IDENTIFICATION OF IMMUNOREACTIVE PROTEIN ENCODING GENES OF THE FISH PATHOGEN *PISCIRICKETTSIA SALMONIS* AND EVALUATION OF THEIR USE IN GENETIC VACCINATION

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ABSTRACT

Since the emergence of *Piscirickettsia salmonis* in the late 1980's, it has become a major disease issue in the salmonid aquaculture and no effective control measures are currently available. As a potential vaccination strategy, genetic or DNA vaccines offer great promise. They function by carrying the gene coding for a protective antigen on a plasmid into the host cells where the antigen is produced by the cells themselves. These vaccines mimic the pathway by which antigens from intracellular pathogens are normally presented to the immune system. To develop a DNA vaccine for *P. salmonis*, genes coding for potentially protective antigens had to be identified although very little is known about the genetic makeup of this pathogen. The most appropriate way to identify protective genes was by generating an expression library of the entire genome of *P. salmonis* and screening this library with anti-*P. salmonis* rabbit antiserum. To obtain large amounts of *P. salmonis* for genomic DNA extraction and antigen production, the growth in culture of *P. salmonis* had to be optimized. To follow the course of infection of *P. salmonis* in CHSE-214 cell culture a sandwich ELISA (s-ELISA) was developed using rabbit anti-*P. salmonis* antiserum to capture *P. salmonis* and monoclonal 10E6.6C5 for detection. This s-ELISA proved to be both sensitive and practical. The optimum time to harvest *P. salmonis* culture was determined to be 10 days post inoculation, with the fastest increase in antigen levels between day 6 and 7. *P. salmonis* genomic DNA without contaminating CHSE-214 DNA was obtained by treating purified *P. salmonis* with DNaseI to remove CHSE-214 DNA before *P. salmonis* lysis and DNA purification. Genomic DNA was used to generate an expression library in the λZapExpress vector. By screening approximately 18,000 clones, 41 immuno-reactive clones were identified. Inserts from these clones were amplified by PCR and sequenced. Ten open reading frames (ORFs) were assigned putative functions by comparing deduced amino acid sequences to sequences available in Genbank. Five clones were selected for showing homologies to promising targets for the immune system. Putative functions of these five ORFs were: 1) the 17 kDa surface antigen from the spotted fever group rickettsia 2) transposase 3) ATP binding cassette type transporter 4) Preprotein translocase subunit SecA and 5) amino acid transporter. Inverse PCR was used successfully to obtain further sequence information on four of these ORFs and the 17kDa homologous gene was completely obtained. This gene was cloned into the expression vectors pcDNA3.1 and pUK-21 and used as a vaccine candidate in coho salmon (*Oncorhynchus kisutch*) challenged with *P. salmonis*. Mortalities were unexpectedly high in all groups, including *P. salmonis* bacterin immunized fish, most likely due to an overly high challenge dose. However, survival analysis of the mortality data showed a protective effect for the pcDNA3.1-17 kDa immunized fish, but there was no effect with the pUK-17 kDa construct or controls. *In vitro* transfection and immuno-histochemistry of CHSE-214 cells with the DNA vaccine constructs showed antigen production in these cells. Comparison of the 17 kDa gene from the type strain to four isolates from both Canadian coasts showed 13 single nucleotide polymorphisms leading to four amino acid changes. Extracellular location of these changes indicates immune pressure on the 17 kDa gene. The Canadian strains could be differentiated in Pacific and east coast isolates with 99.6% similarity to each other and 97.5% similarity to the type strain.

DEDICATION

I would like to dedicate this thesis to the people without which there would have been none: my parents, Jan and Maria Brouwers, but foremost, to Joy Becker, my inspiration and support.

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ABC	-	ATP binding cassette
ABTS	-	2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)
AmpR	-	Ampicillin resistance gene
AP	-	Alkaline phosphatase
APC	-	Antigen presenting cell
ATP	-	Adenosine triphosphate
BM	-	Boehringer Mannheim
BCIP	-	5-Bromo-4-chloro-3-indolyl phosphate
bGH pA	-	Bovine growth hormone poly adenylation signal
bp	-	Base pairs
BSA	-	Bovine serum albumin
°C	-	Degrees Celsius
CBAP	-	Carp β-actin promoter
CHH-1	-	Chum salmon heart -1 cell line
CHSE-214	-	Chinook salmon embryonic -214 cell line
CIAP	-	Calf intestinal alkaline phosphatase
CMV	-	Cytomegalo virus
CPE	-	Cytopathic effect
CpG	-	Palindromic nucleotide sequence centering on CG
CTAB	-	Cetyl trimethyl ammonium bromide
CTL	-	Cytotoxic T lymphocyte
DNA	-	Deoxyribonucleic acid
DNase I	-	Deoxyribonuclease I
DIG	-	Digoxigenin
EDTA	-	Ethylenediaminetetraacetic acid
ELI	-	Expression library immunization
ELISA	-	Enzyme linked immunosorbent assay
EPC	-	Epithelioma papulosum cyprini cell line
FBS	-	Fetal bovine serum
FHM	-	Fathead minnow
FIA	-	Freund's incomplete adjuvant
GC	-	Guanine - Cytosine
HBSS	-	Hank's balanced salt solution
HRPO	-	Horseradish peroxidase
IFAT	-	Indirect fluorescent antibody test
IFN-γ	-	Interferon -γ
Ig	-	Immunoglobulin
IHNV	-	Infectious hematopoietic necrosis virus
IHV-1	-	Channel catfish herpesvirus -1
IL	-	Interleukin
i.m.	-	Intra muscular

i.p.	-	Intra peritoneal
iPCR	-	Inverse polymerase chain reaction
IPTG	-	Isopropyl-β-D-thiogalactopyranoside
ISS	-	Immuno-stimulatory sequences
ITS	-	Internal transcribed spacer region
KanR	-	Kanamycin resistance gene
kDa	-	kilo Dalton
LPS	-	Lipopolysacharide
MAb	-	Monoclonal antibody
MAF	-	Macrophage activating factor
MEM	-	Eagle's minimum essential medium
MEM-10	-	Eagle's minimum essential medium with 10% fetal bovine serum
MHC	-	Major histocompatibility complex
MPL	-	Monophosphoryl lipid A
NBT	-	Nitro blue tetrazolium
NCC	-	Non-specific cytotoxic cell
ND	-	Not determined
NK	-	Natural killer cells
OD	-	Optical density
ORF	-	Open reading frame
OspA	-	Outer surface protein A
PAMP	-	pathogen associated molecular pattern
PBS	-	Phosphate buffered saline
PBS-T	-	Phosphate buffered saline with 0.05% Tween-20
PCR	-	Polymerase chain reaction
PE	-	Post exposure
PRR	-	Pattern recognition receptors
RAG	-	Recombination activating genes
rDNA	-	Ribosomal sequence coding deoxyribonucleic acid
RLO	-	Rickettsia-like organism
RNA	-	Ribonucleic acid
RNase	-	Ribonuclease
RPC	-	Research and Productivity Council
RPS	-	Relative percent survival
RTG-2	-	Rainbow trout gonad cell line -2
s-ELISA	-	Sandwich enzyme linked immunosorbent assay
SDS-PAGE	-	Sodium dodecylsulfate - polyacrylamide gel electrophoresis
SFG	-	Spotted fever group
SHRV	-	Snakehead rhabdovirus
sIg	-	Surface immunoglobulin
SRS	-	Salmonid rickettsial syndrome
SSPE	-	Sodium chloride, Sodium phosphate, EDTA buffer
SVCV	-	Spring viremia of carp virus

Taq	-	*Thermus aquaticus*
TBE	-	Tris boric acid EDTA buffer
TBS	-	Tris buffer saline
TBS-T	-	Tris buffer saline with 0.05% Tween-20
$TCID_{50}$	-	50% tissue culture infective dose
TCR	-	T cell receptor
TDM	-	Trehalose 6,6'-dimycolate
TE	-	Tris EDTA buffer
TGF-β	-	Transforming growth factor -β
Th1	-	T helper cells, type 1
Th2	-	T helper cells, type 2
TIGR	-	The institute for genome research
TNF-α	-	Tumor necrosis factor -α
UK	-	United Kingdom
VHSV	-	Viral hemorrhagic septicemia virus
X-gal	-	5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside

CHAPTER 1: GENERAL INTRODUCTION

1.1 INTRODUCTION

New and emerging diseases confront both human and animal health management. In particular when new species are put into production, or when established species are introduced into new environments, the threat of new or previously unrecognized infectious diseases are significant. This scenario was clearly evident when salmonid aquaculture was established in Chile. The industry rapidly increased production from 1980 and became the second largest producer worldwide in 1990. In 1989 a new disease, salmonid rickettsial septicemia, emerged within this industry and caused significant economic loses. This thesis will focus on the causative agent of this disease, *Piscirickettsia salmonis*, and the development of a vaccine to combat it.

1.2 *PISCIRICKETTSIA SALMONIS*

1.2.1 History and relevance

In 1989, 1.5 million market sized coho salmon (*Oncorhynchus kisutch*) were lost during an outbreak of a previously unrecognized disease in the area of Calbucon, Region X, southern Chile (Cvitanich *et al.*, 1991; Branson and Nieto Diaz-Munoz, 1991). At first the disease was known as 'coho salmon syndrome' and associated losses were as high as 90%, with the average being approximately 60% (Bravo and Campos, 1989; Garces et al, 1991). The disease was thought to be limited to coho salmon in the salt

water stages of production, but further investigations revealed that it could also affect chinook salmon, *O. tshawytscha* (Walbaum) (Lannan et al, 1991), Atlantic salmon, *Salmo salar* L.(Garces et al, 1991) and rainbow trout, *O. mykiss* (Walbaum) in both fresh and saltwater stages (Gaggero *et al.*, 1995; Almendras *et al.*, 1997a). A rickettsia like organism was isolated from infected fish and confirmed as the causative agent. The pathogen was named *Piscirickettsia salmonis* (Fryer *et al.*, 1992) and its infectious potential was confirmed by experiments on the extracellular survival of *P. salmonis* by Lannan and Fryer (1994) and experimental infections by either intra peritoneal injections (Cvitanich *et al.*, 1991) or cohabitation exposure methods (Almendras *et al.*, 1997a). The study by Lannan and Fryer (1994) showed that *P. salmonis* is quite stable in sea water but the authors were not able to recover any infectious particles when *P. salmonis* was suspended in fresh water. Yet the study by Almendras *et al.* (1997b) showed that fish can transmit the bacterium without a vector and without direct contact in fresh water. The reduced survivability of *P. salmonis* in fresh water might explain why outbreaks in fresh water sites are generally less severe then those in sea pens.

1.2.2 Description of *P. salmonis*

The type strain for *P. salmonis* isolated by Fryer *et al.* (1992) was described as a Gram-negative, non-motile, obligately intracellular bacterium. It is pleomorphic, with the diameter of individual cells between 0.5-1.5 μm and replicates within membrane-bound cytoplasmic vacuoles in cells of infected fish and in a number of cultured fish cell lines.

1.2.3 Clinical signs of *P. salmonis* infection

Although not all infected individual show clinical signs during experimental challenges, under aquaculture conditions clinical signs and mortalities can be observed 6-12 weeks after the smolts are placed into seawater pens. Infected fish show darkening of the skin and or skin lesions that range from slightly raised areas less then 50 mm in diameter to hemorrhagic ulcers 2 cm in diameter and firm white nodules measuring up to 1 cm. The fish show respiratory distress and tend to aggregate near or on the surface in the corners of the netpens (Branson and Nieto Diaz-Munoz, 1991). Many of the infected fish appear lethargic with some developing ascites. The gills are pale, as are the internal organs, indicative of anemia. The spleen is mildly enlarged. The liver and kidneys are swollen, and show gray multi focal areas of necrotic tissue in 5 to 10% of infected fish. Petechial hemorrhages are found throughout the swim bladder and viscera. Hematocrits fall to 2 to 20% compared with a normal range of 40 to 45%. Histopathological changes can be seen throughout the internal organs. In the kidney and the spleen the normal haematopoietic and lymphoid tissues are replaced by inflammatory cells with necrosis. The liver shows multifocal to general necrosis and inflammation. *P. salmonis* can be found within degenerating hepatocytes and enlarged macrophages (Cvitanich *et al.*, 1991). Infected macrophages and free *P. salmonis* can be found in the blood. Fibrin thrombi can be found in the smaller blood vessels with necrosis of the epithelium and infiltration of inflammatory cells. The gills show multifocal epithelial hyperplasia, causing fusion of the secondary lamella. Gut tissue is severely damaged, with inflammation of the lamina propria of the large intestine, resulting in necrosis and

sloughing of the mucosal epithelium. Skin lesions show necrosis of the dermis and epidermis, and some degeneration of the underlying musculature. (Cvitanich *et al.*, 1991; Branson and Nieto Diaz-Munoz, 1991; Rodger and Drinan, 1993; Brocklebank *et al.*, 1993; Smith-Schuster *et al.*, 1994; Olsen *et al.*, 1997; Almendras and Fuentealba, 1997)

1.2.4 Diagnosis of *P. salmonis*

Among the diagnostic tests which have been developed, tissue culture is the gold standard for specificity (Evelyn *et al.*, 1998; Fryer and Hedrick, 2003). For culture of *P. salmonis*, kidney is the recommended tissue for isolation of the organism. *Piscirickettsia salmonis* can be grown on a number of fish derived cell lines, including chinook salmon embryo (CHSE-214), chum salmon heart (CHH-1), rainbow trout gonad (RTG-2), epithelioma papulosum cyprini (EPC) and fathead minnow (FHM)(Cvitanich *et al.*, 1991). The organism's optimal growth temperature range is from 15 to 18°C, with growth being retarded below 10°C or above 20°C but exposure to 25°C or above is lethal to *P. salmonis*. If kept in the optimal growth range, cytopathic effect (CPE) can be seen within 6 to14 days, and is complete within 28 days, depending on the number of *P. salmonis* inoculated (Cvitanich *et al.*, 1991). Almendras *et al.* (1997b) reported growth of *P. salmonis* in a brown bullhead (BB) cell line, previously reported as nonpermissive. Onset of CPE was 45 days post infection. This organism grows slowly in culture and isolation methods must occur without the use of antibiotics. This has prompted the development of other tests. Kidney imprints or smears can be stained with Grams, Giemsa, methylene blue, or acridine orange , or studied by serologic methods, including

immunofluorescence (IFAT) and immunohistochemistry (Fryer *et al.*, 1990; Lannan *et al.*, 1991).

A nested PCR reaction developed by Mauel *et al.* (1996) has been used to rapidly detect *P. salmonis* and at less then one tissue culture infectious dose 50 ($TCID_{50}$) of infectious particles. A single PCR, using the 16S-23S rDNA internal transcribed region (ITS) was also developed, which can detect 10 to 100 *P. salmonis* cells per 20 µl of blood serum (Marshall *et al.*, 1998). More recently, a real-time PCR method using the *Taq*Man principle has been described for the detection of *P. salmonis* (Corbeil *et al.*, 2003). The advantage of these PCR based methods is that they generate faster and more sensitive results then tissue culture, however they do require specialized equipment that may be cost prohibitive. (Min *et al.*, 2001). Therefore at this time, it is more useful as a confirmatory and research tool.

Finally, another available diagnostic test available is an enzyme linked immuno sorbent assay (ELISA) which detects antigens of *P. salmonis* (Aguayo *et al.*, 2002). This test can be performed quickly and cost effectively for a large number of samples. Several ELISAs have been developed including a commercial ELISA test developed by Microtek Ltd-Bayer (Sydney, British Colombia), which has been used extensively by Chilean farmers in a broodstock segregation program.

1.2.5 Treatment and control of *P. salmonis*

The first outbreaks of *P. salmonis* in Chile followed non-toxic algae blooms and highest mortalities were seen in pens that were hit by a storm (Branson and Nieto Diaz-Munoz, 1991). In Norway, *P. salmonis* infections were frequently recorded after algal blooms, in smolt pens overstocked with fish in poor condition (Olsen *et al.*, 1997). Together with the fact that Branson and Nieto Diaz-Munnoz (1991) found that fish testing positive for *P. salmonis* can be found in netpens with fish showing no signs of disease or experiencing any mortalities, these observations suggested that stress induced by a number of factors such as overstocking, smolt transfer, algae blooms or temperature changes may play a role in the development of salmonid rickettsial septicemia (SRS). Reducing these stressors, for example by maintaining lower stocking density, reducing the number of times the fish are handled for grading and minimizing the number of net transfers has shown to be effective in reducing mortality rate related to *P. salmonis* (Fryer and Hedrick, 2003). Other control measures include immediate removal of dead and moribund fish, fewer fish per site and fallowing of areas (Evelyn *et al.*, 1998).

In vitro, Piscirickettsia salmonis is sensitive to antibiotics including streptomycin, gentamycin, tetracycline, oxytetracycline, chloramphenicol, erythromycin, flumequine, imequil, oxolinic acid, sarafloxacin and clarithormycin (Cvitanich *et al.*, 1991; Fryer *et al.*, 1992; Smith *et al.*, 1996). A number of these (oxolinic acid, flumequine, and oxytetracyclin) have been used by oral administration to affected fish, but with limited success in controlling outbreaks (Evelyn *et al.*, 1998). Factors such as the variability of antibiotic dosage per fish when administered orally, the intracellular

location of *P. salmonis*, development of resistance and saltwater cation inhibition may contribute to the apparent inefficacy of antibiotics in controlling the outbreaks. Only injection with fluoroquinolones has been used successfully in extreme cases of infection (Evelyn *et al.*, 1998).

Considering the difficulties in treating the disease, the preferred method of control would be through the development of an effective vaccine (Lannan and Fryer, 1993). The most common type of vaccines used against bacterial pathogens in aquaculture are bacterins where a culture of the pathogen is inactivated with formalin and mixed with adjuvant. An 1:2 diluted lysed cell culture supernatant of formalin inactivated *P. salmonis* was found to be protective in coho salmon in initial studies (Smith *et al.*, 1997). However, due to the high cost of culturing *P. salmonis* using host cells, an alternative vaccine strategy is necessary.

An alternative to bacterins is either recombinant or DNA vaccines. Several studies have identified potential *P. salmonis* antigens by western blotting (Kuzyk *et al.*, 1996; Barnes *et al.*, 1998; Jamett *et al.*, 2001). Most antigens have been identified using rabbit polyclonal or mouse monoclonal antibodies, whereas Kuzyk *et al.* (1996) identified protein antigens between 10 and 70 kDa in size and carbohydrate antigen with an apperent size of approximately 11 kDa using convalescent sera from coho salmon and rainbow trout. Using antisera from *P. salmonis* challenged carp (*Cyprinus carpio*), Trevors (1998) found antigens with approximate molecular mass of 160, 97, 86, 62, 32 and 23 kDa. The antigens Kuzyk identified using rabbit antisera were both protein, with the major ones at 65, 60, 54, and 51 kDa and carbohydrate at 11 and 16 kDa. It is

therefore clear that the rabbit, salmonid and carp immune system react differently to the multiple antigens present on *P. salmonis* .

Employing a molecular approach, Kuzyk *et al.* (2001b) used an expression library, screened with rabbit polyclonal antiserum, to identify an immunoreactive clone. From this clone they identified a 17 kDa outer surface protein (OspA) with 62% deduced amino acid sequence homology to the Spotted Fever Group surface antigen of *Rickettsia prowazekii*. It was shown that convalescent coho salmon sera reacted strongly against OspA. A recombinant protein was produced in *Escherichia coli* and used to vaccinate coho salmon (Kuzyk *et al.*, 2001a). When challenged with a lethal dose, a relative percent survival (RPS) of 58.4% was achieved in the vaccinates. To increase the potency of the recombinant vaccine, the authors added T cell epitopes from tetanus toxin and measles as a fusion protein with the recombinant OspA. These antigens are universally immunoreactive in the mammalian system and it was thought they would be recognized by the salmonid immune system as well. When this construct was compared to the unmodified protein, the authors found an improved response to challenge with 30.2% RPS for the unmodified construct and 83% RPS for a construct containing both T cell epitopes. The lower percentage of RPS for the original construct observed in the second trial was thought to be due to a higher challenge pressure.

1.2.6 Geographical spread and other rickettsia like organisms

Isolates from Chile (Cvitanich *et al.*, 1991; Fryer *et al.*, 1992), the Pacific and Atlantic coasts of Canada (Brocklebank *et al.*, 1993; Jones *et al.*, 1998), Norway (Olsen *et al.*, 1997), Scotland (Birrel *et al.*, 2003) and Ireland (Rodger and Drinan, 1993) have been identified as *P. salmonis*. As well there have been a number of Piscirickettsia-like organisms (PLO) and other Rickettsia-like organisms (RLO) found in aquatic species (Chen *et al.*, 1994; Khoo *et al.*, 1995; Fryer and Lannan, 1996; Chen *et al.*, 2000a; Chen *et al.*, 2000b; Steiropoulos *et al.*, 2002; Mauel *et al.*, 2003). As not all isolates have been sequenced, it is now not certain if some of them might be different strains of *P. salmonis,* which present a different pathology or if these are entirely different rickettsial species.

The first occurrence of lesions consistent with what we now recognize as *P. salmonis* was at the Pacific Biological Station in Nanaimo, British Colombia in 1970 (Evelyn *et al.*, 1998). Seawater-reared pink salmon (*O. gorbuscha*) held for experimental purposes were infected with an agent that was morphologically, serologically and culturally indistinguishable from the more recently isolated Chilean type strain of *P. salmonis* and it was therefore concluded that it was the same organism (Evelyn *et al.*, 1998). Olsen *et al.* (1997) describe an outbreak of *P. salmonis* in Norway with the peak of the outbreak in the Fall of 1988 and it lasted from 1988 to 1992. This would predate the first major outbreak of *P. salmonis* in Chile by a year (1989). Other outbreaks have been reported in Ireland and Canada's east and west coasts and in Scotland. All these outbreaks were in salmonid fish, either coho, pink, Atlantic, masou (*O. masou*) or chinook salmon or rainbow trout. Organisms associated with Piscirickettsia-like diseases

have been seen in non-salmonids including grouper, *Epnephelus melanostigma* Schultz (Chen *et al.*, 2000b), and five species of cultured Tilapia in Taiwan (Chen *et al.*, 1994)and Hawaii (Mauel *et al.*, 2003) but *P. salmonis* has only been confirmed in captive white sea bass (*Atractoscion nobilis*) from California, USA (Chen *et al.*, 2000a). In the French Mediterranean, RLO's that react to anti *P. salmonis* polyclonal serum have been seen in the European sea bass (*Dicentrarchus labrax*, L.) (Steiropoulos *et al.*, 2002).

1.3 THE IMMUNE SYSTEM

1.3.1 General introduction to vertebrate immunity

The immune system of jawed vertebrates is generally divided into innate and adaptive immune systems (Janeway, 2001). The innate system is defined as those defense mechanisms that are present before a pathogen is encountered. It encompasses physical and chemical barriers that separate the body from its environment, such as epithelial barrier and mucus; phagocytic cells such as macrophages and neutrophils; cytotoxic cells such as natural killer cells which in fish are known as non-specific cytotoxic cells (NCC) (Ellis, 2001); and proteins and peptides that have either direct anti-microbial activity or can mediate the response, such as the complement system and cytokines. The innate immune system is present before a pathogen is encountered, but becomes activated by pathogen associated molecular patterns (PAMPs) (Medzhitov and Janeway, 1997), which can come in a number of forms. Examples are the lipopolysacharide (LPS) present on Gram-negative bacteria, double stranded RNA from viruses, peptidoglycan from Gram-

positive bacteria and bacterial DNA containing unmethylated palindromic sequences centered around a CG motief (CpGs). These PAMPs are recognized by pattern recognition receptors (PRR's) (Medzhitov *et al.*, 1997) of the innate immune system and cannot differentiate within groups pathogens. Pattern recognition receptors trigger the activation of the innate system following the invasion of a bacterium, virus or protozoan.

If an invading organism survives the initial reaction of the bodies innate system, the second branch of the immune system, the adaptive or acquired immune system comes into play. It takes days and ,in fish depending on temperature, sometimes weeks to develop a full scale response after the first exposure to a pathogen (Bly and Clem, 1994). This response can use two different pathways, leading to different effector cells. The first pathway leads to CD8 positive T lymphocytes known as cytotoxic T-lymphocytes and is initiated by CD4 positive T lymphocytes of the Th1 subtype. This pathway is known as the cell mediated immune response. The second pathway, known as the humoral immune response, leads to antibody secreting B-lymphocytes and is initiated by CD4 positive T lymphocytes of the Th2 subtype. Both these responses are specific to the pathogen. Through clonal expansion and, in the case of the humoral response, isotype switching and affinity maturation, the responses become more effective over time (Janeway, 2001). This way the adaptive immune system can cope with pathogens that have survived the innate response. A second advantage of the adaptive immune system is that it produces memory cells specific to the pathogen and on re-exposure can quickly produce a fast highly specific response to the pathogen.

1.3.2 The 'Infectious Non-Self' model

In the 'Infectious Non-self' model, activation of the adaptive immune system is dependent on the presence of antigen, processed and presented by cells of the innate immune system to CD4+ T lymphocytes (Janeway, 1992). This antigen is presented on the surface of the antigen presenting cell (APC) bound to a major histocompatibility complex (MHC) type II receptor. The T lymphocyte has to recognize this antigen - MHC II complex using its T-cell receptor (TCR). It is the TCR that determines an individual T lymphocytes antigen specificity. The binding of the TCR to the antigen - MHC II complex is not enough to activate the T-lymphocyte. A second signal has to be provided by the binding of the co-stimulatory proteins B7-1 or B7-2 to CD28 present on the T lymphocyte. If this second signal is not provided the T lymphocyte will not become activated, but rather become anergic which can lead to tolerance to the antigen presented (Janeway, 2001). The co-stimulatory B7 proteins are only present on the cells of the innate immune system after they themselves have become activated by binding of their PRR's to the PAMP's present on, or associated with a potential pathogen. Therefore one of the roles of the PRR's is to ensure that B7 receptors are only up-regulated when the peptide presenting cell detects pathogen-specific molecules. Pattern recognition receptors also function as mediators for receptor mediated endocytosis of the pathogens. The pathogen is internalized into a phagosome that fuses with a lysosome providing the mechanisms to break down the pathogen to peptides. These peptides are now bound by MHC receptors and this complex is transported to the cell surface. To summarize, the PRR's are responsible for making pathogen derived peptides available to the immune

system and they make sure the adaptive immune response is only targeting pathogens and not self cells and proteins (Janeway, 1992; Medzhitov and Janeway, 2000; Janeway, 2001; Janeway and Medzhitov, 2002).

1.3.3 The innate immune system of teleost fish

The innate immune system comprises constitutive factors that are always present and inducible factors that are upregulated during an active response, such as inflammatory mediators and acute phase proteins. Both consist of soluble chemical agents and cellular defenses (Jones, 2001; Ellis, 2001). The innate cellular defenses are based on many of the same cell populations as are seen in mammalians or their fish equivalent: monocytes, neutrophils, macrophages, granulocytes and non-specific cytotoxic cells (NK in mammals and NCC in fish) (Watts *et al.*, 2001).

1.3.3.1 Constitutive factors

The most visible part of the innate immune system is the outer barrier between the body and the environment. Epithelial surfaces and the mucous layer form a physical barrier, prevent attachment and penetration by potential pathogens. The regular shedding of the mucus removes microorganisms from the surface of the skin. The mucous layer also contains chemical factors with an antimicrobial action like defensins, which are antibacterial peptides, proteases and high levels of lysozyme. Lysozyme can also be found in fish eggs and intestine. Compared to mammalian lysozyme, fish lysozyme has a broader spectrum of action (Ellis, 2001). Mammalian lysozyme is active against Gram-

positive bacteria, but can only act against Gram-negative bacteria after complement has formed a lesion. Fish lysozymes on the other hand can act directly against Gram-negative organisms. The concentration of lysozyme in fish is often used as a measure or indicator of nonspecific immune function. As lysozyme concentration and production can be increased in response to the presence of a pathogen, it can be considered as both a constitutive and inducible factor (Watts *et al.*, 2001) .

An example of a constitutive cellular defense is the macrophage. This cell type has an ubiquitous distribution within the body, being found in, amongst others, the skin, liver, head-kidney and the intestinal endothelium. Different sub-populations of macrophages are adapted to the micro-environment in which they are found. The distribution throughout the body ensures continuous surveillance of host tissues for foreign invaders. If an invader is detected by the macrophage's PRR's or the presence of 'danger signals', the macrophage will become active and go through the three stages of phagocytosis: 1) chemotaxis, where the macrophage moves to the pathogen directed by a chemical gradient; 2) adherence to the particle to be engulfed; 3) the actual phagocytosis, where the particle is engulfed by the outer membrane of the phagocyte into a phagosome, which is then fused with a lysozome containing the agents responsible for the degradation of the pathogen such as proteases, nucleases, phosphatases, esterases, lipases and antimicrobial peptides (Neumann *et al.*, 2001). Chemotaxis is mediated through biologically active molecules secreted by the pathogen or host cells. Adherence and phagocytosis can be aided by the opsonisation of the particle to be engulfed by for example complement or antibodies. The macrophages themself secrete factors like the

fish equivalent to tumor necrosis factor- α (TNF-α) followed by other pro-inflammatory cytokines like interleukin 1- β (IL1-β) and IL-6 (Secombes *et al.*, 2001). As macrophages are present before the pathogen arrives, but get activated upon invasion, they can be considered to be both constitutive and inducible factors (Neumann *et al.*, 2001).

1.3.3.2 Inducible factors

When the mucus and skin barriers are breached, inflammatory processes are initiated. This follows by and large the same pathway as the mammalian immune response. Pattern recognition receptors both soluble and cell bound, are activated by the PAMPs, such as LPS, CpG containing bacterial DNA, double stranded viral RNA and peptidoglycan present on or induced by the bacteria, virus or parasite (Janeway and Medzhitov, 2002). This leads to opsonisation of the invader, activation of both pathways of the complement system and activation of cell signaling pathways inducing the transcription of important inflammatory mediators. TNF-α is secreted first, followed by IL-1β and IL-6 in turn (Secombes *et al.*, 2001). These regulate the release of chemokines that serve as potent chemoattractants to induce migration of macrophages and neutrophils to the site of infection. Whereas macrophages provide a first line of cell-mediated defenses due to their ubiquitous distributions within the body, neutrophils represent a class of leukocytes seemingly devoted to immune responsiveness. They are recruited to the site of inflammation from circulatory and hematopoeitic storage sites. Upon arriving at the site, they follow the same three stages of phagocytosis as macrophages, but they are 'suicidal', actively engulfing the foreign particle and releasing degradative enzymes,

antimicrobial molecules and toxic metabolites. In doing this, they destroy and contain the potential pathogen to the site of inflammation and recruit other immune cells (Neumann *et al.*, 2001).

At the same time NCC (in mammals NK cells) become activated, killing infected cells and releasing macrophage activating factors (MAF). The release of cytokines upregulates the production and release of a range of serum proteins, the so-called acute phase proteins from the liver. These act to minimize host damage or act directly on the pathogen. In fish, clotting factors, haemoglobin binding protein, antioxidants and antiproteases have been identified, all of which minimize host damage. In mammals, the major acute phase proteins, C-reactive protein, serum amyloid A and serum amyloid P, function to regulate the immune response. Equivalents to these have been found in fish, but it is not certain if they function in the same way as their mammalian counterparts. Complement proteins C3 and C4 are upregulated during the acute phase. A difference between the fish and mammalian complement is that the C3 protein in teleost is present in multiple isoforms that have different binding efficiencies to various complement-activating surfaces. This explains the five to ten times higher activity seen in the teleost antibody independent, or alternative, complement activation pathway. These differences may well give the fish innate immune system a broader recognition capability than its mammalian counterpart (Watts *et al.*, 2001). The activation of the inducible innate immune system is a prerequisite to the initiation of the adaptive immune response.

1.3.4 The adaptive immune system of teleost fish

The adaptive immune system is characterized by the generation of a broad spectrum of antigen specific receptors formed by recombination of genomic recombination in lymphocytes. In T lymphocytes this is the TCR, in B lymphocytes this is immunoglobulin (Ig) antibody. Genes encoding Ig, TCRs, MHC I and II and recombination activating genes (RAG-1 and -2) have been found in teleosts (Schluter *et al.*, 1999). The MHC I and II genes are linked in tetrapods and sharks in a complex on a single chromosome. In teleost fish, these genes seem to be present in several different linkage groups (Dixon and Stet, 2001). The cells of the adaptive immune system can be differentiated in two main subtypes, the B and T lymphocytes, which can be further divided into B1 and B2 lymphocytes and helper and cytotoxic T lymphocytes (Janeway, 2001)

1.3.4.1 T lymphocytes of teleost fish

T-cell receptor genes have been cloned from teleost fish, but monoclonal antibodies to differentiate between the different subpopulations (CD 8+ Cytotoxic T lymphocytes (CTL) and CD 4+ helper Th1 and Th2 T lymphocytes) have been elusive. T lymphocytes are identified in fish as surface Ig (sIg) negative lymphocytes (Koumans-van Diepen *et al.*, 1994). The existence of the other subtypes is deduced from functional assays. Graft rejection and delayed type hypersensitivity seen in teleosts imply specific cell mediated immunity via cytotoxic T lymphocyte activity requiring both CTL and Th1 T lymphocytes. The production of specific antibodies seen in all teleost species so far

studied requires the collaboration of APCs, Th2 lymphocytes and B2 lymphocytes (Watts *et al.*, 2001).

1.3.4.2 B lymphocytes of teleost fish

The first subset of B lymphocytes (B1- B lymphocytes) are known in both the mammalian and teleost immune system. These B lymphocytes are able to respond non-specifically to common ubiquitous pathogen and self antigens (Watts *et al.*, 2001). These antibodies could be seen as part of the innate immune system. They form a first line of defense and function in immune regulation as well. They have a lower affinity than the antibodies produced by B2 lymphocytes and do not require T lymphocyte help to initiate production. They are polyreactive to provide protection against a broad spectrum of pathogens, are of the IgM isotype and do not go through affinity maturation (Janeway, 2001). The polyreactive antibodies in both teleost and humans bind a remarkably similar spectrum of antigens. In some teleosts, particularly Atlantic cod (*Gadus morhua*) they can be found in high concentration (Pilstrom and Petersson, 1991).

The second subset of B lymphocytes (B2 lymphocytes) function in a way more distinct from their mammalian counterparts. Both are dependent on Th2 lymphocyte help and antigen presentation by APCs to produce antigen specific antibodies, initially of the IgM subtype. This is the only type of secreted Ig found in teleost. In the mammalian humoral response a process of isotype switching provides the full range of secreted immunoglobulins (IgA, IgM, IgE and IgG). The humoral response is further magnified by an increase in binding strength of the antibodies by a process called affinity maturation

(Janeway, 2001). These process require specialized lymphoid tissue called germinal centers, not found in fish. However, small increases in antibody affinity have been found in rainbow trout, although the process behind this is unknown (Kaattari *et al.*, 2002).

An important function of B2 lymphocytes is the formation of memory cells to ensure an adequate humoral response on re-exposure to a pathogen, called the secondary response. The secondary response seen in mammals is fast and shows a logarithmic increase in the levels of production of IgG with a higher affinity compared to the initial, or primary response (Janeway, 2001). In teleosts, the differences between the primary and secondary responses are a reduced lag phase in the appearance of specific antibody after exposure and a moderate increase in antibody level (Watts *et al.*, 2001). The goal of vaccination is priming the immune system for memory formation for either or both the cell mediated and the humoral responses.

1.3.5 Conventional vaccines used in aquaculture

The ability of fish to respond to vaccination with both a cellular and a humoral response has been well established (Shao, 2001) and vaccination has become standard practice in aquaculture (Gudding *et al.*, 1999). There are several distinct categories of conventional vaccines: 1) whole inactivated pathogen, commonly mixed with an oil-based adjuvant (Ellis, 1997); 2) live organisms which can be either nonpathogenic organism expressing protective antigens of the pathogen of interest (Noonan *et al.*, 1995), or an attenuated strain of the pathogen (Griffiths *et al.*, 1998; Kim *et al.*, 2000); and 3) vaccines containing recombinant antigens expressed in prokaryotic or eukaryotic cells

which can be grown in large scale cultures under controlled conditions, from which the antigen can be purified (Lorenzen, 1999). The most commonly used vaccines in salmonid aquaculture belong to the first group, mainly due to the low cost of development and production. Live attenuated vaccines carry the risk of a reversion to virulence as well as shedding into the environment of a genetically modified organism for a non-virulent organism expressing foreign antigens (Gudding *et al.*, 1999).

Vaccines can be administered to fish orally, by immersion or by injection. Oral vaccination is the preferred route as the vaccine can be mixed with the feed, reducing handling stress to the fish and labour costs. No effective oral vaccines are currently available for use in aquaculture. Development of oral vaccines have focused on encapsulation of the antigen to protect it from the early stages of the digestive system (Joosten *et al.*, 1997; Irie *et al.*, 2003; Vervarcke *et al.*, 2004). Immersion vaccines are administered by direct immersion in a concentrated vaccine formulation, hyperosmotic immersion (i.e. direct immersion preceded by a brief immersion in a hypertonic solution) or by spray vaccination of larger animals. Although the added benefit of the hyperosmotic immersion has in the past been questioned, recently Huising *et al.* ((Huising *et al.*, 2003) using this technique, showed an increase in uptake of soluble, but not particulate components of vaccine plus a brief stimulation of the innate immune system, increasing the efficacy with which antigens were processed and presented by APCs and increasing the specific mucosal response. For fish, injection of vaccines is the most common method of vaccination. Vaccines are injected *i.p.* and often contain an oil-based adjuvant which prolongs the immune stimulation and activates the innate immune system.

Alternatives to oil are aluminum salt and glucan which produce a more moderate effect in improving the potency (Shao, 2001), but glucan based compounds show a more immediate effect then oil-based adjuvants, which show a longer duration in the response. A double-adjuvant principle combines glucans and oil-adjuvants for both short- and long term protection (Gudding *et al.*, 1999). A fourth, experimental method of administration has been developed by Nakanishi *et al.* ((Nakanishi et al, 2002). They used a method of percutaneous administration by immersion with application of a multiple puncture instrument. This method showed results comparable with *i. p.* injection, but could be used on fish too small and fragile for injection and was more effective then direct immersion.

1.4 DNA VACCINES

1.4.1 Introduction

A revolution in vaccine technology has been underway since the development of what are commonly called DNA vaccines or genetic vaccination. Significantly different from previous vaccines, they do not directly utilize antigen from a pathogen to stimulate the immune response, but rather use genes coding for these antigens. Once relevant genes from the pathogen are identified, they are inserted into a bacterial plasmid containing the proper eukaryotic translation and transcription signals, which is then administered to the host. The plasmid fulfills a dual role in that first, it is taken up by the host's cell where the gene of interest is translated and transcribed into protein antigen and secondly,

because of the prokaryotic origin of the DNA it can also act as an adjuvant. The theoretical advantage of DNA vaccines are manifold. They are stable, cost efficient and can be optimized to induce the most appropriate immune response for each pathogen (Lewis and Babiuk, 1999; Liu, 2003).

1.4.2 Early development of DNA vaccines

During the late 1980's and the early 1990's it became clear that bacterial plasmids were capable of entering mammalian cells and that transcription and translation of genes encoded on these plasmids could occur, as long as they were under control of eukaryotic regulatory sequences (promoters). Although originally envisioned to be useful to gene therapy development, the possibility of using these plasmids to provoke a protective response against pathogen derived genes was quickly recognized.

1.4.3 Characteristics of a DNA vaccine

The backbone of DNA vaccine plasmid contains a number of genes and regulatory sequences. First, it contains a bacterial origin of replication or *ori*, necessary for the replication of the plasmid in its bacterial host. Second, it has a selection marker, for example an antibiotic resistance conferring gene. This ensures that, when the bacterial host is grown for plasmid production and antibiotic is added to the growth medium, the bacterial host can only grow if it maintains the plasmid. One commonly used antibiotic resistance marker is an ampicillin resistance conferring gene. Third, there is a gene encoding the desired protein antigen. This can be a gene of bacterial, viral or eukaryotic

parasitic origin, or a novel synthetic gene, but the gene has to be flanked by eukaryotic transcription regulators. The gene is preceded by a promoter and often a Kozack sequence is added to optimize ribosome binding. The promoter is most often of viral origin, Cytomegalovirus (CMV) for example is commonly used, but in Atlantic salmon the fish derived carp β-actin (CBAP) promoter has been shown equally efficient (Gomez-Chiarri and Chiaverini, 1999). The other end of the gene requires a poly-adenylation signal, required for mRNA stability. An intron can be added to the gene to increase the efficiency of the plasmid. In addition to these essential segments of the DNA vaccine, additional material can be cloned into the plasmid vector to increase the amount of antigen produced and to stimulate and direct the immune response.

1.4.4 Interaction between the immune system and the DNA vaccine

There are two main functions of any vaccine preparation; first it has to provide the immune system with an appropriate antigen and in a location available to the immune system and secondly, it has to stimulate the immune system so it will mount a response. In most traditional vaccines, with the exception of live attenuated pathogens, this would require an adjuvant. In DNA vaccines both functions are provided by the plasmid.

1.4.4.1 Production and localization of antigen

Once produced by the transcription and translation machinery within the host cell, the immune system can interact with the encoded antigen by three main processes. If the plasmid is taken up by myocytes or other non immune cells, part of the translated protein

will enter into the proteosome where it is processed into small peptides. These peptides combine with MHC I and are transported to the outer surface of the cell where it can be sampled and recognized by CD8+ cytotoxic T-Lymphocytes. This process can be stimulated by adding genes encoding cytokines to the plasmid (Kanellos *et al.*, 1999b). Interferon-γ (IFN-γ) is known to increase the function of the proteosome (Janeway, 2001). Alternatively, a signal peptide can be added to the antigen that directs the protein antigen to be secreted from the cell, which will promote a predominantly antibody mediated response. Secondly, APCs in the muscle or the draining lymph node can internalize the plasmid, produce the encoded antigen and present it on MHC II (Liu, 2003). Thirdly, non-antigen presenting cells that have become transfected and have translated the encoded antigen, may become apoptotic. This process of programmed cell death ends with the cell being reduced to a number of apoptotic bodies, small membrane enclosed vesicles. These are taken up by APCs, so providing a method of cross presentation, where the encoded protein is produced in non-immune cells but presented by APCs.

1.4.4.2 Initiating an immune response

As described in section 1.3, the initiation of an adaptive immune response depends on activation of APCs of the innate system, so that they will present the antigen together with the required co-stimulatory signals. These cells become activated through their PRR binding to PAMPs (Medzhitov and Janeway, 1997). As the plasmid is of bacterial origin nucleotide sequences that function as PAMPs will be unmethylated.

Vertebrates have selected against these sequences, as shown by the fact that they appear at one fourth the frequency as expected by random base usage (Meng *et al.*, 2003). The CpGs that are present in vertebrates are highly methylated. This phenomenon is even more significant in fish and amphibians as their genome is twice as methylated as is the mammalian genome (Jabbari *et al.*, 1997). These CpG motifs are therefore an excellent PAMP and the PRR for them has been identified as the Toll-like receptor 9 (TLR-9), although more receptors may be involved (Hemmi *et al.*, 2000). The activating effect of specific CpG motifs on the innate immune system has been shown in grass carp (*Ctenopharyngodon idellus*) (Meng *et al.*, 2003), Atlantic salmon (Jorgensen *et al.*, 2001) and goldfish (*Carassius auratus*) (Kanellos *et al.*, 1999b), and Ishimoto *et al.*. (2004) have been able to clone a non-specific cytotoxic cell receptor 1 (NCCRP-1) type gene from Tilapia after stimulation with specific CpG's. By increasing the number of these specific CpGs, also known as immuno-stimulatory sequences or ISS in the plasmid the adjuvant effect of the plasmid can be increased.

1.4.4.3 Modifying the immune response

The type of immune response provoked by a DNA vaccine which can be either cell mediated or humoral and either systemic or mucosal can be modified in two ways. First, as with all vaccines the mode and location of administration of the vaccine will influence the response (Gomez-Chiarri and Chiaverini, 1999). Administration to mucosal sites can be achieved by nasal spray in mammals or immersion in fish. An intramuscular (*i.m.*) or *i.p.* injection will favor a more systemic response. DNA vaccines by their nature

favor a cell mediated response but as mentioned before, by including signal peptides the antigen can be secreted into the circulation to favor a humoral response.

Second, genes encoding immunologically significant signal peptides or proteins can be included into the plasmid. Examples of these would be transforming growth factor- β (TGF-β) to direct the immune response to a mucosal response (Min *et al.*, 2001), IL12 for a cell mediated type of response (Sakai *et al.*, 2003) and IL-4 for a humoral response (Kim *et al.*, 1998).

1.4.5 DNA vaccines in fish

The potential for DNA vaccination in fish has been studied in a wide range of teleosts such as rainbow trout (Anderson *et al.*, 1996; Lorenzen *et al.*, 2001), goldfish (Kanellos *et al.*, 1999a), glass (*Kryptopterus bicirrhus*) (Dijkstra *et al.*, 2001) and channel catfish (*Ictalurus punctatus*) (Nusbaum *et al.*, 2002), turbot (*Scopthalmus maximus*) (Sommerset *et al.*, 2003) and zebra fish (*Brachydanio rerio*) (Heppel *et al.*, 1998). However, to date the only effective DNA vaccines against infectious agents are limited to members of the rhabdoviruses, infectious hematopoietic necrosis virus (IHNV) (Anderson *et al.*, 1996) and viral hemorrhagic septicemia virus (VHSV) (Boudinot *et al.*, 1998), both belonging to the *Novirhadovirus* genus and the channel catfish herpesvirus (IHV-1) (Nusbaum *et al.*, 2002). In each of these, genes coding for glycoproteins produced the highest levels of protection (Lorenzen *et al.*, 2002) (Nusbaum *et al.*, 2002). The response against VHSV and IHNV can be differentiated in an early, short lived and non-specific response that protects against heterologous viruses and a delayed, long term

and specific response (LaPatra *et al.*, 2001; Lorenzen *et al.*, 2002; Sommerset *et al.*, 2003). This early non-specific response is triggered by the rhabdoviral glycoprotein G of not only VHSV and IHNV, but of the snakehead rhabdovirus (SHRV) and spring viremia of carp virus (SVCV) as well (Kim *et al.*, 2000). This protection is mediated by induction of a potent interferon response and Mx protein expression (Kim *et al.*, 2000; McLauchlan *et al.*, 2003).

The main technical obstacle for the use of DNA vaccines in aquaculture is the method of delivery. Standard practice in experimental vaccines has been delivery by *i.m.* injection which is not practical and/or cost effective for small fish or low-valued species (Heppell and Davis, 2000). In goldfish, a plasmid in oil was injected *i.p.* and was able to elicit an antibody response only two-fold less than if the plasmid was injected *i.m.* (Kanellos *et al.*, 1999a). As most aquaculture vaccines use an oil-based adjuvant and are *i.p.* injected, this suggests that DNA and bacterin vaccines could be mixed and administered in a single dose. Alternatively, new administration methods are being developed where bacteria are used as carrier system to deliver DNA vaccines (Dietrich *et al.*, 2001; Simon and Leong, 2002). To date no protective DNA vaccines against bacterial pathogens have been published.

1.5 HYPOTHESIS AND OBJECTIVES

The hypothesis of this thesis is that it is possible to use DNA vaccines to protect fish from an intracellular bacterial pathogen. To test this hypothesis, five specific aims have to be fulfilled:

1. Monitoring *P. salmonis* production

To ensure sufficient material for DNA purification, large scale production of *P. salmonis* is necessary. To determine the growth dynamics of *P. salmonis* during production in CHSE-214 cell culture, immunological assays are required to reproducibly determine the amount of *P. salmonis* present in the supernatant.

2. Purification of *P. salmonis* DNA free from CHSE-214 host cell DNA

Genomic *P. salmonis* DNA without contamination from CHSE-214 host cell DNA is a prerequisite to any genetic studies of the pathogen. Methods for differential purification of *P. salmonis* DNA and assessment of purity will be developed.

3. Generating a genomic expression library of *P. salmonis*

Very little genetic information on *P. salmonis* was available at the outset of this project. To obtain relevant DNA sequences, the generation of a genomic library was chosen as the most appropriate way to identify *P. salmonis* genes. By using a library vector capable of expressing the *P. salmonis* fragments it contains, immunoreactive clones can be identified by screening with antiserum from rabbits immunized with *P.*

salmonis. This will identify segments of the *P. salmonis* genome coding for immunoreactive proteins. The selection of clones recognized by the rabbit immune system will increase the likelihood of finding inserts coding for protective antigens.

4. Identification and characterization of potential DNA vaccine candidate genes

By sequencing immunoreactive clones from the *P. salmonis* expression library relevant sequence information can be obtained. Analysis of deduced amino acid sequence can provide a putative function by comparison to nucleotide and amino acid sequences present in Genbank. Fragments from genes considered to have the most potential as DNA vaccine by putative function or location on the pathogen will be further investigated. Molecular techniques will be used to further expand the sequence available if only partial genes are available from the library

5. Production and assessment of a possible candidate DNA vaccine

A candidate DNA vaccine will be developed using the gene judged most appropriate. The selected gene will be cloned into an appropriate plasmid vector and evaluated by *in vitro* transfection and in a *P. salmonis* challenge trial.

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CHAPTER 2: MONITORING GROWTH DYNAMICS OF CULTURED *PISCIRICKETTSIA SALMONIS* USING ENZYME LINKED IMMUNOSORBENT ASSAY

2.1 ABSTRACT

The salmonid pathogen *Piscirickettsia salmonis* is a slow growing fastidious rickettsial organism. To optimize the production of antigen from this organism for research and for development of vaccines, an immune-based test was developed to monitor the growth of the bacteria during *in vitro* culture. For this purpose, two polyclonal antisera were raised against *P. salmonis* in rabbits and mouse monoclonal antibodies were developed. Five parental monoclonals reacted with *P. salmonis* and two of these were further subcloned. All monoclonals and subclones were assessed by immunofluorescence and Western blot. The polyclonal antisera and a monoclonal antibody were used to develop a sandwich enzyme linked immunosorbent assay (s-ELISA). The s-ELISA was used to determine the growth dynamics of *P. salmonis* in CHSE-214 cell culture.

2.2 INTRODUCTION

Piscirickettsia salmonis, and the associated syndrome, Salmonid Rickettsial Septicemia (SRS) cause significant losses in the salmonid aquaculture industries (Fryer *et al*, 1992; Mauel and Miller, 2002). The organism has been isolated from fish worldwide,

however losses in cultured fish are most pronounced in Chile (Almendras and Fuentealba, 1997). When isolated, it was the first rickettsia-like organism identified in fish (Cvitanich *et al*, 1991). It is an obligate intracellular pathogen with a replication cycle that takes approximately 14-20 days to achieve 100% cytopathological effect (CPE) in chinook salmon (*Oncorhynchus tshawtscha)* embryonic cell line CHSE-214. Although the organism is sensitive to a range of antibiotics *in vitro*, chemotherapeutic agents have had little success in combating SRS (Evelyn *et al*, 1998), leaving vaccine and vaccination as a necessary treatment option.

Vaccines for *P. salmonis* could be in the form of a bacterin; a product derived from wild type or culture adopted *P. salmonis* that is killed or inactivated, a recombinant vaccine; in which protective antigens are expressed in a more readily grown bacteria like *Escherichia coli*, or a nucleic acid or DNA vaccine in which the gene encoding the protective antigen and the elements regulating its expression form the basis of the vaccine. Large amounts of cultured organism are needed for bacterin production (Smith-Schuster *et al*, 1994). In contrast, a better understanding of the genome of the pathogen is necessary to select the genes of interest for use in recombinant or DNA vaccines. This requires significant amounts of pathogen as starting material for the genetic studies. As *P. salmonis* is fastidious to culture, optimization and monitoring of the growth of *P. salmonis* in culture is required. Common methods that are applicable to *P. salmonis* include the 50% tissue culture infective dose ($TCID_{50}$) or a plaque assay. Unfortunately both methods are very labor intensive and with CPE appearing as late as 2 weeks post inoculation this does not permit a rapid assessment of the amount of *P. salmonis* present.

The enzyme linked immunosorbent assay (ELISA) is capable of quickly determining the amount of free *P. salmonis* antigen present in culture. Results are rapid (~3 hrs) compared to the several days or weeks needed with $TCID_{50}$ or plaque assays. In a direct ELISA, the sample antigen of interest is directly bound, or coated onto a microtiter plate. The bound antigen can be detected and quantified by incubating the plate with antibodies specific to the antigen of interest. These antibodies are then conjugated to an enzyme such as horseradish peroxidase (HRPO) or alkaline phosphatase (AP). After removing the unbound antigen-enzyme conjugate the total enzyme activity remaining can be measured by addition of a substrate to the enzyme giving a colorimetric reaction. The enzyme activity is a measure for the amount of bound antibody- enzyme conjugate and therefore for the amount of antigen present. In an indirect ELISA, the primary antibody bound to the antigen is not conjugated and is detected by a secondary antibody-enzyme conjugate specific to the primary antibody used (Kemeny, 1997) . Unfortunately, not all antigens will bind optimally to the plate at this standard pH of 9.6 because their outer surface structure does not contribute proper electrostatic forces. Additionally the antigen may not bind because of competition with large amounts of non-relevant protein in the sample. To avoid these problems a sandwich ELISA can be used, in which the microtiter plate is first coated with an antibody specific to the antigen of interest and all remaining free binding sites are blocked with a non-relevant protein. When the sample is applied to the antibody coated plates, only the antigen of interest will be captured by the bound capture antibody. After washing the unbound fraction of the sample away, the amount of antigen can be quantified as in a normal ELISA.

The objective of this study was to determine the growth dynamics of cultured *P. salmonis* and develop the tools needed to achieve this in a reproducible fashion.

2.3 MATERIALS AND METHODS

2.3.1 Growth of CHSE-214 cells and *Piscirickettsia salmonis*

The chinook salmon embryo cell line (CHSE-214) (Lannan *et al*, 1984) was used to culture *P. salmonis*. For maintenance, cells were grown in Eagle's minimum essential medium (MEM) with 10% heat inactivated fetal bovine serum (FBS) (MEM-10). The cells were grown in either T-225 or T-75 cell culture flasks. For propagation, monolayers of CHSE cells that had reached confluency no less than three days prior were used. Supernatant was discarded and the monolayer was rinsed with 5 to10 ml of 1:2000 (w/v) trypsin (GibcoBRL) in Hanks balanced salt solution (HBSS), the wash discarded and the monolayer was incubated with another 5 to10 ml aliquot of this buffer. The monolayer was incubated until the cells had just begun to detach form the surface of the flask. The buffer was then discarded and the cells dislocated from the flask by striking the flask with an open hand. The flask was rinsed with MEM-10 to collect the cells and this cell suspension was seeded into flasks to 3 times the original monolayer area so that one T75 flash generated three additional T75 flasks or one T225 flask

Piscirickettsia salmonis was grown in CHSE-214 cells by inoculating the culture with at least 1 ml for a T75 and 5 ml for a T225 of the supernatant from a culture that has shown at least 80% cytopathic effect (CPE) as judged by rounding of the CHSE-124 cells

and detachment of the monolayer. The *P. salmonis* was harvested after complete detachment of the monolayer (100% CPE).

2.3.2 Purification of *P. salmonis* antigen

Fluid from an infected CHSE-214 culture showing 100% CPE was collected and placed in 50 ml sterile polycarbonate tubes (Falcon). Intact CHSE-214 cells and cell fragments were removed by centrifugation at 500 x g for 10 minutes in a Beckman benchtop centrifuge. The supernatant was then gently removed and centrifuged at 15,000 x g in a JA-14 rotor on a Beckman J2-21M/E High speed centrifuge at 4°C. The supernatant was discarded and the pellet in each tube was resuspended in 0.85% sterile saline and centrifuged at 15,000 x g in a JA-20 rotor at 4°C. This was repeated twice and finally the pellet was resuspended in 1.5 ml of sterile PBS.

2.3.3 Generation of polyclonal rabbit serum

The polyclonal rabbit serum was raised as described by Jones *et al* (1998). Briefly, two naive female New Zealand white rabbits were injected intramuscularly with the *P. salmonis* antigen emulsified with an equal volume of Freund's incomplete adjuvant (FIA). Thereafter, rabbits received subcutaneous injections of antigen at regular intervals until sufficient titres were obtained. Serum was collected and the immunoglobulin fraction was purified by precipitation in 50% saturated $(NH_4)_2SO_4$ and anion exchange chromatography (Jones *et al.*, 1998).

2.3.4 Generation of monoclonal antibodies

Antigen was prepared as above and the protein concentration was determined by protein assay (Boehringer Mannheim). A suspension of *P. salmonis* containing 300 μg protein was then concentrated by centrifugation for 60 minutes at 100,000 x g in a Beckman ultra centrifuge at 4°C. The pellet was resuspended in sterile 0.85% saline to obtain 300 μg of *P. salmonis* antigen in 250 μl of saline. This was mixed 1:1 with 250 μl of RIBI™ adjuvant for the initial immunization and with monophosphoryl lipid A and synthetic Trehalose 6,6'-dimycolate (MPL + TDM adjuvant system, Sigma Biosciences) for subsequent booster injections.

The immunization schedule can be found in Table 2.1. Four days after the final boost, one of the mice was sacrificed and spleen cells were fused with FoxNY cells. The second mouse was boosted with 100 μg in 200 μl of purified *P. salmonis* and four days later sacrificed and spleen cells were fused with SP2 myeloma cells (Kennett, 1979).

2.3.5 Screening of potential monoclonal antibodies

2.3.5.1 Immunofluorescence

Culture supernatant from hybridomas arising were screened for reactivity to *P. salmonis* antigen by indirect immunofluorescence (IFAT). Fifty microliters of *P. salmonis* suspension was placed onto a multi well immunofluorescence slide and air dried. The slide was then fixed in methanol for 5 minutes and air dried again. Fifty microliters of supernatant from hybridoma culture were added per well and incubated for 30 minutes in a moist chamber. The slides were washed three times with PBS for 7

minutes per wash. After the third wash excess liquid was blotted off. Fifty microliters of rabbit α- mouse IgG and IgM isotype specific antibodies conjugated to fluorescein isothiocyanate (FITC) and diluted 1:100 in PBS with 1:200 Evan's blue counterstain, were added and incubated for 30 minutes in a moist chamber in the dark. The slides were washed three times with PBS for 7 minutes per wash, in the dark. After the last wash, excess liquid was washed off and mounting fluid was applied before covering the slide with a cover slip. The slides were observed under fluorescent microscopy, and wells scored on a 0 to 3 scale with 0 indicating no fluorescence and 3 the brightest level.

2.3.5.2 Direct ELISA

To determine the optimal binding conditions of *P. salmonis* and evaluate the monoclonal antibodies, a direct ELISA was performed. For this, a 96 well microtitre plate was coated with antigen purified from a culture of *P. salmonis* as described in paragraph 2.3.2 and serially diluted in Na_2CO_3 / $NaHCO_3$ coating buffer with a pH of 9.6. The *P. salmonis* was allowed to bind overnight at room temperature. After three washes with PBS-T, the remaining binding sites were blocked with 5% bovine serum albumin (BSA) in PBS - 0.05% Tween-20 buffer (pH 7.4) (PBS-T / BSA 5%) for one hour at room temperature. The plate was washed three times with PBS-T and 100 µl of undiluted monoclonal supernatant was added per well and incubated for one hour at room temperature. The plate was washed three times with PBS-T, after which 100 µl of Horseradish peroxidase labeled Rabbit α- mouse Ig (IgA, IgG and IgM isotype specific) (Rα-M HRPO) diluted 1:1000 in PBS-BSA 1% was added and incubated for one hour at

room temperature. The plate was washed three times with PBS-T and the 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) - H_2O_2 substrate added and incubated for 30 minutes at 37°C in the dark. The resulting optical density (OD) was then measured as the difference between the absorbency at 405 and 450 nm wavelength, using a Microdevices plate reader. The resulting data was plotted using the SoftPro software package.

The parental monoclonals that showed a signal were titrated in a second ELISA. For this, the optimal dilution of *P. salmonis* found in the first ELISA was used and coated onto a 96 well microtitre plate as described above. The ELISA was performed as above with this difference that the monoclonals were serially diluted from 1:2 to 1:2048 in PBS-T / BSA 1%. The parental monoclonals were subcloned by the process of limiting dilution. For this, hybridoma cells of each clone were collected, centrifuged and resuspended. The cells were counted and diluted to a concentration of 0.7 cells per 100 µl in growth medium. The cell suspension was transferred to 96 well tissue culture plates at 100 µl per well and incubated at 37°C. Wells showing colonies were considered to be derived from a single cell. The subclones of the best reacting monoclonal were tested in the sandwich ELISA described below.

2.3.5.3 Isotyping of monoclonal antibodies

The isotype of the monoclonals was determined using Boehringer Mannheim Isotyping test strips, using the manufacturer supplied protocol.

2.3.5.4 Western blots

Conditions for sodium dodecylsulfate - polyacrylamide gel electrophoresis (SDS-PAGE) were those described by Laemmli (Laemmli, 1970) with a 12.5% running gel, and a 5% stacking gel using a BioRad mini Protean II system. Purified *P. salmonis* from tissue culture was pelleted by centrifugation at 15,000 x g in an Eppendorf 5415C centrifuge. The pellet was dissolved in sample buffer and boiled for ten minutes. After cooling, 50 µl was loaded into 9 of the 12 wells available, one well was loaded with 5 µl of pre-stained molecular weight marker, and the two outer wells were sham loaded with sample buffer. The gel was mounted into a Bio-Rad mini Protean II chamber and run for one hour at 100 Volts. Afterwards, the gel was removed from the chamber and equilibrated with transfer buffer (25 mM Tris.HCl, 192 mM glycine, pH 8.3). A nitrocellulose membrane was applied to the gel and the location of all wells was marked on the filter with pencil. The sandwich of gel and membrane was mounted into the mini Protean II again. An ice pack was added to maintain temperature during transfer. The antigens were transferred from gel to membrane by applying 120 Volts for one hour. After transfer, the remaining binding sites on the nitrocellulose membrane were blocked by incubating the membrane one hour in 2% BSA dissolved in 0.5M NaCl buffered with 20 mM Tris.HCl to a pH of 7.5 (Tris buffered saline or TBS). The membrane was washed twice with TBS with 0.05% Tween 20 (TBS-T) for ten minutes per wash. The position of the pre-stained marker proteins was marked on the blot, the blot was cut in strips with one *P. salmonis* loaded lane per strip and each strip was then transferred to a 10 ml tube. Each strip was incubated for one hour with 200 µl of supernatant from monoclonal

antibody culture, with the tubes horizontally mounted in a rack fixed to a shaker table to assure even distribution of the antibody solution. The strips were washed three times with TBS-T for seven minutes per wash after which 200 µl of Rα-M HRPO diluted 1:200 in TBS-T/BSA 1% was added and incubated for one hour as described above. After three washes with TBS-T, the strips were washed twice with straight TBS, all for seven minutes per wash and the 4-chloro-1-naphtol - H_2O_2 substrate buffer added. The colour reaction was left to develop in the dark until the desired level of signal was achieved after which the reaction was stopped by rinsing the strips with water.

2.3.6 Sandwich ELISA

The method described in paragraph 2.3.5.2 for a direct ELISA by direct binding the *P. salmonis* antigen to the matrix of a 96-well plate had only limited success. Hybridoma supernatant from wells positive on IFAT and Western Blot produced very low signals and *P. salmonis* containing culture needed to be processed to remove excess proteins from the FBS. Therefore, an indirect, sandwich ELISA (s-ELISA), using the polyclonal rabbit antisera as capture antibody and the highly specific monoclonal described above as secondary antibody and a Goat α-Mouse IgG, A, M conjugated with horseradish peroxidase (HRPO) as detector antibody was developed.

The rabbit polyclonal antisera was titrated as follows: serial dilutions of the polyclonal antibody in a Na_2CO_3 / $NaHCO_3$ coating buffer at pH=9.6 were made in a 96 well plate and left overnight at room temperature. The plate was then washed with PBS - T and remaining binding sites blocked by incubating the plate for 1 hour with PBS-T

/BSA 5%. Again, the plate was washed three times with PBS-T. The goat α- rabbit Ig HRPO labeled conjugate was then applied in a 1:1000 dilution in PBS-T /BSA 1% and the plate incubated for 1 hour at 37°C. The plate was washed three times with PBS-T and the ABTS - H_2O_2 substrate added and incubated for 30 minutes at 37°C in the dark. The OD was then measured as described previously.

The optimal coating was determined to be 8.5 μg/ml of antibody in coating buffer. To titrate the detecting antibody, a plate coated with optimal concentration of capture antibody was prepared as above. Supernatant from a culture of *P. salmonis* infected cells showing 100% CPE was centrifuged at 500 x g in a Beckman benchtop centrifuge for ten minutes and 100 μl of the supernatant from this was added to each well of the antibody coated microtitre plate. After one hour of incubation at room temperature, the plate was washed three times with PBS-T. The monoclonal was serially diluted leaving the last column of wells as controls. After one hour of incubation at room temperature, the plate was washed three times with PBS-T. The goat α- mouse Ig HRPO labeled conjugate was then applied in a 1:1000 dilution in PBS-T /BSA 1% and the plate incubated for one hour at 37°C. The plate was washed three times with PBS-T and the substrate added as described above. The results were plotted as optical density at 405 nm minus optical density at 450 nm on the Y-axis and the dilution series on the X-axis using a log scale. The following four parameter equation was fitted to the results:

$$y = \left[\frac{(A - D)}{1 - C\left[x^{1/B}\right]}\right] + D$$

Where "y" is the fitted OD, "x" the concentration of the serial diluted antigen or antibody; A is the OD at the left plateau (concentration approaching 0); D the OD at the right plateau (concentration approaching ∞); "C" is the concentration at which OD is 50% of max and "B" a measure of the steepness of the curve. The value of "C" is the most stable between ELISA's run on different days and as such can be best used to compare ELISA's (Caulfield and Shaffer, 1984).

2.3.7 Application of the s-ELISA

To determine the practicality and reproducibility of the s-ELISA, four tissue culture flasks with a confluent monolayer of CHSE-214 cells were inoculated with *P. salmonis*. Samples were taken immediately before addition of the inoculum, immediately after, after 3 hours and at day 1, 2, 4, 5, 6, 7, 8, 10 and 14 post inoculation. All samples were frozen at -20°C at the time of sampling. Six Immulon 2 Microtiter plates (Dynex technologies) were coated overnight with 100 µl/well of 8.5 µg/ml of rabbit antiserum. An s-ELISA as described above was performed with each of the samples in quadruplicate and in two concentrations (full strength and 1:1 with PBS-BSA 1%). Each plate also contained *P. salmonis* from one particular harvest (F^{10}), previously frozen, titrated from undiluted to 1:64 as internal standard. The amount of *P. salmonis* present in each sample was expressed as relative to the undiluted standard as determined using a four parameter equation present in the SoftPro program. If the undiluted sample was outside the standard range, only the 1:1 was used.

2.4 RESULTS

2.4.1 Monoclonal antibody titer and specificity

The result of the monoclonal fusion can be seen in Table 2.2 with all clones selected showing reactivity in either Western blotting or IFAT. Of the five parental clones or subclones positive in IFAT tested by direct ELISA, only subclones from 3D11 did not show reactivity in the direct ELISA. When titrated with antigen the best response of the other clones was seen at a dilution of *P. salmonis* antigen of 1:80. Using this antigen concentration, the four reactive clones were titrated (Figure 2.1). Clone 10E6 was considered the antibody of choice as it has the best titre with an optimal antibody dilution of 1:80. The 10E6 subclones 10E6.6D2, 10E6.14F2, 10E6.6E3 and 10E6.6C5 were tested in sandwich ELISA (Figure 2.1). The subclone 10E6.6C5 was selected for use in sandwich ELISA

2.4.2 Polyclonal rabbit sera

Both rabbit antisera were able to function as a capture antibody in the sandwich ELISA (Figure 2.2). Background optical density caused by the conjugate binding to the rabbit antibodies was dependent on the concentration of rabbit antibody. To fit the four parameter curve, background levels were subtracted from the results giving an adjusted result. The unfitted and fitted graphs can be seen in Figure 2.2. For rabbit #1, a curve was fitted with a formula of adjusted result = ((0.006 - 0.717) / (1 + [x / 0.202]$^{2.999}$)) + 0.717 and for the fitted curve for rabbit #7, a formula of adjusted result = ((0.025 - 0.897) / (1 + [x / 0.206]$^{2.637}$))+ 0.897, with "x" being the concentration of capturing antibody

with which the well was coated. Serum from rabbit #7 was selected as the capture antibody.

2.4.3 Application of the s-ELISA

Relative amounts of *P. salmonis* were determined for each sample by comparing the OD of samples with that of the plate internal standard (frozen *P. salmonis* from harvest F^{10}). No difference in relative *P. salmonis* quantities were found between the four flasks sampled at each day. Therefore the results were pooled to get an average. The resulting curve is shown in Figure 2.3. Low relative amounts of *P. salmonis* were seen for the first four days, followed by a rapid increase at day six. A shoulder-point can be seen around day seven.

2.5 DISCUSSION

The main objective of this study was to establish the growth dynamics of *P. salmonis* in CHSE-214 culture. Direct ELISA proved to be unsuitable for this purpose due to the inefficient binding of the organism to the microtitre plate. The availability of both *P. salmonis* specific rabbit antisera and monoclonal antibodies allowed the development of a sandwich ELISA. Sandwich ELISA has been used to follow levels of virus produced by cultures of clinical isolates of Dengue fever (Chanyasanha *et al*, 1999). The s-ELISA against *P. salmonis* was shown to be a rapid procedure for quantification of the amount of free organisms in cell culture supernatant. In comparison to the previously

available methods of $TCID_{50}$ and protein assay for quantification of *P. salmonis* antigen, this method is faster and less labour intensive then $TCID_{50}$'s and more specific than protein assay.

Aguayo *et al* (2002) described a similar ELISA for *P. salmonis* using a capture antibody bound to the microtitre plate with the adhesive polyphenolic protein purified from the mussel *Aulacomya ater* and a *P. salmonis* specific monoclonal conjugated to HRPO as the detector. No attempt was made in their study to use the ELISA to determine growth dynamics of *P. salmonis*, but rather they focused on the possible use as a diagnostic test to detect organisms in tissue. No correlation between ELISA values and $TCID_{50}$ was investigated but for the ELISA data a detection threshold, defined as three times background was found to be 15 ng of *P. salmonis* protein. Unfortunately they did not include an internal standard to improve day to day comparisons of ELISA results and the extra steps in coating the plates with adhesion protein and the cumbersome process of directly conjugating the HRPO to the monoclonal antibody which has to be done every six months due to stability of the conjugate, make this test less desirable.

The major disadvantage of s-ELISA over TCID50 is that it does not give a measure of viability of the *P. salmonis* present. There are however applications like vaccine production or DNA purification, where viability is not an issue, and for these the s-ELISA is optimal. As plates coated with the capture antibody and blocked with PBS-BSA can be frozen and stored as can the standard and samples need only to be incubated for one hour to be bound by the capture antibody of pre-coated plated, rather then incubate in coating buffer overnight as is required in direct ELISA. Results are available

in approximately 3 hours instead of 16.

From the growth curve of the *P. salmonis* it can be seen that the amount of antigen in the supernatant slightly dropped three hours after inoculation compared to immediately after inoculation in three out of the four flasks. This is probably due to *P. salmonis* being adsorbed on or taken up into CHSE cells. At day seven, a shoulder point can be seen . which could mean that the infection has at least two waves whereby not all cells were infected with the initial inoculum but become subsequently infected with newly arising organisms.

The drop in antigen levels after day 10 post inoculation confirms the value of monitoring the levels of *P. salmonis*, as maintaining the culture past this optimum time will actually reduce the multiplicity of infection. The reduction in the amount of detectable *P. salmonis* antigen is hypothesized to be due to degradation of the *P. salmonis* in culture. Although The sensitivity of detection is sufficient to follow the infection through its early stages and future research will have to determine if the s-ELISA is sensitive enough to detect *P. salmonis* from infected fish.

2.6 LITERATURE CITED

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Table 2.1. Immunization schedule for 2 female balb/c mice for monoclonal antibody production

IP injection	Day	Quantity per mouse	Adjuvant*
Initial	0	120 μg / 200 μl	RIBI
2nd	22	100 μg / 200 μl	MPL + TDM
3th	35	100 μg / 200 μl	MPL + TDM
Final boost	53	100 μg / 200 μl	None

Table 2.2. Characteristics of monoclonal antibody (MAb) clones generated in the second fusion

MAb Parent	Subclone	Isotype	IFAT	Western Blot
5E7		G	+++	9 kDa band
5E8		G	+++	9 kDa band
9D8		G	+++	9 kDa band
10E6	6G7	G	+++	12 kDa band
	6E3	G	+++	No band seen
	6C5	G	+++	12 kDa band
	6D2	G	ND	12 kDa band
	14F2	G	ND	12 kDa band
	14C6	G	-	ND
3D11	2D4	M	++±	Nil
	10E6	M	++±	Nil
	10F5	M	++±	Nil

MAb = Monoclonal antibody
IFAT = Indirect fluorescent antibody test
ND = Not determined

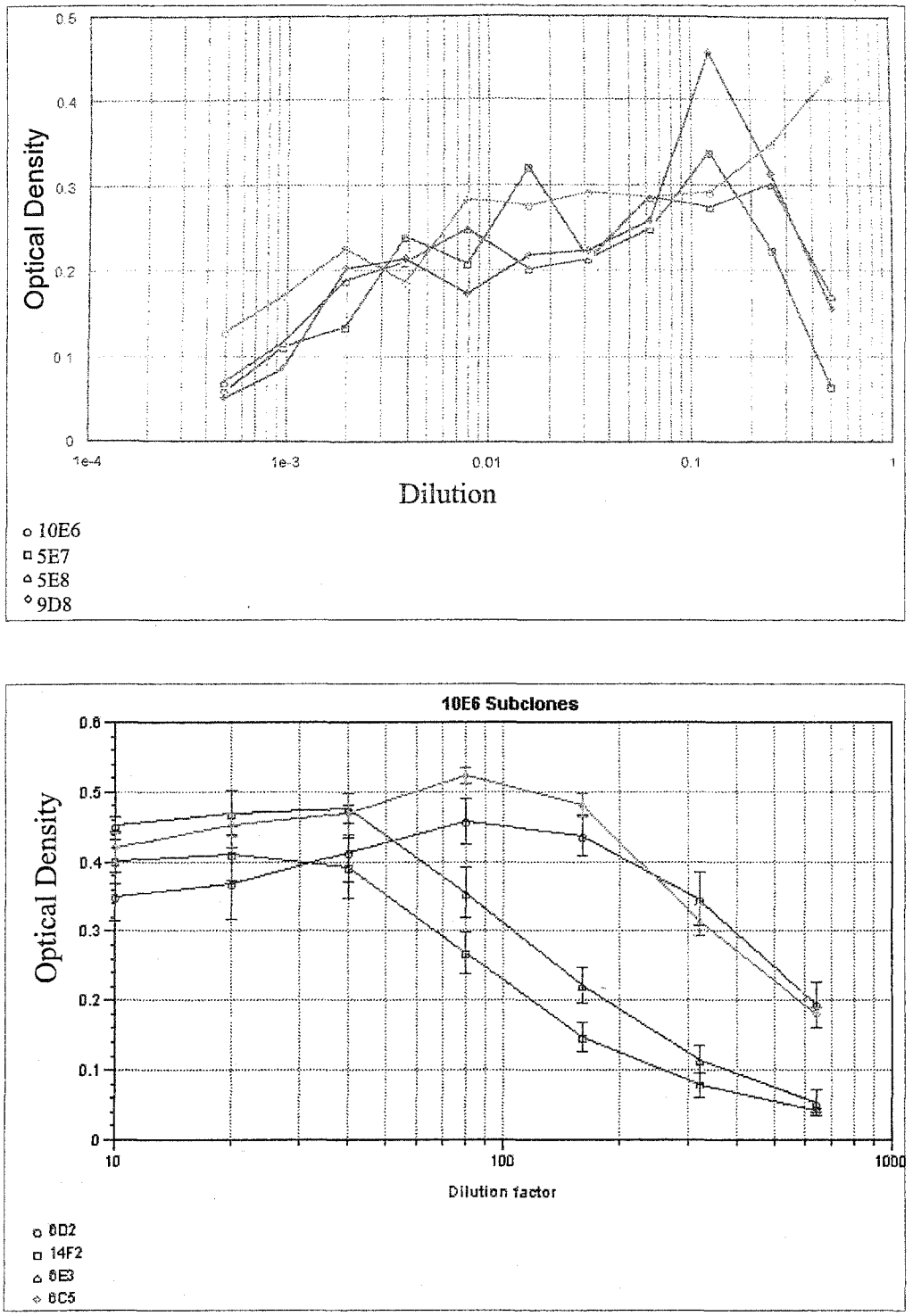

Figure 2.1. *Top*: Direct ELISA of 4 parental clones, 10E6, 5E7, 5E8 and 9D8. Hybridoma supernatant was used undiluted. Plates were coated with *P. salmonis*. *Bottom*: s-ELISA of 4 10E6 subclones to determine their efficiency in detecting *P. salmonis* in sandwich ELISA. Rabbit α-*P. salmonis* from rabbit #7 was used as capture antibody.

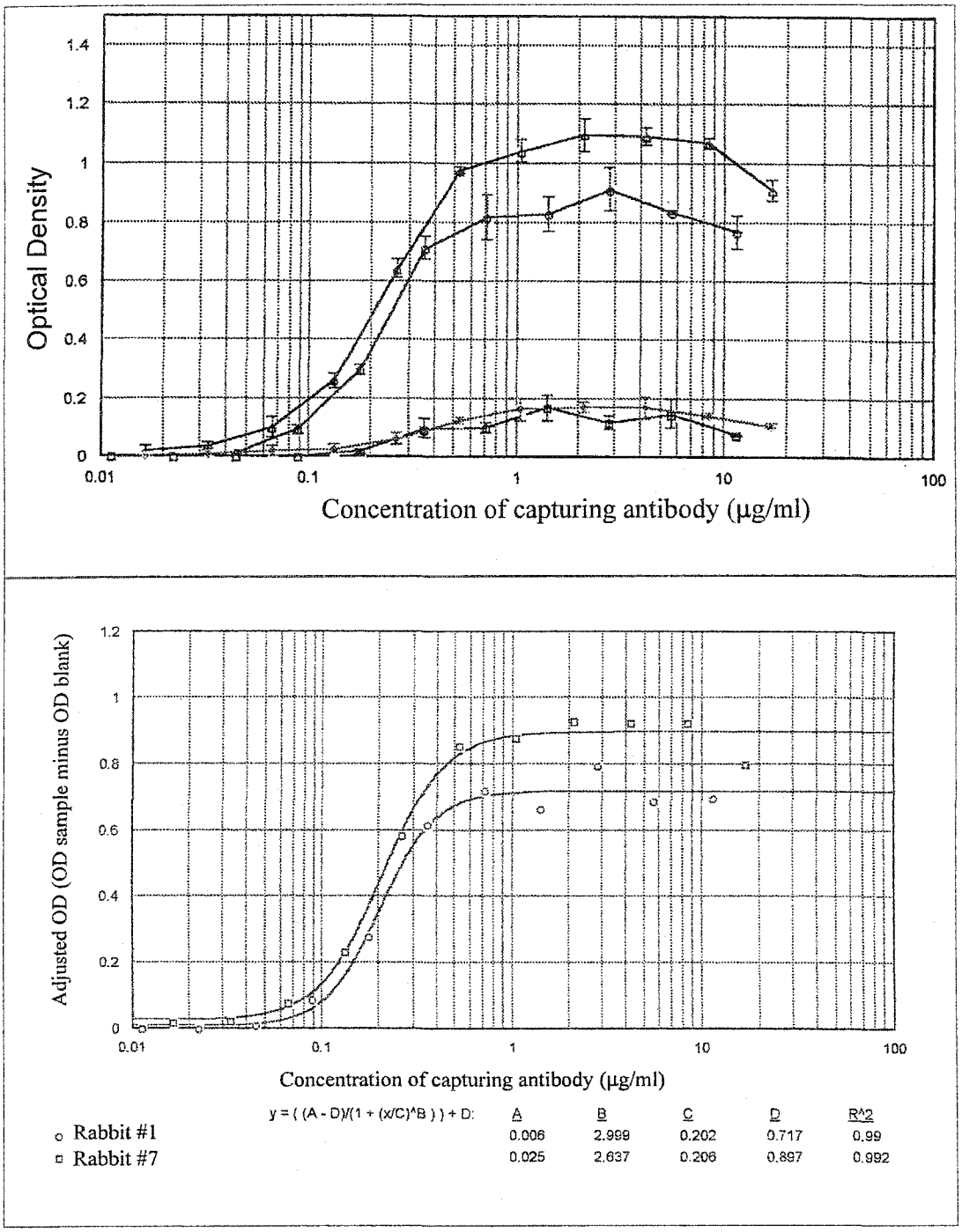

Figure 2.2. Titration of two rabbit antisera as capture antibody in a *P. salmonis* ELISA. Rabbit antiserum was diluted in coating buffer. All wells were incubated with identical *P. salmonis* culture supernatant. Sample wells were subsequently incubated with undiluted α-*P. salmonis* MAb while control wells received PBS-BSA 1%. All wells were incubated with G α-mouse-HRPO at 1:1000
Top: OD's of samples and controls used to generate adjusted results. Rabbit #1: sample ○ - control □; Rabbit #7: sample △ - control ●).
Bottom: Four parameter fitted curve to the adjusted results (sample - control). Rabbit #1:○, Rabbit #7 □

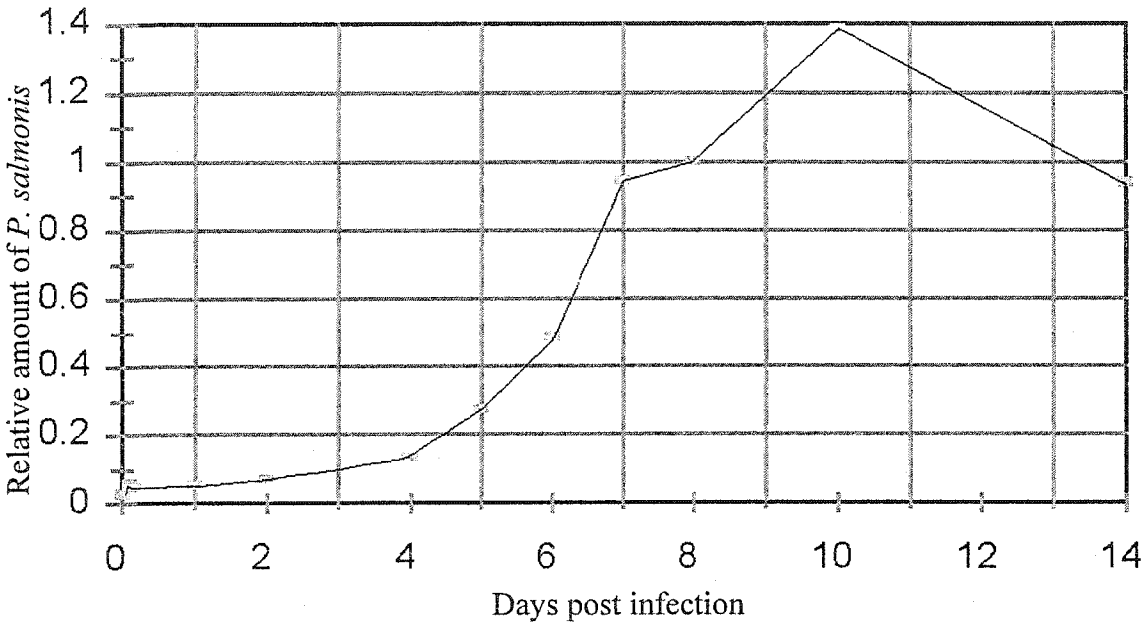

Figure 2.3. Production *P. salmonis* antigen in tissue culture supernatant. Optical densities of samples were compared to the optical density of a internal standard present on each plate (F^{10}) to give a relative amount (y-axis). Each data point on the graph represents the average of four flask, every sample was run in quadruplicate.

CHAPTER 3: IDENTIFICATION OF CANDIDATE GENES FOR DNA - VACCINES BY SCREENING A *PISCIRICKETTSIA SALMONIS* GENOMIC EXPRESSION LIBRARY FOR IMMUNOREACTIVE CLONES

3.1 ABSTRACT

Piscirickettsia salmonis is an intracellular bacterial pathogen of salmonid fish and causes significant losses in the aquaculture industry, particularly in Chile. For effective vaccine development, the identification of genes encoding relevant antigens or epitopes is necessary. A genomic expression library for *P. salmonis* was developed in the λZapExpress vector system. The library was screened using antiserum from rabbits immunized with *P. salmonis*. Forty-three positive plaques were identified. Insert DNA from these clones was amplified by PCR and the amplification product sequenced. Many inserts were short and gave little information on the identity of the gene or its function. Of the clones with enough information to assign putative function, five clones were selected for further investigation. None contained a full open reading frame, however based on the translated amino acid sequence, they were tentatively identified as: 1) an analogue to the 17 kDa spotted fever rickettsia group antigen; 2) an ATP- binding cassette containing transporter; 3) a transposase; 4) an amino acid transporter and 5) the SecA subunit of the protein Translocase system. None of the positive clones contained CHSE-214 DNA, based on sequence analysis, showing that the differential lysis method of purification is suitable for extracting DNA from intracellular bacteria that is free from host cell DNA.

3.2 INTRODUCTION

The recent rapid expansion of aquaculture into new regions of the world led to the introduction of aquaculture species into environments where these species are not native. Aquaculture conditions often entail high stocking densities and high stress, leading to outbreaks of previously unrecognized diseases. One of the most notable disease outbreaks was caused by *Piscirickettsia salmonis* in the Hitu region of Chile in the late eighties and early nineties (Cvitanich *et al.*, 1991; Fryer *et al.*, 1992). Salmonid rickettsial septicemia (SRS), caused mortalities from 40 to 90% in net pet raised coho salmon (*Oncorhynchus kisutch*) (Bravo and Campos, 1989). First identified as a Rickettsia like organism (RLO), the pathogen was further characterized and named *Piscirickettsia salmonis* by Fryer *et al.* (1992). *Piscirickettsia salmonis* was shown to be a Gram-negative, obligate intracellular bacteria. Even though it was shown *in vitro* to be sensitive to a range of antibiotics, SRS does not respond well to antibiotic therapy in a commercial aquaculture environment. Fish management changes which focused on the reduction of stress and the use of less susceptible species have decreased the severity of *P. salmonis* outbreaks, however, there is still a need for more effective therapies. Vaccines have shown the capability to reduce or even eradicate infectious diseases in human and veterinary medicine and have successfully been used in aquaculture for control of such important diseases as vibriosis and furunculosis (Shao, 2001).

Vaccines for bacterial pathogens typically used in aquaculture are adjuvated killed bacterins. Although some encouraging results were shown experimentally with *P. salmonis* obtained from cell culture, the production costs of an inactivated whole cell

vaccine are too high to be applicable in a commercial setting (Smith *et al.*, 1995). Alternative vaccine strategies include recombinant vaccines, where *P. salmonis* antigens are expressed in genetically modified bacteria like *Escherichia coli* (Kuzyk *et al.*, 2001a), and more recently, DNA vaccines. A DNA vaccine consists of a plasmid containing eukaryotic expression control elements regulating the expression of a gene encoding an immunoreactive and protective protein antigen (Lewis and Babiuk, 1999; Liu, 2003). The plasmid is injected into fish, where it is taken up by cells resulting in expression of the gene and presentation of the antigen to cells of the immune system. One advantage of this approach from the practical point of view is that once developed a DNA vaccine can be produced cheaply. Furthermore, DNA is stable, avoiding problems with separation of oil and water phases sometimes seen in adjuvated vaccines, or the need for a cold storage chain for getting the vaccine to the sometimes remote fresh water hatcheries or sea farming net pens (Heppell and Davis, 2000). The antigen present on the plasmid as a gene, is produced as protein by the cells own transcription and translation machinery and, from the immunological point of view, this resembles the way antigen from intracellular pathogens is normally presented to the immune system. This will drive a Th1, or cytotoxic T-cell driven type of immune response, whereas most adjuvated, killed, bacterin type of vaccines normally lead to an antibody driven, or Th2, type of response (Liu, 2003). Given the fact that *P. salmonis* is an intracellular pathogen, a Th1 type of response is expected to be more effective, and therefore more desirable for a *P. salmonis* vaccine.

An important part of the development of any DNA vaccine is the identification of

candidate genes from the pathogen. As very little is known about the genetic makeup of *P. salmonis*, a first step is the identification of genes encoding immunoreactive products. By expressing random fragments of the *P. salmonis* genome in an expression library, and screening the clones for immunoreactivity, potentially protective antigens can be identified. Here, the construction and use of such a genomic expression library from *P. salmonis* is shown. Several immunoreactive clones and their corresponding proteins were identified.

3.3 METHODS AND MATERIALS

3.3.1 Purification of *P. salmonis* DNA from CHSE-214 cells

3.3.1.1 Collection of *P. salmonis*

Piscirickettsia salmonis was cultured as described in paragraph 2.3.1. Six T-225 tissue culture flasks with a confluent monolayer of CHSE-214 cells were infected with 5 ml of *P. salmonis* culture each and grown until 90% CPE was observed. Approximately 300 ml of supernatant from these flasks was collected and pooled. This was divided in two batches of no more than 150 ml. The supernatant was centrifuged 500 x g at 4°C for 10 minutes in a Beckman benchtop centrifuge. The supernatant was carefully collected, leaving the pellet and a 5 ml cushion. These supernatants were pooled and centrifuged in a Beckman J2-21M/E High speed centrifuge using a JA-14 rotor (Beckman) at 15,000 x g, at 4°C for 10 minutes. The pellet was resuspended in 200 ml of sterile 0.85% saline. The centrifugation was repeated, but this time the pellet was resuspended in 25 ml of

sterile 0.85% saline. The suspension was transferred into a JA-20 rotor (Beckman) bucket and centrifuged at 15,000 x g.

3.3.1.2 Digestion of contaminating CHSE-214 DNA with DNase I

To obtain *P. salmonis* DNA free from contaminating CHSE-214 DNA a method originally developed for *Cowdria ruminantium* was adapted (Wilkins and Ambrosio, 1990). The *P. salmonis* pellet from the last step described in paragraph 3.3.2.1 was resuspended in 2 ml of sterile 0.85% saline with 25 (low level) or 250 (high level) $\mu g\ ml^{-1}$ of DNase I and 4.2 mM $MgCl_2$ and incubated for 2 hrs at 15°C. After this incubation 25 ml of sterile saline was added and the *P. salmonis* pellet was washed three times by repeated centrifugation in a Beckman J2-21M/E centrifuge using a JA-20 rotor at 15,000 x g at 4°C for ten minutes and resuspension in 25 ml of sterile saline. After the final centrifugation the pellet was dissolved in 1 ml of 10 mM Tris.HCl, 1 mM EDTA buffer (TE)(pH 8.0), transferred to an Eppendorf vial and centrifuged for 7 minutes at 10,000 x g in a Eppendorf 5415C centrifuge at room temperature.

3.3.1.3 *P. salmonis* DNA isolation

The washed pellet from the paragraph 3.3.1.2 was resuspended in 567 µl of TE buffer (pH 8.0) and 30 µl of 10% SDS and 3 µl of proteinase K (20 $mg\ ml^{-1}$) added to the suspension and incubated for 3 hours at 37°C. After incubation, 100 µl of 5M NaCl was added and mixed well. Eighty microliters of 10% cetyl trimethyl ammonium bromide (CTAB) in a 0.7M NaCl solution was then added, mixed and incubated for 10 minutes at

65°C. The sample was frozen and thawed to complete precipitation of lipopolysaccharides. An equal volume of phenol : chloroform : isoamyl alcohol (25:24:1), was added, mixed and the phases separated by centrifugation at 12,000 x g at 4°C in an Eppendorf 5415C centrifuge. The top layer (aqueous phase) was carefully removed using a blunted pipette tip, and extracted twice more with chloroform : isoamyl alcohol (24:1). The aqueous phase was collected and the DNA precipitated by adding 0.6 volume of 1005 isopropanol at room temperature. The DNA was pelleted by centrifugation at room temperature in an Eppendorf centrifuge at 12,000 x g. The pellet was washed with 70% ethanol and dried under vacuum. The pellet was dissolved in 100 µl of sterile ddH_2O water overnight.

3.3.2 Assessment of purity of genomic *P. salmonis* DNA

The purity of the DNA was assessed by southern blot using both CHSE-214 and *P. salmonis* probes made from digoxigenin (DIG) labeled *Sau*3A I digests.

3.3.2.1 Generation of a DIG-labeled CHSE-214 DNA probe

One T-225 tissue culture flask with a confluent monolayer of CHSE-214 cells was trypsinized and the cells collected by centrifugation at 500 x g in a Beckman benchtop centrifuge at 4°C and resuspended in sterile 0.85% saline. The cells were pelleted again, and resuspended in 567 µl of sterile ddH_2O water and transferred to a 1.5 ml vial. The DNA was then isolated as described in paragraph 3.3.1.4. Three µg of CHSE-214 DNA was digested with 2 units of *Sau*3A I using the manufacturer's buffer for one hour in 50

µl reaction volume. After digestion *Sau*3A I was inactivated by heating the sample to 65°C for 10 minutes and a phenol : chloroform : isoamyl alcohol (25:24:1) extraction was performed and the DNA precipitated by adding 0.1 volume of 3 M sodium acetate (pH 7.3) and one volume of isopropanol. The pellet was washed with 70% ethanol and dried under vacuum. The pellet was resuspended in 16 µl of ddH_2O to which 4 µl of DIG High Prime (Boehringer Mannheim) was added. This was incubated for 20 hours at 37°C after which the reaction was stopped by heating the probe to 65°C for 10 minutes.

3.3.2.2 Southern blot

A 1% (w/v) agarose gel in a Tris.HCl buffered sodium borate-EDTA solution (TBE) (with 2.5 µg ml^{-1} ethidium bromide present in the gel) was loaded with genomic *P. salmonis* purified as described in 3.3.1 with the following adaptations: in one sample DNase I digestion was performed as described in paragraph 3.3.1.3 (250 µg ml^{-1} DNase I), while the second sample was purified using 25 µg ml^{-1} DNaseI. The third sample was gel purified as described in paragraph 3.3.3.5. Other samples loaded where purified CHSE-214 DNA as described in 3.3.2.1 and a 1 kb DNA ladder. All samples were loaded on the gel twice to create identical left and right halves to the gel. The gel was run at 120V for 2 hours and photographed. The gel was then processed for southern blotting by incubating it in 0.5 M NaOH, 1.5 M NaCl for 1½ hours, 0.2 M HCl for 20 minutes, followed by rinsing with ddH_2O and incubation in 1.5 M NaCl, 1 M Tris (pH 7.4) for 30 minutes and one hour respectively. A glass dish was filled with the running buffer (10x standard sodium chloride (0.3 M), sodium phosphate (0.02 M), EDTA (2 mM) or SSPE

buffer at pH 7.4) and over this a glass plate was suspended. A strip of Whatman paper was cut out, big enough to cover the glass plate and dip both ends in the running buffer and was wetted with 1x SSPE and folded over the glass plate. On this the gel was placed, topside down. Parafilm was placed on the edges of the gel to prevent flow of buffer around instead of through the gel. A Nylon filter (Amersham Hybond-N), wetted with ddH_2O and equilibrated by submerging it for 5 minutes in 10x SSPE was placed on top of the gel. This was followed by two pieces of blotting paper wetted in running buffer and nine packs of dry Kimtowel tissue paper. The stack was weighted down and left overnight to blot. After blotting, the filter was removed and cross linked using a Stratagene Stratalinker UV oven at the autocrosslinking setting. The blot was prehybridized with prewarmed hybridization buffer (0.5 M Na_2HPO_4, 7% SDS and 1% BSA, pH 7.2) using a hybridization oven set at 55°C for one hour. The blot was then hybridized with 3.75 ml of hybridization buffer containing 25.6 ng/ml of DIG labeled CHSE-214 probe at 55°C overnight. The next day the probe was collected and frozen for re-use. The filter was washed 3 times with 2 x SSPE, 1% SDS at 55°C for one hour.

3.3.2.3 Assessment of level of contamination by dotblot method

As a quicker alternative to the Southern blot, a dot blot system was developed, to be used for quantification of the amount of CHSE-214 DNA present in the sample. Ten microliters of DNA in TE buffer (pH 8.0) or ddH_2O were pipetted directly onto a Nylon membrane. The filter was cross linked in the Stratagene Stratalinker and processed as described above. The blot was hybridized overnight at 55°C with CHSE-214 probe and

stringency washes were two of one hour, one of 1.5 hour with hybridization buffer (low stringency).

3.3.2.4 Detection of hybridized probe

The protocol supplied with the Boehringer Mannheim High prime labeling and detection kit was used. In short, the filter was washed with maleic acid buffer (0.1 M Maleic acid, 0.15 M NaCl, pH 7.5) for 10 minutes, then blocked with 1 x blocking solution provided with the kit in maleic acid buffer for one hour. The filter was then incubated with the α-DIG-Alkaline Phosphatase conjugated antibody supplied with the kit, diluted 1:5000 in blocking buffer for at least 30 minutes. Unbound conjugate was removed with two washes in maleic acid buffer. After equilibrating the filter with substrate buffer (0.1 M Tris, 0.1 M NaCl, 50 mM $MgCl_2$, pH 9.5) for 10 minutes, the substrate (300 µl of Nitro blue tetrazolium / 5-Bromo-4-chloro-3-indolyl phosphate [NBT/BCIP] stock in 15 ml of substrate buffer) was added. This was incubated with the filter in the dark for one hour or until the desired amount of signal was achieved. The reaction was stopped by repeated rinses of the filter with TE (pH 8.0).

3.3.2.5 Generation of a DIG labeled *P. salmonis* DNA probe

Piscirickettsia salmonis DNA from 150 ml of culture supernatant was purified as described above and loaded in four lanes of a 1% SeaPlaque low melting point agarose gel and electrophoresed, 100 Volts for four hours at 4°C. The high molecular weight band containing the *P. salmonis* DNA was cut from the gel and each piece added to one

ml of prewarmed 0.20 mM Tris.HCl, 1 mM EDTA (pH 8.0) and incubated at 65°C until the agarose was completely molten. The samples were extracted with equal volumes of phenol, then phenol : chloroform (1 : 1) and chloroform. The samples were precipitated with 0.1 volume of 3 M sodium acetate (pH 7.3) and an equal volume isopropanol (100%), the pellet after precipitation was washed with 70 % ethanol. The DNA purified from three lanes was pooled and quantified. Approximately one microgram of this DNA was used as template for the DIG-High prime labeling reaction as described in paragraph 3.3.2.1.

3.3.3 Construction of the λZapExpress library from *P. salmonis* DNA

3.3.3.1 Preparation of *P. salmonis* DNA

Three identical digests were prepared from 16 µg of *P. salmonis* DNA and 16 units of *Sau*3A I enzyme in the appropriate reaction buffer with a volume of 200 µl per sample. The digests were incubated for 1½ hour at 37°C and inactivated at 70°C for 10 minutes. After the digest, the samples were pooled and extracted with an equal volume of phenol : chloroform : isoamyl alcohol (25:24:1), the aqueous phase collected and extracted with an equal volume of chloroform : isoamyl alcohol (24:1). The aqueous phase was separated, removed and the DNA precipitated with 0.1 volume of 3 M sodium acetate (pH 7.3) and 0.6 volume of isopropanol. The DNA was pelleted, the pellet washed with 70% ethanol, dried and dissolved in 100 µl of sterile ddH_2O. The efficiency of the purification and the quantity of DNA retrieved were determined by OD 260/280 ratio.

3.3.3.2 Ligation of *P. salmonis* inserts into the vector

The λZapExpress vector that was chosen as vector for the library had an approximate length of 38,900 base pairs (bp), while from Southern blot analysis the average size of the *Sau3*A I generated fragments was estimated at approximately 1,000 bp. To minimize the chance of having multiple inserts in a clone, a molar ratio of vector to insert of 1 to 1 was used as recommended by the manufacturer of the vector (Stratagene). The ligation reaction consisted of 1 µl of λZapExpress at 1 µg ml^{-1}, 1 µl of *Sau*3A I digested *P. salmonis* DNA at 25 ng μl^{-1}, 0.5 µl of 10x ligation buffer stock, 1.5 µl of sterile, autoclaved ddH_2O and 1 µl of T4 DNA ligase (2 units). The ligation was performed for 12 hours at 4°C.

3.3.3.3 Packaging

After ligation, 4 µl of the ligation mixture was packaged into infective phage particles using the GigaPack III Gold packaging extract (Stratagene) using the manufacturer suggested protocol. Briefly, 1 µl of ligation product as described in paragraph 3.3.3.2 was mixed into one freshly thawed tube of 25 µl packaging extract by gentle stirring with a pipet tip. The tube was immediately centrifuged for 3 seconds at full speed in an Eppendorf 5415C centrifuge and subsequently incubated two hours at room temperature. After the incubation, 500 µl of SM buffer (0.1 M NaCl, 10 mM $MgSO_4$, 50 mM Tris.HCl, 0.1 ‰ gelatin, pH 7.5) was added, followed by 20 µl of chloroform. The tube was briefly centrifuged as before and stored at 4°C until further use. The library is further referred to as λF^{20a}.

3.3.4 Screening of the λF^{20a} library using polyclonal rabbit serum

3.3.4.1 Infecting host cells for expression

To infect host cells so they would express the incorporated fragments, 200 µl of XL-1 Blue MRF' cells (Stratagene) were incubated with 1 µl of phage suspension for 15 minutes at 37°C. A mixture of 15 µl of 0.5 M isopropyl-β-D-thiogalactopyranoside (IPTG) and 3 ml of prewarmed top agar containing NZ amine (10 g l^{-1})(casein hydrolysate), yeast extract (5 g l^{-1}), NaCl (5 g l^{-1}) and $MgSO_4.7H_2O$ (2 g l^{-1}) (NZY medium) was poured over a 100 mm NZY agar plate. All quantities were tripled when 150 mm petri dishes were used.

3.3.4.2 Immuno screening of the λZapExpress λF^{20a} library

After plaques had formed the plate was covered with nitrocellulose and incubated between four hours and overnight at 37°C. The 150 mm plates were screened with a mixture of sera from three rabbits immunized with intact *P. salmonis* as described in paragraph 2.3.3. To minimize the cross reactivity against the *E. coli* host cells, the antiserum mixture was absorbed twice with 1% (w/v) of acetone powder made from the *E. coli* host cell (XL-1 Blue MRF'). Absorptions were done at room temperature, the first for one, the second for three hours.

After the nitrocellulose filter was removed from the plate, remaining binding sites were blocked by incubation with 1x blocking buffer from the BM High prime labeling and detection kit described in paragraph 3.3.2.4. The filter was then incubated with rabbit antiserum diluted 1:100 in blocking buffer, washed three times with maleic acid buffer

for five minutes per wash, incubated with goat α-rabbit antiserum conjugated with alkaline phosphatase diluted 1:5000 in blocking buffer and further developed as the Nylon filters in the southern blot described in paragraph 3.3.2.4. Positive signals on the filter were correlated with plaques on the original plates and the positive plaques excised from the plate and resuspended and re-plated and retested. If every plaque on the new plate was positive, a suspension was made from a plaque of this new plate and used as template for a PCR reaction that would amplify the insert.

3.3.4.3 PCR of the insert in immunoreactive clones

The λZapExpress contains a T7 and a T3 priming site up- and downstream respectively of the insert. By designing primers with these sequences an amplicon can be generated containing the insert plus flanking regions in the vector. The vector specific primers (GibcoBRL) used were T3 Forward (ATT AAC CCT CAC TAA AG) and T7 Reverse (AAT ACG ACT CAC TAT AG). A PCR reaction was set up in 100 µl reaction volume. To 5 µl of plaque suspension 95 µl of master mix was added. For each 95 µl, the master mix contained 5 µl of a stock solution of 10 pmol μl^{-1} of each of the primers, 2 µl of 0.5 M dNTPs, 10 µl of 10x reaction buffer (Amersham Pharmacia Biotech), 0.5 µl (2.5 units) of Taq (Amersham Pharmacia Biotech) and 72.5 µl ddH_2O.

The PCR reaction was performed in a PTC-200 Peltier Thermal Cycler (M.J. Research) using the following protocol: samples were held for two minutes at 94°C followed by 32 cycles of 92°C for 40 seconds, 63°C for 90 seconds an 72°C for five minutes, followed by a final extension of ten minutes at 72°C. The protocol ends by

keeping the sample at 4°C until removed from the cycler. Results were analyzed using horizontal gel electrophoresis in a 1% agarose gel in 1x TBE for one hour at 120 V.

If amplification product was present, it was purified using the High Pure PCR product purification kit (Roche) following the manufacturer supplied protocol with all centrifugation steps performed in an Eppendorf 5415D centrifuge. In brief, binding buffer, is mixed with the sample and then transferred to the upper reservoir of a filter tube. This is centrifuged at 13,000 x g for 30 seconds. The bound DNA was washed twice with 500 µl wash buffer at 13,000 x g for 30 seconds. The DNA was then collected by the addition of elution buffer and another 30 second centrifugation at 13,000 x g. The purified amplicons were sent to the Research and Productivity Council (RPC) in Fredericton, NB for sequencing. The sequences received were analyzed using FramePlot (http://watson.nih.go.jp/~jun/cgi-bin/frameplot.pl) (Ishikawa and Hotta, 1999) and GenBank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) (Altschul *et al.*, 1997).

3.4 RESULTS

3.4.1 Purification of *P. salmonis* DNA

This method yielded between 10 and 25 μg of *P. salmonis* DNA starting from three T-225 tissue culture flasks. The genomic DNA was of sufficient purity for use in generating the expression library as judged by the ratio of absorbance at 260 and 280 nm.

3.4.2 Purity of *P. salmonis* DNA

3.4.2.1 Dot blot analysis of quantity of CHSE-214 DNA contamination

Figure 3.1 shows a dot blot with the samples containing 50 ng in 1 μl per dot and the CHSE-214 DNA standards at doubling dilution starting with 50 ng per dot down to 390 pg per dot. The amount of CHSE-214 DNA can be estimated by comparing the density of the dot to the known concentration of CHSE-214 DNA in the bottom row. The result are shown in Table 3.1. To compare contamination levels within purification runs and between harvests, two purification runs per harvest for two *P. salmonis* harvests (F^{11} and F^{12}) were used. The level of contamination was identical for parallel DNA purifications using the same harvest as starting material, but varied between different harvests of *P. salmonis*.

3.4.2.2 Southern blot assessment of the size of contaminating CHSE-214 DNA fragments

The original agarose gel and the two Southern blots derived from it are shown in Figure 3.2. In Figure 3.2A the blot was probed with a CHSE-214 DNA probe, while in

Figure 3.2B the blot was probed with the *P. salmonis* probe. Three methods of purification are assessed: gel purified high DNase digest DNA in lanes 3a and 3b, high level DNase I digest (250 μg ml^{-1}) in lanes 4a and 4b and low level DNase I digest (25 μg ml^{-1}) in lanes 5a and 5b. To determine if the *P. salmonis* probe cross reacted with the host cell DNA, CHSE-214 DNA was loaded in lanes 2a and 2b. Unrelated DNA (1 kb DNA ladder) was used as a negative control. With the CHSE-214 probe, a clear signal can be seen in the CHSE-214 DNA positive control (lane 2a) and the low level DNase I digest (lane 5a) and a faint signal in the high level DNase I digestion (lane 4a). The gel purified *P. salmonis* DNA shows no detectable signs of CHSE-214 DNA contamination, however less gel purified *P. salmonis* DNA was loaded on the gel, as there was less DNA available. The *P. salmonis* probe was able to detect the *P. salmonis* DNA in lanes 3b, 4b and 5b, and shows that the *P. salmonis* DNA is mainly present in high molecular weight, fragments. The *P. salmonis* probe did not cross react with the unrelated ladder DNA in lane 1b, and shows only a very faint cross reactivity with the CHSE-214 DNA in lane 2b. This may be due to non specific cross reactivity or to traces of the CHSE-214 DNA in the *P. salmonis* DNA used for probe generation.

3.4.3 Identification and characterization of *P. salmonis* sequences from the λF^{20A} library

Six plates with a total of approximately 18,000 plaques were screened for expression of an immunoreactive product. Fifty-four plaques reacted with the *P. salmonis* antiserum. As can be seen in Figure 3.3, positive plaques could be divided in

two distinct types. Positive signal was either confined to an area corresponding to the confines of the plaque (discreet type) or radiate out over a wider area of the filter (diffuse type).

All positive plaques were subcloned and 41 continued to express *P. salmonis* protein upon re-screening. Subcloned plaques were excised from the agar again and 1 µl of phage suspension was used as template in the PCR reaction. If enough amplicon could be obtained, it was sent out for sequencing at RPC in Fredericton, New Brunswick. Sequences obtained from the λF^{20A} library are shown Table 3.2.

The sequences were searched for open reading frames (ORFs) using FramePlot (Ishikawa and Hotta, 1999) and the nucleotide and deduced amino acid sequences were compared to the GenBank Database, using Blast (Altschul *et al.*, 1990) and Blastp (Altschul *et al.*, 1997). No similarities were found for any of the clones if nucleotide sequences were used. If the deduced amino acid sequence of available ORFs were used, we found that most ORF's were too small to give conclusive results. No complete ORF's were found. Insert size, ORF size, and putative function derived from GenBank comparisons can be seen in Table 3.3. As a measure of similarity between the ORF and sequence available from GenBank, the Expect, or E-value is reported. The E-value describes the number of hits one can "expect" to see just by chance when searching a database of a particular size, based on the score of the alignment (Altschul *et al.*, 1990). The lower the E-value, the more significant the match. Clones 3 and 16 had identical inserts and clone 16 is therefore not included in the table. Sequence information obtained from clones 13 and 18 was too ambiguous for further analysis while clone 14 contained

no insert.

3.5 DISCUSSION

Until recently, the only genetic information available on *P. salmonis* were sequences coding for ribosomal sequences and the internal transcribed spacer region (ITS) (Mauel *et al.*, 1999; Casanova *et al.*, 2001). These sequences are useful for phylogenetic studies of an organism, but contain little relevant information to better understand the pathobiology of *P. salmonis*. *Piscirickettsia salmonis* genomic DNA free of contaminating CHSE-214 DNA was required to identify candidate genes for use in a DNA vaccine. However, the CHSE-214 cells used in the culturing of this pathogen contain approximately 6,000 times the nuclear material as a *P. salmonis* cell (Miquel *et al.*, 2003), therefore making purification of uncontaminated *P. salmonis* DNA challenging. Wilhelm *et al.* (2003) attempted to generate a *P. salmonis* library and instead retrieved the full sequence for the mitochondrial DNA of chinook salmon, the species of which the CHSE-214 cell line is derived. The method used in this study was adapted from a protocol previously described to isolate DNA from the causative agent of the cattle disease heartwater, *Cowdria ruminatium*, which was cultured in bovine epithelial cells (Wilkins and Ambrosio, 1990). After a straightforward first step of differential sedimentation to remove intact CHSE-214 cells, a method of differential lysis was used. This method yielded between 10 to 25 μg of total genomic DNA from a total of 675 cm^2 of cell culture.

To assess the quantity and quality of any remaining CHSE-214 DNA

contamination, the dot and Southern blots were shown to be very sensitive and useful tools. The DNase I treatment worked well in selectively digesting the naked host cell DNA in suspensions of whole bacteria obtained from tissue culture. Purity levels varying from 94 to 97% in one set of purifications to >99.2% in a second set were achieved. Good reproducibility was observed between purification performed in parallel from the same harvest of *P. salmonis*, but there was some variability between harvests. Parallel purifications used supernatant from cultures that were all harvested at the same day post infection. The differences in levels of contaminating CHSE-214 DNA observed may therefore be due to day to day variability in purification efficiency or due to differences between supernatant harvested at different times. Depending on the progression of *P. salmonis* infection, more CHSE-214 cells may have lysed releasing their DNA into the culture supernatant. The sandwich ELISA described in chapter 2 can be used to optimize and standardize the time of harvest for successive cultures to increase yield and consistent, high purity levels.

The yield of 10 to 25 μg per purification or approximately 1.5 -3.7 μg per 100 cm^2 compares favourably to other reports that achieve 0.4 and 2 μg per 100 cm^2 (Henriquez *et al.*, 2003, Kuzyk *et al.*, 2001b, respectively). Kuzyk *et al.* (2001b) used 12,000 cm^2 as starting material and Percoll gradient centrifugation to purify the *P. salmonis* before DNA extraction and $CsCl_2$-ethidium bromide gradient centrifugation of the DNA for further purification, making their method both expensive and cumbersome. Henriquez *et al.* (2003) used 3,600 cm^2 as starting material and use a method that incorporates DNase I digestion with 20 units followed by equilibrium centrifugation in iodixanol. These

authors report the purity of their DNA to be almost 99% based on semi-quantitative PCR, but do not comment on day to day variability. Compared to previous reports, the protocol described herein is the fastest, least cumbersome, most economical and gives the highest relative yield at comparable purity levels.

An expression library is a powerful molecular tool in the identification of genes coding for immunoreactive proteins in previously poorly characterized pathogens. These libraries have been used to identify genes from a variety of intracellular or fastidious pathogenic organisms, including *C. ruminatium* (Brayton *et al.*, 1997), *Coxiella burnetii* (Nguyen *et al.*, 1999) and *Bartonella vinsonii* subsp. *berkhoffii* (Gilmore *et al.*, 2003). To generate a genomic expression library for *P. salmonis*, the λZapExpress vector was chosen to take advantage of the phage λ based vector system's efficiency in transfecting cells and the potential for expression in both prokaryotic as well as eukaryotic cells (Short *et al.*, 1988). The λ^{F20a} library was generated from 25 ng of *P. salmonis* DNA ligated into 1 μg of vector DNA. When plated on 150 cm^2 bacterial lawns, approximately 3,000 plaques could be seen per plate. Using IPTG/X-gal screening the insert efficiency could be estimated by counting the blue, no insert plaques. Two hundred and twelve blue plaques were observed on an estimated 3000 total, giving an insert efficiency of 93%. Of the approximately 18,000 plaques screened, 54 were initially found to be immunoreactive with 46 clones remaining reactive after subcloning and re-screening. Two different types of positive plaques could be seen (Figure 3.3) where signal was either diffuse over a wider area of the blot or was only observed within the boundaries of the originating plaque. The significance of these different types of signal has not been investigated, but

might be due to the polypeptide or protein present on the insert being secreted versus cytoplasmic localization. The number of positive clones was higher than reported elsewhere for generation of a *P. salmonis* expression library. Kuzyk *et al.* (2001b) found 18 immunoreactive clones on a total of approximately 10,000 plaques with all inserts encoding an overlapping fragment of the *P. salmonis* genome. This study generated twice as many immunoreactive clones and only one sixth of the sequenced clones did not contain unique sequences. Miquel *et al.* (2003), using a plasmid based rather than phage based vector system for the construction of two libraries, reported an insert efficiency of 80% and a total number of 22,242 and 28,365 clones respectively in their libraries. This study used only 6 µl of a total of 550 µl of library suspension, containing 18,000 clones, therefore the total number of clones in the λF^{20A} can be estimated at 1.6×10^6. This required only 25 ng of *P. salmonis* DNA compared to 25 µg of DNA for each of the libraries generated by Miquel *et al.* (2003). This illustrates the high efficiency of the λZapExpress vector in both insert efficiency and total clone numbers over plasmid based systems and shows the potential of the methods here described in generating a powerful molecular tool for a future new or emerging disease caused by an intracellular pathogen.

The successfully sequenced clones can be found in Table 3.2. Total G + C content for all sequences combined is 41.1%. This A + T bias is significant but not extreme when compared to *Rickettsia prowazekii* at 32.5% in protein coding sequences (Andersson and Sharp, 1996). None of the sequences found showed similarity to *O. tshawytscha* genomic or mitochondrial sequences in GenBank, nor to sequences of the related species *O. mykiss*, that is subject of the Rainbow trout gene idex project of the

Instutue for Genome Researcg (TIGR), providing a further indication for the purity of the genomic DNA used to generate the library. Clone 10 contains two internal *Sau*3A I sites, indicating that this clone contains three separate inserts, one of 362 bp, one of 73 bp and a large, over 2,000 bp insert which is identical to the insert in clone 1. Clone 15 contains one internal *Sau*3A I site, indicating a chimeric sequence with two inserts, one of 190 bp and one of over 1,000 bp. It is not known if there is any correlation between the fact that these clones are chimeric and that they contain some of the larger inserts found in the library. All sequences were compared to the nucleotide sequences available in GenBank, but no significant similarities were found with any insert, indicating the uniqueness of the *P. salmonis* organism.

Sequences obtained from inserts in the λF^{20A} library were analyzed for the presence of ORFs. The deduced amino acid sequence from identified ORFs was compared to available sequences using Blastp. Insert and open reading frame sizes can be found Table 3.3 with the putative function of the open reading frames. A subjective estimate of similarity based on total percentage of homology and presence of areas of high homology is also given. Although all clones are immunoreactive, certain clones such as clone 6, putatively the S19 protein of the 30S ribosomal subunit, and clone 9, containing a DNA binding domain, are most likely housekeeping genes not involved in the pathobiology of *P. salmonis*. As inactivated, whole *P. salmonis* was used to immunize the rabbits used to raise the antisera used for screening, it is likely that some bacteria lysed and exposed these internal proteins to the rabbit immune system. Other clones had inserts which assigned functions made them more likely to be important in the

host - pathogen relationship.

Clone 1 contained an insert with an ORF with a deduced amino acid sequence showing similarity to the 17 kDa surface antigen that is used as group antigen for the spotted fever group (SFG) rickettsia (Anderson and Tzianabos, 1989). These antigens are membrane bound and thus exposed to the immune system during extracellular stages of the pathogen (Anderson, 1990). Deduced amino acid sequences coding for the antigen show a high level of conservation (91.2 to 100%) between different species of the SFG rickettsia, indicating evolutionary pressure on maintaining this lipoprotein (Mcdonald *et al.*, 1997). Kuzyk *et al.* (2001b) concurrently and independently, identified the same 17 kDa surface antigen gene from a *P. salmonis* expression library. From their report, the second ORF present on the clone 1 insert can be identified as a transposase. This is not part of the transposase found in clone 3. Transposases are involved in genetic recombination and as such can aid a potential pathogen in immune evasion and adaption to the host organism through so called pathogenicity islands, phase variation and antigenic variation (Salvatore *et al.*, 2001). The presence of two putative transposases on the *P. salmonis* genome indicates that these processes might play a role in the pathogenesis of this organism.

Clone 7 showed similarity to a wide variety of ATP-binding cassette (ABC) type transporters. Many of the homologous sequences are involved in antibiotic and lantibiotic efflux systems. Although *P. salmonis* is sensitive to antibiotics *in vitro*, chemotherapeutics have only met with limited success in controlling the disease (Cvitanich *et al.*, 1991; Fryer and Hedrick, 2003). Interference with this gene may make

the pathogen more susceptible to treatment chemotherapeutics. Other studies have targeted ABC type transporters for vaccination studies. In humans, during an outbreak of *Enterococcus faecium*, a humoral response to an ABC-type of transporter of the pathogen has been statistically correlated to survival and mice could be protected by immunization with recombinant antigen based on this transporter (Burnie *et al.*, 2002). A knockout strain of the intracellular bacterial pathogen *Brucella abortus*, missing the gene for the ATP transporter *Exs*A, showed decreased survival in a mouse host and this mutant could be used as live vaccine (Rosinha *et al.*, 2002).

Clone 20 showed similarity to amino acid transporters/permease type of proteins involved in transmembrane movement of amino acids (Ames *et al.*, 1990). Given their location in the outer membrane, this protein may provide a suitable target for vaccination.

The insert found in clone 15 showed similarity to the preprotein Translocase subunit SecA. Subunit SecA is the dissociatable nucleotide and preprotein binding subunit of the bacterial Translocase (den Blaauwen *et al.*, 1999). The Translocase system functions by binding a preprotein and promoting its translocation across the bacterial cytoplasmic membrane by nucleotide modulated co-insertion and de-insertion into the membrane (den Blaauwen *et al.*, 1996). Translocase subunits form dynamic complexes in the lipid bilayer and build an aqueous conduit through which preprotein substrates are transported at the expense of energy (Economou, 1998). The proper extra-cytoplasmic localization of proteins is likely to be an important aspect of *P. salmonis* physiology pathogenesis, as it is for other intracellular pathogens, such as *Mycobacterium tuberculosis* (Braunstein *et al.*, 2001). This warrants further investigation of its function

in *P. salmonis* and the possibility of targeting the Translocase system by vaccination or chemotherapeutics.

In conclusion, the generation of a genomic library was a necessary first step in the development of a DNA vaccine for *P. salmonis*. Within this initial step, the development of a consistent method for production of a pure pool of genomic *P. salmonis* DNA was required. The DNA was subsequently used to generate the λF^{20A} library, which provided a wide array of *P. salmonis* inserts of diverse putative functions. A number of inserts showed potential for further studies in the pathobiology of *P. salmonis* and development of a DNA vaccine.

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Table 3.1. Estimated purity by dot blot of three *P. salmonis* DNA isolations containing 50 ng/sample total DNA

Sample	average estimate	% pure
F^{12}	3.13-1.56 ng	94-97
F^{11}	<0.39 ng	>99.2
*Sau*3A I digest for library	6.25-3.13	87.5-94

Table 3.2. Sequences obtained from the λF^{20} library.

clone #	Nucleotide sequence (/N/ = sequence not obtained in initial sequencing)
1	1 GATCAGCAGG ATAAAATAAA GCTAAACCAG AGTTTGGAAA AGGTAAAAGC 51 AGGGCAAGTG ACACGTTGGC GTAATCCAGA TACAGGCAAT AGTTATAGTG 101 TTGAGCCAGT GCGTACTTAC CAGCGTTACA ATAAGCAAGA GCGTCGCCAG 151 CAATATTGTC GAGAATTTCA GCAAAAGGCG ATGATTGCAG GGCAGAAGCA 201 AGAGATTTAC GGCACNTGCA TGCCGGCAAC CGGATGGTCC GTTGGCAAGT 251 CATTTCAACA GAAAAATAAT AATTAGGTTA TGGCTAAATT ATAAAAGTAG 301 CAGTGAATTT ATTTTAATAA TATTTGCTGT AAATTGATAT TTAGAAGACA 351 GCAATAATAA AAAAAATTGA GTTTTATGGT GAATATATTA TTATTTTAGA 401 TGCTAAATGG AATCNTTCTT GAATAGAC /N/ GTAGAGGCCG 451 AGGTAGGTTC GGGTTTTTAG CAGACGGAGT TGCGAAAGCC GTTAGGTCTG 501 TAGCAAGCGT AACGAGTGTC TAAAAATCTA TACGAACCGT AGAGTCATGT 551 GAGAGCACAG TAGTGGAGTG TGCCGCTTCA AGGCACGTAA CGCTGTGTGA 601 CGCGGACAGC CGAGGGTTTA TAGTCGTCGC GTTTTGCTCG GCGATTTTGC 651 ATCATTGGAT GTGCAAAATA CCTACCGAGG TAGCGACGCT TACGCTATAA 701 TCCCCCTTTT TTTTAATAGA ATTTTATCAA TCATTAAAAC CAGCTTATTT 751 TTCATGTTTT TACGAATCTT TGTGATAAGT TGAAGTCCTT TTTCATACAA 801 TTGATC
2	1 GATCAGGCGC GTTACCCATT ATTATTGCTT TGTCAGAATA AACAANGCTA 51 TGACAATTTG TGTAGCTNCT TTCTAANGCT TATCTATANA CCAGAGTTGG 101 TGATGATNGC GATC
3	1 GATCTTTAAT CTCATTAAAA TTTATGTTTC TAATCGCGTG GGTTATTTTC 51 ATCAAATACG GCATTTTTAG GAGGAGGGAT AACAACATCA GCATTAGGTG 101 AGTGGTTTAA AACGGAATCG TAAACATCAT GGCTATCGTA GGCTCCGTCT 151 GCAGTGAAGC GATC
4	1 GATCAAGTAT TAGTCCATTG ATGAGATGGC AGATGTACTA GTTTTCGAGT 51 GTTAATGTTG CTAGAGTGCT GAATATTGAT C
5	1 GATCANGANA ACTATCGTAG AGTTTCTTGT GTCATCCAGT CAGGTTTGTG 51 AGGCTTTTTC CAAATGACAA GGTGANTTTT TGAACCCAAC TTCCTTCCTT 101 TACGAAAGTC ATACTTCCTC TGTGAATGTG CTTTAANAAT ACCATCAGCA 151 CCTATTGACT GCAAATCACC CATCACCGAA AAANCANGGG AAATACCNGA 201 TC
6	1 GATCTGTGAC ATACACAGNG GACATNGCCT GNGCGACCAT TATGAATCGC 51 AATCGTCAAG CCAACCATAT CTGGAAGAAT CATAGAACGG CGAGACCAAG 101 TCTTAATAGG TCGCTTACTG TTAGAAGCAG CAGCTGATTC CACTTTTTTA 151 ATCAAGTGGA GATC
7	1 GATCAANGCC CGCATATTAA TCGACGACCA CGATATTCAA AAGTTAAAAA 51 TTCAAAATAT CCGCCAACAT ATTGCCTATT TACCTCAGCA TGGTGACTTA 101 TTTAATGGCA CGATC
8	1 GATCAATAAT ATAACGAGTT TGAGGTCGNA ATGGGCTTAA GCAAGCTGGT 51 ACGAATTTTA TCTAAAAGAC AGGAACGGAT C

Cont'd

Table 3.2. Sequences obtained from the λF^{20} library.

9

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1   GATCCTGCAT ATTTAAGCTC ATAGACAGGG AGATAATGGT GGAGGAATGT
51  ACGGTCATTA TTATGACTTT AGAGTAGTTA GCAATAATAA TTTCTTTTCT
101 AAAGAAAAAA TTATTTTTTA ACCGTATAGC GAAAATCTCG GGGGATTGCC
151 CCCGTGATGG GCATTGTGGT TCTGTCGCAA TTTGAAATTT TCATGAAACA
201 GGTGGCAGGT AAAGCCTTGG CCACAGATC
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10

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1   GATCCAACCA TGGCGCGTAT ACTGGTGATT CATTACGGGA TAACTGGCTC
51  AGCCGCTAAA AAGCAAGCGA TTTATCATAT ATCATCACCT ACTTATGACA
101 GTTATCTAGG TAAAGGAGTG AGCTATGTGC GTGGGTGTTT AAATGCGCTA
151 ATGTGTAAGC CTGCTTAAGC AGGCTCGTGC NTGTTGCATT TTTTAATAAA
201 ATCACATCAT AAACCTCAAC CTCTTGAGTT TCTAAAATAC TTAACTGTAA
251 CCCTTGAAAT AGTACTAAAA TATAGTACTA TATAAATATG ATACTAAATT
301 TTGGTACTAT ACCTTTGAAT AATAAACACA AGAAGACCTT ACGAGAGATT
351 TATACTGACC CGATCAGCAG GATAAAATAA AGCTAAACCA GAGTTTGGAA
401 AGGTAAAGCA GGCAAGTGCA CGTTGGCGTA TCCAGATCAG G /N/
451 TCATGAGTGC TAGCACGGGG TAGAGGCCGA GGTAGGTTCG GGTTTTAGCA
501 GACGGAGTTG CGAAAGGCCG TTAGGTCTGT AGCAAGCGTA ACGAGTGTCT
551 AAAAATCTAT ACGAACCGTA GAGTCATGTG AACAGCACAG TAGTGGAGTG
601 TGCCGCTTCA AGGCACGGTA ACGCTGTGTG ACGCGGACAG CCGAGGGTTT
651 ATAGTCGTCG CGTTTTGCTC GGCGATTTTG CATCATTGGA TGTGCAAAAT
701 ACCTACCGAG GTAGCGACTG CTTACGCTAT AATCCCCCTT TTTTTTAATA
751 GAATTTTATC AATCATTAAA ACCAGCTTAT TTTTCATGTT TTTACGAATC
801 TTTGTGATAA GTTGAAGTCC TTTTTCATAC AATTGATC
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11

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1   GATCAACTAT CGATGGCGGG TCTAGAAGTC GATGGTATGA CAGCTGTCGC
51  GGCTGAATTT TCTCAGGTAA TTGTGGCAGA GATTACGCAT TGTGAGCAAC
101 ATCCGGATGC TGATC
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12

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1   GATCATGTCT TAACCTGTGA GTTTCATCCC CAAGAATTTA CTGTAAATCC
51  TCTAGAGGTT GATGATTATC AGTGGATC
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15

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1   GATCGGTGGC TTANTGACGC ATCAATTTGG TTATTTACCA CANAAAGGTG
51  AGTCTGTGAT TATAGCAGCA GTATCNATTT ACAGTCCTTA GTACTTCGGG
101 GCGGCGTATT CAACTTTTAC AAGCAGAGCC TGTAAAAAAT TAAAATCAGT
151 GTACAATTGA GAGATTGTAG GCTTAATGCA GGTAGCGCAG ATCATGGCAC
201 TTTTGCCACT CTTGTGTCAA CTTGTCAATT TGGCTNGTCT GTCGCATTTT
251 CAAGCCGTAG CAAGCTGTGC TTTAAGACTG CCCCCTAAAA CAATATCTGT
301 TCCACGGCCG GCCATATTCG TTGCAATAGG TTACTCGACC AGGCTCACCA
351 GCATTAGCGA TAATTTGCGC TTCTTGACCA TGAAACTTTG CGTTTAACAC
401 AGCATGG    /N/        GGGTAAAGAG TATCGATATT GGCGTTTTTT
451 GGCTGTATTT TATGGTTCAG GTTGTGCGAG TACGGTGCCA ACAGGGCGCC
501 TTATTCTGAT TATCCTCATG TGTATGCATG CCCGAATAAG TTAAGTACTT
551 TGTGTTATCG TACAGCGATT GCACCGGTTG GACACTGGTC TCAGTATAAT
601 CAGCTGAGCT TTCAGTTGCC GATTGCTTTG CAAGTACCAT TGCGTCAAGG
651 ACAATTAGAG CTACAAGAGT ATTATGCTAA AAATCCCGTA TTGCCTTCAT
701 CTTTGCCTTT ATCAGGCCCA GGCCCGTTAA CGTCTTATTT ATATCCATTT
751 GGATTGTGTG CAACAAAAAT AATTCGCTTA GAGAGTTTAA CTGATC
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17

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1   GATCGCTGAT TTAGCCTCCT GTCGATTTTT AAAATTCATG TGATGAACTA
51  ACTCCGTTTT TAGTGTATGA AAGAAACTCT CTGAAACAGC GTTATCCCTG
101 TTCTGGTCAA GTCATATTGG ACACTTTTAA TTGAGACTTT TCAAAATTAA
151 TAGGCGATAG GTTACCATTG GCTGTGTGCA ACCGATC
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Cont'd

19

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1   GATCCAGCCA TTGCCGGTAA ACTGCCGCCC CTGATC
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Table 3.2. Sequences obtained from the λF^{20} library.

20	1	GGATCATGCT CATCCTCTAC GTCGATGCGA TGGTCTCACC TTTAGGAACG
	51	GCTTTAGCCT ATACCGGCTC TTCTACACGG ATGCTAACGG CCATGTCTCG
	101	CGAAAAACAG GTTCCGCGTT TCTTTGACCA TGTACACCCC CACTATGGTG
	151	TTTCCCGTCG TTCATTGATC

Table 3.3. Insert and ORF size of clones, plus the deduced function of the insert

Clone #	Insert size in bp	ORF size	Similarity of deduced amino acid sequence	E-value(*)
1	>2 000	272	17kDa Spotted Fever Group Rickettsia surface antigen	$2x10^{-7}$
		117	Transposase	$2x10^{-7}$
2	114	114	domains show similarity to *Chlamydia* DNA polymerase III	N/A
3	164	135	*Vibrio parahaemolyticus* transposase	0.015
4	81	81	No significant similarities found	
5	202	65 + 186	No significant similarities found	
6	164	164	S19 protein of the 30S ribosomal subunit	$4x10^{-14}$
7	115	115	ATP binding cassette type transporters	0.005
8	81	81	No significant similarities found	
9	229	110	Yeast DNA binding domain (Zink finger)	N/A
10	>2 000	multiple	Chimeric insert, contains the same insert as clone 1, plus a second insert containing an 168 bp ORF with no significant similarity	
11	115	93	No significant similarities found	
12	78	78	No significant similarities found	
15	> 1 000	132	Preprotein translocase subunit SecA	$4x10^{-4}$
		124	No significant similarities found	
17	187	81	No significant similarities found	
19	36	36	No significant similarities found	
20	170	168	*Bacillus subtilis* amino acid transporter	$8x10^{-9}$

(*) E value = Expect value. This describes the number of hits one can "expect" to see just by chance when searching a database of a particular size (Altschul *et al.*, 1990).

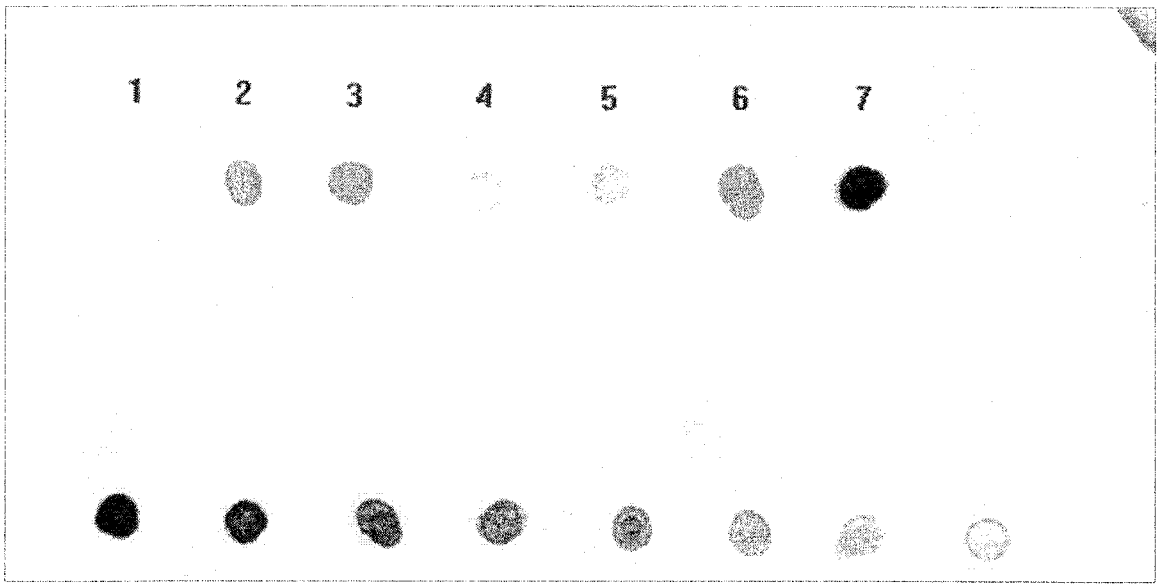

Figure 3.1. Dot blot to quantify levels of contaminating CHSE-214 DNA in samples of purified *P. salmonis* DNA. Samples were directly applied to the filter and hybridized with a DIG labeled CHSE-214 probe as described in the text. *Top row*: 50ng total DNA/dot of: 1) Negative control (DNA ladder); 2) and 3) Two parallel purification batches of *P. salmonis* DNA with *P. salmonis* from the harvest F^{12} used as source; 4) and 5) As samples 2 and 3 but harvest F^{11} was used as source; 6) *P. salmonis* DNA purified from previously frozen *P. salmonis*; 7) CHSE-214 DNA. *Bottom row*: Doubling dilution of purified CHSE-214 DNA, starting with 50 ng/dot and going down to 0.39 ng/dot. Levels of contamination with CHSE-214 DNA were estimated by comparing intensity of sample dots in the top row to the diluted CHSE-214 DNA in the bottom row.

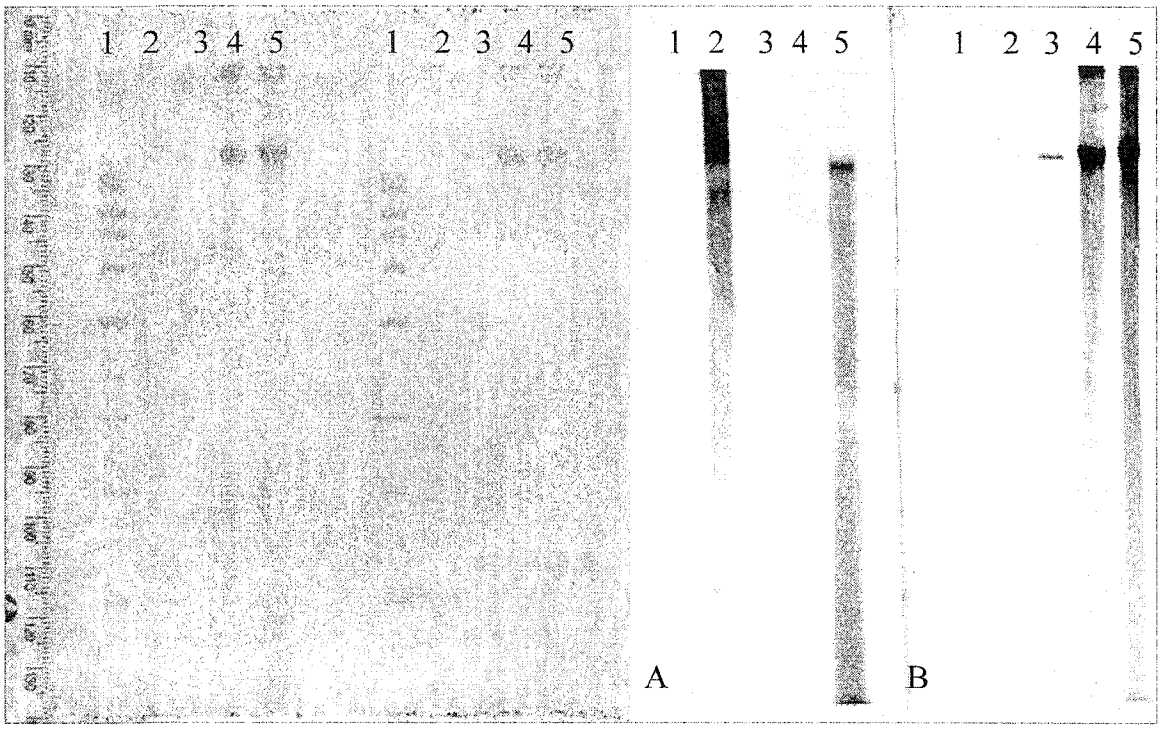

Figure 3.2. Negative image of the originating agarose gel (left) and two Southern blots (right) derived from this gel to determine levels and type of contaminating CHSE-214 DNA in a *P. salmonis* genomic DNA preparation. Samples: 1:) 1kb DNA ladder; 2:) CHSE-214 DNA; 3:) gel purified *P. salmonis* DNA; 4:) *P. salmonis* DNA prepared using 250 µg/ml DNase I digestion to remove contaminating CHSE-214 DNA and 5:) *P. salmonis* DNA prepared using 25 µg/ml DNase I digestion. Blot A was hybridized with DIG-labeled CHSE-214 DNA, blot B with DIG-labeled, gel purified *P. salmonis* DNA as described in the text.

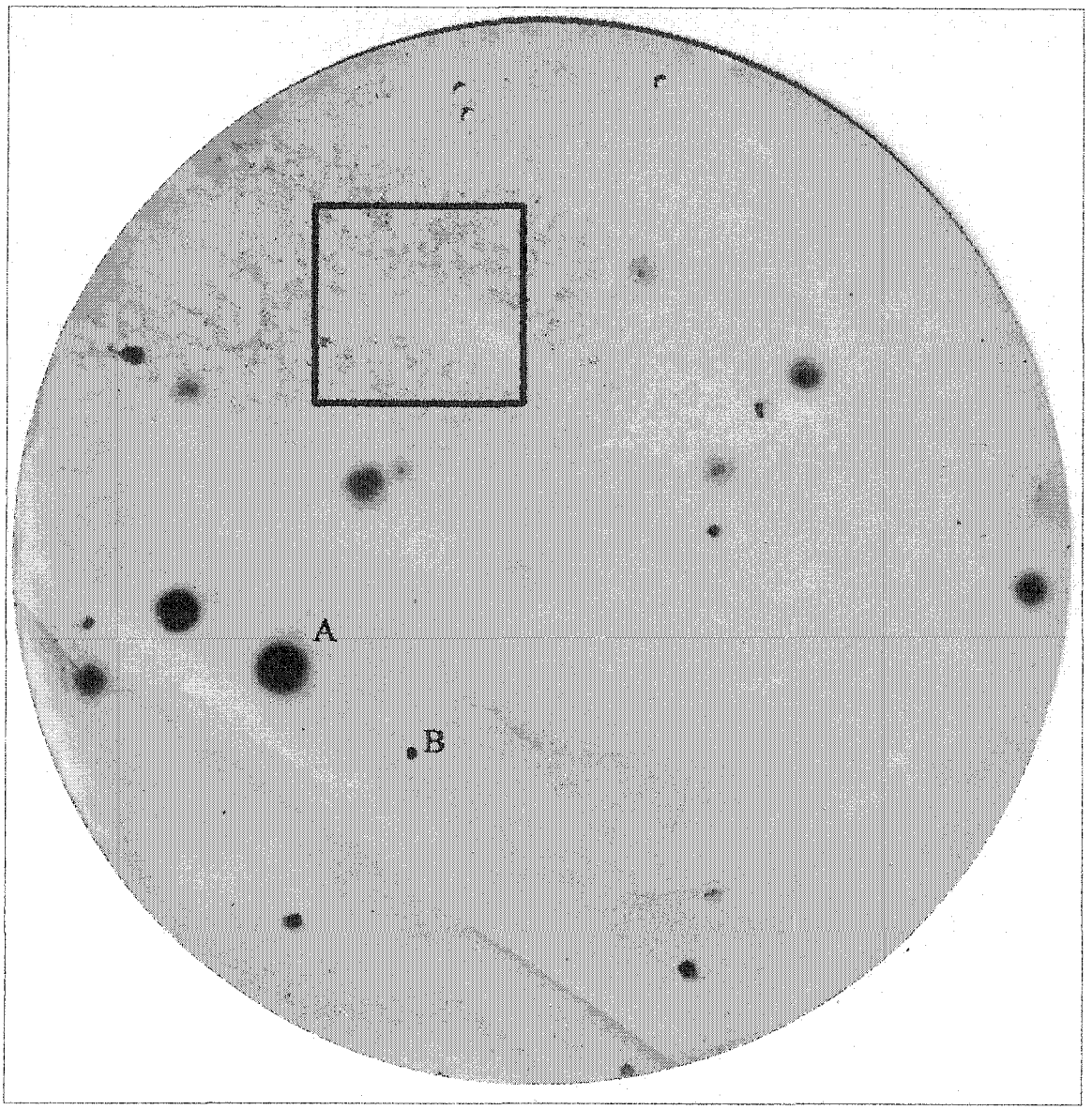

Figure 3.3. Example of a nitrocellulose filter used in immunoscreening of the λ^{20a} library. The density of total plaques can be seen in the boxed area as pale spots in the background. Two types of positive plaques were seen where signal could be either spread out over the filter as at location A or defined within the plaque as at B. The filter was incubated with 1:100 rabbit α- *P. salmonis* antiserum and detected with 1:100 goat α-rabbit-AP conjugate and NBT/BCIP.

CHAPTER 4: DEVELOPMENT OF A DNA VACCINE EXPRESSING A 17 kDa PROTEIN FROM *PISCIRICKETTSIA SALMONIS*

4.1 ABSTRACT

Piscirickettsia salmonis is a significant pathogen in the Chilean salmonid aquaculture industry and no effective treatment is commercially available. In an effort to develop a DNA based vaccine, sequence information obtained from clones from a previously developed expression library (see Chapter 3) was expanded by inverse PCR (iPCR). A full length sequence was obtained for a gene showing a deduced amino acid sequence homology to the 17 kDa genus-specific antigen of the spotted fever group *Rickettsia.* Additional sequence information was generated for clones showing homology to a transposase, an ABC-type protein and an amino acid transporter. The full length gene for the 17 kDa protein was cloned into the pcDNA3.1$^-$ and the pUK21 expression vectors. The constructs, pcDNA3.1-17 kDA and pUK-17 kDa, were analyzed for their potential as DNA vaccines by challenge trial in coho salmon (*Oncorhynchus kisutch*). Total mortalities in the challenge trial, including those injected with adjuvated inactivated *P. salmonis* were high, indicating overwhelming challenge dose. Survival analysis of the mortality data showed significant protection with the pcDNA 3.1 -17 kDa construct but not for the pUK - 17 kDa. Immunohistochemistry performed on *in vitro* transfected CHSE-214 cells with polyclonal rabbit α-*P. salmonis* antisera showed the presence of antigen for both the constructs.

4.2 INTRODUCTION

The expansion of the salmon aquaculture industry in the last 25 years has given a new focus to the development of vaccines aimed at fish pathogens (Shao, 2001). The recent introduction of recombinant DNA technologies has permitted the development of two new vaccine types in addition to the traditional inactivated and live attenuated vaccines. Recombinant vaccines contain single or multiple protein antigens derived from the pathogen, which are produced in non-pathogenic organisms like *Escherichia coli*. DNA vaccines are fundamentally different from other types of vaccine technologies in that they do not contain a protective antigen, but rather the genetic information encoding this antigen (Lewis and Babiuk, 1999; Liu, 2003). The gene coding the antigen is present in a plasmid that is administered to the fish. The plasmid is internalized by cells of the host and these will process the gene by transcription and translation to produce the antigen.

The ability of fish cells to take up plasmids and produce the encoded protein has been well established in a variety of species (Kanellos *et al.*, 1999; Dijkstra *et al.*, 2001; Nusbaum *et al.*, 2002; Romoren *et al.*, 2004). Reporter genes code for enzymes whose activity can be easily visualized, such as luciferase encoding gene *luc*, the *E. coli* β-galactosidase (β-gal) encoding gene *lacZ* and the chloramphenicol acetyltransferase encoding gene CAT. By injecting expression plasmids encoding these genes into fish, the duration of expression can be evaluated. Luciferase is non-immunogenic in fish, however β-gal can elicit an immune response (Heppel *et al.*, 1998). After *intramuscular* (*i.m.*) injection of a plasmid containing the *lacZ* gene, the production of antibodies against β-gal

can be used as a measure of the humoral response, while the cell mediated response can be measured by the accelerated decrease of myofibres expressing β-gal when compared to control plasmid injected muscle (Kanellos *et al.*, 1999). Russel *et al.* (2000) observed a positive correlation between the disappearance of antigen expressing myofibres and the appearance and subsequent increase of an antibody titre in goldfish (*Carassius auratus* L.) held at 22°C. At temperatures where the cytotoxic T cell function is reduced, but where the less temperature sensitive B cell function is not yet impeded (Bly and Clem, 1994), this correlation between number of antigen expressing myofibres and the antibody titre was reversed. The antibody titre was slower to appear but increased above levels seen at 22 or 25°C. This suggests that with the reduction of the rate of myofibres being eliminated by the cell mediated immune system at sub-optimal temperatures, the rate of antigen release was also decreased. Yet, the elimination of myofibres continued for an extended period of time, sufficient for a secondary response to be initiated, resulting in a higher antibody titre. Therefore, it is suggested that a slow, steady production of antigen by DNA vaccines may be more beneficial than short term, higher production levels. Thus DNA vaccine candidates giving less than optimal expression can still be effective in provoking a protective response. Sub-optimal expression leading to protective immunity may be uniquely true for DNA vaccines encoding bacterial antigens. Unlike viral genes that are normally expressed in cells of the host organism, genes of bacterial pathogens are expressed in the context of prokaryotic transcription and translation machinery. There are fundamental differences in codon-bias, regulatory sequences and post-translational modification between the bacterial pathogen and the eukaryotic host cells, possibly

limiting the ability of the host cell to adequately express the bacterial antigen. This may limit the capacity of DNA vaccine plasmids to deliver genes encoding bacterial antigen effectively. However, immunity to intracellular pathogens depends largely on a cell mediated immune response. Cytotoxic T cell mediated immunity typically depends on the recognition of 12 to 20 amino acid oligopeptides presented in the context of host cell MHC (Janeway, 2001). One might predict that post translational modification would have a smaller impact on these relatively short antigenic oligopeptides. Therefore, it is feasible that a DNA vaccine encoding a bacterial antigen can still provoke a protective cell mediated response in the absence of a detectable protective humoral response.

With the exception of live attenuated vaccines, most conventional vaccines favour a humoral response. Intracellular pathogens, like *P. salmonis*, spend a large part of their life cycle hidden from the circulating antibodies elicited by vaccines favouring a humoral response. A cell mediated immune response is required to kill the pathogen or the cells in which it resides (Janeway, 2001; Flynn and Chan, 2001). DNA vaccines are capable of initiating such a cell mediated type of response (Lai and Bennett, 1998; Lewis and Babiuk, 1999; Liu, 2003).

A number of immunoreactive clones in an expression library derived from the intracellular fish pathogen *P. salmonis* have been identified (see chapter 3). From this library, sequence information of selected fragments of the *P. salmonis* genome were characterized. In an effort to characterize the entire ORF of immunoreactive clones, a method for 'walking' the genome was required. Inverse PCR (iPCR) is a method to amplify the regions flanking a known, or core segment (Tchetina and Newman, 1995). In

contrast to standard PCR, the PCR primer sequences for inverse PCR are selected homologous to sequences at the edge of the core sequence, but facing outwards. Genomic DNA is digested with restriction enzymes and ligated again under conditions that favour monomeric circles. The inverse primers are now oriented towards each other so they can generate an amplicon that is then purified and sequenced. The junction between the original upstream and downstream sequences, otherwise ambiguous, can be identified as the restriction site of the restriction enzyme used. The development of iPCR can be subdivided in four different steps: 1) finding a restriction enzyme that will cut the genomic DNA in such a way that the areas flanking the core segment are not too large for amplification or too small to provide useful sequence information; 2) finding the appropriate ligation conditions that favour monomeric circular DNA formation; 3) finding the appropriate amplification condition for PCR amplification; and 4) sequencing of the purified PCR product.

The objective of this study was to expand on the sequence information available from the λF^{20} library described in chapter 3 and to identify full length genes for use in DNA vaccines. The 17 kDa protein of *P. salmonis* was identified as a possible candidate for vaccine development. Inverse PCR was successfully used to determine the complete ORF for the *P. salmonis* gene encoding the 17 kDa spotted fever group surface antigen analogue. The potential of the 17 kDa antigen as a DNA vaccine antigen was assessed by cloning the gene into pcDNA3.1$^-$ and pUK plasmids and testing these constructs in a challenge trial. *In vitro* transfection studies were performed to investigate if the protein expressed by the CHSE-214 cells contained B cell epitopes that could be recognized by

antiserum from rabbits immunized with *P. salmonis*.

4.3 MATERIALS AND METHODS

4.3.1 Inverse PCR

4.3.1.1 Digestion of genomic *P. salmonis* DNA

To determine the appropriate restriction enzyme to use for iPCR two methods where developed. The first method used the clone 1, containing the part of the 17 kDa antigen gene and the preprotein translocase subunit SecA homologous partial gene found in clone 15 as core regions and it was determined which restriction enzymes would yield appropriately sized fragments containing the core segment prior to ligation. Digests of *P. salmonis* genomic DNA were separated by size by electrophoresis on an 1% agarose gel at 120 V and blotted onto a nylon filter. A probe was developed based on the 17 kDa and clone 15 core sequences using the PCR amplification product of core region specific primers in a normal orientation. Using these probes, the size of the restriction fragment containing the core region was determined and only fragments with appropriate size were self-ligated and used as iPCR template. The second method used the partial sequences found in clone 3, a putative transposase, clone 7, an ABC-type transporter and clone 20, an amino acid transporter as the core regions. This method differed from the first in that no preselection of restriction enzyme was performed and the digestion products from all restriction enzymes were self-ligated and used as iPCR template.

The restriction enzymes tested were *Sua*3A I; *Hpa*II; *Mun*I; *Bsp*TI; *Cla*I; *Sac*I; *Xba*I; *Vsp*I; *Pst*I; *Hinf*I; *Eco*RI; *Kpn*2I; *Hind*III (Fermentas). Restriction digestion was performed on 1 μg of purified *P. salmonis* DNA in a total of 50 μl with 2 units of

restriction enzyme using the manufacturer recommended buffer. The digestion was performed for two hours at 37°C and the enzymes were heat inactivated for 20 minutes at 65°C. The only exception was *Kpn*2I where the reaction was performed at 55°C for two hours and heat inactivated at 85°C for 20 minutes. If not used immediately after inactivation, digests were stored at -20°C.

4.3.1.2 Generation of digoxigenin (DIG) labeled probe for the core regions of the 17 kDa gene

With the sequence information acquired from the expression library described in chapter 3, we developed insert specific primers using the Primer3 software available at http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi (Rozen and Skaletsky, 2000). The insert specific custom primers (GibcoBRL) used to generate template for the DIG labeling reaction were 17 kDa-F (5'-GAT CAG CAG GAT AAA ATA AAG CTA AAC-3') and 17 kDa-R (5'-TGC TAC TTT TAT AAT TTA GCC ATA ACC-3'). A PCR reaction was set up in 100 µl reaction volume. One microliter of genomic DNA solution was added to 99 µl of master mix. This master mix contained 5 µl of a stock solution of 10 pmol μl^{-1} of each of the primers, 2 µl of 2 mM dNTPs, 10 µl of 10x reaction buffer (Amersham Pharmacia Biotech), 0.5 µl (2.5 units) of *Taq* (Amersham Pharmacia Biotech) and 76.5 µl ddH_2O.

The PCR reaction was performed in a PTC-200 Peltier Thermal Cycler (M.J. Research) using the following 'Touchdown' protocol: samples were held for one minute at 94°C followed by 20 cycles of 95°C for five seconds, 70°C for 20 seconds but

dropping 0.5°C per cycle and 20 cycles of 95°C for five seconds and 60°C for 30 seconds but extending the time by one second every cycle. The protocol was ended by keeping the sample at 4°C until removal from the cycler.

The amplification product was purified using a High Pure PCR product purification kit (Roche) following the manufacturer's protocol. In brief, binding buffer, supplied with the kit was added to the amplification reaction, transferred to the upper reservoir of a filter tube and centrifuged at 13,000 x g for 30 seconds in an Eppendorf 5415C centrifuge. Remaining impurities were removed by two washes with 500 µl of supplied wash buffer at 13,000 x g for 30 seconds. The DNA was then collected by the addition of elution buffer and another 30 second centrifugation at 13,000 x g. This DNA was used as template for a DIG labeling reaction as described in paragraph 3.3.2.1.

4.3.1.3 Southern blot to determine the size of the restriction fragment containing the core region.

A 1% Agarose gel in 1x Tris/Borate/EDTA buffer (89 mM Tris, 89 mM Boric acid, 2 mM EDTA, pH 8.3) (TBE) was loaded with 300 ng per well of DNA from the restriction digestions described above. The gel was run for two hours at 120 V, photographed and blotted and probed as described in paragraph 3.3.2.2. A scan of the developed blot was aligned with the image of the gel so that the approximate size of bands on the blot could be estimated.

4.3.1.4 Self ligation to produce circular iPCR template

To generate template for the iPCR of the 17 kDa gene, three different concentrations of restriction digest product were used for self ligation, 40 ng μl^{-1}, 800 pg μl^{-1} and 400 pg μl^{-1} final concentration in the ligation reaction to optimize for monomeric self ligation. Ligation reactions were set up with 1 µl of restriction digest product, 1 µl of 5x ligation buffer and 1 unit of T4 DNA ligase (MBI Fermentas) and brought to a final volume of 5 µl with ddH_2O and allowed to ligate overnight at 4°C.

For clones 3, 7, 15 and 20, the protocol was adapted in the following ways: only two concentrations of restriction digest product were used, 40 ng μl^{-1} and 800 pg μl^{-1} and the reaction volume increased to 10 µl with two units of T4 DNA ligase.

4.3.1.5 Inverse PCR

Five microliters of the ligation mixture was used as template for the iPCR and was added to 95 µl of mastermix containing 10 µl of 10x PCR buffer (Amersham Pharmacia Biotech), 2 µl of 2 mM dNTP (Amersham Pharmacia Biotech), 5 µl each of a stock of 10 pmol μl^{-1} of the inverse primers for the particular clone (Table 4.1), 0.5 µl of *Taq* polymerase (2.5 units) (Amersham Pharmacia Biotech) and 72.5 µl ddH_2O for each sample. The PCR reaction was performed in a PTC-200 Peltier Thermal Cycler (M.J. Research) using the following conditions: 94°C for one minute followed by 40 cycles of 94°C for 30 seconds, 50°C for 30 seconds and 70°C for three minutes and a final extension step of ten minutes at 70°C. Samples were stored at 4°C and screened for amplification product by electrophoresis on a 1% agarose gel in 1x TBE buffer for one

hour at 120 V.

4.3.1.6 Sequence analysis

If an amplification product was found after the iPCR, the product was purified from the agarose gel using a modified protocol for the High Pure PCR product purification kit (Roche). Briefly, gel fragments containing bands of interest were excised from the gel and incubated in 300 µl binding buffer for every 100 mg of agarose slice. The sample was vortexed for 15 to 30 seconds to suspend the slice in binding buffer and warmed to 56°C for ten minutes. During this incubation, the sample was vortexed every 2 to 3 minutes. After the gel slice was completely dissolved, 100 µl of isopropanol for every 100 mg of agarose slice was added and the sample vortexed. The resulting suspension was pipetted into the upper reservoir of a filter tube and treated as described in 4.3.1.2. The resulting purified DNA was used as template for sequencing. Sequencing was performed by the Research and Productivity Council (RPC, Fredericton, New Brunswick) using the iPCR primers, dye terminator cycle sequencing chemistry and the ABI PRISM BigDye sequencing kit. Nucleotide sequence was obtained following electrophoresis on an Applied Biosystems model 377 automated sequencer. The sequences were analyzed using Frameplot 2.3 available online at http://watson.nih.go.jp/~jun/cgi-bin/frameplot.pl to determine open reading frames and their deduced amino acid sequence (Ishikawa and Hotta, 1999). The deduced amino acid sequence was submitted for Blastp comparison to sequences available through GeneBank at http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi (Altschul *et al.*, 1990) to determine

homology.

4.3.2 Cloning of the 17 kDa gene into the pcDNA 3.1- expression vector

4.3.2.1 Generating full length 17 kDa gene with flanking restriction sites by PCR

The λZapExpress vector used in the expression library has an *in vivo* excision capability using the ExAssist helper phage. This should generate a plasmid derived from the phage (phagemid). It was found that the phagemids were unstable and did not contain the same insert as the originating clone identified from the λZapExpress library due to recombination. A different cloning strategy was therefore required. Following iPCR the complete sequence for the 17 kDa spotted fever group antigen analogue of *P. salmonis* was determined. Primers were developed using Primer3 software (see above) that flanked the gene but contained mismatched nucleotides that would introduce *Eco*R I, *Eco*R V or *Xho*I restriction sites. The *Eco*R I and *Eco*R V sites were introduced by changing one basepair in the *P. salmonis* sequence, while the *Xho* I site was added to the 5' end of the 17 kDa-EcoR V primer.

The following primers were used (restriction sites in italics): 17 kDa-*Eco*RI: 5'-ATG *AAT TC*A CTT CTA CTT TTA AAT TTA TCC-3'; 17kDa-*Eco*RV: 5'-TTG *ATA TC*A GTG AGA GAA ATA ATG AAC AGA-3' and 17 kDa-*Xho*I: 5'-C*CT CGA G*TT GAT ATC AGT GAG AGA AAT AAT GAA CAG A-3'. Taq generated amplification products may contain *Taq* induced errors in the final construct. However, since sequence mismatches were introduced in the primers to obtain restriction sites, replacement of *Taq* by a proofreading enzyme was not feasible. To address this problem, the Expand High

Fidelity PCR system (Roche), which contains a mix of *Taq* and *Pwo*, a high fidelity thermostable DNA polymerase was used. The PCR cycling conditions were designed to favour *Taq* for the first ten cycles to generate product with the sequence changes. The annealing temperature and extension were raised for the remaining steps to favour the proofreading activity of *Pwo*. Reactions were performed in a 100 µl total volume. Ninety-nine microliters of mastermix were made per sample. The mastermix contained 10 µl of 10x Expand High Fidelity buffer (Roche), 30 µl each of a 10 pmol μl^{-1} stock solution of the 17kDa-EcoRI and 17 kDa-XhoI primers, 2 µl of a 2 mM dNTP stock solution, 2.5 units of Expand High Fidelity DNA polymerase mix and ddH_2O to bring to a volume of 99 µl. The primer concentration is three times higher than standard. It was found that at lower concentrations of primers *Taq* polymerase by itself was capable of generating amplification product, but the Expand High Fidelity DNA polymerase mixture was not. The cycling conditions were as follows: 95°C for three minutes, followed by a total of ten cycles of 92°C for 30 seconds, 50°C for 30 seconds and 72°C for 90 seconds followed by 25 cycles of 92°C for 30 seconds, 55°C for 30 seconds and 72°C for 90 seconds and finally a ten minute extension step. The results were analyzed using horizontal gel electrophoresis in a 1% agarose gel in 1x TBE for one hour at 120 V and amplification product was purified using the High Pure PCR product purification kit (Roche) as described in paragraph 4.3.1.2.

4.3.2.2 Vector and insert preparation and ligation

To prepare the vector for ligation, one microgram of pcDNA3.1$^-$ (Invitrogen) or

pUK plasmid DNA was double digested using one unit each of *Eco*RI and *Xho*I (Fermentas) restriction endonucleases in 2x Y+/Tango buffer (Fermentas) in 30 µl for two hours at 37°C. The reaction was terminated by heat inactivation at 65°C for 20 minutes. To prevent self ligation the 5' ends were dephosphorylated by adding 1 unit of calf intestinal alkaline phosphatase (CIAP) (Fermentas) to the digested DNA and incubating it for 30 minutes at 37°C followed by heat inactivation for 15 minutes at 85°C. The digested, dephosphorylized plasmid was purified using the High Pure PCR product purification kit (Roche) as described in paragraph 4.3.1.2.

One microgram of purified amplification product was double digested using one unit each of *Eco*RI and *Xho*I restriction endonucleases (Fermentas) in 2x Y+/Tango buffer (Fermentas) in 30 µl for two hours at 37°C after which the reaction was terminated by heat inactivation at 65°C for 20 minutes. The double digested insert DNA was purified using the High Pure PCR product purification kit (Roche) as described in paragraph 4.3.1.2.

Purified double digested insert and double digested and dephosphorylated plasmid DNA were added in equimolar amounts to a ligation reaction containing 2 units T4 DNA ligase (Fermentas) 2 µl of 10x ligation buffer (Fermentas) and ddH_2O to a final volume of 20 µl. The reaction was incubated overnight at 16°C, followed by one hour at 22°C and heat inactivation at 70°C for 20 minutes.

4.3.2.3 Transformation of competent cells with the ligated plasmid-insert construct

Ligation products were transferred into the SURE competent cells (Stratagene).

This strain of *E. coli* was engineered for cloning of certain DNA fragments that are "unclonable" in conventional *E. coli*. The strain is restriction minus, endonuclease deficient, and recombination deficient (Manufacturer supplied product information). The protocol recommended by the manufacturer was used. Briefly, SURE cells stored at -80°C were thawed on ice, gently mixed and 100 µl was aliquoted into pre-chilled 14 ml polypropylene round-bottom tubes. To each tube, 1.7 µl of β-mercaptoethanol was added and gently mixed by swirling. The tubes were incubated on ice for 10 minutes, gently swirling every two minutes. Two microliters of ligation mixture (paragraph 4.3.2.2) was added and the tube swirled gently. After incubating the DNA with cells for 30 minutes on ice, the transformation mix was heat-pulsed by suspending the tubes for exactly 45 seconds in a 42°C water bath after which the tubes were immediately put on ice for two minutes. Preheated SOC medium (20 g l^{-1} Tryptone, 5 g l^{-1} Yeast extract, 0.5 g l^{-1} NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM glucose, pH 7) (42°C) was added at 0.9 ml per tube and the tubes were incubated at 37°C for one hour on an orbital shaker set at 225 to 250 rpm. Two hundred microliters of the transformation mixture was plated on LB agar (10 g l^{-1} Tryptone, 5 g l^{-1} Yeast extract, 10 g l^{-1} NaCl, 15 g l^{-1} agar, pH 7) plates containing ampicillin at 100 µg ml^{-1} and these plates were incubated overnight. If colonies were found the next day, they were tested by colony PCR. For cloning of the 17 kDa insert into the pUK-21 vector, LB agar plates containing 50 µg ml^{-1} Kanamycin instead of ampicillin were used.

4.3.2.4 Colony PCR to determine the presence of the 17 kDa insert in transformed cells

Colony PCR was used to screen transformed cells for presence of the 17 kDa insert and pools were made of 12 clones per pool. A flamed, sterile loop was touched on a colony, streaked in a marked square of the pool plate and then twirled in 50 µl of sterile water. The same water was used for each member of the pool. Two separate master mixes were made. One and a half microliters of each pool were added to a 48.5 µl of master mix one, containing 5 µl each a 10 pmol μl^{-1} stock solution of the primers 17 kDa cons-F (5'-GGT GCT GTT TTG GGT GGA T-3' and 17kDa cons-R (5'-ATT GCC TGT ATC TGG ATT ACG C-3'), 2 µl of 2 mM dNTP's (on ice) and ddH_2O to a volume of 48.5 µl. To this 50 µl of master mix two, containing 10 µl 10x buffer (Amersham Pharmacia Biotech) ,0.5 µl *Taq* (2.5 units) (Amersham Pharmacia Biotech) and ddH_2O to a volume of 50 µl, was added and the samples run in a touchdown protocol as described in paragraph 4.3.1.2. One modification was made by extending the initial denaturation step to two minutes at 94°C. As a positive control, 1.5 µl of 1:1000 diluted genomic *P. salmonis* DNA was used as template. A no template reaction was included as negative control. The PCR reaction for each pool was screened for amplification product by electrophoresis on a 1% agarose gel in 1x TBE for 1 hour at 120 V. For pools showing amplification products a suspension of colony material in 50 µl of sterile water was made from the individual clones in the pool and used as template in the same colony PCR. Positive individual clones were replated on LB agar plates containing ampicillin at 100 $\mu g\ ml^{-1}$, incubated overnight at 37°C and stored at 4°C.

4.3.2.5 Miniprep plasmid purification and confirmation of the insert by restriction digest

Clones selected by colony PCR were inoculated in 5 ml of LB medium containing 100 μg ml^{-1} of ampicillin and grown overnight at 37°C with constant agitation. One and a half milliliters of bacterial suspension were centrifuged at 13,000 x g in an Eppendorf 5415C centrifuge for 10 minutes in a 1.5 ml microcentrifuge tube. Plasmid was purified from the pellet using the QIAprep Spin Miniprep Kit (Qiagen) with the manufacturer recommended protocol. In short, the pellet was resuspended in 250 μl of buffer P1 (Qiagen) and 250 μl of buffer P2 (Qiagen) was added and gently mixed by inverting the tube four to six times. After adding 350 μl of buffer N3 (Qiagen) the tube was immediately but gently inverted four to six times. The sample was centrifuged for 10 minutes at 13,000 x g and the supernatant was applied to the upper reservoir of a QIAprep column. The column was centrifuged for one minute and the flow-through discarded. The spin column was washed first by adding 500 μl of buffer PB (Qiagen) and centrifuging for one minute and the flow-through was discarded, then washed again by adding 750 μl of buffer PE (Qiagen) and centrifuging of one minute. After the flow through was discarded, the spin column was centrifuged for an additional minute to remove residual wash buffer. The QIAprep column was transferred to a clean 1.5 ml microcentrifuge tube. Plasmid DNA was eluted from the column with 50 μl of ddH_2O or TE buffer. After standing for one minute columns were centrifuged for one minute at 13,000 x g.

To confirm the presence and orientation of the insert in the plasmids, the plasmids

were digested with *Eco*RI and *Xho*I individually as well as in a double digest and with *Vsp*II as described above. Digestion profiles of the plasmid with insert were compared with the original plasmid.

4.3.3 Evaluation of plasmids containing the 17 kDa gene as a DNA vaccine

To evaluate the 17 kDa gene for use in a DNA vaccine, two approaches were taken. First, CHSE-214 cells were transfected with the vaccine construct and presence of immunoreactive protein was determined using immunohistochemistry. Second, a challenge study was conducted where coho salmon were immunized and subsequently challenged with *P. salmonis.*

4.3.3.1 DNA vaccine plasmid preparation

A single colony was used to inoculate a starter culture of 10 ml LB medium containing ampicillin (100 μg ml^{-1}) for pcDNA3.1 and pcDNA3.1-17kDa and kanamycin (50 μg ml^{-1}) for the pUK and pUK-17kDa. This culture was incubated for 8 hours at 37°C with vigorous shaking on an orbital shaker. The starter culture was diluted 1/500 into 2.5 l of LB medium with the appropriate antibiotic and incubated overnight at 37°C with vigorous shaking. The cells were harvested by centrifugation at 6000 x g for 15 minutes at 4°C in a Beckman J2-21M/E high speed centrifuge with a JA-14 rotor. All plasmids were purified using a Qiagen EndoFree Plasmid Giga Kit, following the manufacturer's recommended protocol. Briefly, the pellets were pooled and resuspended in 125 ml of buffer P1 (Qiagen). Buffer P2 (Qiagen) was added and the sample gently

mixed and incubated for 5 minutes at room temperature. Pre-chilled 125 ml of buffer P3 (Qiagen) was added and gently mixed until a white fluffy material formed and the lysate was no longer viscous. The lysate was immediately transferred to a QIAfilter Mega-Giga Cartridge (Qiagen) and allowed to incubate for 10 minutes at room temperature. A vacuum was applied to the cartridge until all liquid was pulled through the cartridge. Fifty milliliters of buffer FWD (Qiagen) was applied to the QIAfilter Cartridge and the precipitate was gently stirred with a sterile cartridge. The vacuum was re-applied until the liquid was pulled through completely. The filtered lysate was mixed with 30 ml of buffer ER (Qiagen) and incubated for 30 minutes. A QIAGEN-tip 10,000 was equilibrated by passing 75 ml of buffer QBT (Qiagen) through the tip by gravity flow. The filtered lysate was added to the tip and allowed to enter the tip by gravity flow. The tip was washed twice with 300 ml of buffer QC (Qiagen) and the DNA eluted with 75 ml of buffer QN (Qiagen). The purified plasmid DNA was precipitated by adding 52.5 ml of room temperature isopropanol and centrifuged at 15,000 x g for 30 minutes at 4°C. The supernatant was discarded and the DNA pellet washed with 10 ml of endotoxin-free room temperature 70% ethanol and centrifuged at 15,000 x g for 10 minutes. The pellet was air dried and the DNA re-dissolved in endotoxin free TE buffer. The quality of the DNA was determined by gel electrophoresis on a 1% agarose gel for one hour at 120 V and quantified by determining the optical density at 260 nm on a LKB Ultrospec II spectrometer.

4.3.3.2 Transfection of CHSE-214 cells with DNA vaccine plasmids

To determine if pcDNA3.1-17kDa and the pUK-17kDa could express immunoreactive protein, CHSE-214 cells were transfected using LipofectAMINE PLUS system (Life technologies). Eight well chamber slides were seeded with 400 µl of CHSE-214 cells and incubated at 15°C overnight. Two wells per slide were transfected with pcDNA3.1-17kDa, pUK-17kDa or with pcDNA3.1, one was used as positive control (see below) and one received LipofectAMINE without plasmid. The plasmid DNA was pre-complexed with the PLUS-reagent by mixing 1 µl of plasmid DNA (250 ng), 12.5 µl of OptiMEM (Life technologies) and 2.5 µl of PLUS reagent per well and incubating it at room temperature for 15 minutes. During this incubation, 1.25 µl per well of LipofectAMINE reagent was diluted with 23.75 µl of OptiMEM. The diluted LipofectAMINE and precomplexed plasmid DNA were mixed 1:1 and incubated 15 minutes at room temperature. During this incubation, the growth medium on the CHSE-214 cells was removed and the monolayer was washed with 200 µl of OptiMEM medium. Each well received 125 µl of OptiMEM, followed by 50 µl of the LipofectAMINE:DNA mixture. The cells were incubated for three hours at room temperature. One well was used as positive control and received no plasmid DNA, but at the end of the three hour incubation, 25 µl of a *P. salmonis* supernatant from a culture showing 100% CPE was added. The chamber slides were incubated 16 hours at 15°C in a humid chamber. The cells were fixed by washing the wells with 400 µl of 10% buffered formalin, followed by incubation of the wells with 400 µl of 10% buffered formalin for one hour at room temperature.

To detect any immunoreactive protein being produced by the transfected cells, the VECTAstain Universal ABC Kit (Vector Laboratories) was used. Formalin fixed samples on eight well slides were washed twice with 400 µl per well of PBS and permealized with 400 µl per well of 0.1% Triton X-100 in PBS for 30 minutes. After washing the samples twice with 400 µl per well of PBS, the sample was blocked with 400 µl of Blotto buffer at room temperature for one hour. Primary antibody, mixed rabbit antisera raised as described in paragraph 2.3.3, was diluted 1:200 in 1% BSA in PBS-Tween. Wells were incubated with 200 µl per well of primary antibody solution for 90 minutes at 37°C. The wells were washed twice with as much PBS as the wells could hold. The VECTAstain biotinylated universal antibody was diluted 1:50 and approximately 200 µl, were added to each well and incubated for 30 minutes with gentle rocking. During this incubation VECTAstain ABC reagent was prepared by first diluting Reagent A 1:100, then adding 1/100 volume of Reagent B and incubating it for 30 minutes at room temperature. The VECTAstain biotinylated universal antibody was removed and the slides rinsed twice with the maximum amount of PBS the wells could contain. The second wash was allowed to sit for five minutes. One slide was incubated with four drops of VECTAstain ABC reagent conjugated to Alkaline phosphatase (AP), the other slide with an equal amount of VECTAstain ABC reagent conjugated to Horseradish peroxidase (HRPO) for thirty minutes at room temperature with gentle rocking. VIP Substrate Kits for Peroxidase and Alkaline Phosphatase Substrate Kit (Vector Laboratories) were added to the appropriate enzyme and allowed to remain on the sample until desired signal strength was achieved, approximately 25 minutes for HRPO,

approximately one hour for AP. Slides were examined under light microscopy.

4.3.3.3 Challenge study of coho salmon immunized with a DNA vaccine encoding the 17 kDa protein

Coho salmon (*Oncorhynchus kisutch*) with an average weight of eight grams were held in 20 l tanks with flow through freshwater at 9°C. Fish were divided into six groups with each group comprised two tanks containing 25 fish per tank with only 22 and 14 fish per tank in group #5 and 6, respectively. Fish from each group received 0.1 ml of one of the following preparations: 50 μg of (1) pUK, (2) pcDNA3.1$^-$,(3) pUK-17kDa or (4) pcDNA3.1-17 kDa plasmid diluted in saline or (5) formalin inactivated *P. salmonis* mixed 1:1 with adjuvant or (6) saline. Before administration of the vaccine preparation fish were anaesthetized by immersion in 150 mg l^{-1} benzocaine. Fish that received plasmid DNA or saline were injected in the epaxial muscle, just caudal of the dorsal fin. The fish immunized with inactivated, adjuvated *P. salmonis* (Type strain, LF-89) were injected *intraperitoneally* (*i.p.*) with 0.1 ml of 1:1 *P. salmonis* supernatant culture mixed with oil adjuvant. Following acclimation to 15°C by one degree per day, fish were challenged with *P. salmonis* (Type strain LF-89) 104 days (936 degree days) after vaccine or sham injection. For the challenge, fish were anaesthetized and administered 0.1 ml of challenge inoculum by *i.p.* injection with the challenge inoculum consisting of supernatant of a *P. salmonis* culture showing 100% CPE. The fish were followed for 21 days post infection. Mortality data were recorded twice daily and any fish remaining at the end of the study were euthanized by a benzocaine overdose.

4.3.3.4 Statistical analysis

The mortality data for each set of replicates were analyzed using survival analysis as described in Cleves *et al.* (2002). Kaplan-Meier survival curves were generated for each vaccine construct and compared with its respective control using the log rank test. Additionally, a Cox proportional hazard model was generated for each vaccine construct to determine the most effective treatment. A hazard ratio greater than one signified that vaccinated fish were protected against dying from the *P. salmonis* exposure compared to controls. All of the data analysis was performed using Stata 7.0 (Statacorp, College Station, TX) and using a p-value of 0.05 as level of significance. Due to the high rates of mortalities in all groups, no meaningful analysis based on relative percent survival (RPS) could be performed.

4.4 RESULTS

4.4.1 Additional sequence information obtained by inverse PCR

4.4.1.1 Full length nucleotide sequence and deduced amino acid sequence for the 17 kDa gene

Using the 17 kDa specific DIG labeled probe on a southern blot of *P. salmonis* DNA, the following enzymes were chosen based on the size of fragment containing the 17 kDa gene: *Hinf* I ;*Eco*R I; *Hind* III; *Pst* I and *Cla* I (Figure 4.1). The results of the iPCR reaction using the 17 kDa core region from clone 1 can be seen in Figure 4.2. The brightest bands, corresponding to the most amplicon produced, was always seen at a

ligation concentration of 40 ng ml^{-1}, but a non-specific band could be seen for *Hinf*I at this concentration. This might be due to multimer formation or by the PCR reaction going "twice around the loop". Combining the sequence information obtained from the purified iPCR products the entire sequence for the 17 kDa gene could be determined. Table 4.2 shows the full sequence of the 17 kDa gene and the deduced amino acid sequence.

4.4.1.2 Additional sequence information obtained on clones 3, 7, 15 and 20

No preselection in restriction enzymes was used for the remaining clones. Figure 4.3 shows the resulting 1% agarose gel for the iPCR with the clone 7 core region based inverse primers as an example of screening the battery of restriction enzymes produced fragments. The first round of iPCR on the remaining clones 3, 7, 15 and 20 did not produce any full ORFs, but did provide extra sequence information. This was used to develop a second set of inverse and sequencing primers for clones 7 and 20, which can be seen in Table 4.3. This second round of iPCR, did generate additional sequence information, but again did not provide complete open reading frames.

For clone 3, two additional segments of sequence information were found with iPCR that did not overlap the core region. All available sequence information, the deduced amino acid sequence from all ORFs and alignment of the available amino acid sequence with the protein of highest homology can be found in Table 4.4. For clone 7, six additional segments were discovered in two rounds of iPCR. Five of these could be assembled into two separate regions of the gene under investigation, while the 7 *Xho* R2

fragment contained a region of genomic DNA that was not part of the gene identified from the λF^{20} library, but part of a different ORF located in close proximity on the genome. Two of the segments overlapped partially, but no overlap with the core region was seen. All available sequence information, the deduced amino acid sequence from all ORFs and alignment of the available amino acid sequence with the protein of highest homology can be found in Table 4.5. Inverse PCR yielded no additional sequence information for clone 15, while for clone 20, three additional fragments were found, one of which overlapped with the core region, while the other two overlapped with each other but not the core. All available sequence information on clone 20, the deduced amino acid sequence from all ORFs and alignment of the available amino acid sequence with the protein of highest homology can be found in Table 4.6.

4.4.2 Generation of DNA vaccine plasmids

The 17kDa-EcoRI and 17-kDa-XhoI cloning primers were capable of amplifying the 17 kDa gene using genomic *P. salmonis* DNA as template. The colony PCR showed the presence of insert and a number of clones with insert were purified and the orientation of the insert was confirmed by digestion with *Vsp*II, *Eco*RI., *Xho*I and *Pst*I. All digestions confirmed the presence of the 17kDa insert in the proper orientation in both the pUK and the pcDNA3.1$^-$. A map of both plasmids can be seen in Figure 4.4 and 4.5.

4.4.3 *In vitro* expression of 17 kDa antigen in CHSE-214 cells

Cells transfected with the pcDNA3.1-17 kDa and pUK-17 kDa plasmids but not

cells transfected with pcDNA3.1⁻ or only LipofectAMINE were immunoreactive with the rabbit α-*P. salmonis*/VECTAstain immunohistochemistry, when an AP conjugate was used. When a HRPO conjugate was used, background was too severe to differentiate between wells. Positive signal was visible as a red coloration of the cytoplasm of transfected cells and a purple precipitate, mainly concentrated along the interface between cells, while cells in control wells showed pale pink cytoplasma and ill defined cell boundaries (Figure 4.8). As a positive control, a well was incubated with *P. salmonis* infected cell culture supernatant, but no positive reaction was seen in the immunohistochemistry. There was only sixteen hours between start of incubation and fixation of the cells for immunohistochemistry while a normal infection cycle with *P. salmonis* takes ten days to reach maximum levels of *P. salmonis* (Chapter 2). The absence of reactivity in *P. salmonis* infected cells is therefore most likely due to the low level of *P. salmonis* present this early in the infection cycle.

4.4.4 Coho salmon challenge trial

The total mortalities for each tank and averaged for each group can be seen in Table 4.7. Mortalities in all groups were high, which was attributed to an extremely high challenge dose of *P. salmonis* with the bacteria overwhelming the immune system. This made it impossible to draw conclusions based on relative percent survival. An alternative strategy was employed using survivor analysis to compare the hazard ratios of different vaccine preparations compared to their controls. A hazard in this trial was defined as the risk of a fish dying during a single sample period due to *P. salmonis*. Using the daily

mortality number, a model was developed using Kaplan-Meier survival estimates. Fish were held in separate tanks and this clustering was accounted for in the survival analysis by using robust standard errors to identify statistical differences. The survivor plots for all vaccine preparations and controls can be seen in Figures 4.6 and 4.7. There is no significant difference between pUK and the respective controls ($p>0.166$). There is a significant difference in survival between fish immunized with the pcDNA 3.1-17kDa construct and its controls, giving a hazard ratio of 1.54 ($p=0.006$). Therefore, on any given day, a fish injected with the pcDNA3.1-17kDa DNA vaccine is 1.54 times more likely to survive to the next time period than a fish injected with the blank pcDNA3.1$^-$ plasmid without the 17 kDa gene.

4.5 DISCUSSION

With the impact of new and emerging diseases on aquaculture, the development of DNA vaccines against these pathogens, for which little to no genetic information is available will become increasingly important. This study shows a potential pathway to develop such a vaccine. The previously generated *P. salmonis* genomic expression library λF^{20A} provided a number of clones of interest based on the putative function assigned to partial ORFs found in these clones. The library identified a wider range of protein coding sequences than other reported *P. salmonis* expression libraries (Kuzyk *et al.*, 2001b). These authors report 18 inserts, all located within one overlapping region of the genome, containing four complete and one partial ORF. This suggests that the λF^{20A} library contains a better overall representation of the total *P. salmonis* genome at the

expense of obtaining only partial ORFs. To compensate for the smaller insert size, inverse PCR was used to obtain the flanking regions to the inserts or core regions. By using this technique flanking regions were found to four out of the five original core regions previously identified for further study (Chapter 3).

After one round of iPCR on clone 1, the complete ORF for a gene coding a protein with a high degree of homology in deduced amino acid sequence to the 17 kDa spotted fever group (SFG) *Rickettsia* surface antigen was found. This gene was 489 bp in length, with an GC content of 45.6%. The sequence was confirmed using primers outside the gene to amplify the gene and sequencing at RPC (N.B.). The full nucleotide and deduced amino acid for the 17 kDa protein can be seen in Table 4.2. Concurrently and independently, this gene was also reported present in the library developed by Kuzyk *et al.* (2001b) and named outer surface protein A or OspA. The library reported by Kuzyk *et al.* as well as the one developed in this study were derived from the *P. salmonis* type strain LF-89 and nucleotide sequences were 100% identical. Characterization of the OspA protein by Kuzyk *et al.* (2001b) showed the presence of a signal peptide for prokaryotic membrane lipoprotein lipid attachment, confirming its presence in the outer membrane of *P. salmonis*.

Inverse PCR on clone 3 resulted in two additional ORFs. As the core region was 164 bp in size with a relatively low GC content of 39%, suitable primer sequences for the iPCR reaction and subsequent sequencing had to be chosen close to the three and five prime edges of the core region and therefore no overlap between the three fragments was found. The complete sequence information obtained for both nucleotide and amino acid

sequences, plus the alignment with the protein showing the highest homology to the combined ORFs can be seen in Table 4.4. The highest homology was found with a putative transposase of *Photobacterium profundum* (GenBank YP_015504). Alignment of the found ORFs to the *P. profundum* protein suggest that approximately 15 amino acids are missing at either end of the *P. salmonis* sequence, with gaps of missing sequences within the protein of 16 and 3 amino acids respectively at either end of the core region. Using Tax Blast, the compiled ORFs are placed with the *Vibrionaceae* in the gamma-proteobacteria.

For clone 7, two rounds of iPCR generated six additional sequence fragments. Five of the fragments can be assembled into two regions and show ORFs with similarities consistent with an ABC- type transporter. One fragment, XhoR2 contains an ORF that shows significant similarity to galactose-1-phosphate uridyl transferase from a wide variety of species and the *P. salmonis* homolog is most likely located near the transporter on the genome. The remaining five segments could be assembled in two regions, one with two fragments upstream and one with three fragments downstream of the core region. As discussed above for clone 3, no overlap with the core regions was seen. The nucleotide and deduced amino acid sequence for all ORFs as well as an alignment of the assembled amino acid sequence (490 amino acids) with the protein found in GenBank showing the highest level of homology can be seen in Table 4.5. The assembled sequence shows similarity to a variety of ABC-transporters that vary in size from 500 to 900 bp, which makes estimating the percentage of the gene now sequenced not feasible. The last 100 bp of the assembled *P. salmonis* protein does not align with the protein

showing the highest similarity, but if the 7 Xba2-R ORF which contains this section is submitted independently, it shows homology to a different ABC-type bacteriocin-lantibiotic exporter. The protein with the highest similarity score was an ABC-type bacteriocin/lantibiotic exporter from *Magnetospirillum magnetotacticum* (GenBank ZP_00053435). Using Tax blast, homology to proteins from a wide group of different bacteria was seen, with more consistency in function as an exporter or secretory protein than in taxonomy. Most proteins showing similarity play an important role in pathogenesis of the originating organism, for example as a multidrug exporters for *Enterococcus faecium* or toxin transporter for *E. coli* O157:H7. Transporters containing the ATP-binding cassette, have been shown to be immunogenic and efficacious as DNA vaccines against *Mycobacterium tuberculosis* (D'Souza *et al.*, 2002). No full ORF has yet been obtained for this gene..

Two rounds of iPCR on clone 20 were successful in obtaining extra sequence information. The restriction enzyme *Hpa* II in the first round and *Mun* I in the second round produced self circularized fragments small enough to be sequenced completely. The nucleotide and deduced amino acid sequences for all ORFs and the assembled sequences can be seen in Table 4.6, as well as an alignment of the assembled sequence with the protein found to have highest similarity score. Using the conserved domain search option of Blastp (Marchler *et al.*, 2003), the assembled protein could be aligned with the PotE, amino acid transporters (gnl|CDD|10402). From this, the internal gap in the assembled sequence can be determined to be 20 amino acids. Combined with the alignment with the amino acid transporter of *Burkholderia cepacia* (GenBank

ZP_00221678.1), it can be deduced that approximately 70% of this gene is now sequenced. From Tax blast it can be seen that most similarities are seen with proteins from organism from the β- and γ- proteobacteria. No complete ORF of clone 20 was available, but its possible function in pathogenesis and as a potential target for vaccination warrants further investigation. No additional sequence information was obtained for clone 15. None of the clones 3, 7, 15 or 20 nor iPCR derived sequences from these clones show similarity to any *P. salmonis* sequence available in GenBank as of June 2004.

Of the original clones, the one containing partial gene coding for the SFG *Rickettsia* 17 kDa surface antigen showed the highest homology to known proteins of any clone. A complete sequence of the 17 kDa gene was available after just one round of iPCR. It was decided to focus potential DNA vaccine development on this gene. The 17 kDa gene of *P. salmonis* was identified and sequenced. A recombinant version of this protein has been reported to elicit a protective response when used as a subunit recombinant protein vaccine (Kuzyk *et al.*, 2001a). This protection could be optimized by adding xenobiotic promiscuous T-cell epitopes from either the measles virus or the *Clostridium tetani* tetanus toxin (tt) P2 as a fusion protein. Additionally, Kuzyk *et al.* (2001a) reported that a vaccine based on inactivated *P. salmonis* is moderately protective, eliciting a relative percent survival (RPS) of 35 %.

Two potential DNA vaccine plasmids based on the pUK-21 and pcDNA3.1$^-$ plasmids and containing the 17 kDa gene under control of the CMV immediate early promoter were constructed (for plasmid maps, see Figures 4.4 and 4.5). To evaluate the

potential of the DNA vaccine plasmids used in this study to produce a protein in a form that was immunoreactive to rabbit antisera against *P. salmonis*, CHSE-214 cells were transfected with both DNA vaccine plasmids as well as with empty pcDNA3.1 plasmid. Following immunohistochemistry, immunoreactive protein could be seen in DNA vaccine plasmids transfected cell, but not in control plasmid transfected cells (Figure 4.8). In CHSE-214 cells transfected with the pcDNA3.1 based DNA vaccine plasmid, reactivity could be seen as a red colouration of the cytoplasm and the formation of a dark purple precipitate along the plasma membrane. The same reactivity was seen in cells transfected with the pUK based DNA vaccine transcript, but with a darker colouration of the cytoplasm and less precipitate. As the 17 kDa is a membrane protein and contains hydrophobic domains (Kuzyk *et al.*, 2001b), the level of precipitate formation may be an indication for the amount of protein produced as the formation of aggregates requires a minimum concentration. No reactivity was seen in vector transfected cells or *P. salmonis* infected cells within the 16 hours of the experiment. As *P. salmonis* is a slow growing organism, this was most likely due to the short duration of the experiment.

A challenge trial was performed using coho salmon. Fish in all groups showed an unexpectedly high level of mortalities, including fish immunized with an inactivated *P. salmonis* based vaccine. By gross necropsy the fish were judged to have died from *P. salmonis* infection. Therefore, we concluded that the challenge dose overwhelmed the immune system as no other pathogens than *P. salmonis* were observed during the trial and challenged fish showed clinical signs of *P. salmonis* infection. Previous experiments with a challenge of 100 µl of *P. salmonis* suspension from a heavily infected culture have

been successful in our laboratory. Unfortunately, the determination of when a culture is at optimum challenge dose is subjective and lacking a method to determine viability and quantity of the *P. salmonis* at the time of challenge, some variability between challenges is to be expected. A further refinement of the s-ELISA described in Chapter 2 and correlating the relative antigen load to $TCID_{50}$ may provide a fast, reproducible measure for determining the amount of *P. salmonis* present, thus allowing more accurate selection of the challenge. Recently, survival analysis has been used as an alternative method to relative percentage survival (RPS) to assess efficacy in challenge trials (Raida *et al.*, 2003). Rather than focusing on the final state of fish at the end of the challenge (e.g. alive or dead), survival analysis is concerned with the amount of time it takes until this change in state from alive to dead takes place. From these data, a Cox proportional hazards regression model was developed, where the hazard was defined as the risk of a fish dying during any specific time frame. By comparing the hazards of vaccinated fish to their controls a hazard ratio was obtained (Cleves *et al.*, 2002). This hazard ratio signifies how much more likely a fish in one particular group (e.g. 'vaccinated') is to survive to the next time frame compared to fish in a different group (e.g. 'control'). By analyzing the data using survival analysis instead of RPS, we determined that the inactivated *P. salmonis* and the pcDNA3.1-17 kDa plasmid provided protection against *P. salmonis* challenge, although 88% of the fish died due to *P. salmonis*. Such protection was not observed with the pUK-17 kDa plasmid or the saline or no insert plasmid controls. Fish vaccinated with the pcDNA- 17 kDa DNA vaccine were 1.54 times more likely to survive a single sample period compared to fish injected with the empty plasmid or saline. The

difference in efficiency with both plasmid strains may be due to the difference in CpG content. The ampicillin resistance gene found in pcDNA3.1- contains two CpG's while the kanamycin resistance gene used as selective marker in pUK contains none. These CpG's function as pathogen associated molecular patterns (PAMP) in mammals as well as teleost (Joergensen *et al.*, 2001; Meng *et al.*, 2003; Mutwiri *et al.*, 2003). These PAMPs are needed to activate the immune system for an adaptive response.

Using an expression library immunization (ELI) approach, Miquel *et al.* (2003) reported a reduction in cumulative mortality and the presence of *P. salmonis* specific antibodies in coho salmon immunized twice with 20 and 10 μg of total library plasmid DNA. Their study and the study here reported are the only examples in the current literature of DNA vaccines against a bacterial pathogen showing any level of protective effect in fish. Currently, all other effective DNA vaccines in fish are against viral pathogens, with DNA vaccine based on the glycoproteins of rhabdoviruses being the best characterized (Corbeil *et al.*, 1999; Nusbaum *et al.*, 2002; Lorenzen *et al.*, 2002). These viral glycoprotein based vaccines are capable of producing an immediate, non-specific response that confers protection against heterologous viruses and a delayed, more specific response (Kim *et al.*, 2000; LaPatra *et al.*, 2001; Sommerset *et al.*, 2003). The early protecion seen was by induction of an interferon α/β type of response as characterized by Mx protein induction (Kim *et al.*, 2000). Further study of the pcDNA3.1-17kDa vaccine should determine levels of acute phase proteins that might be induced by the plasmid. The combination of results from the challenge trial and the *in vitro* transfection study indicates that the pcDNA3.1-17 kDa plasmid is a good candidate for future vaccine

development. The efficacy of the vaccine can be further improved by, for example, recoding the gene for the codon bias found in salmonids rather than that of *P. salmonis* (Lewis and Babiuk, 1999) or by adding the coding sequence for the promiscuous T-cell epitope from the measles virus that was used by Kuzyk *et al.* (2001a) to improve protection levels of a recombinant vaccine based on the 17 kDa protein.

In conclusion, the use of iPCR allowed us to expand on the sequence information obtained from the λF^{20A} library. The sequences found for clones 1, 3, 7 and 20 showed a variety of putative functions and potential vaccine targets in these clones. The full ORF for the 17 kDa was obtained while the large segments of the genes found on the clones 3, 7 and 20 can now be assigned the functions of a transposase, an ABC-type exporter most likely involved in pathogenesis and a PotE homologous amino acid transporter. The 17 kDa full ORF was successfully incorporated in two DNA vaccine vectors and assessed in a coho salmon challenge trial. Even with an extremely high challenge dose, we observed a statistically significant level of protection from the pcDNA-17 kDa vaccine plasmid. Additionally, the DNA vaccine groups did have the lowest overall mortality at 88% for the pcDNA based and 86% for the pUK based DNA vaccines. This warrants further investigation with a more appropriate challenge dose. It is crucial to estimate the amount of *P. salmonis* present before the challenge by the s-ELISA described in chapter 2, as an addition to the tissue culture infectious dose 50% ($TCID_{50}$), as the latter does not provide direct results and can only provide after the fact data. A further indication for the potential of these DNA vaccines was their ability to produce immunoreactive protein in transfected CHSE-214 cells.

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Table 4.1. Primer sequences used for iPCR, derived from the sequence of immunoreactive clones in the λF^{20} expression library (Chapter 3).

Clone	Primer 1 (5' to 3')	Primer 2 (5' to 3')
17kDa	GCC CTG CTT TTA CCT TTT CC	GTC CGT TGG CAA GTC ATT TC
20	AAA GGT GAG ACC ATC GCA TC	CGC GTT TCT TTG ACC ATG TA
7	CGT GGT CGT CGA TTA ATA TGC	CCG CCA ACA TAT TGC CTA TT
3	ACC CAC GCG ATT AGA AAC AT	GCT ATC GTA GGC TCC GTC TG
15	TGT GGT AAA TAA CCA AAT TGA TGC	AAT TTG CGC TTC TTG ACC AT

Table 4.2. Nucleotide and deduced amino acid sequence of the 17 kDa as determined from the λZapExpress library and iPCR. Sequence obtained by iPCR in italics, start and stop codons in bold.

Nucleotide sequence 5' to 3'	1	***ATG****AACAGAG GATGTTTGCA AGGTAGTAGT CTAATTATTA TCAGTGTGTT*
	51	*TTTAGTTGGC TGTGCCCAGA ACTTTAGTCG TCAAGAAGTC GGAGCTGCGA*
	101	*CTGGGGCTGT TGTTGGCGGT GTTGCTGGCC AGCTGTTTGG TAAAGGTAGT*
	151	*GGTCGAGTTG CAATGGCCAT TGGTGGTGCT GTTTTGGGTG GATTAATTGG*
	201	*TTCTAAAATC GGTCAATCGA TGGATCAGCA GGATAAAATA* AAGCTAAACC
	251	AGAGTTTGGA AAAGGTAAAA GCAGGGCAAG TGACACGTTG GCGTAATCCA
	301	GATACAGGCA ATAGTTATAG TGTTGAGCCA GTGCGTACTT ACCAGCGTTA
	351	CAATAAGCAA GAGCGTCGCC AGCAATATTG TCGAGAATTT CAGCAAAAGG
	401	CGATGATTGC AGGGCAGAAG CAAGAGATTT ACGGCACTGC ATGCCGGCAA
	451	CCGGATGGTC GTTGGCAAGT CATTTCAACA GAAAAA**TAA**
Deduced amino acid sequence	*1*	*MNRGCLQGSS LIIISVFLVG CAQNFSRQEV GAATGAVVGG VAGQLFGKGS*
	51	*GRVAMAIGGA VLGGLIGSKI GQSMDQQDKI* KLNQSLEKVK AGQVTRWRNP
	101	DTGNSYSVEP VRTYQRYNKQ ERRQQYCREF QQKAMIAGQK QEIYGTACRQ
	151	PDGRWQVIST EK

Table 4.3. Additional PCR primers developed from sequence information obtained from iPCR for clones 7 and 20. Primer with names containing INV were used for additional iPCR, other primers were used for sequencing.

Name	Sequence 5' to 3'
Clone 7*Mun*I-INV-F	ACT GTA AGC GGT TCC AGA CG
Clone 7*Mun*I-INV-R	TGG CAC ATG CAG ATA AAA TC
Clone 7-*Not*I	AGC GGC CGC ATG TCC TCT GTA GTT ATT ATT ACA GCC
Clone 7-*Eco*RI	GGA ATT CGC TCC TGG GCG GTT AGT GC
Clone 20-*Not*I	GCG GCC GCA TGA ATT GGC TCG GTA TTG TTG C
Clone 20-*Eco*RI	GGA ATT CAC GGC GAG AGC CAG CAT AAG
Clone 20-II-F	CAT GAT CAT ATG CAG GCC TAA
Clone 20-II-R	GAT CAA TGA ACG ACG GGA AA
Clone 20-INV-II	TGC ACC AAT AAA TGA CAC TTG A

Table 4.4. Sequence information available on the ORF present in clone #3, a putative transposase, after iPCR.

Fragment	Nucleotide sequence of open reading frames 5' to 3'
Core region	1 GATCGCTTCA CTGCAGACGG AGCCTACGAT AGCCATGATG TTTACGATTC 51 CGTTTTAAAC CACTCACCTA ATGCTGATGT TGTTATCCCT CCTCCTAAAA 101 ATGCCGTATT TGATGAAAAT AACCCACGCG AT
3 *Pst*-R	1 ATAAGCCTAG ATGATGATAT TGCGGGAATA NCCATTGATT NACAGGCCTT 51 AAGCGTTTTG GCCGTGACGA TGTGGCACCA AGAAAAATAC AAGATATCAG 101 CAAAGCGCAG CTGGCGTAAA CTTCATGTGG CCGTTGATGA TGATCANTAT 151 ATTCAAGCCG CACTCATCAC CGATCGCTAT GAAGCAGATG AGGAG
3 *Apa*-F	1 TATGAGATTA AAGATCATGG TAGAATGCAC TGGCAAAAGA CACGACAATA 51 CGGCAAGCGT AATTATTCTG AGTTGGCGAT TCAGCGTTAC AAACGCATTT 101 TGGGCAACAC GATGCAGTCC AGAGACATAT CGCGACAGAA AAATGAAGGA 151 CTAATTGGCG CGGGTATTTT AAATAGAGAT GACCANTCTC GGCATGCCGG 201 TGACAATAAT GTA

Combined deduced amino acid sequences in aligned order, 3 *Pst*-R, core region, 3 *Apa* -F. (/**X**/ = missing regions)

```
1   ISLDDDIAGI XIDXQALSVL AVTMWHQEKY KISAKRSWRK LHVAVDDDXY
51  IQAALITDRY EADE  /X/  DRFTADGAYD SHDVYDSVLN HSPNADVVIP
101 PPKNAVFDEN NPRD /X/   YEIKDHGRMH WQKTRQYGKR NYSELAIQRY
151 KRILGNTMQS RDISRQKNEG LIGAGILNRD DXSRHAGDNN
```

Alignment of combined amino acid sequence with sequence in GenBank of highest similarity.

```
>ref|YP_015504.1| putative transposase [Photobacterium profundum]
 emb|CAG17988.1| putative transposase [Photobacterium profundum]
          Length = 214
Score =  142 bits (359), Expect = 2e-33
Identities = 77/183(42%), Positives = 107/183 (58%), Gaps = 17/183
(9%)
Query: 4 DDDIAGIXIDXQALSVLAVTMWHQEKYKISAKRSWRKLHVAVDDDXYIQAALITDRYEAD
         DD IA I ID   L       WHQEK+K++AKRSWRK H AVD+  +IQ+A++T +   D
Sbjct: 19DDKIAAIAIDSMGLKRFGKDEWHQEKHKVNAKRSWRKAHFAVDEAHFIQSAVLTGKNTMD

Query: 64-------------EXDRFTADGAYDSHDVYDSVLNHSPNADVVIPPPKNAVFDE-NNPRD
                    + +  +AD  YD++ VY ++  H P+A++VIPP  N   DE ++P+
Sbjct: 79AQVVGTLCQFIITDVEHVSADKMYDTNAVYQTLDAHFPDAEIVIPPKDNTFADEAHHPKR

Query:110XYEIKDH---GRMHWQKTRQYGKRNYSELAIQRYKRILGNTMQSRDISRQKNEGLIGAGI
           +      G + WQ  RQYGKRN SE A+QRYK+I+GNT+  R I  Q  E L+G  +
Sbjct:139MSNLIGCFALGIIGWQSVRQYGKRNISETAMQRYKKIIGNTLHIRKIENQSKEMLLGCSV

Query:167 LNR 169
          LNR
Sbjct:199 LNR 201
```

Table 4.5. Sequence information available on the ORF present in clone #7, a putative ABC type transporter after iPCR.

Fragment	Nucleotide sequence of open reading frames, 5' to 3', overlap in italics.
Core region	1 ATCAANGCCC GCATATTAATC GACGACCACG ATATTCAAAA GTTAAAAATT 51 CAAAATATCC GCCAACATAT TGCCTATTTA CCTCAGCATG GTGACTTATT 101 TAATGGCACG ATC
7 *Xba*-R	1 CACGCATATC GATACCTAAC GTCNTTTCAA GAACAAAAAT ATTATAAACA 51 AGCCATAGAA GTCAGCCAAT TACTTGGCCT TGACTCAA*TT ATTGAGCGCT* 101 *TGCCCAAAGG CTATCACACT CCTGTTGCCA ATCATGCC*
7 *Mun*-R	1 *TTATTGAGCG CTTGCCCAAA GGCTATCACA CTCCTGTTGC CAATCATGCC* 51 ATGGNAGTCG CTACCTCGCG GTATCATTCA GCGCATTGCG ATTGCCCGTG 101 CCCTGATTCA TAAGCCACCA ATCGTCCTAT TCGATGAGGC CAATACGGCC 151 ATGGACATGC AAGGTGATAC CATCTTAATT AATGTGCTTG AACAACTTAA 201 AGGCACCTGC ACACTCATCC TCGTCTCTCA TCGCCCATCA TTGCTGGCAC 251 ATGCAGATAA AATCTTTATC CTCGAGAATA AAAA*TCTGGT GGAGAAAGTC* 301 *ACA*
7 *Xba2*-R	1 *TCTGGTGGAG AAAGTCACA* TGAGCTCTGCA CTAACCGCCC AGGAGCATAA 51 TATTCGCACT GCGTTTATTA ACAGCCTCGA ACCACTGTTA ACTGCATTAG 101 GCTGGCAGGG CGATCAACGG GCGCTTAATG AAAATTTGCC ACACTTTACC 151 GAAGGACTAG ATTTATCTTC ATTTCGCCAG GTCATGCTAA ATTTACGCTA 201 TAGCTGCACA ACAATGAGAA CCCAACTCAA TAAAATTGAT AATCGACTTT 251 TACCCTGTTT ATTTATTTCT GATGAAGGCC GTGTACTGAT TGTCATCTCC 301 GTTAATCAAT TTAATCAATA TACCGCCTTT GATGGCGCTG CGAATATTCA 351 ACTCACCCCT GCCTGA
7 *Mun*-F	1 *GCACCGTNAC TGTCAGGGCG CTGTNNA CAG CCAATTAATC GTGCNACCTC* 51 CGTCTGGAAC CGCTTACAGT CGATTGGTAG TGCTGAAGAT AAANTACAAG 101 AAGTCTTTAA ACTCCCTCCA GAGTATGTTC CCACGCAAAA GCAACTCGCC 151 CATATTGATG GCCACATTTG CTTTGAACAT GTCAATTTTC GTTATGATAA 201 AAATAATAAT ACAAGCACAA ATGAAGATGA TGAAAATTGG CNATTTACCA 251 ATATTAGCCT TGATATTCCA NCAACA
7 *Xba2*-F	1 TCCTCTGTAG TTATTATTAC AGCCTTTATT TTATTCACAA TTTTTATCAY 51 CCAGCACTTA AAAGCCTTAC TGATGCAACG GCGTGAACAT GAAGACCGCC 101 GCCTTAATTT TATTATTGAA GTGTTACAAA GCATCCATAC AATCAAATCC 151 ATGACCATGG AAGCCCAAAT GCTGCGCCGC TACGAACGCC TACAAGGCAG 201 CAGTGCAATC AATGACCACA AACTTAGCAT TAAAGCCTCC CAGGTGATGT 251 CCTTTGGCTT GCTAGTCTCA CAATTGAGTA TGGTCGCGAC CGTTGCAGTT 301 GGTGCCCTGC TGGTCTTACA AGGACAGTTA ACAATTGGTG GTCTTGCCGC 351 CTGCACCCTA *CTGTCAGGGC GCTGTCTACA GCCAATTAAT CGT*

Combined deduced amino acid sequences in aligned order 7 *Xba2*-F -7 *Mun*-F, core region, 7 *Xba* -R, 7 *Mun*-R, 7 *Xba2*- R, (**/X/** = missing regions)

```
1   SSVVIITAFI LFTIFIXQHL KALLMQRREH EDRRLNFIIE VLQSIHTIKS
51  MTMEAQMLRR YERLQGSSAI NDHKLSIKAS QVMSFGLLVS QLSMVATVAV
101 GALLVLQGQL TIGGLAACTL LSGRCLQPIN RXTSVWNRLQ SIGSAEDKXQ
151 EVFKLPPEYV PTQKQLAHID GHICFEHVNF RYDKNNNTST NEDDENWXFT
201 NISLDIPX   /X/         IXARILIDDH DIQKLKIQNI RQHIAYLPQH
251 GDLFNGT   /X/          THAYRYLTXF QEQKYYKQAI EVSQLLGLDS
```

Cont'd.....

Table 4.5. Sequence information available on the ORF present in clone #7, a putative ABC type transporter after iPCR.

```
301 IIERLPKGYH TPVANHWXSL PRGIIQRIAI ARALIHKPPI VLFDEANTAM
351 DMQGDTILIN VLEQLKGTCT LILVSHRPSL LAHADKIFIL ENKNLVEKVS
401 SALTAQEHNI RTAFINSLEP LLTALGWQGD QRALNENLPH FTEGLDLSSF
451 RQVMLNLRYS CTTMRTQLNK IDNRLLPCLF ISDEGRVLIV ISVNQFNQYT
501 AFDGAANIQL TPA
```

Alignment of combined amino acid sequence with sequence in GenBank of highest similarity.

```
gi|46202308|ref|ZP_00053435.2|   COG2274: ABC-type bacteriocin/lantibiotic
exporters, contain an N-terminal double-glycine peptidase domain
          [Magnetospirillum magnetotacticum]
          Length = 558

 Score =  273 bits (699), Expect = 4e-72
 Identities = 153/389 (39%), Positives = 221/389 (56%), Gaps = 21/389 (5%)

Query: 3   VVIITAFILFTIFIXQHLKALLMQRREHEDRRLNFIIEVLQSIHTIKSMTMEAQMLRRYE 62
           + I+  F L  I +   L+  L +R   +DRR NFIIEVL  IH++K+  MEAQM+RRYE
Sbjct: 149 ITILVLFCLTAILLGGKLRGALQKRMIADDRRFNFIIEVLGGIHSVKAFAMEAQMVRRYE 208

Query: 63  RLQGSSAINDHKLSIKASQVMSFGLLVSQLSMVATVAVGALLVLQGQLTIGGLAACTLLS 122
           RLQ + A   +++++ +S  M      SQ + V     GAL+VL G++T GGLAAC++L+
Sbjct: 209 RLQETCAEGAYQVALNSSTAMGVSSFFSQATTVCVAMFGALVVLNGEMTTGGLAACSMLA 268

Query: 123 GRCLQPINRXTSVWNRLQSIGSAEDKXQEVFKLPPEYVPTQKQLAHIDGHICFEHVNFRY 182
           GR + PI +   VW R QS   A  +  E+FKL PE      ++    G +  +  +FRY
Sbjct: 269 GRAMAPIQKALGVWTRFQSFMLARHRLSELFKLKPESAKNLPKMTSPKGALELKDCSFRY 328

Query: 183 ---------DKNNN---------TSTNEDDENWXFTNISLDIPXIXARILIDDHDIQKLK 224
                 D + N          +  N   +    T +   I      +L+D   +   +
Sbjct: 329 GEKLPVIIHDASINIREGECIAISGGNGSGKTTLLTLMQGAIKPTKGEVLVDGQPMTMFE 388

Query: 225 IQNIRQHIAYLPQHGDLFNGTHAYRYLTXFQEQKYYKQAIEVSQLLGLDSIIERLPKGYH 284
            Q++R HIA+LPQ G LF GT    + +T F+++ +   A+E + LLGLD ++  LP  GY
Sbjct: 389 PQSVRDHIAFLPQMGVLFQGT-ILQNITMFRKE-FDDVAVETAALLGLDEVVATMALGYD 446

Query: 285 TPVAN-HWXSLPRGIIQRIAIARALIHKPPIVLFDEANTAMDMQGDTILINVLEQLKGTC 343
           TPV +  + SLPRGI QRIAIARAL++ P +VLFDEANTA+D  GD  L   LE+ KG
Sbjct: 447 TPVGDGAYDSLPRGIKQRIAIARALVNNPRVVLFDEANTAVDTTGDNFLRVWLERAKGKR 506

Query: 344 TLILVSHRPSLLAHADKIFILENKNLVEK 372
           TL+LV+ RPSL   AD++F L+   L+ K
Sbjct: 507 TLVLVTPRPSLSKMADRVFDLKEGTLIPK 535
```

7 *Xho* R2 (region on the genome flanking the gene)

```
1   CGCGAACGCA CCGTTGTCGA AAATAATGAT TGGATTACGG TTGTACCTTA
51  TTGGGCCTTA TGGCCCTATG AGACTTTATT ACTCCCTAAA TTTCCAGTGC
101 AACATTTGCC TGCCCTTTCA AATGATTTAC GCCATTCTCT CGCCCAGCTA
151 TTAAAATCAT TATTAGTAAA ATATGATCAA TTATTTAACC ACTCATTTCC
201 TTATTCAATG GGTTGGCACG GTGCGCCTTG CCAACCTACG GCCACAGAAA
251 TAGAGKCGAA AACCACGCAT TGGCAGCTGC ATGCCCACTT TTATCCACCA
301 TTATTACGCT CAGCTACGGT TAAAAAATTT ATGGYCGGCT ATGAAATGCT
351 CGCTGAAGCA CAACGAGAYT TAACG
```

Table 4.6. Sequence information available on the ORF present in clone #20, a putative amino acid transporter after iPCR.

Fragment	Nucleotide sequence of open reading frames 5' to 3' (overlap in italics)
Core region	1 ATCATGCTCA TCCTCTACGT CGATGCGATG GTCTCACCTT TAGGAACGGC 51 TTTAGCCTAT ACCGGCTCTT CTACACGGAT GCTAACGGCC ATGTCTCGCG 101 AAAAACAGGT TCCGCGTTTC TTTGACCATG TACACCCCCA CTATGGT*GTT* 151 *TCCCGTCGTT CATTGATC*
20 *Hpa*	1 *GTTTCCCGTC GTTCATTGAT* CTTTAATATT GCGCTGGCAA TTGTCTTTTT 51 ATTTAGTTTC AGAAGCTGGM CTTCCCTCGC ACAAATCCTT AGCTTATTTC 101 ATATCCTTTC CTTTATGACG GTACCACTTG CCCTGGTCGT CTTTAGAAAA 151 ACAGTCGCCC CAAAAGAATT TACTTTTAAG ATTATAGGAG GACGCTTGAT 201 TGCAGCACTA CTGTTTATTG MCTTCAGCTA TCTTTTTACC ATCGGCCACT 251 TTTCTATCGT CTTTGAACTT ATTTTATTAA TTACTATTTT CCAGGGTGCG 301 TTCATCGCCC TACAATCTTC TTCACTGCAA AGTGCAGGCT GTGCCATCAA 351 AAAATGCGGC TTATTGTTCC TTTATTTCTA TGCACTTACA ATCTTATGCT 401 GGCTCTCGCC G
20 *Mun*	1 AATTGGCTCG GTATTGTTGC GGTCATTCCT ATGGAAGCTG ACGCAACCAT 51 TCAATATCTT ATCAGCTTAT TTCCAAGTAT GAAGTCTTAC TTATGGGTCA 101 ATGACTCCTT AGCTCCTTTA GGGTTGATCT TTTCCCTCGT ATTAATTGTT 151 ATTTATTGCT TGATTAACTA CTGGGGCGTA CGCTCATTAA CAAAAGCTAA 201 TAATGCCATT GCAATCTTTA AAATTTTTGT CCCCTTAATT ACCGCTATTA 251 TTGTCATTGC AGTAAGTTTT CACGCCAGCA ACTTTACAGC AGTCGATCAT 301 AATTATATGC CTTATGGCTG GAACTCTGTC TTCACTACCA TTATTACCAC 351 TGGCATTATC GTAGCGTTTA ATGGCTTTCA ATCTATTATT TCGTTTTCCA 401 GTGAGATT*TC AAACCCAAAA CGCAATATRC CGCTCTCTCT GGTCCTCTCG* 451 *ATTGTAATCT GCTTAGTG*
20 *Vsp* F	1 *TCAAACCCAA AACGCAATAT CCCGCTCTCT CTGGTCCTCT CGATTGTAAT* 51 *CTGCTTAGTG* ATTTACTTGC TCTTGTCAAG TGTCATTTAT TGGTGCACTA 101 CCGCCAAGTA TGCT

Combined deduced amino acid sequences in aligned order 20 *Mun*, 20 *Vsp F*, core region, 20 *Hpa*, (**/X/** = missing regions)

```
1   NWLGIVAVIP MEADATIQYL ISLFPSMKSY LWVNDSLAPL GLIFSLVLIV
51  IYCLINYWGV RSLTKANNAI AIFKIFVPLI TAIIVIAVSF HASNFTAVDH
101 NYMPYGWNSV FTTIITTGII VAFNGFQSII SFSSEISNPK RNIPLSLVLS
151 IVICLVIYLL LSSVIYWCTT AKY /X/    IMLILYVDAM VSPLGTALAY
201 TGSSTRMLTA MSREKQVPRF FDHVHPHYGV SRRSLIFNIA LAIVFLFSFR
251 SWXSLAQILS LFHILSFMTV PLALVVFRKT VAPKEFTFKI IGGRLIAALL
301 FIXFSYLFTI GHFSIVFELI LLITIFQGAF IALQSSSLQS AGCAIKKCGL
351 LFLYFYALTI LCWLS
```

Cont'd.....

Table 4.6. Sequence information available on the ORF present in clone #20, a putative amino acid transporter after iPCR.

Alignment of combined amino acid sequence with sequence in GenBank of highest similarity.

```
>ref|ZP_00221678.1| COG0531: Amino acid transporters [Burkholderia
cepacia R1808]
          Length = 518
 Score =  252 bits (643), Expect = 1e-65
Identities = 138/381 (36%), Positives = 218/381 (57%), Gaps = 26/381
(6%)

Query: 1   NWLGIVAVIPMEADATIQYLISLFPSMKSYLWVNDSLAPLGLIFSLVLIVIYCLINYWGV
           NW+ IV+VIP+EA+A+IQY+ +        L+VN  L+  GL+ S VL+VIY L+NYWGV
Sbjct: 79  NWIAIVSVIPIEAEASIQYMSTWPYPWAHALFVNGELSTPGLLLSAVLVVIYFLLNYWGV

Query: 61  RSLTKANNAIAIFKIFVPLITAIIVIAVSFHASNF-TAVDHNYMPYGWNSVFTTIITTGI
           ++  +AN AI IFK  +P +T + ++  SFH+ N  TA + ++ PYGW++V T + T+GI
Sbjct:139 KAFARANTAITIFKFLIPGLTILGLMLSSFHSENLGTASNASFAPYGWSAVLTAVATSGI

Query:120 IVAFNGFQSIISFSSEISNPKRNIPLSLVLSIVICLVIYLLLSSVIYWCTT---------
           + AFNGFQS ++ + E  NP R++P +++ SI++ LVIY+LL
Sbjct:199 VFAFNGFQSPVNLAGEARNPSRSVPFAVITSILLALVIYVLLQMAYIGSVNPADVAKGWA

Query:171 --------AKYXIML-------ILYVDAMVSPLGTALAYTGSSTRMLTAMSREKQVPRFF
                  A+  I L        +LYVDA +SP GT   Y  ++TRM+ AM R   +P+ F
Sbjct:259 HFNFSSPFAELAIALNLNWLAILLYVDAFISPSGTGTTYMATTTRMIYAMERNNTMPKMF

Query:216 DHVHPHYGVSRRSLIFNIALAIVFLFSFRSWXSLAQILSLFHILSFMTVPLALVVFRKTV
           +VHP YGV R+++ FN+ ++ VFLF FR W SLA ++S+  ++S++T P++L+  R+
Sbjct:319 GNVHPIYGVPRQAMWFNLLVSFVFLFFFRGWSSLAAVISVATVISYLTGPISLMALRRAA

Query:276 APKEFTFKIIGGRLIAALLFIXFSYLFTIGHFSIVFELILLITIFQGAFIALQSSSLQSA
              E    I   +LIA   F+  S +     + +  E+ILL+ +    +   Q+ +
Sbjct:379 TDIERPLSIPLMKLIAPFAFVCASLILYWAKWPLTGEIILLMVVALPVYFFFQAKAGWGG

Query:336 -GCAIKKCGLLFLYFYALTIL 355
            G  +K    L  Y   + +L
Sbjct:439 WGADLKAAWWLVAYLPTMAVL 459
```

Table 4.7. Total mortalities in vaccinated coho salmon *(Onchorynchus kisutch)* 22 days following *intraperitoneal* injection with cultured *Piscirickettsia salmonis* (strain PS02).

Treatment	Fish per tank	Mortalities per tank (two tanks)	Average mortality (%)
Saline	22	22 & 20	95.4
Inactivated *P. salmonis* + oil	14	13 & 13	92.9
pcDNA3.1$^-$	25	24 & 24	96.0
pUK	25	25 & 22	94.0
pcDNA3.1-17kDa	25	22 & 22	88.0
pUK-17kDa	25	21 & 22	86.0

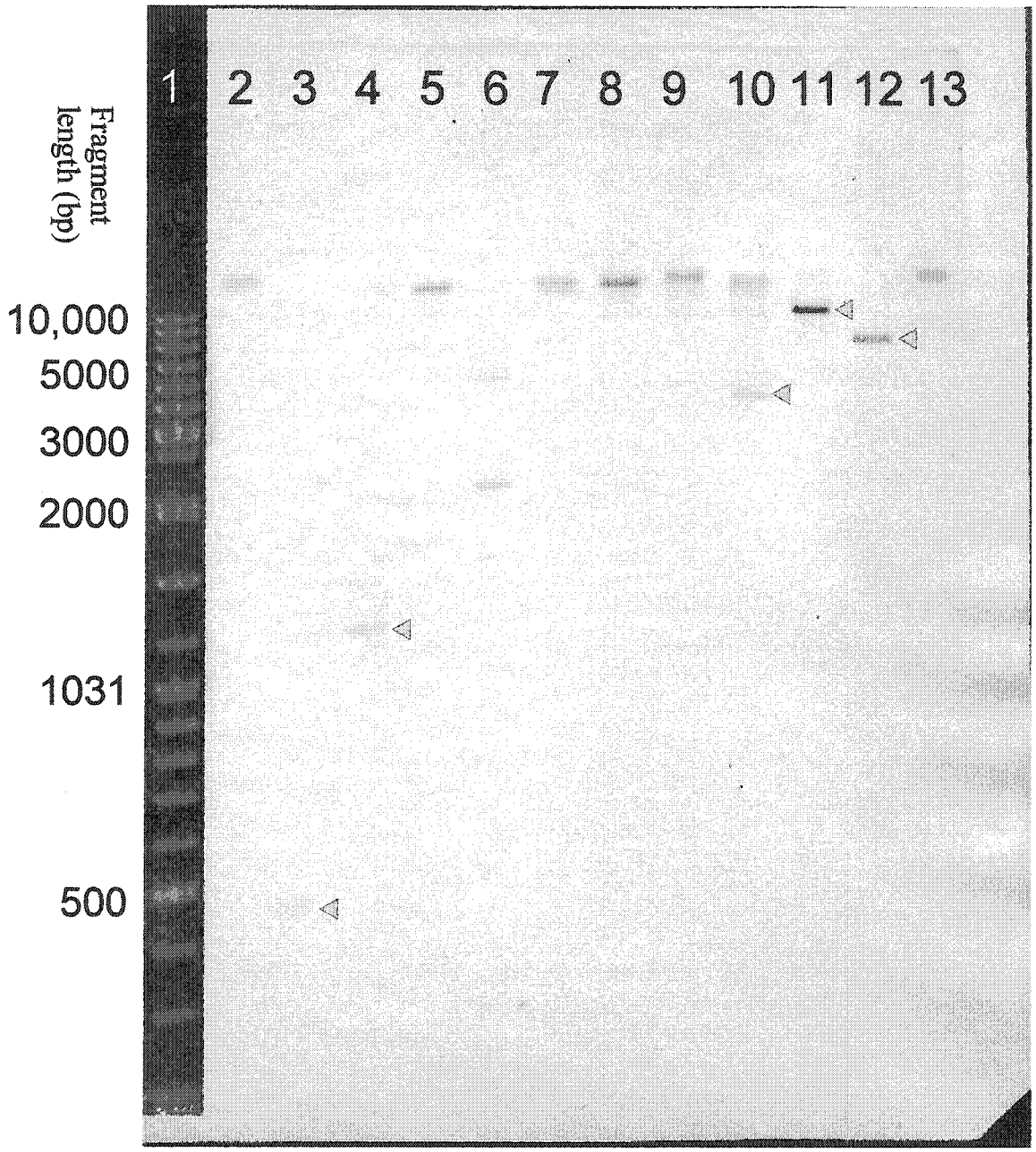

Figure 4.1. Southern blot of *P. salmonis* DNA digested with various restriction enzymes and probed with a DIG labeled 17 kDa gene specific probe. Lane 1 was photographed from the originating gel before blotting. Bands within the desired 100-10,000 molecular weight range are marked with ◁ Lanes:1: 5 µl GeneRuler (Fermentas); 2:*Bam*H I; 3:*Cla* I; 4:*Hinf* I; 5:*Sac* I; 6:*Sau*3A I; 7:*Xba* I; 8:*Xho* I; 9:*Avr* II; 10:*Hind* III; 11:*EcoR* I; 12:*Pst* I; 13:undigested.

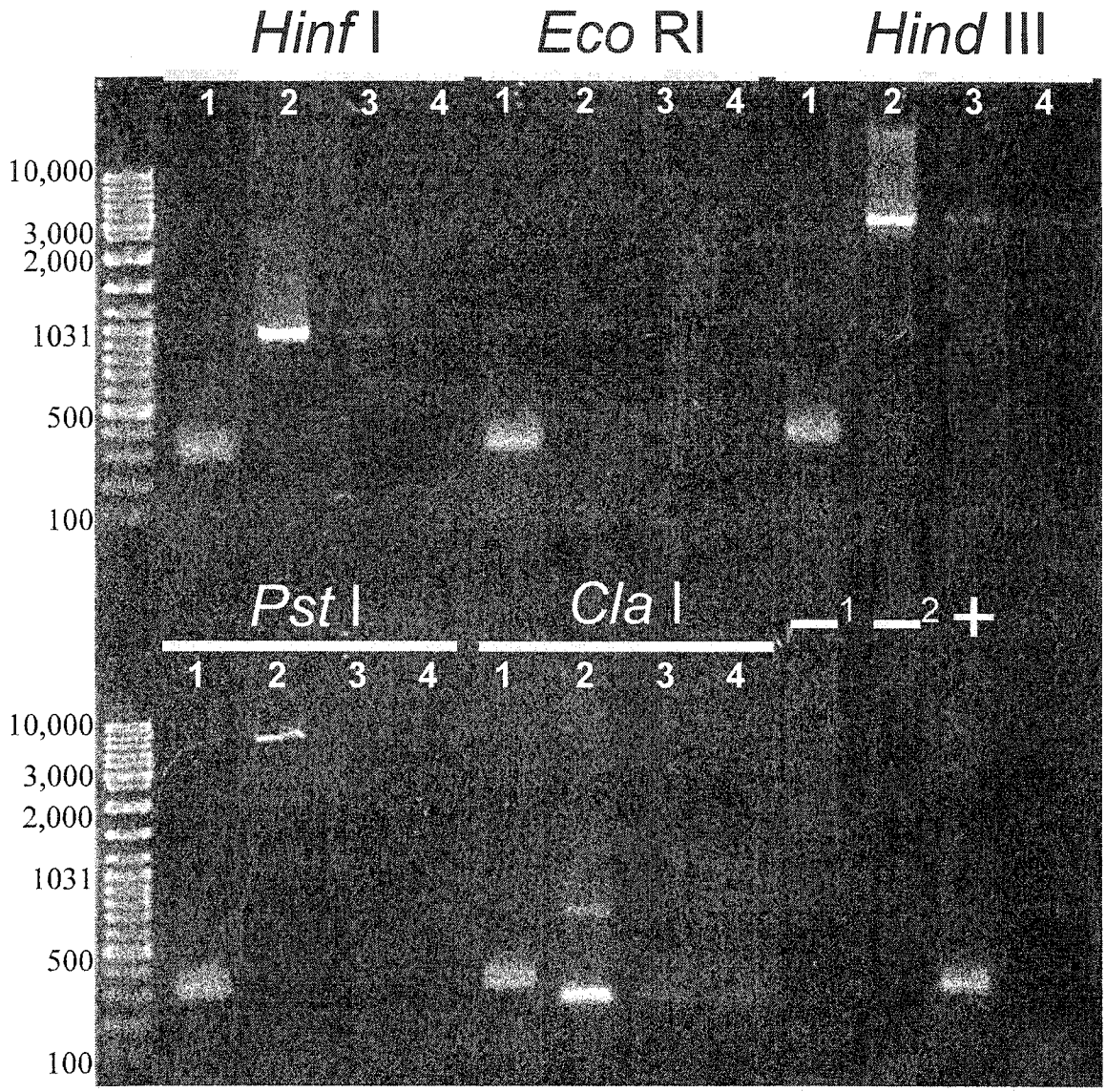

Figure 4.2. Results of the iPCR using the 17 kDa inverse primers and selected digests. For each enzyme 4 samples were run. 1) positive control using the 17 kDa insert specific regular primers; 2) through 4) using the inverse primers with either 40 ng/ml (lane 2), 800 pg ml^{-1} (lane 3) or 400 pg ml^{-1} of digested DNA in the ligation reaction. The negative control for the insert specific primers is on the left ($-^{1}$), the negative control for the inverse primers is on the right ($-^{2}$). The positive control (+) used undigested *P. salmonis* genomic DNA as template.

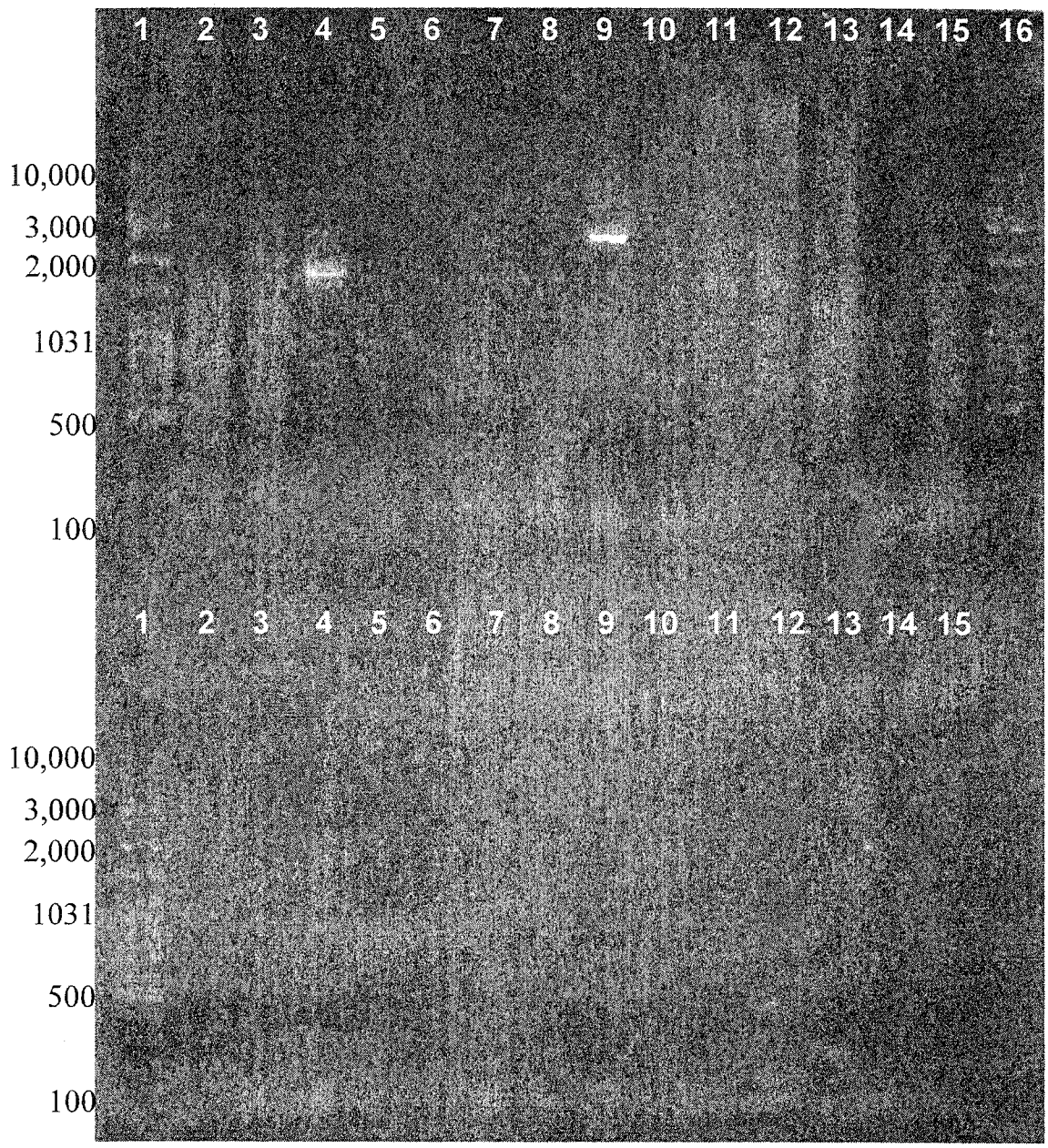

Figure 4.3. Results of the iPCR using the clone 7 based inverse primers and various restriction enzymes. Top: iPCR using as template a ligation reaction with 40 ng ml^{-1} digested *P. salmonis* DNA; Bottom: iPCR using as template a ligation reaction with 800 pg/ml digested DNA. Lanes:1:GeneRuler (Fermentas); 2:*Sau*3A I; 3:*Hpa* II; 4: *Mun* I; 5:*Bsp* TI 6; *Bam* HI; 7: *Cla* I; 8: *Sac* I; 9: *Xba* I; 10:*Vsp* I; 11:*Pst* I; 12:*Eco*R I; 13:*Kpn* 2I; 14:*Hind* III; 15:Neg control; 16:GeneRuler

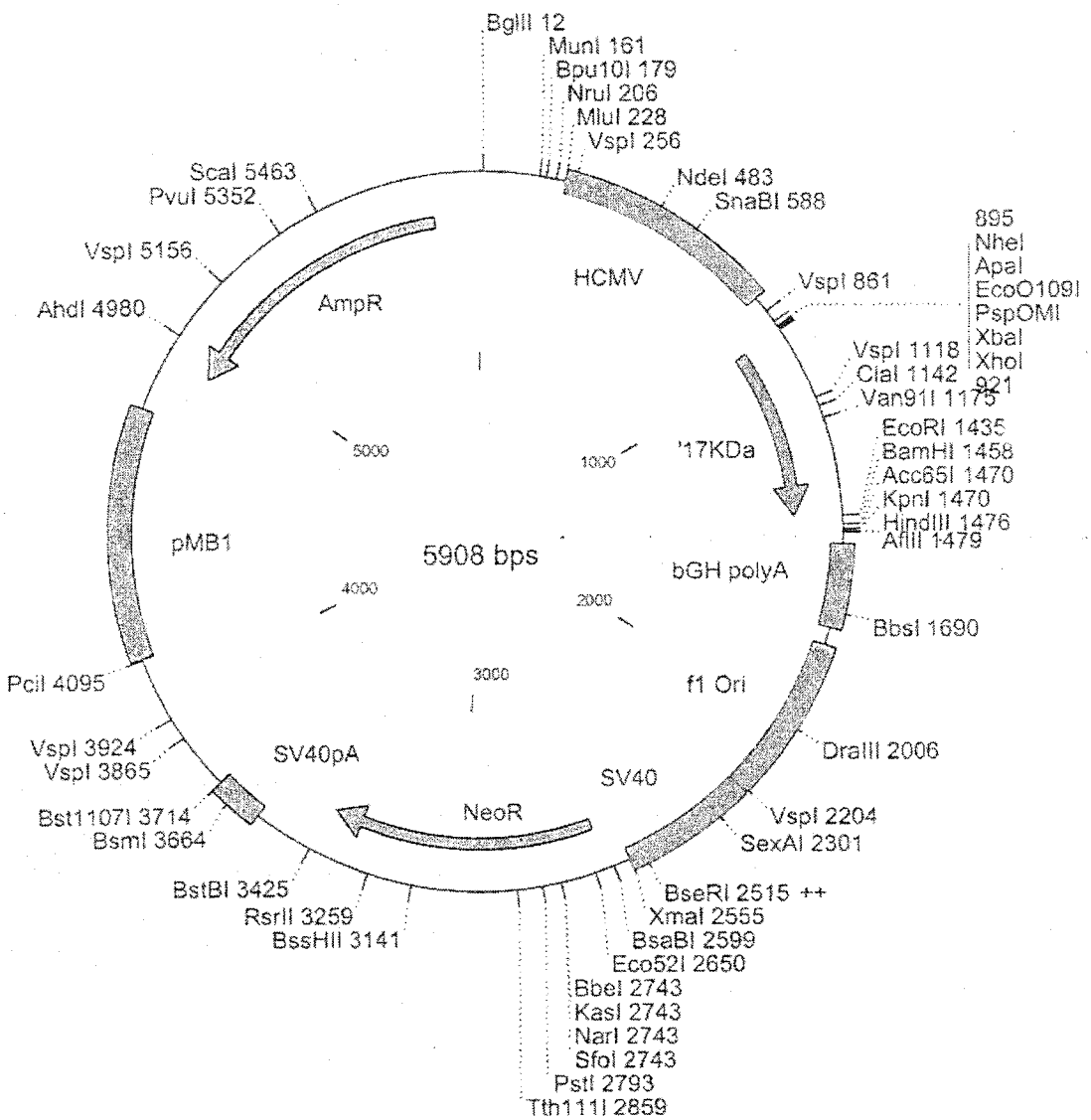

Figure 4.4. Plasmid map of the pcDNA3.1- 17kDa plasmid with restriction sites, open reading frames and control elements.

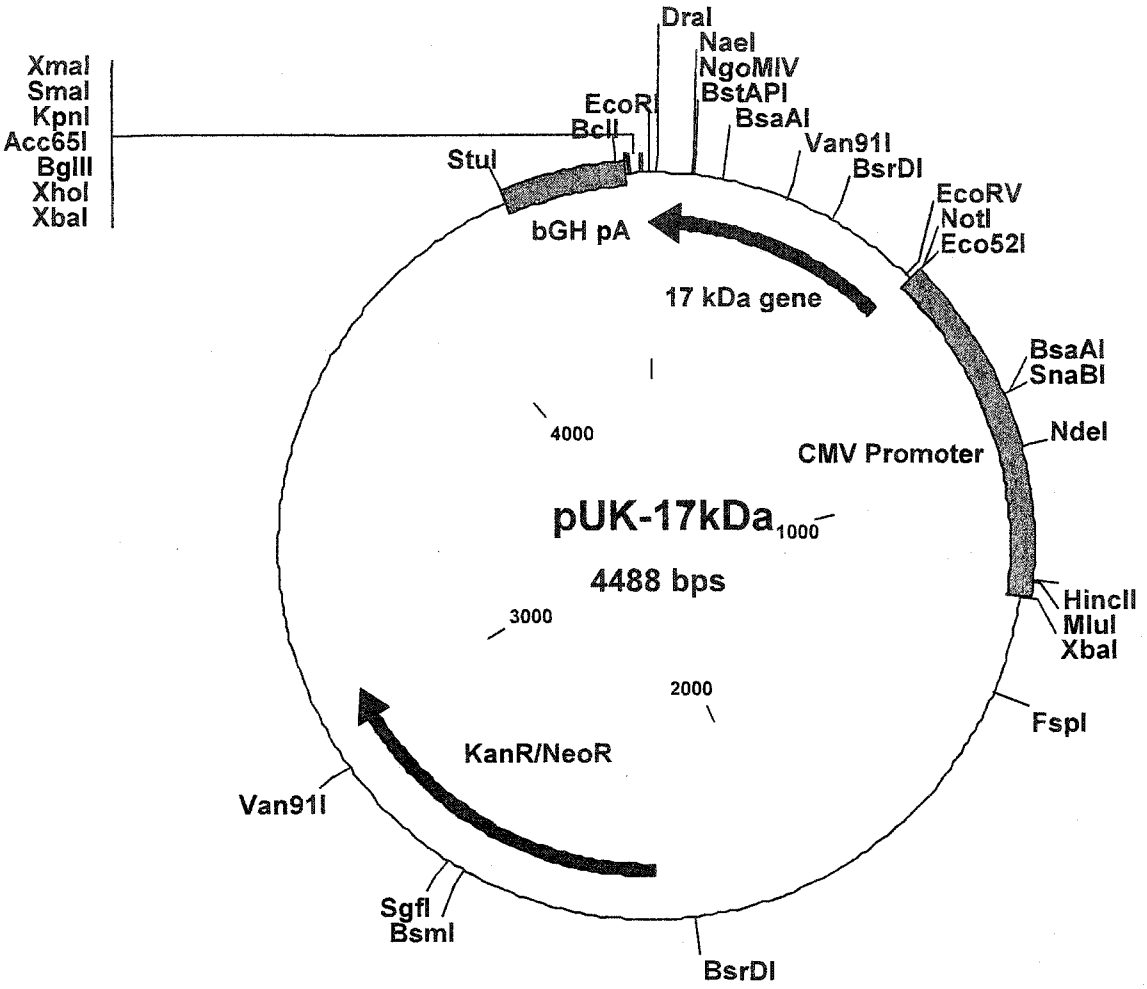

Figure 4.5. Plasmid map of the pUK-17 kDa plasmid showing restriction sites, open reading frames and control elements regulating the expression of the 17 kDa gene

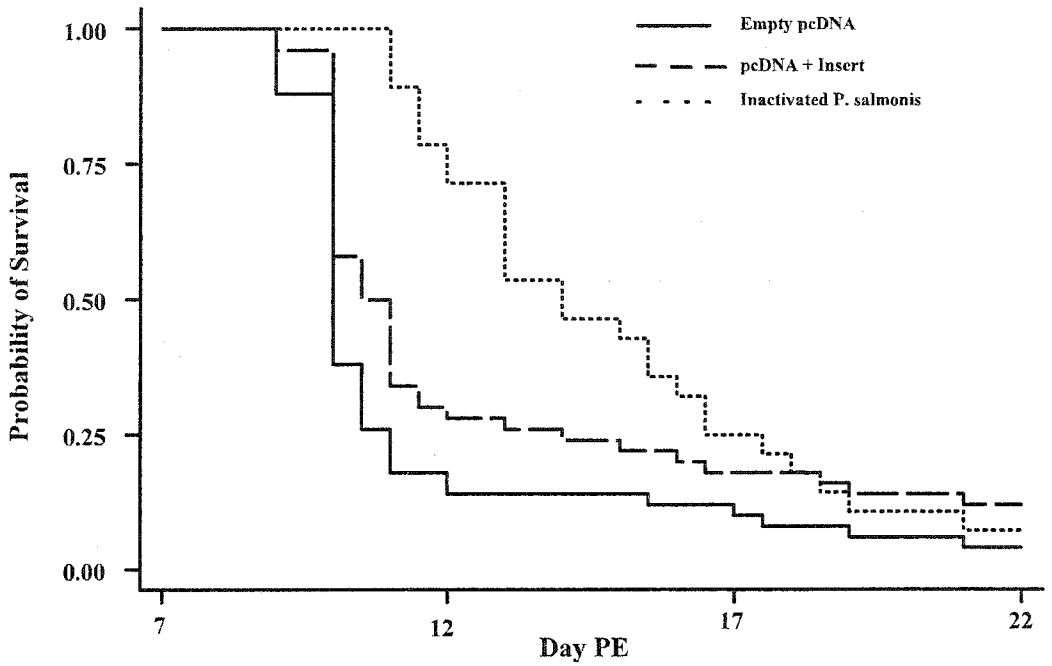

Figure 4.6. Kaplan Meier survivor curve of fish immunized with either 0.1 ml of inactivated adjuvated *P. salmonis* (dotted line), pcDNA3.1 plasmid (solid line) or pcDNA3.1 containing the 17kDa insert (dashed line). On the x-axis, Day PE = days post exposure. Both the pcDNA 3.1-17kDa and the inactivated *P. salmonis* are significantly different from the control with a p-value of 0.006 for the DNA vaccine construct.

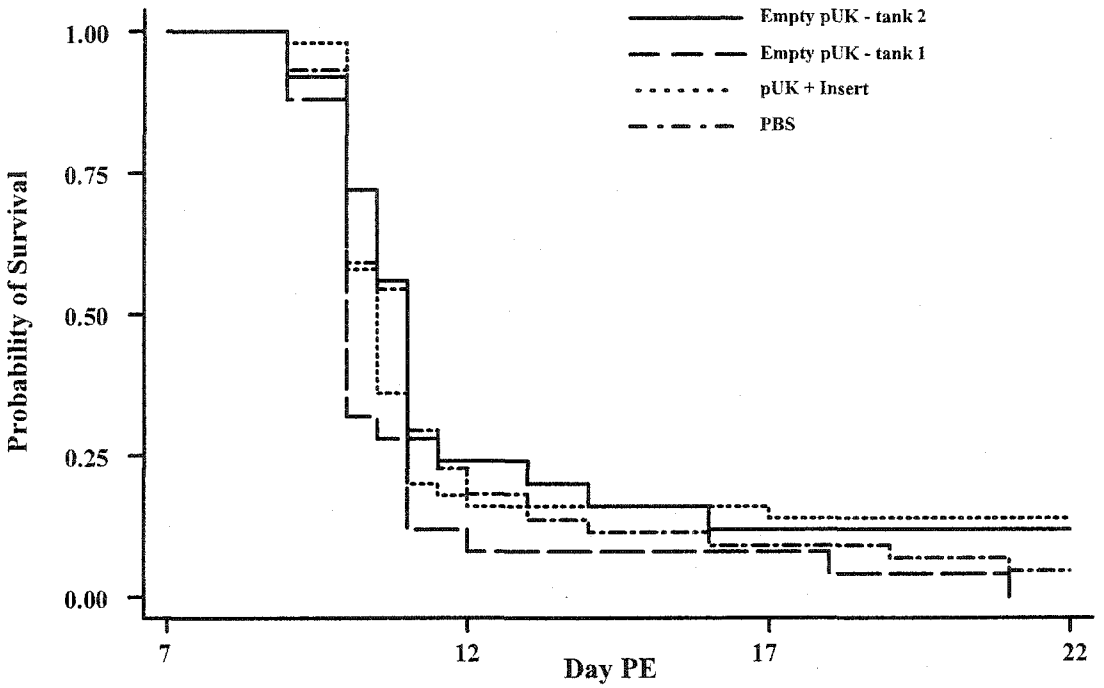

Figure 4.7. Kaplan Meier survivor curve of fish immunized with 0.1 ml of PBS (dot - dash line), pUK containing the 17 kDa gene (dotted line) and two control tanks immunized with empty pUK. On the x-axis, Day PE = days post exposure. There was no significant difference among the groups ($p>0.166$)

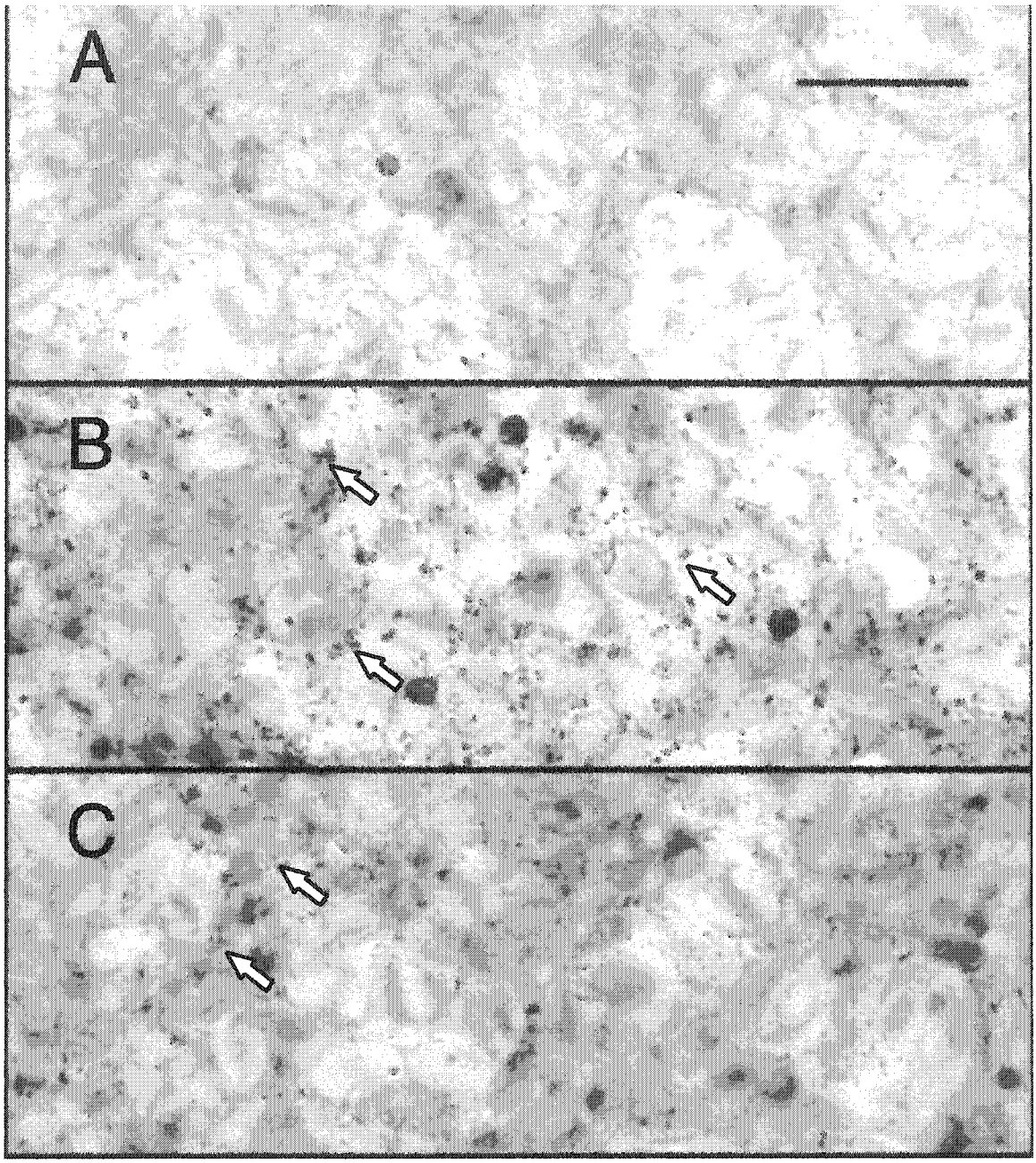

Figure 4.8. CHSE-214 cells transfected with plasmids used in the DNA vaccine challenge trial. Cells were transfected using the LipofectAMINE Plus system with pcDNA3.1$^-$ (A), pcDNA3.1-17kDa (B) or pUK-17kDa (C). Expressed 17 kDa was detected 24 hours after transfection with 1:200 rabbit α-*P. salmonis* antiserum and the VECTAstain Universal ABC and VIP alkaline phosphatase substrate kits (Vector laboratories). Positive signal can be seen as dark red coloration of the cytoplasm and purple precipitate (arrows). Bar = 50 μm.

CHAPTER 5: GENETIC DIFFERENCES IN THE 17 KDA GENE FROM *PISCIRICKETTSIA SALMONIS* TYPE STRAIN LF-89 AND ISOLATES FROM THE CANADIAN PACIFIC AND ATLANTIC COASTS

5.1 ABSTRACT

Piscirickettsia salmonis is an intracellular pathogen and since its emergence in Chilean aquaculture in the late 1980s has become a source of major economic losses and is widespread in areas of salmonid aquaculture. As previously described (chapter 4) a full length gene, homologous to the 17 kDa genus-specific antigen of the spotted fever group (SFG) *Rickettsia* on deduced amino acid sequence was identified and evaluated for its potential as a DNA vaccine. DNA vaccines depend on a single protein as antigen and therefore a high level of variability within such a protein between strains will reduce the usefulness in the field. Because the 17 kDa antigen was used for phylogenetic studies in SFG *Rickettsia*, the sequence of the 17 kDa gene for four Canadian isolates was determined, three from the Pacific coast and one from the Atlantic and these were compared to Chilean type strain LF-89. Thirteen single nucleotide polymorphisms (SNPs) were identified, leading to four amino acid changes. The effect of these amino acid changes to the predicted structure of the protein were determined, as well as the changes in antigenic index of the protein. All Pacific isolates showed identical 17 kDa genes and a similarity analysis for the Pacific, Atlantic and type strain showed that the Canadian isolates were more closely related to each other than they were to the type strain.

5.2 INTRODUCTION

The intracellular fish pathogen, *Piscirickettsia salmonis* has become a major source of economic losses to the Chilean salmon aquaculture industry since it was first characterized in the late 1980's and early 1990's (Bravo and Campos, 1989; Cvitanich *et al.*, 1991; Mauel and Miller, 2002). The syndrome caused by this pathogen, salmonid rickettsial septicemia (SRS) was first described based on outbreaks and isolates from Chile (Branson and Nieto Diaz-Munoz, 1991; Fryer *et al.*, 1992). Since then *P. salmonis* has been isolated from numerous locations around the world including Norway (Olsen *et al.*, 1997), Scotland, Ireland (Rodger and Drinan, 1993) and both coasts of Canada (Jones *et al.*, 1998; Evelyn *et al.*, 1998). Interestingly, Olsen *et al.* (1997) described an outbreak of *P. salmonis* in Norway that occurred concurrently or might even have predated the first severe outbreaks described in Calbucon, Region X, southern Chile (Branson and Nieto Diaz-Munoz, 1991) in 1989. At the Pacific Biological Station on Vancouver Island on the Canadian Pacific coast, an essentially identical disease was first observed in 1970 in seawater-reared pink salmon (*Oncorhynchus gorbuscha*) that were kept for experimental purposes. The causative agent was morphologically, serologically and culturally indistinguishable from *P. salmonis* isolated in Chile and this is therefore considered to be the earliest known outbreak of *P. salmonis*. The disease was recorded again in pink salmon in 1978, in chinook salmon *(O. tshawytscha)* in 1983, 1984 and1986 and in coho (*O. kisutch*), but always incidental to other disease problems (Evelyn *et al.*, 1998). More recently in British Columbia (B.C.), *P. salmonis* has been isolated from several production farms raising Atlantic (*Salmo salar*) and chinook salmon, either coincidental

with other diseases such as bacterial kidney disease (BKD) or in epizootics where it was the primary cause of mortality (Brocklebank *et al.*, 1993). On the Atlantic coast of Canada, *P. salmonis* has been isolated from farm-reared Atlantic salmon (Jones *et al.*, 1998).

Most comparisons between isolates of *P. salmonis* have focused on antigenic differences as determined by Western blot or the genetic difference based on the areas of the genome coding for the 16 and 23S ribosomal subunits and the internal transcribed spacer (ITS) (Mauel *et al.*, 1999; Heath *et al.*, 2000; Casanova *et al.*, 2003). The advantage of looking at the genetic differences within these regions is that procedures for PCR and sequencing are well established in other species and information on conserved rDNA sequences between species is available, allowing for the design of degenerate oligonucleotide primers to amplify homologous parts of the *P. salmonis* genome. However, a disadvantage of basing phylogenetic criteria on ribosomal or spacer regions is that in most bacteria, these sequences are not involved in the virulence of a pathogen. Genetic diversity among genes involved in the virulence of a pathogen, collectively known as the virulome, may be more relevant in differentiating strains of *P. salmonis*.

As described in chapters 3 and 4, the sequence for an analogue of the spotted fever group (SFG) *Rickettsia* common 17 kDa antigen in *P. salmonis* was determined and its potential as a DNA vaccine evaluated. The next step in determining the usefulness of the 17 kDa DNA vaccine is to determine the level at which this gene is preserved amongst isolates. If the variability is high among isolates, a DNA vaccine based on the type strain 17 kDa might not be effective against all *P. salmonis* strains. To determine

the variability of the 17 kDa gene, four isolates were compared to the Chilean type strain by sequencing the 17 kDa gene in each of these isolates. Three of the isolates were from the Canadian Pacific coast (BC-RLO, PNGR-1 and PNGR-2), the fourth was isolated during an outbreak on the Canadian Atlantic coast (ECR0811) described by Jones *et al.* (1998). The sequence of the 17 kDa genes from each isolate was compared to the known sequence of the type strain for the presence of insert or deletion events (indels) and single nucleotide polymorphisms (SNPs). If any differences were found between the nucleotide sequences, comparisons were made of the deduced amino acid sequence from the different isolates. The impact of any differences between deduced amino acid sequences on the predicted secondary structure of the 17 kDa and antigenicity was further analyzed using an array of bioinformatics tools.

5.3 MATERIALS AND METHODS

5.3.1 Purification of *P. salmonis* genomic DNA and PCR products

Piscirickettsia salmonis genomic DNA was purified from each of the different isolates using the protocol described in paragraph 3.3.1. The genomic DNA was used as template in a PCR reaction using the 17-kDa-*Eco*RI (5'-ATG AAT TCA CTT CTA CTT TTA AAT TTA TCC-3') and 17 kDa-*Eco*RV (5'-TTG ATA TCA GTG AGA GAA ATA ATG AAC AGA -3') primers and the PCR was performed as described in paragraph 4.3.1.2. The results of the PCR reaction were analyzed by horizontal gel electrophoresis using a 1% agarose gel in 1x TBE. The amplification product was excised from the gel

and purified using the High Pure PCR purification kit for sequencing as described in paragraph 4.3.1.6.

5.3.2 Sequencing and sequence analysis tools

Purified PCR product was sent to the Research and Productivity Council (RPC), (Fredericton, NB, Canada) for bidirectional sequencing using the same primers as were used in the PCR reaction. Resulting electropherograms were analyzed. Sequences were aligned using Macaw 2.0.5 software (NCBI, Maryland) and open reading frame analysis was performed using the Frameplot 2.3.2 online software (http://watson.nih.go.jp/~jun/cgi-bin/frameplot.pl) (Ishikawa and Hotta, 1999). The deduced amino acid sequence was used to generate a predicted secondary structure using the PredictProtein server (http://www.embl-heidelberg.de/predictprotein/predictprotein.html) and a graphical representation of the predicted protein and confidence of prediction was generated using PSIPRED, transmembrane region predicted using MEMSAT 2 available at http://bioinf.cs.ucl.ac.uk/psiform.html. An antigenicity index plot was generated using the Java Molecular Biologist Workbench available at http://hometown.aol.com/lucatoldo/myhomepage/JaMBW/3/1/7/index.html, which uses the algorithm developed by Hopp and Woods (1981).

5.4 RESULTS AND DISCUSSION

The 17 kDa protein of *P. salmonis* has been shown to be an important potential candidate for use in a recombinant (Kuzyk *et al.*, 2001a) or DNA based vaccine (chapter 4). The homologous genes coding for the 17 kDa protein of the SFG *Rickettsia* show at least 89.1% DNA similarity between species of this group, with a maximum of 100% between *Rickettsia conorii* and *R. rickettsii* (Roux and Raoult, 1997). This indicates that these genes have an important function with genetic selection pressure on their maintenance (McDonald *et al.*, 1997). It was therefore decided to compare the DNA sequences from a number of *P. salmonis* isolates to that of the LF-89 type strain. Geographically distinct locations were chosen from the Canadian Atlantic and Pacific coasts, with one isolate from the Atlantic coast (ECR0811) and three from British Columbia (BC). Two of the BC strains were concurrently isolated from one site (PNGR-1 and 2), while the third was isolated previously (BC-RLO).

For comparison, the 17 kDa sequence from the type strain LF-89 was considered the standard. A 519 basepair (bp) product was amplified from all isolates (Figure 5.1). Each amplification product was bidirectionally sequenced and the sequence compared to the standard sequence. The first 30 bp of the 17 kDa gene were not considered as this region is too near the 5' sequencing primer and contains ambiguous sequencing results. Thirteen single nucleotide polymorphisms (SNP) were identified. No sequence differences were seen among the isolates from the Pacific coast. The sequence alignments can be seen in Table 5.1 and are summarized in Table 5.2. No insertions or deletions were seen and no stop codons were introduced when compared to the type strain

17 kDa open reading frame. Of the 13 observed SNPs, nine were synonymous polymorphisms while four were replacement polymorphisms, leading to amino acid changes. Eleven SNPs represented transitions where a purine was changed to purine or pyrimidine to pyrimidine. The majority of nucleotide replacements, eight out of thirteen, tended to either generate or destroy palindromic sequences of four basepairs or longer.

The SNPs were not distributed evenly over the gene but appeared to be clustered within certain regions of the gene with the second half of the gene containing 11 out of 13 SNPs and three regions of 20, 32 and 26 basepairs respectively containing 3, 3 and 4 SNPs, i.e. 16% of the gene contains 76% of the SNPs (Figure 5.2). Using protein structure prediction software, all but one of the SNPs are found in regions predicted to code for extracellular domains of the protein (Figures 5.3 and 5.4). If all SNPs were due to random mutations of the genome, a more uniform spread along the length of the gene would be expected. It was speculated that *P. salmonis* may have a mechanism to specifically increase the mutation rate in the extracellular domain to increase the chance of non- synonymous mutations that can cause antigenic drift and therefore aid in the evasion of host defenses. Four of the polymorphisms lead to amino acid changes between the type strain and the East Coast isolate, three of which were also present in the Pacific isolates. All of the amino acid replacements were located in areas predicted to be extracellular. Three of the four amino acid changes observed occur in regions of the protein with a high antigenicity index (Figure 5.5), indicating that these regions are more likely to contain epitopes recognized by the immune system. The fourth replacement at amino acid #22 was adjacent to a consensus pattern for prokaryotic membrane lipoprotein

lipid attachment, with the Cys-21 as the predicted acylation site (Kuzyk *et al.*, 2001b). The predicted secondary structure seen in Figure 5.4 showed changes in the secondary structure of the 17 kDa of the type strain and that of the Canadian isolates, with a small shift in the location of a stretch of helix in the amino acid 64 to 76 region.

In bacterial pathogens, a variety of SNPs have been discovered that result in enhanced pathogenicity of the organism. These random genetic mutations that confer a strong selective advantage upon the clone are known as pathoadaptive and can broadly be divided in three groups (Sokurenko *et al.*, 1999). First, they can enhance the tropism for a suitable tissue, second they can enhance the uptake of available nutrients and lastly they can enhance mechanisms to evade or overcome antibacterial defenses. Recent studies of enterobacterial adhesins of *Escherichia coli* and *Salmonella typhimurium* have shown the importance of SNPs in the determination of host specificity and tissue tropism. By determining the location of the amino acid replacements due to SNPs, these can be used as a tool to study protein function at the molecular level (Weissman *et al.*, 2003). With *P. salmonis*, the contribution of the 17 kDa protein to virulence may be determined by comparing the SNP profile of different isolates of *P. salmonis* to their relative virulence. An isolate from BC (ATL-4-91) was previously been shown to be less virulent than the LF-89 type strain (House *et al.*, 1999). Given that there was no variation in the 17 kDa between the isolates in the study herein, one can speculate that the isolate studied by House *et al.* (1999) contained the B.C. version of the 17 kDa gene. As well, outbreaks in B.C. are reported less severe then those typically seen in Chile (Brocklebank *et al.*, 1993). Further study on the relation between the profile of SNPs in geographically varied

locations and their SNP profile is warranted.

The fact that a number of SNPs created or destroyed palindromic sequences in the 17 kDa gene makes them useful as genetic markers. The restriction enzymes *Ngo*M IV, *Hpa*II, *Rsa*I, *Psi*I and *Taq*I each recognized at least one sequence that was changed by a SNP. The restriction sequence and the SNP involved as identified in Table 5.2 can be seen in Table 5.3 for each of these enzymes. A graphical representation of the number and location of all restriction sites for each enzyme in either wild type or the Canadian isolates can be seen in Figure 5.6. By digesting 17 kDa gene PCR amplification product with some or all of these restriction enzymes, unknown isolates can quickly be assessed for the presence of some or all of the seven SNPs that are part of a restriction pattern. Even though SNP 8 is part of a ten basepair palindromic sequence (G/ACAATATTGT), there is currently no restriction enzyme that has this sequence as its restriction pattern. This is unfortunate as SNP 8 is unique to the Pacific isolates. Casanova *et al.* (2003) used electrophoretic analysis of amplification products using conserved regions of the ITS as primers to differentiate Chilean isolates of *P. salmonis*. However, products from this region of the genome are not under selective pressure from the immune system and they were able to identify only two different patterns.

The usefulness of the 17 kDa gene in either detecting (Tange *et al.*, 1994; Beninati *et al.*, 2002) or determining phylogenetic relationship between rickettsial isolates has been well established (Anderson and Tzianabos, 1989; Baird *et al.*, 1996; Ishikura *et al.*, 2003). For determining phylogenetic trees, the 17-kDa gene has been mostly used in conjunction with rickettsial outer membrane protein A (rOmpA) and rickettsial citrate

synthase (gltA) (Ishikura *et al.*, 2003) as well as 16S rDNA genes (Baird *et al.*, 1996). Even though the 17 kDa of *P. salmonis* shows a high level of homology to SFG *Rickettsia* on the deduced amino acid level, no significant homology is seen on the nucleotide level. The only sequences available to determine phylogenetic relationships were those acquired in this project. A similarity table between the different sequences was generated with DINADIST, available online at http://evolution.gs.washington.edu/phylip.html and the output can be seen in Table 5.4. Similarity was highest between the Canadian isolates at 99.6%, while both Canadian isolates showed an equal similarity to the type strain of 97.5%. Mauel *et al.* (1999) found between 97.8, 98.7 and 98.5% similarity between LF-89 and the BC isolate ATL-4-91 when a sequence from the ITS and two from the 23S rDNA were used, respectively. The slightly higher levels of similarity observed may be due to reduced immune pressure on these fragments or to the fact that there may be some differences between the 17 kDa gene of the ATL-4-91 and the 17 kDa gene here described for all our BC isolates.

In conclusion, this study shows the usefulness of the SNPs in the 17 kDa gene as genetic markers. Further investigations on the impact the SNPs have on the overall antigenicity and epitopes of the 17 kDa are warranted as three of the observed amino acid changes occur in areas likely to be epitopes.

5.6 LITERATURE CITED

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Table 5.1. Sequence alignment of *P. salmonis* isolates from the Canadian Atlantic (EASTC) and Pacific coasts (BC) with the LF-89 type strain (TYPE). SNPs are marked in bold, SNPs leading to amino acid changes are marked with ⇑.

```
TYPE :1    ATGAACAGAGGATGTTTGCAAGGTAGTAGTCTAATTATTATCAGTGTGTTTTTAGTTGGC 60
EASTC:1    ATGAACAGAGGATGTTTGCAAGGTAGTAGTCTAATTATTATCAGTGTGTTTTTAGTTGGC 60
BC   :1    ATGAACAGAGGATGTTTGCAAGGTAGTAGTCTAATTATTATCAGTGTGTTTTTAGTTGGC 60

TYPE :61   TGTGCCCAGAACTTTAGTCGTCAAGAAGTCGGAGCTGCGACTGGGGCTGTTGTTGGCGGT 120
EASTC:61   TGTACCCAGAACTTTAGTCGTCAAGAAGTCGGAGCTGCGACTGGGGCTGTTGTTGGCGGT 120
BC   :61   TGTACCCAGAACTTTAGTCGTCAAGAAGTCGGAGCTGCGACTGGGGCTGTTGTTGGCGGT 120
                ⇑
TYPE :121  GTTGCTGGCCAGCTGTTTGGTAAAGGTAGTGGTCGAGTTGCAATGGCCATTGGTGGTGCT 180
EASTC:121  GTTGCTGGCCAGCTGTTTGGTAAAGGCAGTGGTCGAGTTGCAATGGCCATTGGTGGTGCT 180
BC   :121  GTTGCTGGCCAGCTGTTTGGTAAAGGCAGTGGTCGAGTTGCAATGGCCATTGGTGGTGCT 180

TYPE :181  GTTTTGGGTGGATTAATTGGTTCTAAAATCGGTCAATCGATGGATCAGCAGGATAAAATA 240
EASTC:181  GTTTTGGGTGGATTAATTGGTTCTAAAATCGGTCAATCGATGGATCAGCAGGATAAAATA 240
BC   :181  GTTTTGGGTGGATTAATTGGTTCTAAAATCGGTCAATCGATGGATCAGCAGGATAAAATA 240

TYPE :241  AAGCTAAACCAGAGTTTGGAAAAGGTAAAAGCAGGGCAAGTGACACGTTGGCGTAATCCA 300
EASTC:241  AAGCTAAACCGGAGTTTAGAAAAGGTAAAAACAGGGCAAGTGACACGTTGGCGTAATCCA 300
BC   :241  AAGCTAAACCGGAGTTTAGAAAAGGTAAAAACAGGGCAAGTGACACGTTGGCGTAATCCA 300
                     ⇑                    ⇑
TYPE :301  GATACAGGCAATAGTTATAGTGTTGAGCCAGTGCGTACTTACCAGCGTTACAATAAGCAA 360
EASTC:301  GATACCGGCAATAGTTATAGTGTTGAGCCAGTGCGTACTTACCAGCGTTATAATAAGCAA 360
BC   :301  GATACCGGCAATAGTTATAGTGTTGAGCCAGTGCGTACTTACCAGCGTTATAATAAGCAA 360

TYPE :361  GAGCGTCGCCAGCAATATTGTCGAGAATTTCAGCAAAAGGCGATGATTGCAGGGCAGAAG 420
EASTC:361  GAGCGTCGCCAGCAATATTGTCGGGAATTTCAGCAAAAGGCGATGATTGCAGGGCAGAAG 420
BC   :361  GAGCGTCGCCAACAATATTGTCGGGAATTTCAGCAAAAGGCGATGATTGCAGGGCAGAAG 420

TYPE :421  CAAGAGATTTACGGCACTGCATGCCGGCAACCGGATGGTCGTTGGCAAGTCATTTCAACA 480
EASTC:421  AAAGAGATTTATGGTACTGCATGCCGACAACCGGATGGTCGTTGGCAAGTCATTTCAACA 480
BC   :421  CAAGAGATTTATGGTACTGCATGCCGACAACCGGATGGTCGTTGGCAAGTCATTTCAACA 480
           ⇑
TYPE : 481 GAAAAATAA 489
EASTC: 481 GAAAAATAA 489
BC   : 481 GAAAAATAA 489
```

Table 5.2. Nucleotide sequence differences in the 17 kDa protein from different isolates of *P. salmonis*. Pos = location of the SNP ; Dif = which isolate shows difference from the type strain, with 1 = Pacific isolates and 2 = East coast isolate; Bp = nucleotide switch from the type strain; AA = amino acid change if one took place; Pd = size of the palindromic sequence created or destroyed by the nucleotide change

SNP	1	2	3	4	5	6	7	8	9	10	11	12	13
Pos	64	147	251	258	271	306	351	372	384	421	432	435	447
Dif	1+2	1+2	1+2	1+2	1+2	1+2	1+2	1	1+2	2	1+2	1+2	1+2
Bp	G→A	T→C	A→G	G→A	G→A	A→C	C→T	G→A	A→G	C→A	C→T	C→T	G→A
AA	A→T		Q→R		A→T					Q→K			
Pd	4	2	4	2	2	4	6	10	4	2	2	4	8

Table 5.3. Restriction enzymes that recognize palindromic sequences involved in SNP's of the 17 kDa gene

	Restriction sequence	No of the SNP(s) involved as found in Table 5.2
*Hpa*II	C'CG_G	3, 6, 13
*Taq*I	T'GC_A	9
*Rsa*I	GT'AC	1, 12
*Ngo*M IV	G'CCGG_C	13
*Psi*I	TTA'TAA	7

Table 5.4. Output of the DinaDist phylogenetic distance program. Similarity is on a scale of 0 to1.

Table of similarity between sequences

	East	BC
East	*	
BC	0.995910	*
Type	0.975460	0.975460

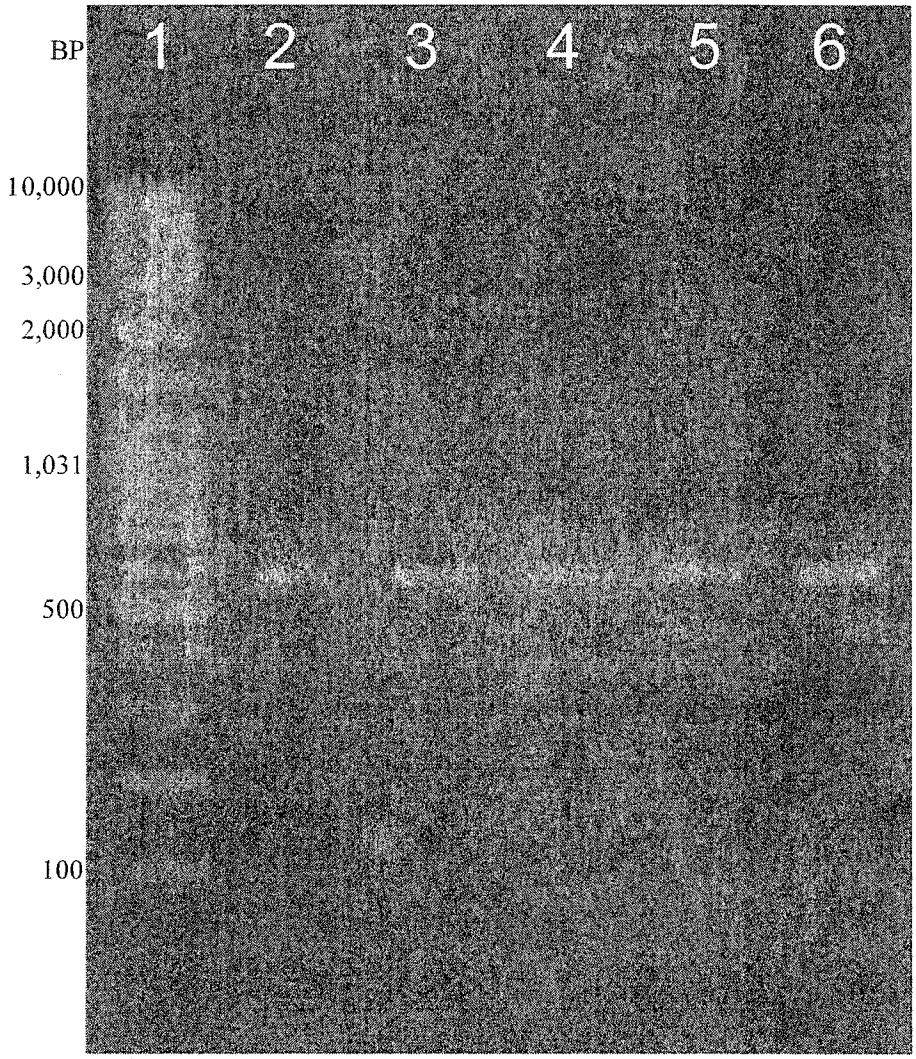

Figure 5.1. Results of PCR amplification of the 17 kDa gene comparing the type strain to different Canadian isolates. The electrophoresis was performed for 1 hr at 120V. 1: GeneRuler (Fermentas); 2: LF-89 Type strain; 3: East Coast Isolate; 4: BC-RLO; 5: PNGR-1; 6: PNGR-2

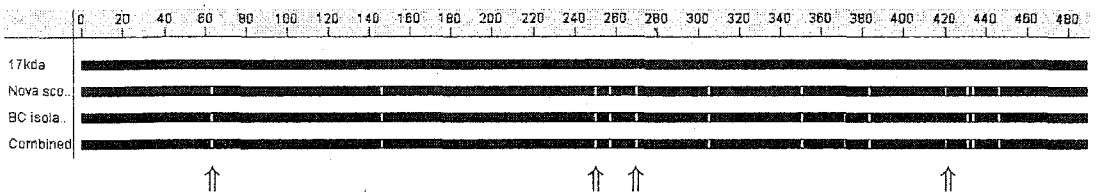

Figure 5.2. Sequence alignment of isolates from three geographical locations and graphical representation of the location of nucleotide changes between the type strain and the Canadian isolates (white bars). SNPs leading to amino acid changes are marked with ⇑.

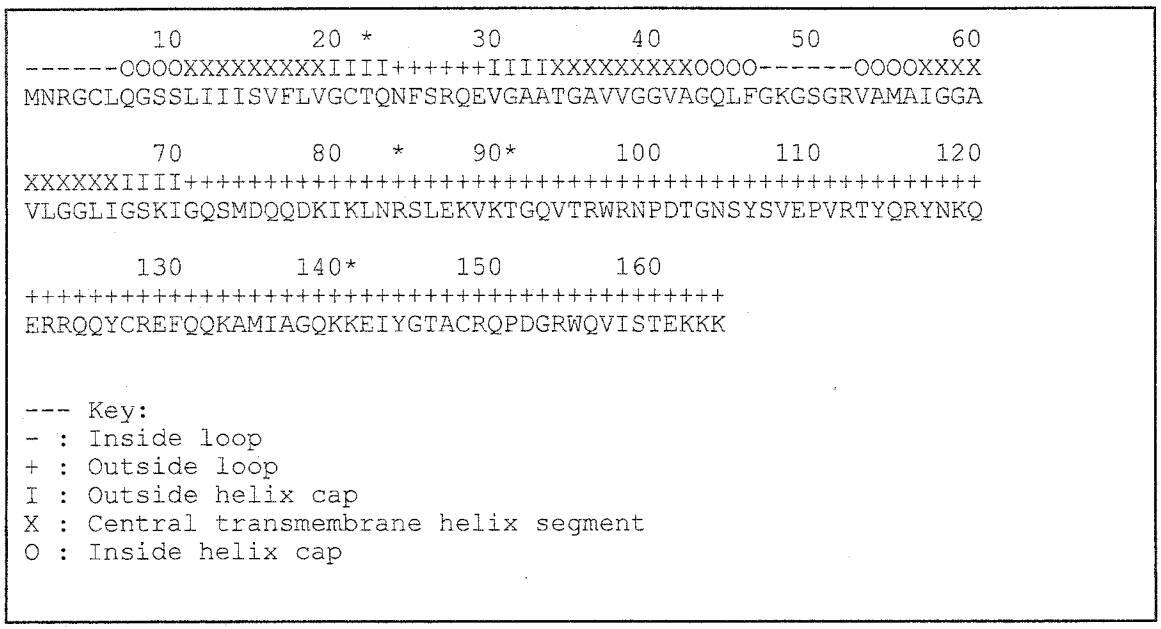

Figure 5.3. MEMSAT2 prediction of transmembrane regions of the 17 kDa gene product. Positions were amino acid polymorphisms are found are marked "*".

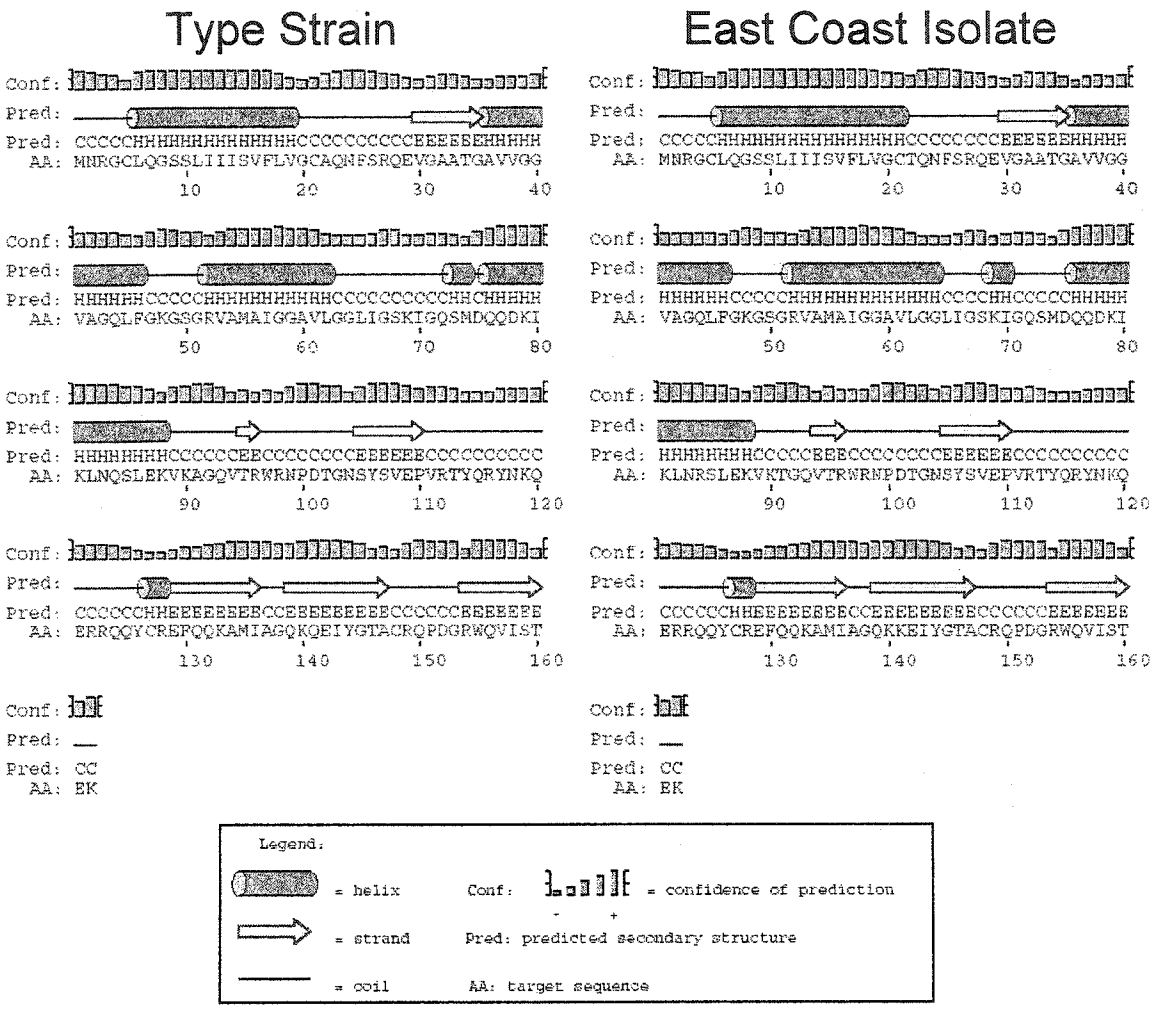

Figure 5.4. Predicted secondary structure of the type strain 17 kDa and the east coast isolate. No secondary structure differences were seen between the east coast and Pacific coast isolates.

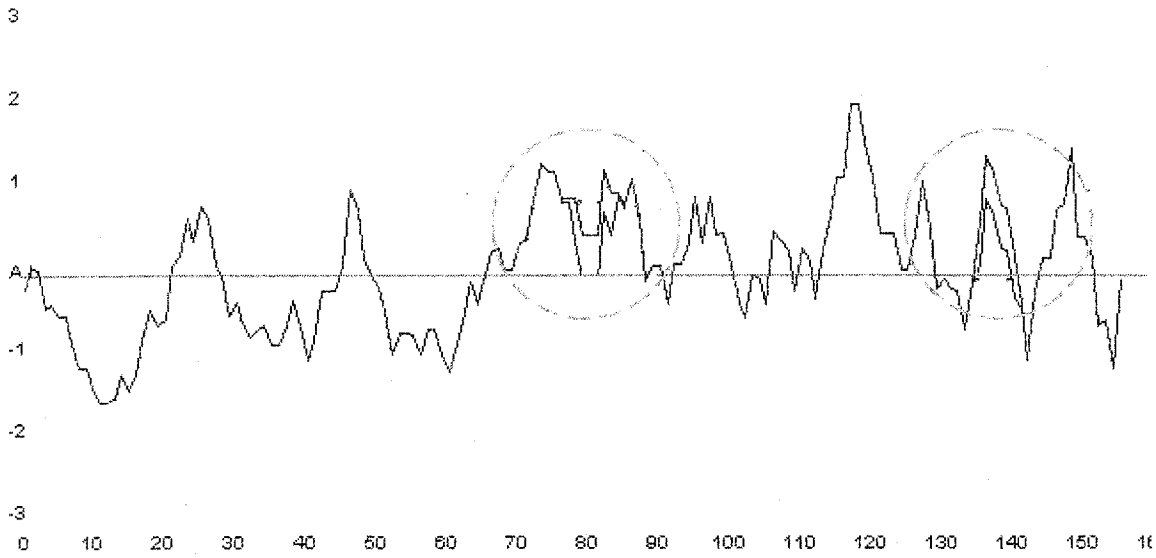

Figure 5.5. Antigenicity plot of the 17 kDa protein differences between both Canadian isolates and the type strain are circled on the left, differences unique to the East coast isolate are circled on the right. In both locations, the type strain is the lower line. The y-axis represents the antigenicity index, with positive values representing a higher likely hood of the presence of an epitope, the x-axis represents the amino acid position.

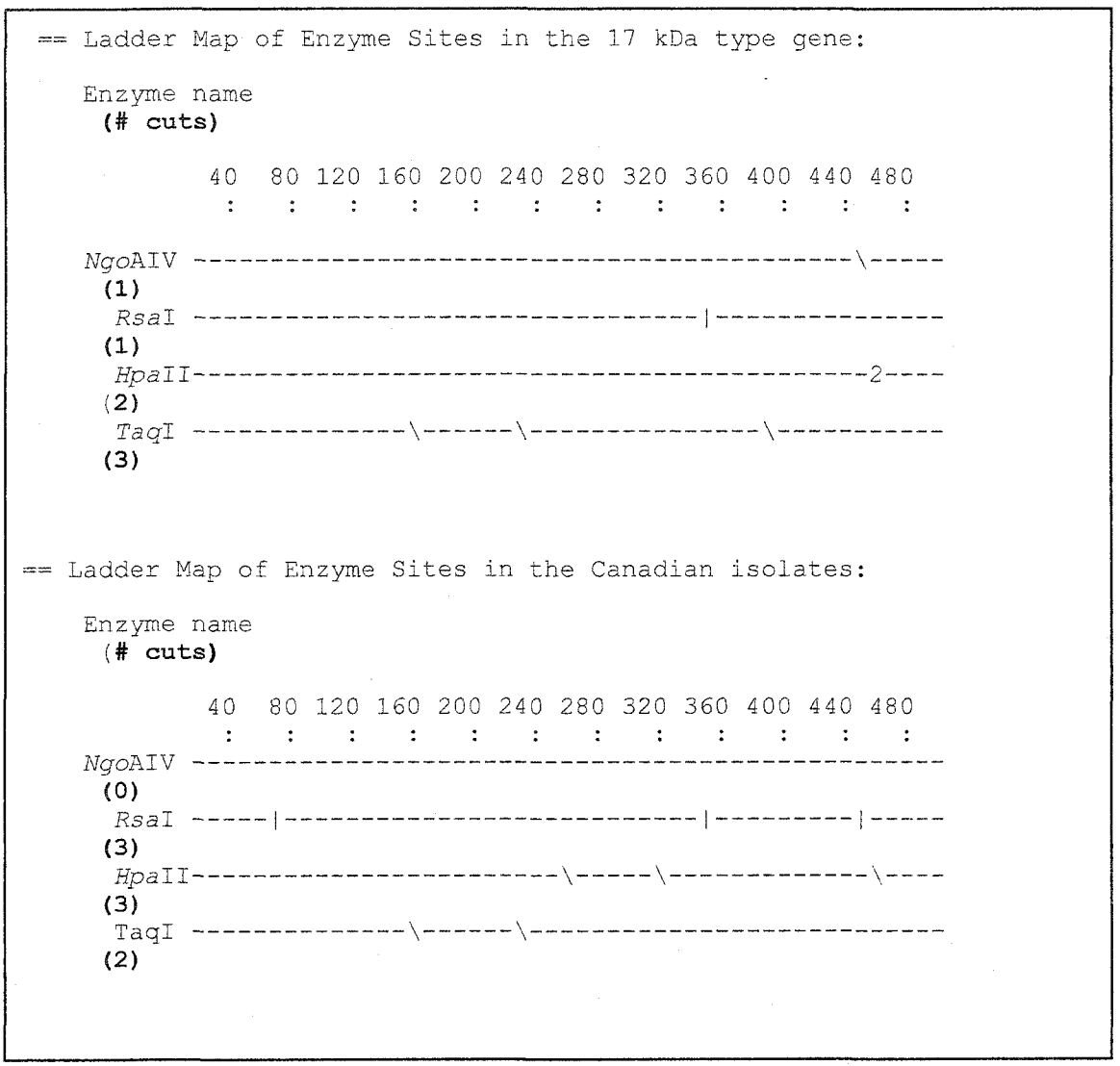

Figure 5.6. Graphical representation of the location of restriction sites within the open reading frame (ORF) of the 17 kDa gene for selected enzymes in the wild type or the Canadian isolates of *P. salmonis*. Cut locations are marked from the start codon of the ORF with | denoting restriction enzymes generating blunt ends and \ restriction enzymes generating cohesive ends. The number 2 in the ladder map indicates two restriction sites located in close proximity.

CHAPTER 6: GENERAL DISCUSSION

The focus of this thesis is on molecular and immunological properties of the intracellular bacterial pathogen *Piscirickettsia salmonis* to enable development of a DNA vaccine. Since the first reported outbreaks in 1989, the organism has quickly established itself as one of the major bacterial diseases in Chilean salmonid aquaculture (Bravo and Campos, 1989; Cvitanich *et al.*, 1991) with lesser outbreaks reported in most geographical centers of salmonid aquaculture including both the Pacific and Atlantic coasts of Canada (Brocklebank *et al.*, 1993; Jones *et al.*, 1998; Evelyn *et al.*, 1998), Norway (Olsen *et al.*, 1997), Scotland (Birrel *et al.*, 2003) and Ireland (Rodger and Drinan, 1993). Piscirickettsia-like organisms have been observed causing mortalities in white seabass (*Atactoscion noblis*) (Chen *et al.*, 2000), European sea bass (*Dicentrarchus* sp.) (Steiropoulos *et al.*, 2002), tilapia (*Oreochromis*, *Tilapia* and *Sarotherodon* spp.) (Chen *et al.*, 1994; Mauel *et al.*, 2003) and blue-eyed plecostomus (*Panaque suttoni*) (Khoo *et al.*, 1995). Therefore, *P. salmonis* is considered a new or emerging disease in aquaculture.

To study the molecular and immunological properties of a novel pathogen, a source of reasonably pure organism has to be available. Pure cultures of most bacterial pathogens can be obtained using the appropriate culture media and conditions. *Piscirickettsia salmonis* is an obligate intracellular pathogen and requires an eukaryotic host cell culture, typically the CHSE-214 cell line, which makes obtaining sufficient amounts of *P. salmonis* for research or vaccine purposes labour and time intensive.

Therefore, optimization of culture conditions to maximize the harvest is desirable. The standard method to determine *P. salmonis* levels in culture is by tissue culture infectious dose 50% ($TCID_{50}$) which is labor intensive and yields results only after seven to 10 days. The sandwich enzyme linked immunosorbent assay (s-ELISA) described in chapter 2 provides a faster, less laborious tool to monitor the growth in culture of *P. salmonis*. Compared to other studies reporting on the development of ELISA methods for *P. salmonis*, the use of an internal standard in the protocol here described makes plate to plate and day to day comparisons of *P. salmonis* possible (Aguayo *et al.*, 2002). Samples of cell culture supernatant can be frozen for later analysis, alleviating the need to conduct experiments at each sampled time point as would be required if the amount of *P. salmonis* in the supernatant was determined by $TCID_{50}$ assays. The observed drop of *P. salmonis* antigen levels ten days post inoculation suggested that free *P. salmonis* degrades under typical cell culture conditions and that the maximum amount of *P. salmonis* might be present before 100% CPE is reached. The survival of *P. salmonis* in tissue culture supernatant was investigated by Lannan and Fryer (1994). In that study the $TCID_{50}$ of semi-purified *P. salmonis* dropped quickly after four days at 15°C. By using a s-ELISA that contains a capture antibody, the problem of high levels of soluble protein in tissue culture medium out-competing the *P. salmonis* for binding sites on the substrate of the microtitre plate can be avoided and samples can be applied directly from culture. Additionally, by using a monoclonal antibody to detect the captured *P. salmonis* a high level of specificity can be achieved. This is significant as unwanted reactivity of the polyclonal rabbit antiserum against the host cell material can be problematic in a standard

ELISA (Kemeny, 1997). Since the $TCID_{50}$ is only a measure of viable bacteria while s-ELISA will theoretically measure total antigen, further studies to correlate the relative amount of *P. salmonis* determined by s-ELISA and $TCID_{50}$ are recommended

The genetic information available for *P. salmonis* at the initiation of this project was limited to ribosomal sequences and the internal transcribed spacer (ITS) region (Mauel *et al.*, 1999). Methods for expanding this information were crucial for an attempt to develop a DNA vaccine for *P. salmonis*. The use of an expression library to identify complete or partial genes coding for immunoreactive products combined with inverse polymerase chain reaction (iPCR) to expand fragments of identified partial genes, proved to be a valuable combination. Standard protocols for generation of expression libraries focus on generating clones with inserts of appropriate size to contain complete or even multiple genes. Inverse PCR negates the need to have the complete gene, as the sequence of the full gene can be determined using iPCR primers developed from the fragment found in an immunoreactive clone (Ochman *et al.*, 1988). The advantage of this approach is that more unique fragments of the genome are identified. With the increasing amount of sequences available in GenBank for comparison, assigning putative functions to the full length genes and their translated products has become more feasible. A further selection of the available fragments based on this comparison was made, weighing the likelihood that the gene was relevant to a protective response based on location of the gene product (e.g. outer membrane or cytoplasmic) and function (e.g. virulence factors such as hemolysins or lectins as opposed to housekeeping genes). The most promising candidates were then selected to be expanded upon by iPCR. In a direct comparison,

Kuzyk *et al.* (2001) used the traditional approach in generating a λ ZAP II based library of *P. salmonis* and identified 18 immuno reactive plaques, but on further analysis showed that they all contained a common region of DNA. This was determined to be a 4,983 basepair (bp) fragment of DNA containing five open reading frames (ORFs), four of which were complete. In comparison, the methods reported in chapter 3 generated a total of 54 immunoreactive plaques and 20 non overlapping ORFs of much smaller size were identified.

Using comparisons with available sequences in GenBank, putative functions could be assigned to nine ORFs found in the library described in chapter 3. Based on putative function and insert size, ORFs present on five inserts were chosen for further analysis by inverse PCR (iPCR) to determine the flanking genomic regions to the inserts found in the library. Four out of five inserts were successfully expanded upon, with one yielding the full gene sequence of the *P. salmonis* homolog to the spotted fever group (SFG) *Rickettsia* 17 kDa group antigen. The additional sequence information obtained by iPCR on the ORFs found in clones 3, 7 and 20 allowed them to be assigned the following functions: a transposase, an ATP-binding cassette (ABC) containing exporter and an amino acid transporter, respectively. Even though the full length sequences for these genes have not been identified, the tentative role of these proteins in the pathobiology of *P. salmonis*, as judged by the function of homologous proteins, and their location in the outer membrane of *P. salmonis*, make these genes potential candidates for future incorporation in DNA vaccines.

The full length sequence of the 17 kDa gene obtained by the iPCR was identical to

the sequence obtained independently by Kuzyk *et al.*.(2001), which confirms the validity of the methods here used. However, a concern when working with PCR products is the error rate of *Taq* DNA polymerase. For sequencing, this is less of a concern, assuming any errors in the copy made by *Taq* would be random and any particular error made would be a minority within the total population of copies. In the sequencing reaction the peak generated by the copies containing the original base for any specific location will be much larger then the one generated by copies containing the erroneous base.

In cloning experiments, one single fragment of DNA is taken out of the population and cloned into one single molecule of plasmid and grown and multiplied in transformed *Escherichia coli*. The entire population of the plasmid from one colony of transformed cells will therefore contain any error present in the one fragment of DNA generated by PCR. To minimize the risk of errors, an Expand High Fidelity PCR system mix of polymerases was used to generate the template for cloning and the minimum number of cycles needed to generate enough amplicon for cloning was determined.

The potential for the 17 kDa containing plasmid as a DNA vaccine against *P. salmonis* was investigated by experimental exposure challenge as described in chapter 4. The challenge experiments were conducted before any *in vitro* expression was determined. It was hypothesized that due to the intracellular location of the *P. salmonis*, a Th1, or cell-mediated type of response might be induced and be protective without seeing a clear Th2 or antibody driven type of response. T cell epitopes are comprised of fragments of the entire protein and might still be present, while the intact protein produced in an eukaryotic cell might be mis-folded to the point where the antiserum used

to screen the library can no longer recognizes the original B cell epitopes. When unexpectedly high mortalities were observed in the challenge experiment, even in groups immunized with whole cell bacterin, the question of whether expression had occurred immediately was raised. To address this, an *in vitro* expression experiment was performed to investigate if any B cell epitopes were being expressed. The demonstration of *in vitro* expression of *P. salmonis* antigen by transfected CHSE-214 cells in an immunoreactive form strengthened the results from the survival analysis performed on the trial that showed a positive response in fish vaccinated with 17 kDa containing DNA vaccine. These results combined warrant future trials with the 17 kDa gene containing DNA vaccines with emphasis placed on determining the appropriate challenge dose by establishing the correlation between $TCID_{50}$ and the s-ELISA and subsequently following the levels of *P. salmonis* in the challenge culture using the s-ELISA. The ability to detect immunoreactive protein expressed by vaccine plasmids by transfection of CHSE-214 cells allows future pre-screening of the (partial) genes coding for the transposase, ABC-type exporter and amino acid transporter for use in DNA vaccines, although a possible inability of immunohistochemistry to detect antigen should not automatically disqualify a potential DNA vaccine. Absence of B cell epitopes may be due to different post-translational processing of the originally prokaryotic gene being expressed in eukaryotic cells, rather than due to absence of expression or lack of immunogenicity of the protein being expressed. The protective response to intracellular pathogens is often dependant on a T cell driven response and screening for T cell epitopes by proliferation assay can identify potentially protective antigens (Gormley *et al.*, 1999). As typical T cell epitopes

are small polypeptides (Janeway, 2001), they are less dependent on proper folding of the protein and therefore more likely to be maintained when expressed in eukaryotic cells. Proliferation assays are more cumbersome than immunohistochemistry and the suggested sequence for screening would therefore be to initially screen by immunohistochemistry and subsequently screen by proliferation assay if no initial immunoreactivity has been found.

The potential of the 17 kDa gene to be used to differentiate between isolates from different geographic locations was investigated as described in chapter 5. Different Canadian strains of *P. salmonis* were compared to the type strain by analyzing the sequence of the 17 kDa gene, 13 single nucleotide polymorphisms (SNPs) were observed. The Pacific and Atlantic coast isolates showed 11 SNPs in common when compared to the LF-89 type strain. Two SNPs were found that were unique to either the Atlantic or the Pacific isolates. Of the total SNPs, four led to amino acid replacement and all these amino acids were predicted to be located extracellularly and therefore visible to the immune system. Immune pressure may be driving this variability especially when considering that the three amino acid changes that are present in the main extracellular domain are located in areas predicted to have the highest antigenicity and lead to changes to this predicted antigenicity. *Piscirickettsia salmonis* infections in British Columbia (B.C.) have become the leading pathogen identified by the fish health auditing and surveillance program of the provincial Ministry of Agriculture, Food and Fisheries for Atlantic salmon (*Salmo salar*) in the most recent published report (2001), available online at http://www.agf.gov.bc.ca/fisheries/health/fhasp.htm. Outbreaks in B.C. are reported as

less severe than those seen in Chile (Brocklebank *et al.*, 1993) and the B.C. isolate ATL-4-14 was reported to have decreased virulence when compared to the LF-89 type strain (House *et al.*, 1999). The predicted higher antigenicity of the *P. salmonis* 17 kDa protein described in chapter 5 for the Pacific isolates may contribute to an increased ability of Atlantic salmon to mount an adequate response to this pathogen. This emphasizes the need to further investigate the 17 kDa gene as a DNA vaccine candidate. The changes the SNP introduced in palindromic sequences make the 17 kDa suitable for quick taxonomical assessment of new *P. salmonis* isolates. Restriction digestion of PCR fragments can determine the presence or absence of 7 of the 13 SNPs without the need for sequencing. The other method used for taxonomical assessment of *P. salmonis* was sequencing of the DNA coding for ribosomal subunits and the internal transcribed spacer (ITS) region or by studying the ITS electrophoretic migration pattern. These methods are either more time consuming and costly as is the case with sequencing or provide less detail when looking only at the migration pattern. Further directions for this method would be to obtain geographically unique isolates and to assess them for the presence or absence of these SNPs so that a phylogenetic tree based on the 17 kDa gene can be obtained. By comparing the SNP profile with the virulence of these isolates, it can be determined if these SNPs are random genetic mutations that confer a strong selective advantage upon the bacterial strain containing them or a so-called pathoadaptive mutation (Sokurenko *et al.*, 1999). In enterobacterial adhesins the study of such SNPs has provided information on how bacterial genes determine host specificity, tissue tropism and molecular level information on protein function (Weissman *et al.*, 2003).

Recent trends in the development of sustainable aquaculture include the investigation of alternative species and introduction of established species to new geographic locations. Both of these trends can lead to the rise of new or emerging diseases. When fish are raised in a new location, they are exposed to a new environment that contains a spectrum of naturally occurring pathogens to which the fish may be susceptible. Conversely, if new species are introduced to the intense rearing conditions of aquaculture with high stocking densities leading to high levels of stress, pathogens already present at a low level in the population can exploit these conditions and lead to epidemics. *Piscirickettsia salmonis* can be used as a model of a new or emerging disease and this thesis demonstrated one approach to development of a vaccine after the initial identification of a new disease. First, the tools to optimize culturing and recovery of the pathogen were developed using a s-ELISA. The genetic make up of the pathogen was studied using an expression library and this knowledge was applied to the development of vaccine candidates and to study the genetic differences between different isolates. The large potential benefit of an effective DNA vaccine against *P. salmonis* warrants further studies into the 17 kDa gene containing plasmid as a vaccine candidate and testing of the other immuno reactive proteins coding genes identified as DNA vaccine inserts. A repeat of the challenge trial with an appropriate challenge dose should show if the 17 kDa gene based DNA vaccine is efficacious. The methodology developed and described in this thesis is an effective template for the investigation of future new and emerging diseases in salmon aquaculture.

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