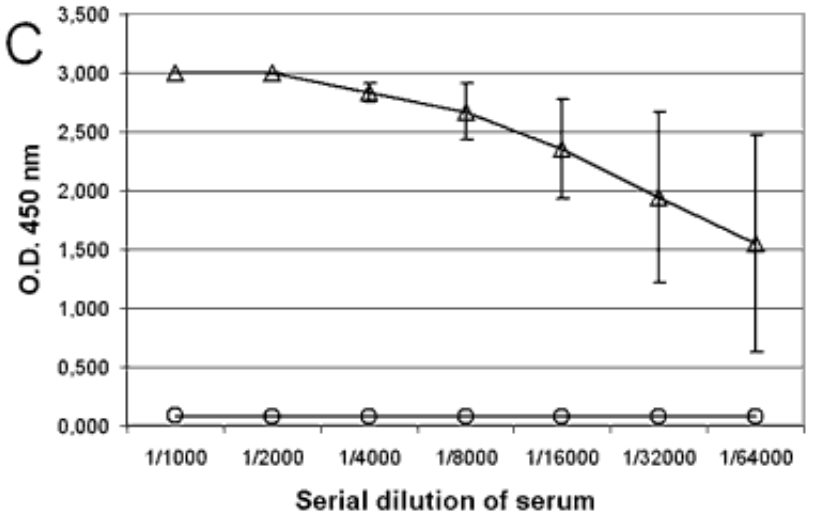


Figure 7. Measurement of antibodies to Trx-MltB-N, Trx-MltB-C and Trx-TbpB-N by ELISA in serum of salmon immunized with the three recombinant proteins.
A: Antibodies against MltB-N in immunized (Ð) and control fish (O).
B: Antibodies against MltB-C in immunized (Ð) and control fish (O).
C: Antibodies against TbpB-N in immunized (Ð) and

control fish (O).

REFERENCES

ADU-BOBIE J, LUPETTI P, BRUNELLI B, GRANOFF D, NORAIS N, FERRARI G, GRANDI G, RAPPUOLI R, PIZZA M (2004) GNA33 of *Neisseria meningitidis* is a lipoprotein required for cell separation, membrane architecture, and virulence. Infect Immun 72: 1914-1919
[Medline]

ALEXANDER J, INGRAM G (1992) Noncellular non-specific defence mechanisms of fish. Ann Rev Fish Dis 2: 249-279

AGUAYO J, MIQUEL A, ARANKI N, JAMETT A, VALENZUELA PDT, BURZIO LO (2002) Detection of *Piscirickettsia salmonis* in fish tissues by an enzyme-linked immunoabsorbent assay using specific monoclonal antibodies. Dis Aquat Org 49: 33-38

BINDER S (1995) Mitochondrial nucleic acid purification and analysis. Methods Mol Biol 49: 383-389

BRANSON EJ, NIETO DÍAZ-MUÑOZ D (1991) Description of a new disease condition occurring in farmed coho salmon, *Oncorhynchus kisutch* (Walbaum) in South America. J Fish Dis 14: 147-156

BROCKLEBANK JR, EVELYN TPT, SPEARE DJ, ARMSTRONG RD (1993) Rickettsial septicaemia in farmed Atlantic and Chinook salmon in British Columbia: Clinical presentation and experimental transmission. Can Vet J 34: 745-748

DESPREAUX CW, MANNING RF (1993) The *dacA* gene of Bacillus stearothermophilus coding for D-alanine carboxypeptidase: cloning, structure and expression in *Escherichia coli* and *Pichia pastoris*. Gene 131: 35-41
[Medline]

EHLERT K, HOLTJE JV, TEMPLIN MF. (1995) Cloning and expression of a murein hydrolase lipoprotein from *Escherichia coli*. Mol Microbiol 16:761-768
[Medline]

FRYER JL AND MAUEL MJ (1997) The Rickettsia: an emerging group of pathogens in fish. Emerg Infec Dis 3: 137-144

HÖLTJE J-V, TUOMANEN E (1991) The murein hyfdrolases of *Escherichia coli:* properties, functions and impact on the course of infections *in vivo*. J Microbiol 137: 441-454

HOUSE ML, BARTHOLOMEW JL, WINTON JR, FRYER JL (1999) Relative virulence of three isolates of *Piscirickettsia salmonis* for coho salmon *Oncorhynchus kisutch*. Dis Aquat Org 35: 107-113

JAMETT A, AGUAYO J, MIQUEL A, MULLER M, ARRIAGADA R, BECKER MI, VALENZUELA P, BURZIO LO (2001) Characteristics of monoclonal antibodies against *Piscirickettsia salmonis* J Fish Dis 24: 205-215

JONES SR, MACHAM RJ, GROMAN DB, CUSACK RR (1998) Virulence and antigenic characteristics of a cultured Rickettsia-like organism isolated in farmed Atlantic salmon *Salmo salar* in eastern Canada. Dis Aquat Org 33: 25-31

KAMMLER M, SCHON C, HANTKE K (1993) Characterization of the ferrous iron uptake system of *Escherichia coli*. J Bacteriol 175: 6212-6219
[Medline]

KÖHLER G, MILSTEIN C (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256: 495-497
[Medline]

LAEMMLI UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685
[Medline]

LANNAN CN, FRYER JL (1993) *Piscirickettsia salmonis*, a major pathogen of salmonid fish in Chile. Fish Res 17: 115-121

MCMICHAEL JC (2000) Vaccines for *Moraxella catarrhalis.* Vaccine 19; S101-S107

PIZZA M, SCARLATO V, MASIGNANI V, GIULIANI M, ARICÒ B, COMANDUCCI M, JENNING G, BALDI L, BARTOLINI E, CAPECCHI B, GALEOTTI C, LUZZI E, MANETTI R, MARCHETTI E, MORA M, NUTI S, RATTI G, SANTINI L, SAVINO S, SCARSELLI M, STORNI E, ZUO P, BROEKER M, HUNDT H, KNAPP B, BLAIR E, MASON T, TETTELIN H, HOOD D, JEFFRIES A, SAUNDERS N, GRANOFF D, VENTER J, MOXON E, GRANDI G, RAPPUOLI (2000) Identification of vaccine candidates against serogroup B meningococcus by whole-genome sequencing. Sci 287: 1816-1820

RENAULD-MONGENIE G, LINS L, KRELL T, LAFFLY L, MIGNON M, DUPUY M, DELRUE RM, GUINET-MORLOT F, BRASSEUR R, LISSOLO L (2004) Transferrin-binding protein B of *Neisseria meningitidis*: Sequence-based identification of the transferring-binding site confirmed by site-directed mutagenesis. J Bacteriol 186: 850-857
[Medline]

RETZER MD, YU RH SCHRYVERS AB (1999) Identification of sequences in human transferrin that binds to the bacterial receptor protein, transferrin-binding protein B. Mol Microbiol 32: 111-121
[Medline]

SANGER F, NICKLEN S AND COULSON AR (1977) DNA sequencing with chain terminating inhibitors. Proc Nat Acad Sci USA 74: 5463-5467
[Medline]

SIMS KL AND SCHRYVERS AB (2003) Peptide_peptide interactions between human transferring and transferring-binding protein B from *Moraxella catarrhalis*. J Bacteriol 185: 2603-2610
[Medline]

SMITH PA, CONTRERAS JR, LARENAS JJ, AGUILLÓN JC, GARCES LH, PÉREZ B, FRYER JL (1997) Immunization with bacterial antigens: Piscirickettsiosis. Dev Biol Stand 90: 161-166
[Medline]

SPRATT BG, BOYD A, STOKERN (1980) Defective and plaque-forming lambda transducing

bacteriophage carrying penicillin-binding protein-cell shape genes: genetic and physical mapping and identification of gene products from the lip-dacA-rodA-pbpA-leuS region of the *Escherichia coli* chromosome. J Bacteriol 143: 569-581
[Medline]

STOVER CK, PHAM X-QT, ERWIN AL, MIZOGUCHI SD, WARRENER P, HICKEY MJ, BRINKMAN FSL, HUFNAGLE WO, KOWALIK DJ, LAGROU M, GARBER RL, GOLTRY L, TOLENTINO E, WESTBROOK-WADMAN S, YUAN Y, BRODY LL, COULTER SN, FOLGER KR, KAS A, LARBIG K, LIM RM, SMITH KA, SPENCER DH, WONG GK-S, WU Z, PAULSEN IT, REIZER J, SAIER MH, HANCOCK REW, LORY S, OLSON MV (2000) Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. Nature 406: 959-964
[Medline]

TISCHLER ND, FERNÁNDEZ J, MÜLLER I, MARTÍNEZ R, GALENO H, VILLAGRA E, MORA J, RAMÍREZ E, ROSEMBLATT M, VALENZUELA PDT (2003) Complete sequence of the genome of the human isolate of Andes virus CHI-7913: comparative sequence and protein structure analysis. Biol Res 36: 201-210
[Medline] [Lilacs] [SciELO Chile]

VALENZUELA P, BURZIO LO, ROSEMBLATT M, YUDELEVICH A, BERNALES S, ENGEL E, ERAZO E, HERNANDEZ C, HUARACAN B, ARAYA P, MARTINEZ R, MIQUEL A, MORALES C, SOZA C, VILLEGAS J, WILHELM V (2001) Sequence and applications of the *Piscirickettsia salmonis* genome. Biol Res 34: 17

VAN ASSELT EJ, DIJKSTRA BW (1999) Binding of calcium in the EF-hand of Escherichia coli lytic transglycosylase Slt35 is important for stability FEBS Lett 458: 429-435
[Medline]

VAN ASSELT EJ, KALK KH AND DIJKSTRA BW (2000) Crystallographic studies of the interactions of *Escherichia coli* lytic transglycosylase Slt35 with peptidoglycan. Biochem 39: 1924-1934

WEBB D, CRIPPS A (1999) Immunization with recombinant transferring binding protein B enhances clearance of nontypeable *Haemophilus influenzae* from rat lung. Infect Immun 67: 2138-2144

WEST D, REDDIN K, MATHESON M, HEALTH R, FUNNEL S, HUDSON M, ROBINSON A, GORRINGE A (2001) Recombinant *Neisseria meningitidis* transferrin binding protein A protects against experimental meningococcal infection. Infect. Immunol 69: 1561-1567

WILHELM V, VILLEGAS J, MIQUEL A, ENGEL E, BERNALES S, VALENZUELA PDT, BURZIO LO (2003a) The complete sequence of the mitochondrial genome of the Chinook salmon, *Oncorhynchus tshawystscha*. Biol Res 36: 223-231
[Medline] [Lilacs] [SciELO Chile]

WILHELM V, HUARACAN B, MARTÍNEZ R, ROSEMBLATT M, BURZIO LO, VALENZUELA PDT (2003b) Cloning and expression of the coding regions of the heat shock proteins HSP10 and HSP16 from *Piscirickettsia salmonis*. Biol Res 36: 421-428
[Medline] [Lilacs] [SciELO Chile]

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