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Development of an Efficacious Recombinant Vaccine for the Obligate Intracellular Salmonid Pathogen *Piscirickettsia salmonis*

by

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B.Sc., University of Victoria, 1994

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

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We accept this dissertation as conforming
to the required standard

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ABSTRACT

Piscirickettsia salmonis is the aetiological agent of salmonid rickettsial septicaemia (SRS), an economically devastating rickettsial disease of farmed salmonids. SRS responds poorly to antibiotic treatment and no effective vaccine is available for its control. A molecular biology approach was used to characterize and identify antigens of *P. salmonis* that would be suitable to use as a recombinant subunit vaccine to aid in the control of SRS.

A system for routine and reliable growth of *P. salmonis* was established using a chinook salmon (*Oncorhynchus tshawytscha*) embryo cell line. A purification protocol to separate *P. salmonis* from host cell material was devised using a combination of differential and Percoll density gradient centrifugation. Purified *P. salmonis* was used to generate polyclonal rabbit antisera. Indirect immunofluorescence microscopy, immunogold transmission electron microscopy, and biotin labeling of intact *P. salmonis* confirmed that *P. salmonis* was effectively separated from host cell debris and that immunoreactive antigens identified by rabbit antisera were surface associated. Rabbit anti-*P. salmonis* sera recognized the lipooligosaccharide component of bacterial lipopolysaccharide, and 7 protein antigens with relative mobilities of 27, 24, and 16 kDa and 4 migrating between 50-80 kDa. *P. salmonis* lipopolysaccharide was observed to be predominantly low m.w., but less abundant high m.w. species containing O-antigen were present.

Genomic DNA was isolated from purified *P. salmonis* and used to construct an expression library in lambda ZAP II. In the absence of preexisting DNA sequence, rabbit polyclonal anti-*P. salmonis* serum was used to identify immunoreactive clones. A lambda clone encoding an immunoreactive 17 kDa outer surface protein (OspA) of *P. salmonis* was identified. The 4,983 bp insert contained a high molar percentage of adenine and thymine, encoded four intact ORF's, and represented the first non-ribosomal DNA sequence data from *P. salmonis*. OspA is modified as a bacterial lipoprotein in *Escherichia coli* and is most closely homologous to a rickettsial 17 kDa surface lipoprotein previously only observed within the genus *Rickettsia*. A codon optimized version of *ospA* was constructed and the lipoprotein nature of OspA was determined to be a limiting factor in its production in *E. coli*. High level production of immunoreactive OspA targeted to inclusion bodies was achieved

in *E. coli* by combining OspA with an N-terminal fusion protein. The OspA fusion was recognized by convalescent salmon sera thereby identifying OspA as an excellent candidate for a recombinant vaccine against *P. salmonis*.

Vaccine preparations using *P. salmonis* bacterins were found to elicit variable immune responses in coho salmon (*Oncorhynchus kisutch*) that resulted in either protection or immunosuppression of vaccinates which varied with antigen dosage. Recombinantly produced OspA elicited an astonishing level of protection in vaccinated coho salmon with a relative percent survival (RPS) as high as 59%. In an effort to further improve the efficacy of the OspA recombinant vaccine, T cell epitopes (TCE's) from tetanus toxin and measles virus fusion protein which are universally immunogenic in mammalian immune systems were incorporated into an OspA fusion protein. Addition of the TCE's dramatically enhanced the efficacy of the OspA vaccine, reflected by a 3-fold increase in the number of coho salmon protected (83% RPS). These results represent an effective monovalent recombinant subunit vaccine for the rickettsial pathogen, *P. salmonis*.

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A	Adenine
aa	Amino acid
A_{600}	600 nm Absorbance
Ap	Ampicillin
ATCC	American Type Culture Collection
BKD	Bacterial kidney disease
bp	Base pair
C	Cytosine
CDR	Complementarity determining regions
CHSE-214	Chinook salmon embryo cell line
cm	Centimetre
Cm	Chloramphenicol
CPE	Cytopathic effect
dATP	Deoxyadenosine 5'-triphosphate
dil.	Dilution
dsDNA	Double stranded deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EPC	Epithelial carp cell line
ERM	Enteric red mouth
FAO	Food and Agriculture Organization of the United Nations
Fig.	Figure
FITC	Fluorescein isothiocyanate
g	Grams
G	Guanine
h	Hour
Ig	Immunoglobulin
IHNV	Infectious haematopoietic necrosis virus
IP	Intraperitoneal
IPNV	Infectious pancreatic necrosis virus
IPTG	Isopropyl-β-D-thiogalactoside
ISAV	Infectious salmon anaemia virus

kbp	Kilobase pair	PBS	Phosphate buffered saline
kDa	KiloDaltons	PCR	Polymerase chain reaction
KLH	Keyhole limpet hemocyanin	PK	Proteinase K
		PKD	Proliferative kidney disease
Kn	Kanamycin		
L	Litre	pmol	Picomolar
LD_{50}	50% lethal dose	ppm	Parts per million
LOS	Lipooligosaccharide	PTA	Phosphotungstic acid
LPS	Lipopolysaccharide	RBS	Ribosome binding site
M	Molar	RPS	Relative percent survival
MEM	Minimal essential media	rRNA	Ribosomal ribonucleic acid
mg	Milligram	s	Second
MHC	Major histocompatibility complex	sIg	Surface immunoglobulin
		spp.	Species
ml	Millilitre	SRS	Salmonid rickettsial septicaemia
mM	Millimolar		
MVF	Measles virus fusion protein	T	Thymine
		TB	Terrific broth
m.w.	Molecular weight	TBS	Tris-buffered saline
ng	Nanogram	TCE	T cell epitope
oligo.	Oligonucleotide	$TCID_{50}$	Tissue culture infectious dose
ORF	Open reading frame		
OspA	Outer surface protein A		

TEM	Transmission electron microscopy
TS-buffer	Tris-sucrose buffer
tt	Tetanus toxin
v	Volume
VHSV	Viral haemorrhagic septicaemia virus
w	Weight
µg	Microgram
µl	Microlitre
µm	Micrometre
µM	Micromolar

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INTRODUCTION

1.1 AQUACULTURE

Aquaculture encompasses the cultivation of aquatic plants and animals. Since 1950, aquaculture has grown from relative obscurity into a global industry. Aquaculture provides an important alternate source of meat protein for an increasing world population. Since 1985, the aquaculture industry has expanded more rapidly than any terrestrial meat industry, experiencing an average annual growth of 10.9% (Currie, 2000). Aquaculture produced 28.8 million tonnes of food fish in 1997 which accounted for one third of all food fish destined for human consumption (Currie, 2000). The wild capture fisheries currently produce 62 million tonnes annually but have experienced very little growth since 1990. A 1999 report by the FAO (Food and Agriculture Organization of the United Nations) on the state of world fisheries and aquaculture estimated that 25% of all natural stocks targeted by wild capture fisheries are depleted and another 44% are currently fished at their biological limits (Currie, 2000). Until wild stocks are managed efficiently and over-fishing is reversed, it is unlikely that the production from capture fisheries will expand beyond its current levels. By the year 2010, the annual world demand for food fish is estimated by the FAO to exceed 105 million tonnes, 18 million tonnes more than the world fisheries currently produce (Currie, 2000). Clearly, aquaculture is the only sector of the world fisheries that can grow sufficiently to meet this demand.

Aquaculture of salmonid species is a high value global food fish industry. The salmonid aquaculture industry practices livestock inventory control, good animal husbandry, and has grown at a rate faster than aquaculture as a whole since 1981. The global salmonid aquaculture harvest in 1981 was 17,000 tonnes and has grown to an annual harvest in

excess of 1 million tonnes in 1999. Aquaculture production of salmon and trout has exceeded the production of wild capture fisheries since 1997 and currently provides 55% of the annual world supply. The largest salmon and trout producing nations, in descending order, are Norway, Chile, the United Kingdom, and Canada.

1.2 Aquaculture and disease

Future expansion of the aquaculture industry is anticipated to occur primarily through gains in productivity (Benmansour and de Kinkelin, 1997). Disease is one of the predominant obstacles slowing the growth of aquaculture and remains the largest cause of economic losses. The economic impact of disease extends beyond lost fish stock, expenses are also incurred from lost labour, production time, treatment, disinfection, and restocking (Meyer, 1991). The World Bank estimates that in 1997 alone, the global aquaculture industry lost US$3.02 billion to disease. As the aquaculture industry grows and new species of fish are intensively cultured, the range of diseases expands similarly.

1.3 Economically significant diseases in salmonid aquaculture

Cultured salmonids suffer from a variety of bacterial, viral, parasitic, and fungal infections (Table 1). A number of diseases have plagued salmonid aquaculture since its beginnings and the industry did not expand until efficacious vaccines were readily available to control them. Vaccines are not available for many recently identified diseases and the development of vaccines for many of them has proven to be difficult. To worsen matters, no effective commercial vaccines are available against any viral or parasitic fish pathogens. Only with the development of vaccines against these recalcitrant pathogens will

Table 1. Major diseases of salmonid aquaculture.

Disease	Causative Agent	Geographic Distribution
Bacterial diseases		
Enteric red mouth	*Yersinia ruckeri*	North America, Europe, South America
Vibriosis - Vibriosis - cold water vibriosis	 *Vibrio anguillarum, V. ordalii* *V. salmonicida*	 Worldwide Norway, Faroe Islands
Furunculosis	*Aeromonas salmonicida*	North America, Europe, South America
Bacterial kidney disease	*Renibacterium salmoninarum*	North America, Europe, South America
Motile aeromonad septicaemia	*Aeromonas hydrophila, A. caviae, A. sobria*	Asia, Europe, United States
Salmonid rickettsial septicaemia	*Piscirickettsia salmonis*	Chile, United Kingdom, Norway, Canada
Rainbow trout fry syndrome	*Flavobacterium psychrophilum*	United States, Europe, Japan
Nocardiosis	*Nocardia asteriodes, N. kampach*	Canada
Clostridial infections	*Clostridium botulinum*	Europe, United States
Columnaris disease	*Flexibacter columnaris, F. maritimus*	North America, Asia, Japan, Europe
Bacterial gill disease	*Cytophaga* spp., *Flexibacter* spp., *Flavobacterium bronchiophilia*	North America, Japan, Europe
Viral diseases		
Infectious pancreatic necrosis	Birnavirus	Europe, Chile
Viral haemorrhagic septicaemia	Rhadbovirus	Japan, North America, Europe
Infectious haematopoietic necrosis	Rhadbovirus	Japan, North America
Infectious salmon anaemia	Orthomyxovirus	Norway, Canada
Parasitic diseases		
Sea lice	*Lepeophtheirus salmonis, Caligus elongatus*	Northern hemisphere
Proliferative kidney disease	*Tetracapsula bryosalmonae*	United Kingdom, Europe, North America
White spot disease	*Ichthyophthirius multifilus*	Worldwide
Myxosporeans	Range of pathogenic species, includes *Kudoa* spp.	Worldwide
Costiasis	*Ichthyobodo necator*	Worldwide

aquaculture be able to further increase its productivity and meet increasing global demands for food fish.

1.3.1 Viral diseases

Infectious haematopoietic necrosis (IHN) was first observed in hatchery raised sockeye salmon (*Oncorhynchus nerka*) in the United States in the 1950's (Rucker *et al.*, 1953). IHN, caused by an enveloped rhabdovirus (IHNV), is one of the most significant viral diseases of cultured salmonids and can cause extensive mortalities in small fish (losses up to 80-100%) (Winton, 1997). In larger fish, IHN is more chronic and mortalities can reach 25% (Winton, 1997). The geographic distribution of IHNV has spread from North America to Europe and Asia with the trade of infected eggs and fish. Protective immunity has been demonstrated with both killed preparations of IHNV and recombinantly expressed IHNV glycoprotein (Winton, 1997). Disadvantages to these vaccines being developed for small fish are that the inactivated virus vaccines must be administered by injection to be effective and recombinant glycoprotein must be produced using expensive eukaryotic expression systems to be immunogenic. The only control measures currently available against IHN are avoidance of exposure and destruction of infected stocks.

Viral haemorrhagic septicaemia virus (VHSV) is a an enveloped, negative strand RNA virus that belongs to the rhabdovirus family (Lorenzen and Olesen, 1997) and was first identified in Denmark in 1965 (Jensen, 1965). Rainbow trout (*Oncorhynchus mykiss*) are the primary species affected by VHS and are susceptible at all ages, but higher mortality occurs in smaller fish (losses up to 90%). Obstacles similar to those facing the development of an IHN vaccine prevent the production of a VHS vaccine. Vaccines based on both

inactivated VHSV and recombinantly expressed VHSV glycoprotein are protective but the cost of their production is prohibitive (Lorenzen and Olesen, 1997).

Infectious pancreatic necrosis (IPN) is a disease of young rainbow trout and Atlantic salmon (*Salmo salar*) that costs the Norwegian industry, for example, an estimated US$60 million annually. IPN was first reported as a viral disease in 1955 in U.S. hatchery-raised brook trout (*Salvelinus fontinalis*) (Wood *et al.*, 1955) and has since spread to every salmon producing nation. IPN is caused by a birnavirus, IPNV, and is capable of causing acute mortality (losses up to 90%) in salmonid fry at start of feeding and can also induce a carrier state (Benmansour and de Kinkelin, 1997). IPN also occurs as a chronic disease in more mature salmon causing mortalities for up to a year following transfer to saltwater (Christie, 1997). A recombinant subunit vaccine based on the VP2 protein of IPNV is commercially available in Norway for administration by injection, but fish most susceptible to the disease (<10 g) can not be immunized by injection (Press and Lillehaug, 1995).

Infectious salmon anaemia virus (ISAV) is a recent addition to the repertoire of viral salmonid pathogens. ISA was first observed in Atlantic salmon in 1985 but was not successfully isolated until 1994 (Mjaaland *et al.*, 1997). ISAV is a complex, enveloped, influenza-like virus with a segmented, negative strand RNA genome that appears to comprise a new genus within the *Orthomyxoviridae* (Krossøy *et al.*, 1999). ISAV has also been isolated in Scotland and on the east coast of Canada, in association with extensive mortalities (losses up to 90%). ISAV is the only disease on the European Union list of most dangerous fish diseases and there is currently no vaccine available to help in its control.

1.3.2 Parasitic diseases

A diverse collection of parasitic diseases afflict aquaculture, but only a select few are geographically widespread and cause high mortalities among farmed salmonid species. No commercial vaccines are currently available against any parasitic fish disease. Parasites have complex life cycles that often involve multiples stages in definitive and intermediate hosts and the aquatic environment. Distinct antigenic profiles are often associated with these morphologically different stages. Identification and characterization of these life cycle stages is an important step in vaccine development for parasites because antigens of the infectious stage of the life cycle are often good candidate vaccine antigens (Woo, 1997).

Sea lice (*Lepeophtheirus salmonis* and *Caligus elongatus*) is the most economically significant parasitic disease of Atlantic salmon and can cause very high losses. Sea lice have very complex life cycles containing 10 different stages (Woo, 1997). Extended sea lice infection can result in large wound formation with subepidermal haemorrhages and cranial bone exposure (Woo, 1997). These wounds are highly susceptible to secondary bacterial and fungal infection. Although no vaccine for sea lice currently exists, 20 candidate antigens of *L. salmonis* are currently being investigated for vaccine potential.

Ichthyophthiruis multifiliis, the aetiological agent of white spot disease, does not demonstrate host specificity and is a problem in global freshwater aquaculture including salmonid species (Woo, 1997). Protective immunity has been demonstrated against *I. multifiliis* following immunization with the trophont stage of its life cycle (Burkart *et al.*, 1990). Immunity to *I. multifiliis* in channel catfish (*Ictalurus punctatus*) has been

correlated with surface immobilization antigens and a recombinant subunit vaccine is under development (Woo, 1997).

Proliferative kidney disease (PKD), caused by *Tetracapsula bryosalmonae*, is responsible for high losses among farmed trout in Europe (Scholz, 1999). *T. bryosalmonae* infects the kidney and spleen and disease is caused by the intense immune reaction mounted against the parasite (Scholz, 1999). *Kudoa thyrsites* is the aetiological agent of soft flesh disease, an economically significant myxosporean parasitic disease that impacts cultivation of Atlantic salmon. Economic loss from soft flesh disease results from rapid deterioration of fillets harvested from salmon infected with *K. thyrsites* (Moran *et al.*, 1999). The molecular pathogenesis of both *T. bryosalmonae* and *K. thyrsites* is poorly understood.

1.3.3 Bacterial diseases

Vibriosis

The genus *Vibrio* contains the most significant marine bacterial fish pathogens and *Vibrio* spp. are ubiquitous throughout the marine environment. *Vibrio* spp. are gram negative, motile rods and three species, *V. anguillarum*, *V. ordalii* and *V. salmonicida*, are the primary agents of vibriosis, a septicaemic infection (Toranzo *et al.*, 1997). *V. anguillarum* is the most virulent *Vibrio* spp. and produces a variety of proteases responsible for ulcerative lesions in skeletal muscle. Outbreaks of vibriosis resulting in mortalities usually occur soon after smolts are transferred to saltwater and when ocean temperatures are high (Press and Lillehaug, 1995). Ten serovars of *V. anguillarum* exist, but only serovars O1 and O2 cause significant disease in salmonids (Toranzo *et al.*, 1997). *Vibrio* spp. have highly immunogenic lipopolysaccharides that elicit strong protective immune

responses in salmonids (Toranzo *et al.*, 1997). Commercial vaccines based on bacterin preparations of *Vibrio* spp. are highly effective and generate long term immunity. Control of vibriosis is often credited with enabling the intense marine farming of salmon (Press and Lillehaug, 1995).

Furunculosis

Furunculosis is caused by *Aeromonas salmonicida*, a gram negative, non-motile rod. Furunculosis is likely the most commonly occurring disease amongst farmed salmonids and is encountered wherever salmonids are farmed. *A. salmonicida* is also capable of inducing a carrier state in exposed fish and disease can occur in fish of all ages, but the most serious economic losses occur in salmonids during the saltwater stage of their life cycle. Pathology of furunculosis can vary from acute to chronic with fish dying with few external signs to chronic infections exhibiting localized hemorrhaging and tissue necrosis in the gills, gut and muscle (Ellis, 1997). *A. salmonicida* produces a large number of well characterized virulence factors including a hydrophobic protein surface layer (A-layer), a polysaccharide capsule, serine and metallo-proteases, a hemolytic cytotoxin, and a glycero-phospholipid cholesterol acyl transferase (Ellis, 1997). *A. salmonicida* is generally not as immunogenic as *Vibrio* spp., but, bacterin preparations of *A. salmonicida* do elicit a protective immune response. Protection from an *A. salmonicida* bacterin is improved when *A. salmonicida* is supplied as a component of a polyvalent vaccine that contains bacteria that are more immunogenic (Evelyn, 1997).

Enteric red mouth disease

Enteric red mouth (ERM) disease is caused by *Yersinia ruckeri*, a gram negative, motile rod. ERM is a characterized by a haemorrhagic septicaemia and the symptomatic red mouth is not always present. ERM can occur in acute, chronic and carrier forms and affects all species of salmonids, but rainbow trout are particularly susceptible with mortalities ranging from 10-60% (Romalde and Toranzo, 1993). *Y. ruckeri* has eight serovars classified by LPS O-antigen diversity (Stevenson, 1997). For vaccination purposes serovars O:1 and O:2 are the most significant and bacterin vaccines against ERM and induce strong, protective immunity. Interestingly the immune response to serovar O:2 resembles that of *Vibrio* spp. with a strong antibody response against LPS and O-antigen, but serovar O:1 elicits a negligible antibody response (Stevenson, 1997).

Bacterial kidney disease

The aetiological agent of bacterial kidney disease (BKD) is a gram positive, non-motile rod, *Renibacterium salmoninarum*. BKD occurs worldwide causing major economic losses in salmonid aquaculture. The pathology of BKD is a chronic, systemic and granulomatous infection characterized by necrotic abscesses in the kidney (Kaattari and Piganelli, 1997). External symptoms of disease are not usually evident until terminal stages of the disease. *R. salmoninarum* can be transmitted horizontally and vertically through gametes (Wood and Kaattari, 1996). Multiple efforts to construct a vaccine based on various bacterin preparations have failed to protect salmon from BKD (Wood and Kaattari, 1996). Later vaccine development work focused on a highly expressed surface protein (p57), but, p57 is immunosuppressive with leukoagglutinating and hemagglutinating properties (Wood and Kaattari, 1996). Development of a vaccine for BKD is further hindered by slow growth

and a poor challenge model (Press and Lillehaug, 1995). The only control for BKD is avoidance and routine broodstock screening.

1.3.4 Emerging diseases

Emerging pathogens are continually being identified as the cause of various fish diseases considered "new" to the scientific community. "New" pathogens are often identified when previously unrecognized diseases increase in incidence or cultured fish species are introduced to geographic regions previously foreign to them (Austin, 1999). *Flavobacterium* and *Flexibacter* spp. have emerged as a group of fastidious gram negative bacteria capable of inflicting extensive mortalities (losses up to 80%) in various salmonid species (Bernardet, 1997). Other bacterial pathogens identified as responsible for recent significant losses in salmonid aquaculture are: *Streptococcus difficilis*, *Aeromonas caviae*, *Moritella marina*, *M. viscosa*, *Vibrio logei*, and *Yersinia intermedia* (Austin, 1999).

Piscirickettsia salmonis, a gram negative obligate intracellular pathogen of salmonids, is the aetiological agent of salmonid rickettsial septicaemia (SRS). SRS was only recently described in Chile (Bravo and Campos, 1989), but SRS is now recognized as a highly significant disease. *P. salmonis* has been isolated in Canada, Norway and the United Kingdom since its initial isolation in 1989.

1.4 Salmonid rickettsial septicaemia

Chile is the world's second largest producer of farmed salmon (156,000 tonnes in 1999). Chile suffers annual losses from 3 predominant pathogens, all of which lack effective vaccines for their control: IPNV, *R. salmoninarum*, and *P. salmonis*. *P. salmonis* is

the most economically significant, uncontrolled bacterial pathogen in Chilean aquaculture and costs the industry in excess of US$150 million annually.

P. salmonis was first isolated from a moribund coho salmon (*Oncorhynchus kisutch*) in 1989 in the Puerto Montt region of Chile (Fryer *et al.*, 1990). *P. salmonis* is an obligate intracellular bacterial pathogen of salmonids and is the first rickettsia-like bacterium to be isolated from an aquatic poikilotherm. Very little research has been conducted on *P. salmonis* since its initial isolation. Only studies that have focused on the pathology of disease caused by *P. salmonis*, diagnostic methods for its detection, and its taxonomic placement have been published.

SRS was first described in 1989 as coho salmon syndrome and Huito disease when coho salmon stocks in saltwater net pen sites in the Puerto Montt region of Chile suffered mortality of unknown aetiology (Bravo and Campos, 1989). In May of 1989, coho salmon mortalities peaked at 90% at some sites and were 60% on average (Branson and Nieto Diaz-Munoz, 1991). To date, *P. salmonis* still causes 40-80% cumulative mortality in problem regions (Smith *et al.*, 1997). *P. salmonis* LF-89 (type strain) was first isolated in cell culture in a chinook salmon (*Oncorhynchus tshawytscha*) embryo cell line (CHSE-214) from the kidney of a moribund coho salmon in late 1989 (Fryer *et al.*, 1990). *P. salmonis* was confirmed as the aetiological agent of SRS upon its reisolation from an experimentally infected salmon (Cvitanich *et al.*, 1991). *P. salmonis* has since been isolated from Atlantic salmon (*Salmo salar*), chinook salmon, and rainbow trout (*Oncorhynchus mykiss*) (Cvitanich *et al.*, 1991; Garcés *et al.*, 1991). *P. salmonis* has also been observed in farmed salmon on both coasts of Canada (Brocklebank *et al.*, 1993; Jones *et al.*, 1998), Scotland, Ireland (Rodger and Drinan, 1993), and Norway (Olsen *et al.*, 1997). Thus *P. salmonis*

appears to be an emerging pathogen of salmonids with serious implications for the global aquaculture industry.

1.4.1 Morphologic characteristics of *P. salmonis*

P. salmonis is a gram negative, non-motile, rickettsia-like bacterium. *P. salmonis* cells are pleomorphic but generally coccoid ranging from 0.5-1.5 μm in diameter. *P. salmonis* cannot be grown on standard bacteriological media and must be grown in cell culture (Cvitanich *et al.*, 1991; Fryer *et al.*, 1990). Growth of *P. salmonis* is morphologically similar to members of the tribe *Ehrlichieae* growing within membrane bound cytoplasmic vacuoles of host cells (Fig. 1); this combined with its obligate intracellular nature was the basis of its preliminary classification as a member of the order *Rickettsiales* (Fryer *et al.*, 1990).

A variety of teleost derived cell lines, primarily from salmonids, support infection and growth of *P. salmonis* (Cvitanich *et al.*, 1991; Fryer *et al.*, 1990). *P. salmonis* is routinely grown on monolayers of cell line CHSE-214 at 15-18°C. *P. salmonis*-infected monolayers begin to display a cytopathic effect (CPE; Fig. 1) 4-6 days following infection with full CPE occurring within 11-15 days and *P. salmonis* titres reaching 10^6-10^7 50% tissue culture infectious dose ($TCID_{50}$)/ml (Fryer *et al.*, 1990). *P. salmonis* is capable of surviving and remaining infective in an extracellular state for over 14 days in a saltwater environment, but is almost instantly inactivated in freshwater (Lannan and Fryer, 1994).

1.4.2 Phylogenetic analysis of *P. salmonis*

Historically, all obligate intracellular bacteria were referred to as rickettsia and species were classified based on morphological, pathogenic, and antigenic characteristics (Roux

Figure 1. Micrographs of *P. salmonis*. **(A)** Phase contrast light microscopy of the cytopathic effect caused by *P. salmonis* on an infected CHSE-214 monolayer. Bar = 10 μm. **(B)** Light microscopy of a CHSE-214 monolayer infected with *P. salmonis*, 3 days post-infection. Bar = 10 μm. **(C)** Transmission electron microscopy of *P. salmonis* undergoing division (arrow) within a cytoplasmic vacuole of a CHSE-214 host cell. Bar = 1 μm. Adapted from Fryer *et al.* (1990).

and Raoult, 1997). The term rickettsiae is still defined as any obligate intracellular bacterium, but the advent of recombinant DNA technologies allowed refinement of rickettsial phylogeny and demonstrated that they encompass a diverse collection of bacteria both closely and distantly related. Thus, obligate intracellular bacteria that do not belong to the order *Rickettsiales* are still described as rickettsiae.

Phylogenetic studies using the 16S rRNA sequence of *P. salmonis* placed it within its own genus and species (Fryer *et al.*, 1992). The closest relative of *P. salmonis* was identified as *Coxiella burnetii* (Fryer *et al.*, 1992). Although *P. salmonis* morphologically resembles members of the tribe *Ehrlichieae*, 16S rRNA analysis placed the genus *Piscirickettsia* in the gamma subdivision of *Proteobacteria* while the order *Rickettsiales* lies within the alpha subdivision of *Proteobacteria*.

Subsequent phylogenetic studies of five *P. salmonis* isolates from Chile, Norway, and Canada using both 16S and 23S rRNA have confirmed the taxonomic placement of *P. salmonis* and further refined its position by adding it to the *Francisella* group of bacteria (Mauel *et al.*, 1999). Mauel *et al.* also showed that *Legionella pneumophila* (89.2% similar) is almost as closely related to *P. salmonis* as *C. burnetii* (89.5% similar).

1.4.3 Pathology of SRS

Clinical *P. salmonis* infection is not normally observed in salmon during the freshwater stage of their life cycle. Onset of SRS mortalities usually occurs 6-12 weeks after transfer of salmon to saltwater rearing pens (Fryer *et al.*, 1990). Stress is considered to be a major factor in triggering the onset of massive SRS mortalities with outbreaks often following smolt transfer, water temperature changes, algal blooms, and severe storms (Almendras and Fuentealba, 1997). Moribund salmon suffering from SRS are generally lethargic and

found swimming near the surface of the water in the corners of pens with a darkened body colour (Bravo and Campos, 1989; Cvitanich *et al.*, 1991). The most common symptom found in SRS infected salmon is pale gill colour (Fig. 2) suggesting anaemia, but this symptom is not diagnostic. Internally fish have lowered hematocrit values (also indicative of anaemia), swollen kidneys, enlarged spleens, and occasionally have grey mottled lesions on the surface of the liver (Fig. 2) (Bravo and Campos, 1989; Cvitanich *et al.*, 1991; Fryer *et al.*, 1990). In acute cases of SRS, death is the only gross sign of disease.

P. salmonis is observed in tissue smears of kidney, spleen, liver, muscle, skin, heart, blood, brain, ovaries, ovarian fluid, testes, intestines and gills in heavily infected fish, characteristic of the systemic nature of SRS (Cvitanich *et al.*, 1991). During the early stages of infection *P. salmonis* is primarily found infecting macrophages, later spreading to be found in endothelial cells of almost every organ.

Natural reservoirs of *P. salmonis* remain unknown. Although *P. salmonis* has been observed in several regions of the world, no common link other than the marine environment has suggested that a certain aquatic organism is responsible for transmission of *P. salmonis* to salmon. *P. salmonis* has been shown to be horizontally transferred in both freshwater and saltwater environments, likely by direct physical contact (Almendras and Fuentealba, 1997; Almendras *et al.*, 1997). Members of the order *Rickettsiales* are transmitted by arthropod vectors (Azad and Beard, 1998). Other rickettsia-like bacteria like *C. burnetii*, the closest relative of *P. salmonis*, are transmitted by aerosols (Maurin and Raoult, 1999).

C. burnetii exhibits a complex infection cycle that gives rise to spore-like forms that represent the extracellular form of the bacterium. These spore-like forms are metabolically inactive, and are resistant to desiccation and osmotic changes (Maurin and Raoult, 1999).

Figure 2. Gross pathology of SRS. Coho salmon infected with *P. salmonis*, note the ring-like lesions on the enlarged liver (A), enlarged spleen (B), and pale gills (C). Adapted from Fryer and Mauel (1997).

Although no spore-like phase has been observed for *P. salmonis* it is likely that it doesn't require a spore-like phase for extracellular survival because of its aquatic environment (Fryer and Mauel, 1997). Based on current evidence it seems quite probable that *P. salmonis* does not require a vector for transmission.

1.4.4 Control of SRS

When tested *in vitro*, *P. salmonis* LF-89 exhibits antibiotic sensitivity to streptomycin, gentamycin, tetracycline, cloramphenicol, erythromycin, oxytetracycline, clarithromycin, and sarafloxycin (Cvitanich *et al.*, 1991; Fryer *et al.*, 1990). *P. salmonis* LF-89 is resistant to penicillin, penicillin G, and spectinomycin *in vitro* (Almendras and Fuentealba, 1997). In practice, heavy antibiotic treatment of *P. salmonis* infected farmed salmon has had unpredictable results and there are reports of antibiotic resistant *P. salmonis* strains emerging (Almendras and Fuentealba, 1997). Broodstock salmon are often intraperitoneally injected with antibiotics and antibiotics are added to water during egg hardening in an effort to control potential vertical transmission (Almendras and Fuentealba, 1997).

The only hope for an effective control strategy of *P. salmonis* relies on the development of an efficacious vaccine. At present, no efficacious vaccines against *P. salmonis* are commercially available. A study using formalin inactivated bacterin preparations of *P. salmonis* reported induction of a minor protective immune response in vaccinated coho salmon, but challenge pressure was low at the field sites used in the study and some vaccinated groups experienced higher cumulative mortalities than unvaccinated control fish (Smith *et al.*, 1997).

1.5 Current state of vaccinology in salmonid aquaculture

Vaccines are the most effective and inexpensive method of prophylaxis for aquaculture. Presently, every smolt in Norway receives at least one vaccine injection before going to sea. As vaccine usage has increased, a direct decrease in antibiotic usage has occurred.

Aquaculture's interest in vaccination as an alternative to chemotherapy began in the mid-1970's as interest in marine fish farming increased. The popularity of antibiotic chemotherapy was waning as the frequency of antibiotic resistant isolates and viral fish pathogens increased (Evelyn, 1997). From a fish health perspective, vaccination is preferable to chemotherapy because of its preventative rather than curative approach to disease control. Antibiotics are very expensive and only provide short term protection requiring multiple treatments while vaccines are capable of conferring long term protection from a single treatment (Ellis, 1985). Vaccines are also theoretically capable of controlling disease caused by any pathogen including viruses and parasites.

Vaccine development for aquaculture is complicated by the cost to the end user. Fish farmers vaccinate millions of smolts, so the cost per dose must be low and products must be reliable. Vaccines must also provide long term protection under intensive rearing conditions (Adams *et al.*, 1997). These considerations initially deterred vaccine companies from investing heavily in the research and development of aquaculture vaccines. Vaccines were only developed for diseases that were common to many fish producing countries and were easily controlled with simple bacterin vaccine preparations like those for diseases such as vibriosis, ERM, and furunculosis (Ellis, 1985; Evelyn, 1997). The complexity of vaccine development for recalcitrant fish pathogens is approaching that of current human vaccine development, therefore a larger cost burden from research and development

investment faces the industry as it attempts to sustain its growth by further controlling disease.

1.5.1 Routes of vaccine administration

Several methods of vaccine administration must be evaluated when developing a vaccine. These routes differ in the amount of stress and handling that fish must undergo and the amount of time and labour required.

Intraperitoneal injection is the most common method used for vaccination. Injection ensures that each fish receives an equal amount of vaccine and is the only method appropriate for the adjuvant formulated vaccines that dominate the vaccine market. Injection uses less vaccine than other methods, but is only suitable for fish over 10 g (Press and Lillehaug, 1995). Injection vaccination is labour intensive, requires anesthesia of the fish, and subjects fish to handling stress.

Vaccination by immersing fish for 20-30 sec in a bath of diluted vaccine is fast and minimizes stress to the fish (Press and Lillehaug, 1995). Immersion is well suited for small fish, with antigen uptake occurring primarily through the gills. Immersion uses more vaccine, and disposal of waste vaccine becomes an issue (Austin, 1984). Most vaccines currently available are less efficacious when administered by immersion.

Oral administration of vaccine is viewed as the ultimate choice for immunization of fish. Unfortunately, protection currently obtained from oral vaccination is poor and formulating feed with vaccine is expensive and consumes more vaccine that immersion and injection (Press and Lillehaug, 1995).

1.5.2 Bacterin vaccines

The first demonstration of a protective immune response in a salmonid species was by Duff (Duff, 1942) using chloroform-inactivated *A. salmonicida* as an immunogen in cutthroat trout (*Oncorhynchus clarki*). Approximately 30-40 years would pass before the salmonid immune response was further explored largely because of a preoccupation with antibiotics (Evelyn, 1997). The first commercially produced aquaculture vaccine was licensed in 1976 against ERM (Evelyn, 1997). Commercial vaccines are currently available against *A. salmonicida*, *V. salmonicida*, *V. anguillarum*, *V. ordalii*, and *Y. ruckeri* and are based on inactivated whole cell bacterin preparations formulated with adjuvant (Ellis, 1985). Bacterin preparations represent the simplest and traditional approach to vaccine development against fish pathogens (Austin, 1984; Winton, 1998). Bacterin vaccines are formulated with adjuvant to maximize immunogenicity, thereby limiting their administration to intraperitoneal injection. An immersion or oral vaccination strategy is a prerequisite for many viral diseases because they predominantly afflict juvenile fish too small for injection. Although bacterin vaccines were originally found to be very effective against several bacterial diseases, many diseases have since proven resistant to this simplistic approach to vaccine development (Austin, 1984).

1.5.3 Molecular approaches to vaccine development

The bacterin approach to early vaccine development was largely empirical and the result of a direct lack of knowledge regarding the pathogenic mechanisms of disease causing organisms (Austin, 1984). As the bacterin approach began to fail with subsequent salmonid pathogens it was realized that conditions favouring the generation of a protective immune response must be understood. Vaccine development must be more systematic

and should involve investigation of the pathogenesis of an organism within the context of the humoral and cellular components of the fish immune system (Austin, 1984; Evelyn, 1997).

Identification of virulence factors of a pathogen is considered an important step in modern vaccine development. Many virulence factors are protective antigens making them desirable components of recombinant vaccines. Identification of protective antigens has not proven an easy task with the observation that some organisms do not express certain virulence factors under standard *in vitro* conditions (Evelyn, 1997; Thornton *et al.*, 1993). The advent of recombinant DNA technologies has greatly aided in the development of vaccines against pathogens that have proven resistant to traditional approaches (Lorenzen, 1999). A recombinant DNA approach to vaccine development also allows the construction of elegant multivalent vaccines based on protective epitopes from two or more pathogens and the incorporation of molecular adjuvants and targeting components (Lorenzen, 1999). But, recombinant vaccines currently face ethical questions and must overcome regulatory hurdles before their full potential can be realized by the aquaculture industry.

Subunit vaccines

By definition subunit vaccines are based on a component of a pathogen that can elicit a protective immune response (Winton, 1998). In practice, purification of protective immunogens directly from an organism is labor intensive and cost prohibitive. Subunit vaccines are usually based on the expression of all or part of a gene encoding a protective antigen in a foreign bacterial, viral or eukaryotic expression system (Lorenzen, 1999). After initial research and development costs, subunit vaccines can be used to inexpensively produce large amounts of antigen by fermentation. The relative safety of subunit vaccines

is very high because no infectious agents are present during production. When choosing an expression system for production of subunit vaccines the nature of the pathogen must be taken into consideration. Expression systems using *Escherichia coli* are limited in their ability to correctly fold foreign proteins and are unable to glycosylate proteins, thereby potentially reducing the immunogenicity of certain proteins (Winton, 1998). In general, common expression systems also produce proteins at temperatures higher than fish pathogens normally grow which can also affect protein folding (Lorenzen and Olesen, 1997).

Gilmore *et al.* were the first to use a subunit vaccine approach to a fish pathogen when they expressed a fusion protein encoding a portion of the IHNV glycoprotein in *E. coli* and vaccinated rainbow trout (Gilmore *et al.*, 1988).

A baculovirus vector has been used to express the VHSV glycoprotein in insect cell lines to allow glycosylation (Lorenzen and Olesen, 1997). The recombinantly produced VHSV glycoprotein protected rainbow trout and elicited neutralizing antibodies when vaccinated by injection, but failed to protect when administered by immersion (Leong *et al.*, 1997). Baculovirus expression systems also generate undesirable amounts of new recombinant viral particles that are difficult to inactivate without compromising vaccine antigen.

Only one recombinant subunit vaccine has been licensed for aquaculture and is currently available in Norway for IPNV (Lorenzen, 1999). The recombinant IPN vaccine is administered by injection to smolts before going to saltwater and is based on the VP2 protein of IPNV produced in *E. coli* (Christie, 1997). It appears that improved protection against IPNV can be been obtained with a combination of recombinantly produced VP2,

VP3 and NS proteins, protecting 60% of vaccinated rainbow trout when administered by immersion (Winton, 1998).

Live recombinant vaccines

Live recombinant vaccines encompass avirulent strains of fish pathogens with defined genetic attenuations and avirulent bacterial and viral vectors capable of carrying and expressing recombinant DNA encoding protective antigens of fish pathogens (Lorenzen, 1999). Traditional methods of serial passage in culture and mutagenesis have been used to create attenuated vaccines for fish pathogens, but residual virulence in salmonids and virulence in feral fish species has often been a problem (Benmansour and de Kinkelin, 1997). Live vaccines are generally considered superior to subunit vaccines because they can be administered by immersion and their ability to replicate in fish is suspected to elicit a more robust humoral and cellular immune response (Winton, 1998).

Surface disorganized attenuated strains of *A. salmonicida* have been shown to elicit very strong protection in salmonids against furunculosis, which has traditionally been a difficult disease to control with bacterin vaccines (Thornton *et al.*, 1991). There are currently no avirulent vectors analogous to poxvirus systems in mammals that are capable of infecting and expressing recombinant DNA in fish. But, an avirulent strain of *A. salmonicida* lacking a 1.4 kbp region of the surface A protein gene has been used to express fragments of the VHSV and IHNV glycoprotein genes (Noonan *et al.*, 1995). Rainbow trout immunized by immersion with the live recombinant *A. salmonicida* strain were moderately protected against viral challenge (Noonan *et al.*, 1995).

There has been a reluctance to accept attenuated vaccine strains in aquaculture because of the release of live organisms into an aquatic environment and fear of reversion to virulent form. No live vaccines have been licensed for use in aquaculture.

Genetic vaccines

Genetic vaccines are based on the injection of plasmid DNA encoding genes of pathogens under the control of eukaryotic promoters into skeletal muscle of fish (Lorenzen, 1999; Winton, 1998). Production of antigen for presentation to the host immune system requires uptake and *in situ* expression of the plasmid-encoded gene by host cells (Lorenzen, 1999). This method of producing protein antigens directly in host cells allows appropriate folding and post-translational modification for the induction of antibodies specific to topographically assembled epitopes, and aids in the induction of cellular immune responses. DNA vaccines are capable of eliciting highly effective immune responses against bacterial and viral pathogens in higher animals (Donnelly *et al.*, 1997). Non-methylated bacterial DNA sequences also have an adjuvant effect in mammals which adds to the immunogenicity of DNA vaccines (Lorenzen, 1999). A DNA vaccine approach seems well suited to viruses and intracellular pathogens with presentation of protein antigens to the immune system in a manner similar to that in natural infections (Lorenzen, 1999). DNA vaccines are non-infectious, stable and relatively easy and inexpensive to produce on an industrial scale, but current delivery methods are impractical for field use in aquaculture.

Viral fish pathogens have largely been the focus of DNA vaccine research. DNA vaccines using the glycoprotein-encoding genes of VHSV and IHNV controlled by cytomegalovirus promoters protect high percentages of vaccinated rainbow trout (Leong *et al.*, 1997; Lorenzen *et al.*, 1998). Very little is currently understood about the function

of DNA vaccines in fish and information is lacking regarding the duration of protection. It will likely be many years before DNA vaccines are licensed for use in aquaculture. No DNA vaccine has been licensed for any veterinary application to date. But, concerns regarding use of DNA vaccines in aquaculture will likely ease as DNA vaccines are developed and licensed for other animals.

Future of recombinant vaccines in aquaculture

The ideal fish vaccine should elicit strong, long lasting immunity, without side effects and without induction of a carrier state, while remaining inexpensive and easy to administer. Economically significant diseases that are geographically widespread will likely continue to receive greater attention for vaccine development because the high development costs associated with recombinant vaccines will probably continue to be borne by the developer and not the end user. A shift toward vaccines based on recombinant DNA technology is inevitable in aquaculture as traditional approaches repeatedly fail against emerging diseases. Serious questions regarding the ethical and environmental implications of widespread use of attenuated strains of pathogens and DNA vaccines will have to be addressed before these promising technologies are granted licenses by regulatory agencies for use in aquaculture.

1.6 The fish immune system

Immunity is considered to involve two major systems: the innate system and the adaptive immune system (Warr, 1997). Innate immunity is ancient in origin, while the adaptive immune system is only observed in vertebrates above agnathan (jawless) fish (van Muiswinkel, 1995; Warr, 1997). Fish appear in the fossil record 350-400 million years ago

and are the oldest animal group that possess an immune system with characteristics similar to that of birds and mammals (van Muiswinkel, 1995). Although many aspects of the fish immune system remain uncharacterized, sufficient information is available to conclude that fish have the basic mechanisms and molecules possessed by immune systems of higher vertebrates (Warr, 1997). But, similarities to bird and mammalian immune systems do not imply that fish have immune responses like those of birds and mammals. A major difference between fish and higher vertebrates is the poikilothermic nature of fish. The fish immune system is strongly influenced by environmental temperature. Immune responses mediated by T helper cells, and the cytotoxic activity of non-specific cytotoxic cells in salmonids is delayed at temperatures below 4°C (Le Morvan *et al.*, 1998).

1.6.1 Innate immunity

Fish have an array of innate defense mechanisms similar to those of other vertebrates. Epithelial barriers and secretions represent the most significant physical barriers for fish. Maintaining the integrity of the epithelial barrier is essential to prevent entry of invading microorganisms and to maintain osmoregulation (van Muiswinkel, 1995). Fish epithelial barriers are covered with a mucous layer that contains lysozyme, immunoglobulin (Ig), and complement factors (Press and Lillehaug, 1995; van Muiswinkel, 1995).

A variety of serum factors found in the innate immune systems of other vertebrates are also present in fish (van Muiswinkel, 1995). Transferrin minimizes the availability of bloodstream iron to invading microorganisms. Fish possess a variety of lytic enzymes, including lysozyme, enzyme inhibitors, and a fully functional complement cascade which can be initiated via classical and alternate pathways. Virus-infected cells produce interferon. Lectins and C-reactive protein participate in the inactivation of microorganisms and

enhance their phagocytosis. C-reactive protein is also capable of activating complement in a fashion similar to Ig.

The fish innate immune system is capable of eliciting an acute inflammatory response to infection and tissue damage characterized by rapid infiltration of neutrophils and macrophages to the site of infection or damage (van Muiswinkel, 1995). Phagocytosis of antigen by macrophages and neutrophils is an important aspect of the innate immune system and is also capable of initiating the adaptive immune response. Fish also have non-specific cytotoxic cells that display a non-induced lytic activity similar to natural killer cells in birds and mammals (Nakanishi *et al.*, 1999).

1.6.2 Lymphoid cells and organs of the adaptive immune system

The adaptive immune system is orchestrated by lymphocytes and is capable of remarkable specificity and immunological memory. Fish lack certain primary lymphoid organs of higher vertebrates: lymph nodes, bone marrow, and the Bursa of Fabricius of birds (Warr, 1997). Many of the classical studies that allowed demonstration of discrete lymphocyte subpopulations in birds in mammals are not currently possible with fish for lack of an isogenic strain of any species (Warr, 1997). Primary lymphoid organs of fish are the thymus, head kidney (pronephros), and spleen (van Muiswinkel, 1995; Warr, 1997).

Fish have a well differentiated thymus and are capable of mounting a cell-mediated immune response involving thymus derived lymphocytes. Thus far, fish T cells have only been demonstrated by physiological properties and as lymphocytes lacking surface Ig (sIg^-) (Partula, 1999). The presence of a lymphocyte population equivalent to mammalian T cells is supported by the proliferative response of sIg^- cells to concanavalin A (Partula, 1999). In mixed leukocyte reactions sIg^- lymphocytes proliferate with monocytes as accessory cells

(Miller *et al.*, 1986) and sIg$^-$ lymphocytes are required for anti-hapten humoral responses to T-dependent antigens (Miller *et al.*, 1985). Fish also display allograft rejection and delayed type hypersensitivity (Partula, 1999). Despite considerable efforts, monoclonal antibodies have not been successfully generated against T cell specific markers, and it is still not possible to distinguish subpopulations of sIg$^-$ lymphocytes (Partula, 1999).

1.6.3 The humoral immune response

The humoral immune response is the most studied aspect of the fish immune response. Teleost fish display typical primary and secondary humoral immune responses to antigen with peak numbers of Ig-producing cells observed in the kidney and spleen (van Muiswinkel, 1995). The temporal nature of the fish humoral immune response is influenced by environmental temperature, dose, and type of antigen (van Muiswinkel, 1995). Generally, teleost humoral immune responses are slower to develop and lower in magnitude than mammalian responses. Secondary humoral responses show increased Ig titres and an accelerated response to antigen (Arkoosh and Kaattari, 1991; Houghton *et al.*, 1992). The increase in magnitude of secondary responses over primary responses is not as dramatic as in mammals, with Ig titres usually increasing only 10-20 fold (Arkoosh and Kaattari, 1991). A memory humoral response has been attributed to a direct increase of the population of antigen specific B cell precursors (Arkoosh and Kaattari, 1991).

Fish have a single class of Ig, a tetrameric molecule with heavy and light chains, that is referred to as IgM because of its polymeric structure resembling mammalian IgM (Press and Lillehaug, 1995; van Muiswinkel, 1995). The genetic organization of Ig genes in teleost fish resembles that of mammals with V, D, J, and C regions (Ghaffari and Lobb, 1991). Teleosts are capable of generating a diverse repertoire of antibodies using

combinatorial diversity of V, D, and J segments, junctional imprecision, and insertion of P and N nucleotides (Warr, 1997). Interestingly, teleosts lack affinity maturation of antibodies during secondary humoral responses. Both isotype switching and hypersomatic mutation do not occur in teleosts (Arkoosh and Kaattari, 1991; van Muiswinkel, 1995; Warr, 1997). Generally, humoral immune responses in teleosts are of lower affinity and specificity than in mammals (Warr, 1997).

1.6.4 Major histocompatibility complex molecules

Structure and function of teleost MHC molecules has largely been inferred from expressed genes (Stet *et al.*, 1996). Advent of the polymerase chain reaction (PCR) has allowed the identification of teleost major histocompatibility complex (MHC) genes using oligonucleotides based on conserved regions of mammalian MHC genes (Stet *et al.*, 1996; Warr, 1997). A variety of MHC molecules have been identified in more than 25 species of teleosts and elasmobranchs: MHC class I-α chain, β_2-microglobulin, MHC class II-α and -β chains, and TCR-α and -β chains (Stet *et al.*, 1996).

A lack of monoclonal antibodies against fish T cell markers has hampered demonstration of functional antigen presentation by MHC class I and II complexes. But, high level expression of functional mRNA transcripts from MHC class II genes has been detected in the head kidney, spleen, and hind gut of Atlantic salmon following vaccination (Koppang *et al.*, 1998). Although teleost β_2-microglobulin has not been confirmed to associate with MHC class I, coordinate upregulation of β_2-microglobulin and MHC class I expression has been observed in rainbow trout gonad cell lines infected with IHNV (Nakanishi *et al.*, 1999).

MHC class I molecules can be divided into classical (class Ia) and non-classical (class Ib). In mammals, MHC class Ia genes are highly polymorphic, encoded by only a few loci, and are expressed in all nucleated cells (Nakanishi *et al.*, 1999). MHC class I genes have been identified in a number of teleost species including rainbow trout, Atlantic salmon, and pink salmon (*Oncorhynchus gorbuscha*). Studies of MHC polymorphism in teleosts is still in its infancy because of difficulties associated with discerning loci of genes from alleles and the lack of an isogenic strain of fish (Stet *et al.*, 1996). It seems reasonable to assume that MHC molecules play an antigen-presenting role in the fish immune system analogous to mammalian MHC molecules.

1.6.5 T cell receptors

Although monoclonal antibodies against teleost TCR's have proven to be difficult to make, a fair amount is known regarding the genetic organization and diversity of TCR genes in both rainbow trout and Atlantic salmon. TCR-α and -β genes have been described in a number of teleost fish, but TCR-γ and -δ genes have only been identified in cartilaginous fish (elasmobranchs) (Partula, 1999). Similar to current knowledge regarding teleost MHC molecules, structure and function of teleost TCR's has been inferred from gene sequences. The basic structure of the teleost TCR consists of two extracellular Ig-like domains (variable and constant), a transmembrane segment, and a short cytoplasmic domain (Partula, 1999). The most notable difference between the teleost and mammalian TCR is the shorter constant domain of the teleost TCR. Three complementarity determining regions (CDR) have been identified in fish TCR's. CDR1 and 2 have limited diversity and occur within the V gene segment, and CDR3 displays greater diversity and occurs at the V-J and V-D-J junctions of α and β chains, respectively (Partula, 1999). In mammals, CDR1

and 2 appear to contact the MHC molecule at the ends of the peptide binding region and CDR3 of TCR-α and -β chains interact with the MHC-bound peptide (Garcia *et al.*, 1996). Mechanisms used to generate TCR diversity have been studied extensively in both rainbow trout and horned shark (*Heterodontus franscici*) and both species exhibit considerable TCR variability (Partula *et al.*, 1996). Combinatorial diversity of TCR genes is obtained through somatic rearrangement of V and J and V, D, and J gene segments for TCR-α and -β genes respectively (Partula *et al.*, 1996). A large number of genetically distant V and J gene segment families have been identified in rainbow trout: 4 Vβ families with less than 39% amino acid identity, 6 Vα families (27-55% aa identity), 10 Jβ families, 32 Jα families, and only one Dβ family (Partula *et al.*, 1996). Junctional diversity occurs at V-J, V-D, and D-J junctions and random addition of nucleotides also contributes to diversity, particularly to CDR3 (Partula *et al.*, 1996). Finally, TCR diversity is multiplied by the pairing of TCR-α and -β chains (Partula *et al.*, 1996).

1.6.6 Lymphocyte accessory molecules

Increasing evidence indicates that fish lymphocytes use molecular mechanisms similar to those of mammals to mediate interactions between lymphocytes and signal transduction (Warr, 1997). Accessory molecules CD3, CD4, and CD8 remain to be identified in fish. Regions of the teleost MHC class I molecule, TCR, and surface Ig receptor of B cells contain conserved amino acids and motifs known to interact with accessory molecules to form fully functional receptor complexes in mammals (Stet *et al.*, 1996). Recent analysis of a leukocyte cDNA library constructed from Japanese flounder (*Paralichthys olivaceus*) undergoing a viral infection lead to the cloning and identification of several cytokines (Nam *et al.*, 2000). Further identification, and biochemical and genetic characterization of

accessory molecules and cytokines is required to help determine their role in modulating the fish immune response.

1.7 Summary

Despite the massive annual economic impact of *P. salmonis* on salmonid aquaculture, SRS remains a largely uncontrollable disease. Chemotherapy and vaccination with bacterin preparations of *P. salmonis* have resulted in incomplete and variable control of SRS. Development of an efficacious vaccine against this obligate intracellular pathogen is a priority. A recombinant DNA approach appears to be the only logical and viable option for the development of an efficacious *P. salmonis* vaccine.

CHAPTER 1

PURIFICATION AND ANTIGENIC CHARACTERIZATION OF *P. SALMONIS*

Introduction

Inherent difficulties accompany the investigation of obligate intracellular bacteria; rickettsiae have poorly defined genetic systems (Mallavia, 1991), and grow slowly in cell culture making them difficult to produce cells *en masse* and separate from host cell material. Therefore, it is impractical to obtain purified antigens from rickettsiae for molecular characterization studies and vaccine preparation (Schuenke and Walker, 1994). An efficient purification protocol is an essential prerequisite to the molecular research of any obligate intracellular microorganism. Historically, characterization of rickettsial pathogenesis and the discrimination of rickettsial antigens has largely relied upon antibody inactivation studies of rickettsial surface proteins (Li and Walker, 1998; Messick and Rikihisa, 1994; Seong *et al.*, 1997b). Recombinant DNA technologies have since allowed a variety of rickettsial genes to be cloned and characterized (Anderson, 1990; Andersson *et al.*, 1998; Ching *et al.*, 1998; Dunbar and Winkler, 1997; Emelyanov, 1993; Emelyanov and Demyanova, 1999; Hahn *et al.*, 1993; Li and Walker, 1998; Matsumoto *et al.*, 1996; Schuenke and Walker, 1994; Seong *et al.*, 1997a). Studies conducted using recombinantly produced rickettsial antigens have shown protection against rickettsial infection, thereby establishing a research strategy that can be applied to vaccine development against other obligate intracellular bacteria (Emelyanov, 1993; Gilmore *et al.*, 1989; Hickman *et al.*, 1993; Mahan *et al.*, 1998; Musoke *et al.*, 1996; Sumner *et al.*, 1995; Vishwanath *et al.*, 1990).

A protocol for the purification of *P. salmonis* from infected CHSE-214 monolayers was devised using a combination of differential and Percoll density gradient centrifugation. Immune sera from rabbits immunized with purified, whole cell *P. salmonis* reacted with 7

major protein antigens and one carbohydrate antigen. The carbohydrate antigen appears to be core-region lipooligosaccharide (LOS) of bacterial LPS. Indirect immunofluorescence microscopy, immunogold transmission electron microscopy (TEM), and biotin labeling of intact *P. salmonis* cells suggest that the immunoreactive antigens identified with rabbit antisera are surface exposed.

Materials & Methods

Bacterial strains, cell lines, and growth

Type strain *P. salmonis* LF-89 was obtained from the American Type Culture Collection (ATCC VR-1361) and is herein referred to as *P. salmonis*. *P. salmonis* was routinely passaged on CHSE-214 (ATCC CRL-1681) monolayers, at 15-18°C, using a 10% v/v inoculum of *P. salmonis* cell culture supernatant. Freezer stocks of *P. salmonis* were made from infected CHSE-214 cell culture supernatants with dimethylsulfoxide added as a cryopreservative (10% v/v).

CHSE-214 monolayers were grown in cell culture flasks that varied in surface area from 25-6,320 cm^2 (Nalge Nunc International). CHSE-214 cells were grown in minimal essential media (MEM) Auto-Mod (Sigma) with Earles salts supplemented with heat inactivated newborn bovine calf serum (10% v/v), L-glutamine (2 mM), HEPES (10 mM), and sodium bicarbonate (0.2% v/v). CHSE-214 monolayers were maintained by treatment with 0.5% tryspin-EDTA (Sigma) and split at a 1:3 ratio with fresh MEM.

For purification purposes, *P. salmonis* was grown in Nunc cell factories (6,320 cm^2; Nalge Nunc International) seeded with cell line CHSE-214 in 1 L of MEM. CHSE-214 monolayers in Nunc cell factories were infected with 400 ml of cell culture supernatant

from fully disrupted CHSE-214 monolayers infected with *P. salmonis*. *P. salmonis* infection was allowed to progress at 15-18°C until CPE disrupted the entire monolayer, usually within 14-17 days.

Determination of *P. salmonis* titres

Falcon 24 well cell culture plates (Becton Dickinson) were seeded with CHSE-214 cells and allowed to achieve confluency for 2 days at 18°C. *P. salmonis* samples were serially diluted 10^{-1}-10^{-7} in incomplete MEM (no serum). Media was removed from the CHSE-214 monolayers. *P. salmonis* samples (100 µl) of each dilution were added to wells of CHSE-214 monolayers in replicates of 4 and allowed to adhere for 1 h at 15°C. MEM (2 ml) was added to each well and plates were covered and sealed with tape. *P. salmonis* dilution series plates were incubated at 15°C until CPE was clearly evident, usually within 8-11 days. The number of *P. salmonis* infected and uninfected wells at each dilution were recorded and the protocol of Reed and Muench (Reed and Muench, 1938) was used to calculate the $TCID_{50}$ of the *P. salmonis* sample.

Standard rickettsial plaque assays using semisolid methyl cellulose overlays were also used to determine *P. salmonis* titres (Wike *et al.*, 1972). *P. salmonis* suspensions were serially diluted 10^{-1}-10^{-7} in incomplete MEM and allowed to adhere to confluent CHSE-214 monolayers in Falcon 6 well cell culture plates (Becton Dickinson) for 1 h. Following adherence of *P. salmonis*, monolayers were overlaid with 5 ml of methyl cellulose MEM (1% w/v methyl cellulose). Cell culture plates were incubated at 15°C until individual plaques of CPE were evident in the monolayer, usually within 5-8 days. *P. salmonis* titres were determined by direct plaque counts.

Purification of *P. salmonis*

A protocol for purifying *P. salmonis* was developed by combining and modifying the protocols of Tamura (Tamura *et al.*, 1982) and Weiss (Weiss *et al.*, 1975). For purification purposes *P. salmonis* was grown on CHSE-214 monolayers in Nunc cell factories until full CPE was observed. Prior to collecting *P. salmonis* infected cell culture supernatant from the cell factory, remaining adherent host cells were dislodged by gently shaking the flask for 5 min. Approximately 1.4 L of cell culture supernatant was collected and centrifuged at 10,000 × *g* for 30 min at 4°C in 6 × 250 ml centrifuge bottles using a type JA-14 rotor and Beckman J-21C centrifuge (Beckman). Supernatant was discarded and each pellet was resuspended in MEM (5 ml). Remaining intact CHSE-214 cells were disrupted using a Wheaton 40 ml Dounce tissue homogenizer (VWR). The homogenized suspension was centrifuged at 200 × *g* for 10 min at 4°C in a JA-20 rotor (Beckman) to pellet large host cell debris.

Supernatant was collected and centrifuged at 17,600 × *g* for 15 min at 4°C. Supernatant was discarded and the pellet was resuspended in 12 ml of TS-buffer (33 mM Tris-HCl, 0.25 M sucrose; pH 7.4). Samples (2 ml) were loaded onto 6 self-forming 40% continuous Percoll (Amersham Pharmacia) gradients (10.4 ml stock Percoll solution, 13.6 ml TS-buffer) and centrifuged in a fixed angle rotor (type JA-20) at 20,000 × *g* for 60 min at 4°C. Bands were collected by aspiration and the *P. salmonis* containing band was identified by negative staining with 0.5% phosphotungstic acid (PTA) and analysis by TEM on a Phillips EM 300 at an accelerating voltage of 75 kV. The *P. salmonis* containing bands from the 6 density gradients were collected by aspiration and pooled. Purified *P. salmonis* was centrifuged at 100,000 × *g* in a type 50.2 fixed angle rotor (Beckman) for 2 h at 4°C to

remove Percoll from the *P. salmonis* sample. *P. salmonis* was collected with a syringe from the interface between the Percoll pellet and supernatant, diluted with phosphate buffered saline (PBS, pH 7.4) (Sambrook *et al.*, 1989) and centrifuged at 17,600 × *g* for 10 min at 4°C using a type JA-20 rotor. Supernatant was discarded and the *P. salmonis* pellet was washed and centrifuged using PBS two more times in a 1.5 ml Eppendorf tube. The purification routinely yielded a ~100 µl pellet volume of purified *P. salmonis*.

Antibody generation

Anti-*P. salmonis* rabbit serum was prepared against *P. salmonis* obtained from the ATCC and purified from mycoplasma-free CHSE-214 cells obtained from the ATCC. Percoll purified whole cells of *P. salmonis* were both formalin inactivated (5% formalin in PBS v/v) overnight at 4°C and killed by repeated freeze thaws. Two New Zealand white rabbits were administered ~250 µg of either formalin inactivated *P. salmonis* (82CR) or freeze thaw killed *P. salmonis* antigen (IPA) emulsified in Freund's complete adjuvant by subcutaneous and intramuscular injections. Prebleed serum was collected prior to the primary immunization. Rabbits were boosted with 230 µg of *P. salmonis* protein antigen emulsified in Freund's incomplete adjuvant at intervals 3 and 5 weeks after the primary immunization.

Sera were collected and titred by enzyme-linked immunosorbent assay (ELISA) (Engval and Perlmann, 1972) as previously described (Collinson *et al.*, 1991). Briefly, 1 µg per well of purified whole cell *P. salmonis* was used as antigen in 96 well flat bottom plates. Polyclonal rabbit antibody samples were serially diluted 1:500 through 1:1,024,000 in Tris-buffered saline with Tween (TBS; pH 7.4, 0.5% Tween-20) (Sambrook *et al.*, 1989) and incubated with antigen for 1 h at room temperature. Goat anti-rabbit IgG conjugated to

alkaline phosphatase (Caltag Laboratories) was used as the second antibody (dil. 1:4000). ELISA's were developed with *p*-nitrophenyl phosphate (Sigma) in diethanolamine buffer and absorbances were measured at 405 nm with a reference wavelength of 490 nm.

Immunofluorescence microscopy

Air-dried samples of purified *P. salmonis* were fixed in -20°C acetone/ethanol (60:40 v/v) for 20 min. Samples were incubated for 30 min with rabbit anti-*P. salmonis* serum (dil. 1:200 in PBS) then washed 3 × 5 min with PBS. Slides were incubated with a second antibody, goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC) (Caltag Laboratories) in PBS (dil. 1:40) for 45 min. Slides were washed 3 × 5 min with PBS. Preparations were observed using a photo-microscope equipped with an ultraviolet light source and excitation and barrier filters for fluorescein isothiocyanate.

Preparation of SDS-PAGE samples

Samples of cell lines were prepared for SDS-PAGE from 75 cm^2 monolayers. Medium was decanted and cells were collected using a cell scraper. Cells were washed with PBS twice and resuspended in 1 ml of PBS. SDS-PAGE sample buffer (4×) was added to the cell suspension at a ratio of 3:1.

P. salmonis SDS-PAGE samples were prepared from 75 cm^2 cultures of *P. salmonis* infected CHSE-214 cells. Cells that had remained adhered to the flask bottom were dislodged by gently striking the flask several times. *P. salmonis* and CHSE-214 cell suspensions were collected and homogenized with 10 strokes of a Wheaton 10 ml Dounce tissue homogenizer. Disrupted cell suspensions were centrifuged at 4°C for 10 min at 200 × *g* in a type JA-20 rotor (Beckman). Supernatant was collected and centrifuged at

17,600 × *g* for 10 min at 4°C. The pellet was resuspended in PBS and washed twice. Following the final centrifugation, the pellet was resuspended in 300 µl of SDS-PAGE sample buffer.

SDS-PAGE Analysis

Protein samples were separated by SDS-PAGE using a discontinuous SDS gel system following the protocol of Laemmli as modified by Ames (Ames, 1974; Laemmli, 1970). SDS-PAGE samples were boiled for 5 min prior to loading. Whole cell lysates of cell lines and *P. salmonis* were routinely analyzed using 12% polyacrylamide gels with 5% stacking gels.

Silver staining

SDS-PAGE samples were digested with 1 µl of stock proteinase K (PK) solution (1 mg/ml) per 10 µl of SDS-PAGE sample for 1 h at 60°C. PK digestion was stopped by boiling for 10 min. A 12-17% continuous gradient polyacrylamide gel was used for silver staining and immunoblot analysis of PK digested samples following the method of Fling and Gregerson (Fling and Gregerson, 1986). Silver staining followed the method of Tsai and Frasch (Tsai and Frasch, 1982).

Western blot analysis

Western blotting was carried out as previously described (Collinson *et al.*, 1991). Briefly, antigens were analyzed by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes (BioRad) using an LKB Multiphor II Electrophoresis System (Amersham Pharmacia). Membranes were blocked using skim milk TBS-Tween (5% w/v

skim milk). *P. salmonis* antigens were detected using rabbit anti-*P. salmonis* serum (dil. 1:2000) followed by goat anti-rabbit IgG conjugated to alkaline phosphatase (dil. 1:4000) (Caltag Laboratories). Immunoreactive antigens were visualized using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium.

Biotin labeling of surface proteins

Percoll purified *P. salmonis* cells and freshly harvested CHSE-214 cells were surface labeled with the impermeant modification reagent, sulfo-NHS-biotin (Pierce), and analyzed following the method of Dooley and Trust (Dooley and Trust, 1988). To minimize potential CHSE-214 host cell protein contamination of the *P. salmonis* preparation, purified *P. salmonis* cells were washed, centrifuged, and resuspended with PBS a minimum of 4 times before biotinylation was conducted.

Immunogold electron microscopy

Samples prepared for TEM were fixed with 2.5% glutaraldehyde and post-fixed with 1% osmium tetroxide in 0.2 M sodium cacodylate buffer. Fixed samples were dehydrated in a series of graded ethanol steps and propylene oxide and embedded in epoxy resin Epon 812. Thin sections of samples were mounted on formvar coated nickel grids. Osmium was destained from sections using 0.2% sodium metaperiodate. Sections were washed three times with water then blocked with PBS containing 3% bovine serum albumin. Sections were incubated with rabbit anti-*P. salmonis* serum 82CR (dil. 1:200), washed three times with PBS, then incubated with goat anti-rabbit IgG conjugated to 5 nm gold particles (dil. 1:50) (Caltag Laboratories) and then washed three times with PBS. Samples were fixed

briefly with 4% glutaraldehyde in PBS and stained with 1% uranyl acetate in 50% ethanol. Thin sections were viewed using a Phillips EM 300 at an accelerating voltage of 75 kV.

RESULTS

Growth of *P. salmonis*

CHSE-214 monolayers infected with *P. salmonis* began to show evidence of CPE within 4-6 days post-infection. To completely disrupt a CHSE-214 monolayer *P. salmonis* required 11-15 days. Typical growth of *P. salmonis* would cause an increase in the acidity of the culture, indicated by a colour shift of phenol red to an orange colour.

Determination of *P. salmonis* titres in a reproducible manner was best achieved using the method of $TCID_{50}$ (Reed and Muench, 1938). Standard rickettsial plaque assays produced highly variable results even when modified by the addition of semisolid overlays. *P. salmonis* routinely achieved titres of $10^{6.3}$ $TCID_{50}$/ml when grown using CHSE-214 monolayers.

Purification of *P. salmonis*

P. salmonis was separated from CHSE-214 debris by differential sedimentation and density gradient centrifugation. High purity *P. salmonis* was required to generate polyclonal rabbit serum with low cross reactivity to host cell material. A 40% Percoll density gradient gave the greatest separation of *P. salmonis* from CHSE-214 debris (Fig. 3A). PTA negative staining of the homogenized cell culture sample before density gradient centrifugation showed that it contained a large amount of cellular debris (results not shown) of either *P. salmonis* or CHSE-214 origin making the preparation unsuitable for generation of high

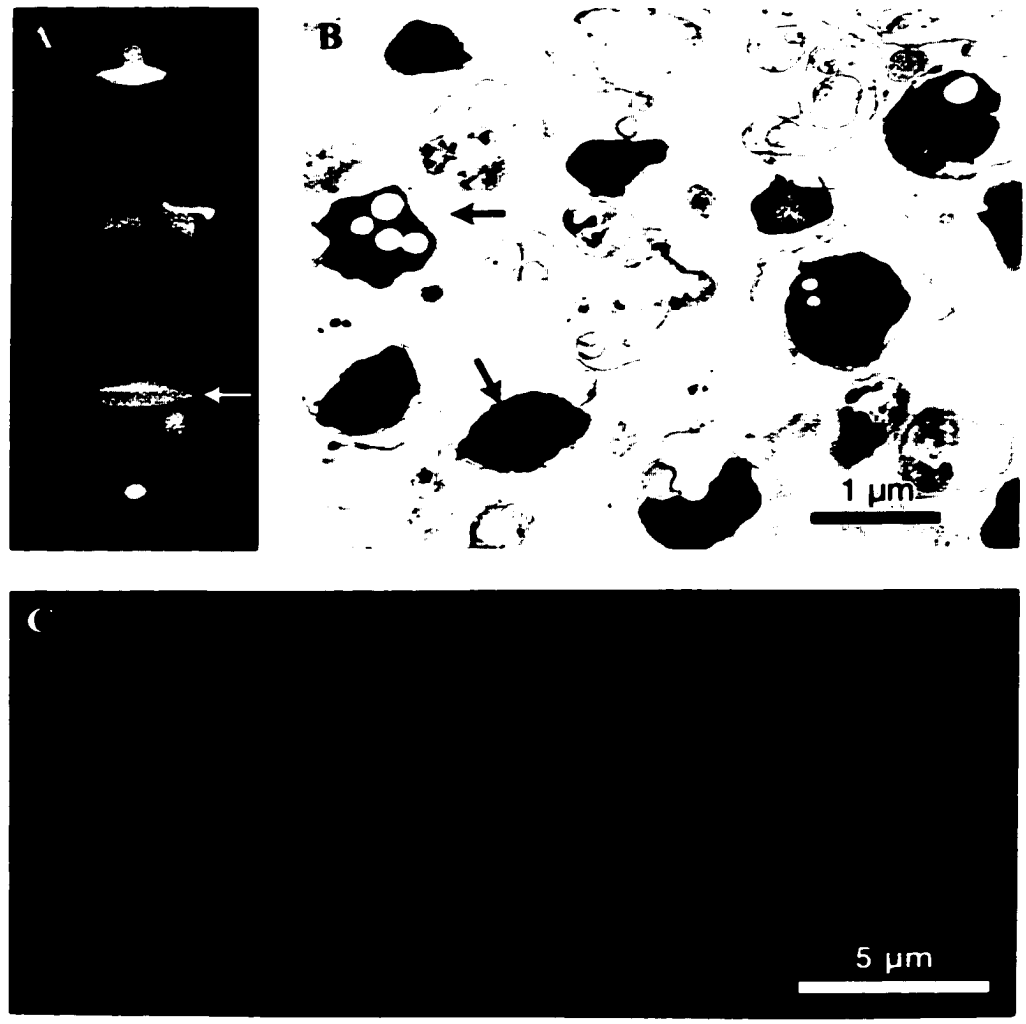

Figure 3. Percoll purification of *P. salmonis*. **(A)** Percoll density gradient separation of *P. salmonis* from CHSE-214 host cell debris. The arrow denotes the high density, *P. salmonis* containing band. **(B)** Thin sections of the high density band from the *P. salmonis* Percoll purification analyzed by TEM. Arrows point to both electron dense material that is likely *P. salmonis* and membranous vesicular material. Magnification × 15,800 (bar = 1 µm). **(C)** Indirect immunofluorescence microscopy of *P. salmonis*. The high density band collected from the Percoll density centrifugation of *P. salmonis* is shown. Antigen was detected with rabbit anti-*P. salmonis* serum. Goat anti-rabbit IgG conjugated to FITC was used as the second antibody. Magnification × 4,700 (bar = 5 µm).

specificity antiserum. Following density centrifugation, a minor well resolved low density band near the top of the gradient and a major diffuse higher density band in the lower half of the gradient were collected separately. PTA negative staining and TEM of thin sections demonstrated that the low density band was devoid of whole cell material that resembled *P. salmonis*. The high density band was mainly composed of material consistent with the size and morphology of *P. salmonis* and some less abundant vesicular material (Fig. 3B). The high density band containing whole cell *P. salmonis* was used to generate polyclonal rabbit antiserum against formalin inactivated (82CR) and freeze thaw killed (IPA) *P. salmonis*.

Immunofluorescence light microscopy using anti-*P. salmonis* 82CR rabbit serum was conducted on the high density band (Fig. 3C). The predominant immunoreactive material in the preparation were coccoid cells ranging from 0.7-1.2 μm in diameter consistent with the size and morphology of *P. salmonis*. The anti-*P. salmonis* 82CR rabbit serum also reacted with *P. salmonis* isolated from kidney of moribund salmonids suffering from SRS by immunofluorescence microscopy and immunoblot analysis (results not shown).

Surface exposed antigens of *P. salmonis*

In order to characterize the antigenic profile of *P. salmonis*, western blot analysis of *P. salmonis* was carried out using anti-*P. salmonis* rabbit serum (Fig. 4). PK digestion was used to determine if any immunoreactive antigens were carbohydrate. Rabbit anti-*P. salmonis* 82CR serum reacted with six *P. salmonis* immunoreactive antigens. Four antigens had relative mobilities ranging between 50-80 kDa and the remaining 2 antigens were 16 and ~11 kDa (Fig. 4). Anti-*P. salmonis* IPA rabbit serum recognized 4 major *P. salmonis* antigens (Fig. 4), with relative mobilities of 54, 27, 24, and ~11 kDa.

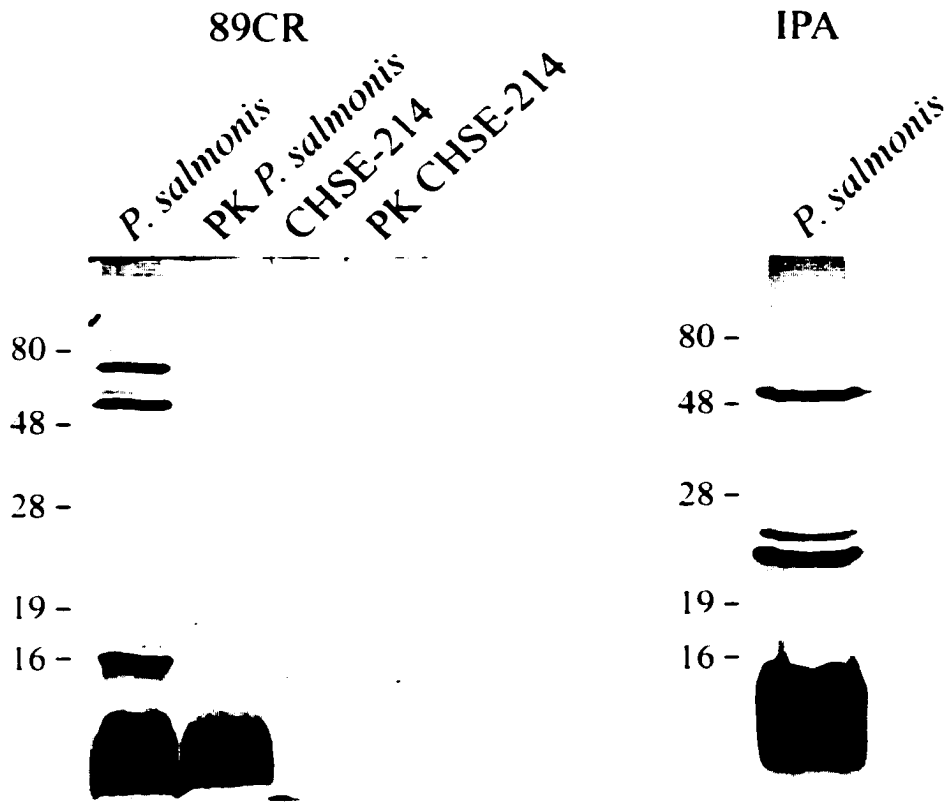

Figure 4. Western blot analysis of *P. salmonis*. Whole cell lysates and proteinase K digest samples of *P. salmonis* and CHSE-214 were separated by 12% SDS-PAGE and reacted with 89CR and IPA anti-*P. salmonis* rabbit sera followed by immunochemical detection. Both sera 89CR and IPA recognize different antigens of *P. salmonis*. The ~11 kDa antigen of *P. salmonis* was not susceptible to PK digestion. Molecular mass is on the left in kDa.

PK digestion destroyed all immunoreactive antigens except the 11 kDa antigen recognized by the 82CR serum (Fig. 4). Neither anti-*P. salmonis* sera cross-reacted with CHSE-214 proteins (Fig. 4) even when Western blots were allowed to develop overnight. Rabbit anti-*P. salmonis* serum also did not react with CHSE-214 host cells or salmonid kidney tissue by analysis of infected cells with immunofluorescence microscopy (results not shown).

Percoll purified *P. salmonis* and freshly harvested CHSE-214 cells were surface biotinylated using an impermeant alkylating reagent, sulfo-NHS-biotin, to help evaluate the purity of *P. salmonis* and determine if any immunoreactive proteins comigrated with major *P. salmonis* surface proteins. Biotinylated proteins were detected by streptavidin-conjugated alkaline phosphatase (Fig. 5). One major *P. salmonis* protein was clearly evident around 28 kDa and four major proteins between the 45 and 80 kDa ranges (Fig. 5), although several other minor proteins were also evident. These five major proteins were easily differentiated from CHSE-214 proteins. Control non-biotinylated samples of *P. salmonis* and CHSE-214 did not demonstrate any streptavidin binding.

Immunogold TEM of CHSE-214 cells infected with *P. salmonis* was conducted with anti-*P. salmonis* 82CR rabbit serum to determine if the antibodies reacted with antigens localized to the surface of *P. salmonis* cells. Rabbit anti-*P. salmonis* 82CR serum specifically labeled the surface region of *P. salmonis* (Fig. 6). Gold labeling of surrounding host cell membranous and cytoplasmic material was negligible.

Analysis of *P. salmonis* carbohydrate antigens

PK digested *P. salmonis* was silver stained to determine if *P. salmonis* has a lipopolysaccharide (LPS)-like component and what its characteristics are (Fig. 7). PK

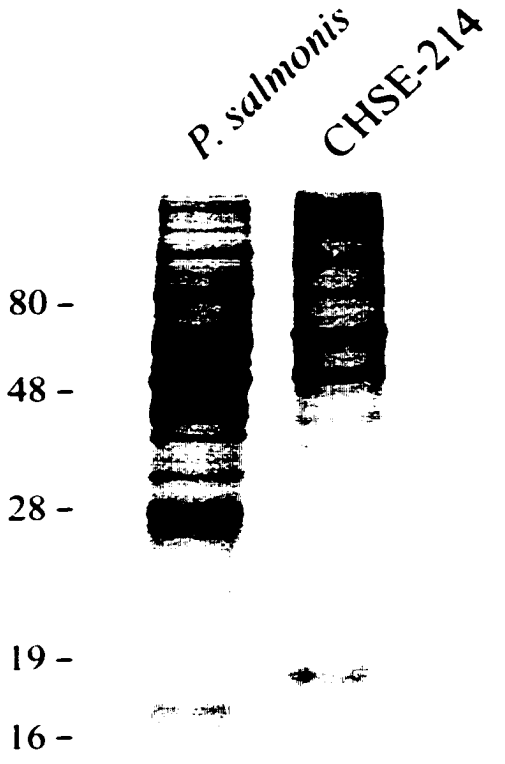

Figure 5. Analysis of surface exposed proteins of *P. salmonis*. Biotinylated *P. salmonis* and CHSE-214 proteins were separated by 12% SDS-PAGE and detected by streptavidin conjugated alkaline phosphatase. Note how the banding pattern of *P. salmonis* differs from CHSE-214 host cells. Molecular mass is on the left in kDa.

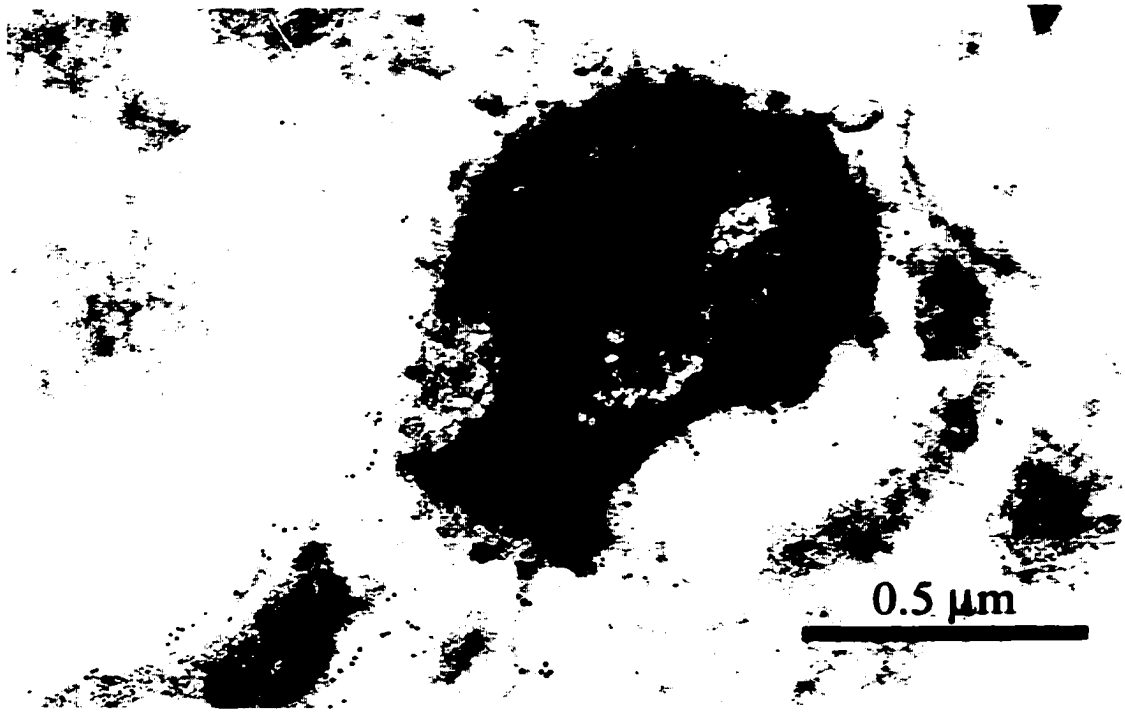

Figure 6. Immunogold stained TEM of *P. salmonis*. Thin sections of CHSE-214 cells infected with *P. salmonis* were reacted with rabbit anti-*P. salmonis* serum and labeled with goat anti-rabbit IgG conjugated to 5 nm gold particles. Magnification × 70,000 (bar = 0.5 µm).

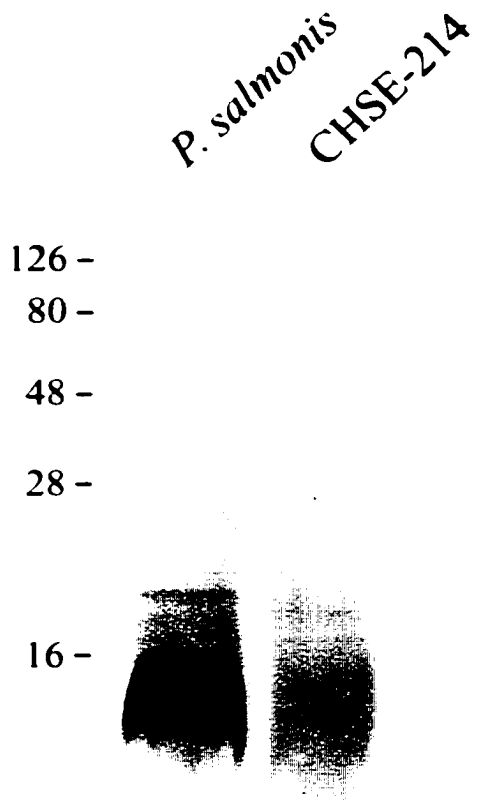

Figure 7. Silver staining of *P. salmonis* for bacterial LPS. PK digested *P. salmonis* and CHSE-214 samples were analyzed by 12-17% gradient SDS-PAGE. A faint banding pattern, that diminishes as molecular mass increases, is evident above the strongly stained lipooligosaccharide component of bacterial LPS in the *P. salmonis* sample. Molecular mass is on the left in kDa.

digested *P. salmonis* was analyzed by SDS-PAGE with a 12-17% polyacrylamide gradient. When silver stained for LPS, PK digested *P. salmonis* revealed a very faint, ladder-like banding pattern of carbohydrates ranging from 16 to 35 kDa in size with a discrete band around 20 kDa and an intensely stained major band around 11 kDa (Fig. 7).

Discussion

This chapter describes a purification protocol for *P. salmonis* from cell line CHSE-214 and the preliminary identification of likely surface exposed *P. salmonis* antigens. No previous reports have described the antigenic composition of *P. salmonis* since it was first isolated in 1989 (Fryer *et al.*, 1990). Past efforts to purify *P. salmonis* did not produce pure *P. salmonis* (Lannan *et al.*, 1991), thus previous attempts to generate high specificity polyclonal antiserum for antigen identification and the screening of genomic expression libraries have likely been unsuccessful.

Obtaining any rickettsial cells in amounts large enough to allow purification of proteins or preparation of vaccines has repeatedly proven to be extremely difficult and cost prohibitive. Inherent difficulties associated with the investigation of rickettsia-like bacteria has made the establishment of effective purification protocols essential. Morphological characteristics of *P. salmonis* growth resemble members of the tribe *Ehrlichieae* because of their growth within cytoplasmic vacuoles, unlike members of the tribe *Rickettsieae* which grow freely within the cytoplasm and nucleus of host cells (Rikihisa, 1991; Tamura, 1988). Rickettsiae tend to be very fragile and susceptible to osmotic changes (Schuenke and Walker, 1994; Tamura *et al.*, 1982), therefore a purification strategy must be relatively gentle.

Differential sedimentation and density gradient centrifugation were used to successfully separate *P. salmonis* from CHSE-214 host cell material (Fig. 3). Immunofluorescence and TEM of thin sections of purified *P. salmonis* showed that the preparation only contained material consistent with the size and morphology of *P. salmonis* and some vesicular material (Fig. 3). Material observed in thin sections of the *P. salmonis* preparation by TEM (Fig. 3) was consistent with material observed in Percoll purified preparations of *Orientia tsutsugamushi* (Tamura *et al.*, 1982). Vesicular immunoreactive material (Fig. 3C) most likely originated from *P. salmonis* because Western blot analysis using rabbit anti-*P. salmonis* sera demonstrated that the sera did not cross-react with CHSE-214 proteins (Fig. 4). Since rickettsiae are generally unstable when separated from their host cells, vesicular material might have been generated by plasmolysis and outer membrane blebbing of *P. salmonis* during the homogenization step of the purification. SDS-PAGE analysis of biotinylated proteins from purified *P. salmonis* cells also suggests that the purified cells are free of membranous CHSE-214 material (Fig. 5). If the vesicular material in the purified *P. salmonis* preparation was of CHSE-214 origin, some major biotinylated proteins would be common to both the *P. salmonis* and CHSE-214 samples. The observed banding patterns of major biotinylated proteins from *P. salmonis* and CHSE-214 differed significantly (Fig. 5). However, the multiplicity of minor biotinylated proteins suggests that some *P. salmonis* damage occurred during purification.

Rabbit antisera generated against either formalin inactivated purified *P. salmonis* (82CR) or freeze thaw killed purified *P. salmonis* (IPA) recognize a total of eight predominant antigens (Fig. 4). Both anti-*P. salmonis* 82CR and anti-*P. salmonis* IPA rabbit sera appear to recognize common and unique *P. salmonis* antigens with high specificity

(Fig. 4). Both anti-*P. salmonis* 82CR and IPA rabbit sera recognize antigens with relative mobilities of 54 and ~11 kDa. Anti-*P. salmonis* rabbit serum IPA, which was generated against *P. salmonis* at a later date than serum 82CR, recognizes a unique pair of antigens with relative mobilities of 29 and 24 kDa (Fig. 4).

Silver staining of PK digested *P. salmonis* confirmed that an ~11 kDa immunoreactive antigen was carbohydrate while the remainder were protein. The 11 kDa carbohydrate antigen is most likely the LOS component of LPS. LPS is a primary component of the outer lipid leaflet of outer membranes of rickettsiae and other gram-negative bacteria (Amano *et al.*, 1993). The chemical composition of spotted fever group rickettsiae LPS displays an enterobacteria-like banding pattern (Amano *et al.*, 1993). A faint repeating pattern of a high molecular weight carbohydrate species that decreased in abundance as its mass increased was present within the silver stained PK digested sample of *P. salmonis* (Fig. 7). The high molecular weight carbohydrates most likely represent core-region LOS plus O-antigen with a diminishing number of repeat units (Amano *et al.*, 1993). A carbohydrate banding pattern in which carbohydrate becomes less abundant as molecular weight increases is characteristic of random polymerization of O-antigen repeating units (Schnaitman and Klena, 1993).

Molecules involved in bacterial adhesion and invasion, and evasion and activation of the host immune response are often surface exposed and contribute to the overall virulence of an organism (Roth, 1988). Characterization of surface exposed proteins that lend to the molecular pathogenesis of *P. salmonis* is a crucial step in the development of a vaccine against *P. salmonis* as virulence factors are often protective antigens (Evelyn, 1997). Rabbit

serum was generated against purified *P. salmonis* to aid in the identification, and future cloning and characterization of *P. salmonis* antigens.

Anti-*P. salmonis* rabbit serum 82CR appears to recognize only surface exposed antigens of *P. salmonis*. Immunofluorescence of purified *P. salmonis* with rabbit anti-*P. salmonis* 82CR serum showed a large amount of fluorescence over the entire cell with noticeably brighter fluorescence around the cell perimeter which is characteristic of surface labeling (Fig. 3C). Surface biotinylation of purified *P. salmonis* labeled major surface proteins around the 16 and 50 kDa ranges which correlate well with the m.w. of the two major immunoreactive protein antigens recognized by Western blot at 16 and 51 kDa (Fig. 4). Immunogold TEM of *P. salmonis* infected CHSE-214 cells clearly demonstrated the high specificity of the anti-*P. salmonis* serum 82CR for surface localized antigens (Fig. 6).

Cloning and characterization of *P. salmonis* genes relies upon the ability to isolate genomic *P. salmonis* DNA free of host cell DNA. In the absence of protein and DNA sequence data, screening of *P. salmonis* genomic expression libraries will also require highly specific antiserum against *P. salmonis* for the identification of immunoreactive clones. *P. salmonis* was highly purified from CHSE-214 host cells using differential sedimentation and Percoll density gradient centrifugation. Rabbit antisera generated against purified *P. salmonis* recognized LOS, and 7 likely surface exposed proteins with relative molecular weights of 27, 24, and 16 kDa and 4 migrating between 50-80 kDa. *P. salmonis* LOS and LPS was observed to be predominantly low molecular weight, but less abundant high molecular weight species containing O-antigen were present.

CHAPTER 2

MISDIAGNOSIS OF *P. SALMONIS* ANTIGENS IN MYCOPLASMA INFECTED CELL LINES

Introduction

Mycoplasmas are common cell culture contaminants that can easily go undetected. The term mycoplasma encompasses four genera, *Mycoplasma*, *Acholeplasma*, *Anaeroplasma*, and *Ureaplasma*, that all belong to the class *Mollicutes* (Ruuth and Praz, 1989). Mycoplasmas are ubiquitous throughout nature and are small, coccoid prokaryotes that range from 0.3–1.0 μm in diameter and cause a variety of subclinical diseases in plant and animal hosts (Ruuth and Praz, 1989). Mycoplasmas lack a rigid cell wall preventing them from readily being removed from media and solutions by standard filter sterilization methods.

Recent work focusing on identification of proteins contributing to the pathogenesis of *P. salmonis* has been the subject of several publications (Barnes *et al.*, 1998; Jones *et al.*, 1998; Kuzyk *et al.*, 1996). *P. salmonis* infected fish cell lines were reported to display LPS typical of gram negative bacteria (Jones *et al.*, 1998). We demonstrate that such diagnostic patterns are erroneously derived from fish cell lines infected with mycoplasma, typically producing lipoglycans that differ from bacterial LPS. These results illustrate some of the difficulties that must be taken into consideration when researching an obligate intracellular pathogen and also emphasize the need for strict precautions to ensure that mycoplasma free cell lines are used for the cultivation of viruses and intracellular bacteria.

Growth of *P. salmonis*.

P. salmonis was cultured as described in Chapter 1. *P. salmonis* was also passaged using an epithelial carp cell line (EPC).

Isolation of *P. salmonis* from Atlantic salmon.

Atlantic salmon fry were experimentally infected with *P. salmonis* by 100 µl intraperitoneal injections of cell culture supernatant from a CHSE-214 culture infected with *P. salmonis* (~$10^{6.3}$ $TCID_{50}$/ml). *P. salmonis* was isolated in cell culture on a 25 cm^2 CHSE-214 monolayer from kidney tissue of a dead Atlantic salmon fry following the protocol of Lannan and Fryer (Lannan and Fryer, 1991).

Antibody generation

Polyclonal rabbit serum 79CR was generated against formalin inactivated *P. salmonis* that was purified from mycoplasma contaminated CHSE-214 cells using the Percoll density gradient centrifugation and rabbit immunization protocols described in Chapter 1.

Western blot analysis

SDS-PAGE, PK digestion of SDS-PAGE samples, and Western blot analysis was conducted as previously described in Chapter 1.

Polymerase chain reaction (PCR) and DNA electrophoresis

Mycoplasma were detected using mycoplasma specific primer sets supplied with the ATCC mycoplasma detection kit (cat. no. 90-1001K). Nested PCR was conducted using

the recommended protocol provided with the product. The detection principle is a two-step, nested PCR reaction targeted to a 16S-23S rRNA gene spacer region which differs in size, sequence and even repetitions between mycoplasma species. The kit uses appropriate sequence-specific primers to detect the presence of at least 20 of the most commonly occurring *Mycoplasma* and *Acholeplasma* species.

PCR products were analyzed by 1.5% agarose gel electrophoresis using a tris-acetate EDTA buffer system (Sambrook *et al.*, 1989). Samples and standards were detected by ethidium bromide staining.

Results & Discussion

Mycoplasma are responsible for a multitude of human diseases and have also been shown to infect both tench and trout, causing gill epithelial necrosis (Stadtlander and Kirchhoff, 1990, 1995; Stadtlander *et al.*, 1995). Mycoplasma are also insidious and common contaminants of cell cultures with incidences of contamination varying from 5-87%, and over 20 different species isolated (Chastel, 1995). Detection of mycoplasma contamination has often proven difficult because mycoplasma do not cause turbid growth or visibly affect cell line monolayers. Mycoplasma often cause covert infections of cell lines that can cause unreliable experimental results and unsafe biological products. Persistant mycoplasma contamination of viral and obligate intracellular bacterial stocks is a serious concern, therefore, it is imperative that cell lines used in research and in production work be tested frequently and sensitively (Chastel, 1995).

During preliminary work on the antigenic characterization of *P. salmonis* we observed a PK resistant, ladder-like immunoreactive pattern in our *P. salmonis* samples (Fig. 8)

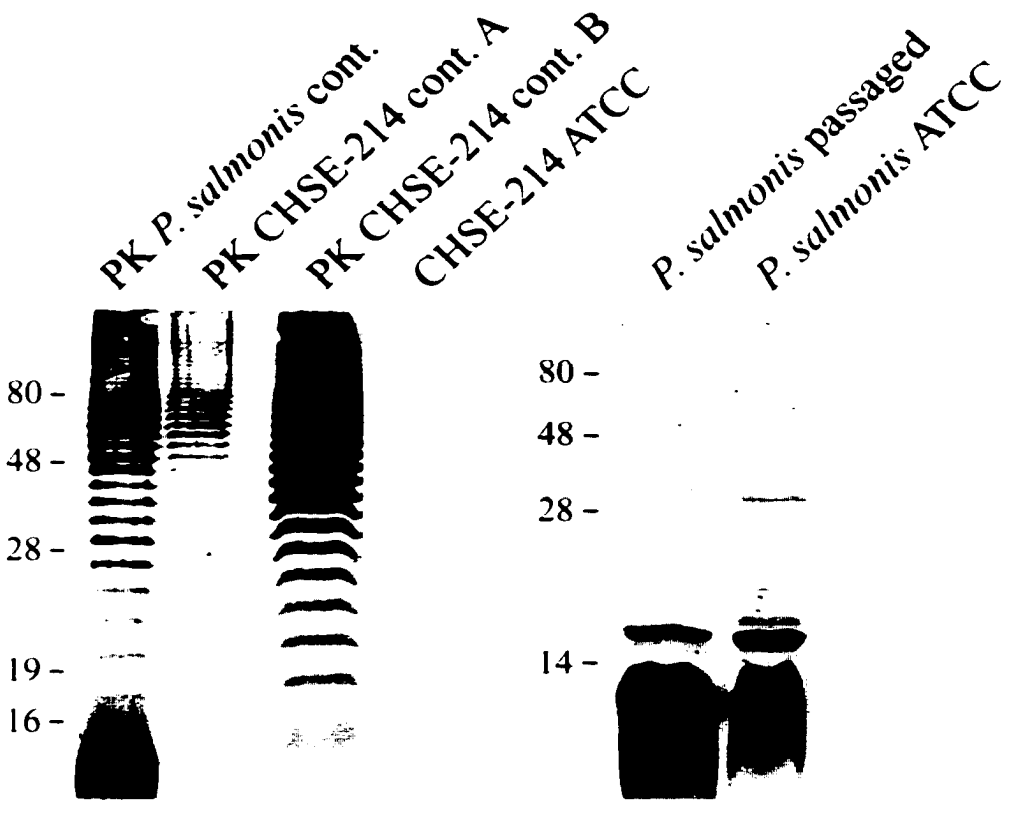

Figure 8. Western blot analysis of mycoplasma contaminated cell lines. All blots were developed with anti-*P. salmonis* 79CR rabbit serum. Contaminated CHSE-214 samples, A and B, contain an immunoreactive banding pattern similar to that in the *P. salmonis* infected CHSE-214 sample. Western blot analysis of contaminated CHSE-214 B was allowed to develop overnight and displays the entire ladder pattern indicating a high level contaminant. Samples in the first three lanes are PK digested. The ATCC CHSE-214 cells lack the contaminating immunoreactive banding pattern. *P. salmonis* passaged in an Atlantic salmon and certified *P. salmonis* from the ATCC grown on the ATCC CHSE-214 cell line also lack the banding pattern. Molecular mass is on the left in kDa.

not unlike that recently reported as a *P. salmonis* antigen (Jones *et al.*, 1998). A fainter, identical immunoreactive pattern was also observed in control samples of uninfected CHSE-214 (Fig. 8) and EPC (results not shown) cell lines. Subsequent Western blot analysis of fresh SDS-PAGE samples of CHSE-214 and EPC cell lines clearly indicated that both cell lines contained an immunoreactive pattern identical to the ladder-like pattern observed in *P. salmonis* samples (Fig. 8). The cell lines either contained a subclinical *P. salmonis* infection, or another contaminant microorganism.

A new sample of the CHSE-214 cell line was obtained from the ATCC. The CHSE-214 ATCC sample did not contain the immunoreactive pattern that was observed in the previous laboratory stocks of CHSE-214 and EPC (Fig. 8). When *P. salmonis* was isolated from the kidney of an experimentally infected Atlantic salmon mortality on new ATCC CHSE-214 monolayers *P. salmonis* isolate no longer contained the immunoreactive ladder-like antigen pattern (Fig. 8). Subsequently, a certified stock of *P. salmonis* LF-89 obtained from the ATCC also lacked the ladder-like immunoreactive pattern (Fig. 8) clearly indicating that the immunoreactive ladder-like pattern was not of *P. salmonis* origin.

Contaminated CHSE-214 and EPC cell lines were analyzed by PCR to determine if the cell lines were contaminated with mycoplasma (Fig. 9). *Mycoplasma* specific PCR products were amplified from all cell line samples that contained the contaminating immunoreactive ladder pattern (Fig. 9). Interestingly, the immunoreactive ladder pattern resembles the immunoreactive pattern of two different common mycoplasma antigens: lipoglycans and a variable surface antigen (V-1) (Simmons *et al.*, 1996; Smith, 1984; Watson *et al.*, 1989). Lipoglycans are surface exposed polysaccharides covalently bound to lipids and exhibit structural and physiological similarities to LPS from gram negative

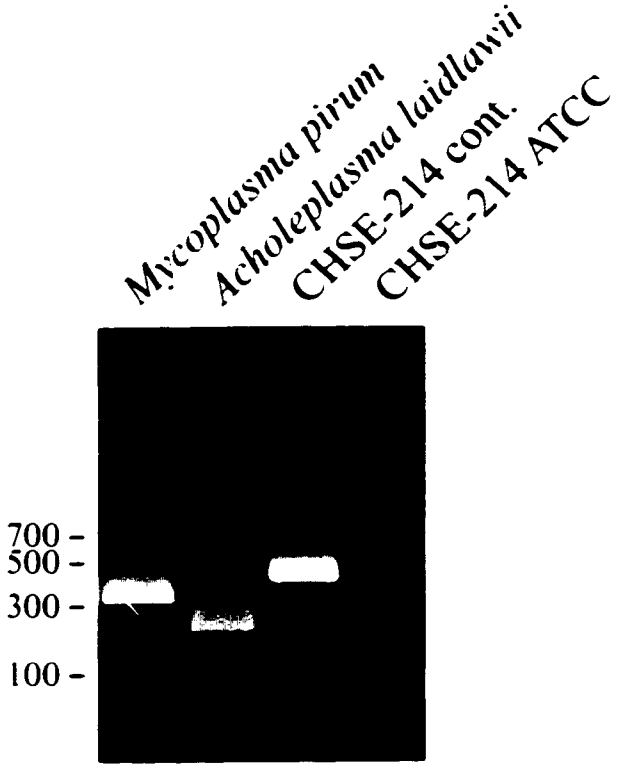

Figure 9. PCR detection of mycoplasma contamination. Contaminated and uncontaminated CHSE-214 cell lines were tested using an ATCC mycoplasma detection kit. *Mycoplasma pirum* and *Acholeplasma laidlawii* samples were run as positive controls producing DNA products with sizes of 323, 426, and 219 bp, respectively. The contaminated CHSE-214 sample tested positive for mycoplasma, producing a product approximately 420 bp in size. The ATCC CHSE-214 sample tested negative for mycoplasma contamination. Molecular mass standards are on the left in bp.

bacteria (Smith, 1984). Strikingly similar to the ladder-like pattern reported here and that observed by Jones is the pattern of the *Mycoplasma pulmonis* V-1 antigen (Jones *et al.*, 1998; Watson *et al.*, 1989). The *M. pulmonis* V-1 antigen displays a ladder-like pattern seemingly identical to the contaminant antigen found within the CHSE-214 and EPC cell lines (Simmons *et al.*, 1996; Watson *et al.*, 1989). Based on the similarity of the contaminating antigen to described mycoplasma antigens and the PCR detection of mycoplasma in cell lines containing the antigen, it appears that mycoplasma was the likely source of the contaminating immunoreactive pattern.

CHAPTER 3

CONSTRUCTION AND IMMUNOLOGICAL SCREENING OF A *P. SALMONIS* GENOMIC LIBRARY

Introduction

A renaissance in the identification of obligate intracellular pathogens as aetiological agents of many poorly understood diseases and as emerging pathogens has been made possible by the advent of recombinant DNA technology (Anderson, 1997, Azad and Beard, 1998, Azad *et al.*, 1997, Davis *et al.*, 1998, Fryer and Mauel, 1997, Stenos *et al.*, 1998). The first rickettsial gene sequences were only reported in 1987 (Anderson *et al.*, 1987, Wood *et al.*, 1987). A few novel rickettsial antigens have been identified and characterized further upon sub-cloning into *E. coli* (Anderson *et al.*, 1990, Anderson *et al.*, 1987, Carl *et al.*, 1990, Ching *et al.*, 1992, Ching *et al.*, 1996, Hahn and Chang, 1996, Musoke *et al.*, 1996). But, functional characterization of novel rickettsial genes has been hindered by the inability to introduce and exchange genetic material in rickettsiae (Mallavia, 1991). Exciting prospects for the future of research of rickettsiae have been raised with completion of the *Rickettsia prowazekii* genome sequence (Andersson *et al.*, 1998) and successful transformation of both *Rickettsia typhi* (Troyer *et al.*, 1999) and *R. prowazekii* (Rachek *et al.*, 1998).

In an effort to identify and clone *P. salmonis* antigens for use in protection studies, a small quantity of genomic DNA was isolated from purified *P. salmonis*, and used to construct a genomic DNA expression library. In the absence of available *P. salmonis* DNA sequence, anti-*P. salmonis* rabbit serum was used to identify immunoreactive clones. An immunoreactive 4,983 bp clone was identified encoding 1 partial and 4 intact predicted open reading frames (ORF's), representing the first non-ribosomal DNA sequence from *P. salmonis*. A 486 bp ORF, *ospA*, encoding a 17 kDa antigenic outer surface protein (OspA) had 62% aa sequence homology to a *R. prowazekii* genus common 17 kDa

outer membrane lipoprotein. Post-translational lipid modification of OspA in *E. coli* was confirmed. Codon usage of *ospA* was optimized to permit high level production of OspA in *E. coli* using an N-terminal fusion partner. A strong antibody response against OspA was detected in convalescent sera from coho salmon, thereby identifying OspA as a prospective recombinant vaccine candidate.

Materials & Methods

Bacterial strains, growth, and isolation

The description of the growth and purification of *P. salmonis* is described in Chapter 1.

E. coli DH5α, SOLR, XL1-Blue, XL1-Blue MRF' (Stratagene), and BL21 (Amersham Pharmacia) were used for general cloning and lambda phage propagation. *E. coli* was routinely grown using either LB, Terrific Broth (TB), or MacConkey media (Sambrook *et al.*, 1989). When required, supplements were added to media at the following concentrations: ampicillin (Ap), 100 μg/ml; chloramphenicol (Cm), 28 μg/ml; kanamycin (Kn), 28 μg/ml; maltose, 0.2% (w/v); $MgSO_4$, 10 mM; X-gal, 40 μg/ml; IPTG, 1 mM; glucose, 1% (w/v).

E. coli cells were electroporated using a BioRad Gene Pulser II (BioRad). Electrocompetent *E. coli* cells were made following the BioRad Electroprotocol supplied with the Gene Pulser II.

All lambda phage manipulations were carried out following protocols and using media described in the documentation provided with the Stratagene Gigapack III Gold packaging extract.

DNA isolation and analysis

Plasmid DNA was routinely prepared using a standard alkaline lysis procedure (Sambrook *et al.*, 1989). Qiagen kits were also used to obtain lambda genomic DNA and plasmid DNA for sequence analysis. DNA preparations and restriction endonuclease digests were analyzed by Tris-acetate agarose gel electrophoresis (Sambrook *et al.*, 1989).

DNA ligations

All DNA ligation reactions were conducted using T4 DNA ligase (Life Technologies) following the manufacturer's suggested protocol. Proteins were routinely extracted from DNA samples using an equal volume of Tris-buffered phenol/chloroform (1:1 v/v) (Sambrook *et al.*, 1989). DNA samples were precipitated using 0.3 M sodium acetate and 3 volumes of 95% ethanol (Sambrook *et al.*, 1989).

PCR

All PCR reactions were carried out using *Taq* DNA polymerase (Boehringer Mannheim) and standard PCR conditions unless otherwise stated (Giovannoni, 1991).

Purification of genomic DNA and gene library construction

P. salmonis was purified by density gradient centrifugation from 12,000 cm^2 of CHSE-214 cells exhibiting full CPE 14 days after *P. salmonis* infection. A single step DNA isolation solution, DNAzol (Life Technologies), was used to obtain genomic DNA from the purified *P. salmonis*. Genomic DNA was further purified by equilibrium centrifugation using a CsCl-ethidium bromide gradient to yield 250 μg of *P. salmonis* genomic DNA (Sambrook *et al.*, 1989).

P. salmonis DNA was partially digested using serially diluted *Eco*R I (New England Biolabs). Digests containing an average fragment size of 10 kbp were chosen for creation of a *P. salmonis* gene expression library using a Stratagene predigested lambda ZAP II cloning kit.

Immunological screening of the gene library

Approximately 10,000 plaques of the *P. salmonis* lambda expression library were screened per round with a desired density of 1,000 plaques per 80 mm petri dish. Plaque lifts were conducted in duplicate using 80 mm nitrocellulose discs (BioRad) impregnated with 10 mM isopropyl-β-D-thiogalactoside (IPTG; Boehringer Mannheim). Immunological screening followed the protocol of Sambrook *et al.* (1989) using anti-*P. salmonis* rabbit serum 82CR. Immunoreactive plaques were picked and rescreened until pure phage cultures were obtained.

Excision of pBluescript clones from lambda ZAP II phagemids

Amplified liquid stocks of *P. salmonis* lambda clones were created for pBluescript clone excision. Stratagene ExAssist interference resistant helper phage were used to excise the pBluescript clones into *E. coli* strains SOLR and ABLE K. A glucose supplement (1% v/v) was required in all media used for selection of excision clones.

Antibody generation

Antibodies were generated in New Zealand white rabbits against 10 and 20 amino acid (aa) synthetic peptides based on residues 110-129 of the predicted OspA sequence. Peptides were glutaraldehyde conjugated to keyhole limpet hemocyanin (KLH) using a 40:1 molar

ratio of peptide:KLH in standard phosphate buffered saline (pH 7.5). Conjugation was carried out for 1 h at 4°C in a 10 ml reaction volume with 500 µg/ml KLH and 1% glutaraldehyde. For the primary immunization, rabbits received 250 µg of conjugated peptide mixed 1:1 with Freund's complete adjuvant. Each rabbit was boosted three times at 2 week intervals with 250 µg of conjugated peptide per boost mixed 1:1 with Freund's incomplete adjuvant. *E. coli* specific antibodies were adsorbed out of polyclonal antibody samples using *E. coli* lysates (Sambrook *et al.*, 1989).

Convalescent sera were collected from coho salmon fry that had been challenged with *P. salmonis* but did not succumb to infection.

SDS-PAGE and Western blot analysis

Whole cell lysates were separated by SDS-PAGE as described in Chapter 1. Protein samples were visualized using GelCode Blue Stain Reagent (Pierce) and quantified with AlphaEase software (Alpha Innotech Corp.).

Western blotting was conducted as described in Chapter 1. *P. salmonis* antigens were detected by using rabbit anti-*P. salmonis* serum 82CR (diluted 1:2,000) and rabbit anti-OspA peptide serum (dil. 1:100) followed by goat anti-rabbit IgG conjugated to alkaline phosphatase (dil. 1:3,000). Convalescent coho salmon anti-*P. salmonis* sera (dil. 1:30) was also used, followed by an anti-rainbow trout Ig (heavy chain) monoclonal Ab IPA2C7 (dil. 1:100; Immuno-Precise Antibodies) and goat anti-mouse IgG_1 conjugated to alkaline phosphatase (dil. 1:3,000) (Caltag Laboratories).

DNA dot blot analysis

DNA samples (0.5 and 1.0 μg) from CHSE-214 cells, immunoreactive *P. salmonis* lambda clones, and excised pBluescript clones were applied to a nylon membrane, in duplicate (Hybond-N+; Amersham). Blots were denatured in 1.5 M NaCl, 0.5 M NaOH, neutralized in 1.5 M NaCl, 0.5 M Tris (pH 8.0), and rinsed in 0.2 M Tris (pH 7.5), 2× SSC (Sambrook *et al.*, 1989).

Insert DNA from clone pB12 was used as a nucleotide probe. The pB12 insert was agarose gel purified using Sephaglas (Pharmacia) and labeled with [α-^{32}P]dATP by nick translation (Amersham Pharmacia). Blots were hybridized with probe under standard conditions at 60°C for 18 h (Sambrook *et al.*, 1989). Following stringency washes, blots were visualized using a storage phosphor screen imaging system (Molecular Dynamics).

Construction of deletion clones and DNA sequence analysis

A directional deletion library of *P. salmonis* clone pB12 (Fig. 10A) was constructed to facilitate DNA sequence analysis. An *Exo* III/S1 nuclease double-stranded nested deletion kit (Amersham Pharmacia) was used to create deletions in the direction of *lacZ* on the pBluescript vector. Restriction endonucleases *Eco*R I and *Sac* I were used to generate opposing single stranded DNA overhangs to protect the vector from *Exo* III digestion. Following *Exo* III digestion, plasmid DNA was religated and insert sizes were screened by agarose gel electrophoresis following digestion with restriction endonuclease *Xho* I (New England Biolabs). 32 deletion clones were selected that represented the entire pB12 insert and differed in size by 100-500 bp.

Double stranded plasmid DNA samples were sequenced using a combination of dye primer (Sequenase; Amersham Pharmacia) and dye termination (Dynamic ET Terminator;

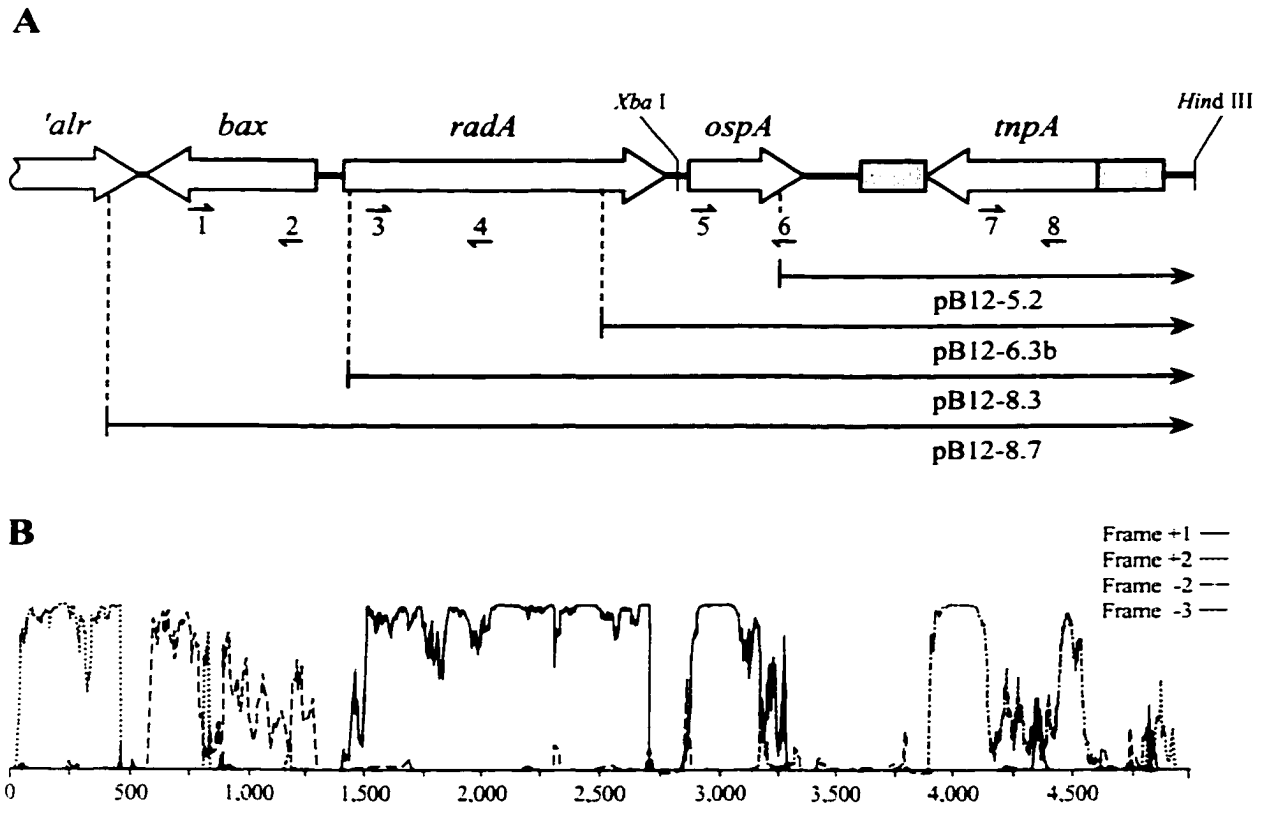

Figure 10. The *P. salmonis* pB12 insert. **(A)** Physical map of the 4,983 bp pB12 insert. Open arrows depict predicted ORF's and their coding direction. The shaded regions represent a 288 bp direct repeat that flanks the predicted transposase ORF, *tnpA*. The entire pB12 insert has a G+C content of 41%. The regions of clone pB12 encoded by deletion clones pB12-5.2, pB12-6.3b, pB12-8.3, and pB12-8.7 are represented by black arrows. The *Xba* I and *Hind* III restriction sites were used to subclone the *ospA* into pBC. Primers 1-8 (Table 2) were used to PCR amplify probes for Northern blot analysis. **(B)** Coding predictions were made using Borodovsky's Markov method (Borodovsky *et al.*, 1995). Insert legend denotes reading frame of coding predictions.

Amersham Pharmacia) sequencing reactions. Sequencing reactions were analyzed using an automated sequencer (ABI 377; Perkin-Elmer). The entire pB12 insert (Appendix 1) was sequenced with an average redundancy of 4× coverage with problem regions sequenced in both directions at a minimum. Sequence data were assembled and analyzed using the Lasergene software package (DNASTAR).

In vitro transcription and translation

E. coli S30 lysates (Promega) for transcription and translation of plasmid DNA were used to analyze protein expression from *P. salmonis* inserts in clone pB12 and deletion clones pB12-5.2, pB12-6.3b, and pB12-8.7 (Fig. 10A). Each reaction used 5 μg of plasmid DNA and L-[U-^{14}C]leucine (Amersham Pharmacia) was used as a radiolabel. Protein was acetone precipitated and rehydrated in SDS-PAGE sample buffer for analysis by 12% SDS-PAGE. Gels were vacuum dried at 80°C and visualized using a storage phosphor screen imaging system.

RNA isolation and Northern blot analysis

Qiagen RNeasy columns were used to isolate total RNA from *E. coli* DH5α and clones pB12, and pB12-6.3b. Total RNA, 10 μg per sample, was analyzed by 1.2% formaldehyde-agarose gel electrophoresis. Gels were vacuum blotted onto nylon membranes (Hybond-N+; Amersham Pharmacia), air dried and baked for 1 h at 80°C. Coding regions of the four predicted ORF's of clone pB12 (Fig. 10A) were PCR amplified using primers listed in Table 2. Prior to radiolabeling, probes were agarose gel purified using Sephaglas to ensure removal of template DNA. Probes were labeled with [α-^{32}P]dATP by nick translation.

Table 2. PCR primers used to amplify probes for Northern blot analysis.

PRIMER	SEQUENCE
***bax* ORF**	
1	TGC TCA ACA ACC CTT ATT CTC AG
2	TAG GCG TTA TGT GTA TTT AGT GTA TGT ATG
***radA* ORF**	
3	TAA GTG AAG TAA AAG AAG TGC TGC CAA ATC
4	CGT GTT TAT TTT TAG TCG GTC ATG TGA
***ospA* ORF**	
5	ACA GAG GAT GTT TGC AAG GTA GTA GTC TAA T
6	ATG GTC GTT GGC AAG TCA TTT CAA
***tnpA* ORF**	
7	AAA AAC GTA ATA AGC CTT CAC AAA TGT CAA C
8	AAA GGC CAT TAA CTC TCC CAT ATC ATT CAC

Blots were hybridized with probes for 18 h at 65°C under standard conditions. Following stringency washes, blots were visualized using a storage phosphor screen imaging system.

Creation of *E. coli* optimized *ospA* and subsequent signal peptide constructs

The products of all completed PCR strategies were initially subcloned into either pBC KS(+) (Stratagene) or pGEM-5Zf(+) (Promega) and electroporated into XL1-Blue. Following confirmation of positive clones by DNA sequencing, inserts not already in pET21a(+) (Novagen), were sequentially subcloned into pBC KS(+) then pET21a.

pBC-17kDa

The *Xba* I/*Hin*d III fragment of deletion clone pB12-6.3b (Fig. 10A) was subcloned into vector pBC KS(+) to place wild type *ospA* under T7 RNA polymerase promoter control. Following ligation, pBC-17kDa was electroporated into *E. coli* XL1-Blue MRF'.

pET-17E2

The EditSeq application, of the Lasergene software suite (DNASTAR), was used to reverse translate the OspA aa sequence using optimal non-degenerate codons for strong expression in *E. coli*. The synthetic *ospA* gene sequence (herein referred to as 17E2) was then modified to reduce the number of instances identical codons were used by replacing them with equally favoured codons wherever possible. Oligonucleotides of 17E2 were synthesized as six overlapping regions (94-151 bp in length) (Fig. 11) using standard techniques on a PCR-MATE 391 DNA synthesizer (ABI). *Taq* DNA polymerase (Boehringer Mannheim) was used throughout the construction of 17E2.

The central region of 17E2 was created in PCR reaction 1-1 using 0.5 pmol of oligonucleotides #3 and #4 (Table 3) as templates with 2.5 pmol of oligonucleotides #2 and #5 (Table 3) as primers (Fig. 11). PCR reaction 1-1 was carried out for 30 cycles (92°C

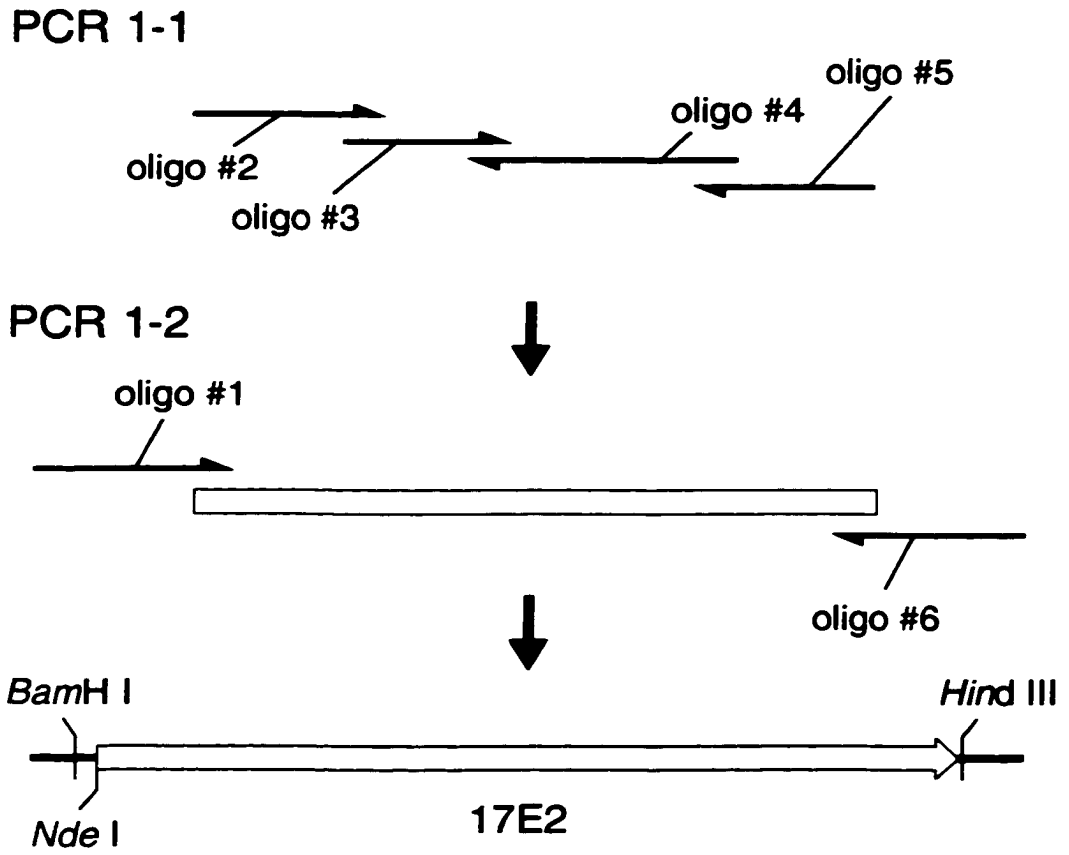

Figure 11. Construction of an *E. coli* codon optimized version of *ospA*. A schematic illustration of the PCR strategy used when constructing an *E. coli* codon optimized, synthetic version of the *ospA* gene, 17E2. Assembly of the gene was conducted using two sequential PCR reactions, in which the product of the reaction 1-1 served as the template for reaction 1-2. The sequences of oligonucleotides 1-6 are listed in Table 3.

Table 3. Oligonucleotides used to construct a synthetic version of *ospA* optimized for high level expression in *E. coli*. Bold nucleotides represent regions homologous to primers for PCR amplification of the final product.

OLIGO.	SEQUENCE
1	**CGC CAG GGT TTT CCC AGT CAC GAC** GGA TCC GTC TCA TAT GCG TGG TTG CCT GCA GGG CAG CTC TCT GAT CAT TAT CTC TGT TTT CCT GGT GGG TTG CGC CCA GAA CTT CAG CCG CCA G
2	TGG GTT GCG CCC AGA ACT TCA GCC GCC AGG AAG TTG GCG CGG CCA CCG GTG CGG TTG TGG GCG GTG TTG CCG GCC AGC TGT TCG GTA AAG GCT CTG GTC GTG TGG CGA TG
3	AAA GGC TCT GGT CGT GTG GCG ATG GCC ATC GGC GGT GCG GTT CTG GGC GGT CTG ATT GGC TCT AAA ATC GGT CAG AGC ATG GAC CAG CAG GAT A
4	CCT GTT TGT TGT AAC GCT GGT AGG TGC GAA CCG GTT CCA CAG AGT AGC TGT TAC CGG TGT CCG GAT TAC GCC AAC GAG TAA CCT GGC CGG CTT TCA CTT TTT CCA GAG ACT GGT TCA GTT TGA TTT TAT CCT GCT GGT CCA TGC TCT GAC C
5	GGT GCC GTA GAT TTC CTG TTT CTG ACC TGC GAT CAT GGC TTT CTG CTG AAA TTC GCG GCA GTA CTG CTG ACG GCG TTC CTG TTT GTT GTA ACG CTG GTA GGT
6	**CGT CCT CTC GTC CTG GTC CCA** GAT AAG CTT AAT TAA TTT TTT CGG TGC TAA TCA CCT GCC AGC GGC CAT CCG GCT GAC GGC ACG CGG TGC CGT AGA TTT CCT GTT TCT GAC

30 s, 55°C 30 s, 72°C 30 s). All PCR reaction volumes were 100 µl and contained 200 µM of each dNTP, 10 µl of 10× reaction buffer (100 mM Tris/HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8.3) and 2 units of *Taq* DNA polymerase.

Full length 17E2 was constructed in PCR reaction 1-2 using 1 µl of the completed first reaction (dil. 1:100) as template. The 100 µl reaction contained 2.5 pmol of oligonucleotides #1 and #6 (Table 3) as primers (Fig. 11). The first cycle of PCR reaction 1-2 had an annealing temperature of 55°C which was raised to 70°C for the remaining 29 cycles; denaturing and elongation temperatures were the same as for PCR 1-1.

The full length product was selectively amplified with primers FOR (5'-CGC CAG GGT TTT CCC AGT CAC GAC-3') and END (5'-TGT GAA GCT TAT TTT TCG GTG CTA ATC ACC TGC CA-3'), both complementary to regions outside the 17E2 coding region, in a third PCR reaction. The synthetic 17E2 product was digested with *Bam*H I and *Hin*d III and ligated into vector pBC KS(+). The ligation mixture was electroporated into XL1-Blue and plated on MacConkey agar (Cm) for selection overnight at 37°C. Positive clones were confirmed by DNA sequencing. The optimized 17E2 insert was then subcloned from pBC-17E2 into pET21a using the *Nde* I and *Hin*d III restriction sites to construct pET-17E2.

pET-C17E2

The *Bam*H I/*Hin*d III fragment of pBC-17E2 was subcloned into fusion vector pETC to place 17E2 in frame with an N-terminal fusion partner, Protein C. The pET-C17E2 ligation mixture was electroporated into *E. coli* BL21. Transformants were selected on MacConkey agar (Ap) and confirmed to be positive upon plasmid purification and excision of the insert with *Bam*H I/*Hin*d III digestion.

pET-Δ17E2

A truncated version of 17E2 lacking its predicted signal sequence was created by PCR using a forward primer encoding *Bam*H I and *Nde* I restriction sites (5'-CTT GGA TCC GTC TCA TAT GGC CCA GAA CTT CAG CCG CC-3'). The 38 bp forward primer was complementary to only 19 bp of 17E2 in the region of codons 22-27. The 17E2 Cys-21 codon was replaced with an ATG start codon. The reverse primer, 17E2 REV (Table 4), encoded a *Sac* I restriction site immediately following the 17E2 TAA stop codon. The 50 µl volume PCR reaction contained 100 ng of pET-17E2 as template, 10 pmol of each primer, 200 µM of each dNTP, 5 µl of 10× *Pfu* buffer (200 mM Tris/HCl, 20 mM $MgCl_2$, 100 mM KCl, 1% Triton X-100) and 1.25 units of *Pfu* DNA polymerase (Stratagene). PCR was carried out for 30 cycles (94°C 60 s, 55°C 60 s, 72°C 60 s). The Δ17E2 PCR product was digested with *Bam*H I and *Nde* I and ligated into pET21a(+) and confirmed by DNA sequencing to create pET-Δ17E2.

pET-lppospA

A 114 bp oligonucleotide, LPPSIG (Table 4), was synthesized encoding the 21 residue signal sequence of *E. coli* Braun's lipoprotein (*lpp*; GenBank AE000263) with optimized *E. coli* codon usage, an *Nde* I site overlapping the ATG start codon, and flanking primer sites for amplification (Fig. 12). A 21 bp region complementary to codons 2-8 of Δ17E2 was added to the 3' terminus of the LPPSIG oligo in reaction 2-1 (Fig. 12) using primers FOR SYNTH and LPPOSP REV (Table 4). A 23 bp region complementary to the 3' region of LPPSIG was also added to the 5' region of Δ17E2 in reaction 2-2 (Fig. 12) using primers OSPLPP and 17E2 REV (Table 4). Reaction conditions were identical to those used during construction of pET-Δ17E2. The first cycle of PCR was allowed to anneal at 55°C to allow

Table 4. Primers used to construct *ospA* genes encoding foreign signal sequences. Bold nucleotides represent the coding regions of the *R. rickettsii* and Braun's lipoprotein signal sequences.

PRIMER	SEQUENCE
Addition of Braun's lipoprotein signal sequence	
LPPSIG	CGC CAG GGT TTT CCC AGT CAC GAC ATA **TGA AAG CGA CCA AAC TGG TTC TGG GCG CGG TTA TCC TGG GTT CTA CCC TGC TGG CGG GTT GC**G AGC TCG GAC CAG GAC GAG AGG ACG
LPPOSP REV	CCT GGC GGC TGA AGT TCT GGG CGC AAC CCG CCA GCA GGG TAG AAC
OSPLPP FOR	GTT CTA CCC TGC TGG CGG GTT GCG CCC AGA ACT TCA GCC GCC AGG
Addition of *R. rickettsii* signal sequence	
RICSIG	CGC CAG GGT TTT CCC AGT CAC GAC ATA **TGA AAC TGC TGT CTA AAA TCA TGA TCA TCG CTC TGG CTA CCT CTA TGC TGC AGG CTT GC**G AGC TCG GAC CAG GAC GAG AGG ACG
RICOSP REV	CCT GGC GGC TGA AGT TCT GGG CGC AAG CCT GCA GCA TAG AGG TAG CC
OSPRIC FOR	GGC TAC CTC TAT GCT GCA GGC TTG CGC CCA GAA CTT CAG CCG CCA GG
Primers common to both procedures	
FOR SYNTH	GCG AAA TTA ATA CGA CTC ACT ATA GGG G
17E2 REV	GCC GAG CTC TTA TTT TTC GGT GCT AAT CAC CTG CC

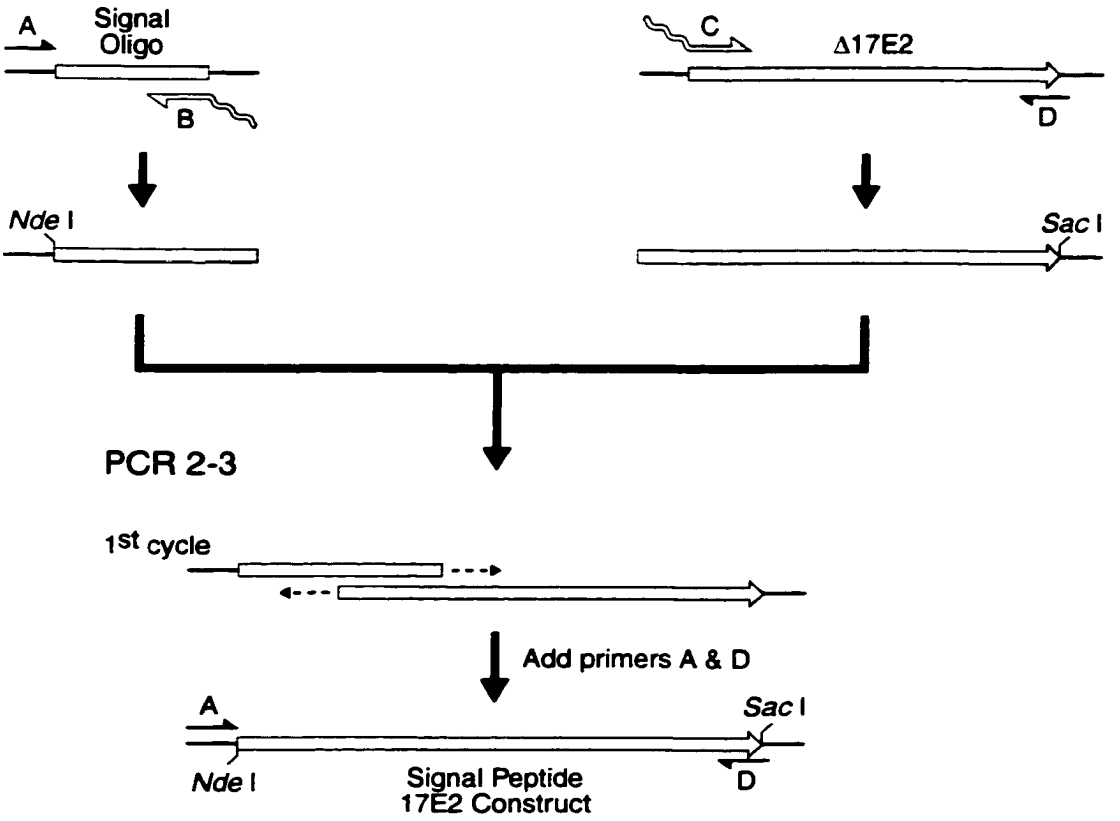

Figure 12. Addition of heterologous signal sequences to *ospA*. A schematic illustration of the PCR strategy used to add heterologous signal sequences to the truncated version of optimized *ospA*, Δ17E2. In two separate initial PCR reactions, 2-1 and 2-2, homologous regions of Δ17E2 and the signal oligo. (LIPPSIG, RIC SIG) are added to each other using primers B (LPPOSP REV, RICOSP REV) and C (OSPLPP FOR, OSPRIC FOR) (Table 4), respectively. The products of these two reactions are used as primers to each other in the initial round of amplification in PCR reaction 2-3. During the denaturation step following the first cycle of reaction 2-3, primers A (FOR SYNTH) and D (17E2 REV) (Table 4) are added to allow amplification of the full length product in the remaining 29 cycles.

all non-homologous regions of primers to be filled in. The subsequent 29 cycles annealed at 65°C (94°C 60 s, 65°C 60 s, 72°C 60 s).

The *lpp* signal sequence was added to Δ17E2 using the products of reactions 2-1 and 2-2 as templates in PCR reaction 2-3 (Fig. 12). PCR reaction conditions remained unchanged, and 1 µl of reactions 2-1 and 2-2 diluted 1/10 were used as template. The first cycle of PCR contained no primers allowing the 44 bp overlap of the two templates to anneal at 55°C and *Pfu* DNA polymerase to synthesize the complementary regions. Following the first cycle, 10 pmol of primers FOR SYNTH and 17E2 REV were added and the remaining 29 cycles annealed at an elevated temperature of 65°C (94°C 60 s, 65°°C 60 s, 72°C 60 s).

The product of reaction 2-3 was ligated into pGEM-5Zf(+) using the incorporated *Nde* I and *Sac* I restriction sites. Ligation mixture was electroporated into XL1-Blue and clones were confirmed by DNA sequencing. The confirmed lppospA insert was then subcloned into pET21a to create pET-lppospA.

pET-ricospA

A 111 bp oligonucleotide, RICSIG, was synthesized encoding the 21 residue signal sequence of *R. rickettsii* 17 kDa lipoprotein (GenBank AAA26381) with optimized *E. coli* codon usage, an *Nde* I site overlapping the ATG start codon, and a flanking primer sites.

Addition of the *R. rickettsii* 17 kDa signal sequence to Δ17E2 followed the same strategy as used when creating the pET-lppospA construct. Only the primers used to create overlaps between the *R. rickettsii* signal sequence and Δ17E2 differed (Table 4). The primer RICOSP REV was used to add a 21 bp Δ17E2 overlap to the 3' terminus of the *R. rickettsii* signal sequence (Table 4, Fig. 12). The primer OSPRIC FOR was used to add a

25 bp region complementary to the *R. rickettsii* signal sequence to the 5' terminus of Δ17E2 (Table 4, Fig. 12).

Following subcloning into pGEM-5Zf(+) and DNA sequence confirmation, the 17E2 construct encoding an *R. rickettsii* signal sequence was subcloned into pET21a to create pET-ricospA.

Induction of expression and [^{14}C]palmitate incorporation

For induction experiments, pGP1-2 encoding T7 polymerase (Tabor and Richardson, 1985) was cotransformed with each pET21a-based construct into *E. coli* BL21. Cotransformants were selected overnight at 30°C on MacConkey media (Kn/Ap). pBC-17kDa was costransformed with pGP1-2 into XL1-Blue MRF' and selected at 30°C on MacConkey media (Kn/Cm).

For expression studies of the *ospA* constructs, 3 ml TB cultures containing appropriate antibiotics were grown overnight at 30°C and subcultured (1:100) into 5 ml TB broth. Once cultures were actively growing (A_{600} 0.5) temperature was shifted to 42°C for induction for 3 h. Inclusion bodies were collected by sonicating induced cultures of pET-C17E2 3 × 60 s followed by centrifugation at 10,000 × *g*.

Toluene was removed from [1-^{14}C]palmitate (Amersham Pharmacia) under vacuum and the palmitate was dissolved in 0.2 vol. of isopropanol. The [^{14}C]palmitate was added to cultures after being shifted to 42°C at a final concentration of 5 μCi/ml. Labeling was carried out for 3 h before samples were collected for SDS-PAGE. Samples were analyzed by 15% SDS-PAGE and the gels were vacuum dried at 80°C and visualized using a storage phosphor screen imaging system.

Primary structure analysis of OspA

SignalP v2.0 (www.cbs.dtu.dk/services/SignalP) was used to analyze potential signal peptide regions of OspA, pET-lppospA and pET-ricospA chimeric OspA constructs. SignalP uses neural networks trained on proteins with characterized signal peptides to determine if query sequences have signal peptides (Nielsen *et al.*, 1997).

The OspA protein sequence was queried against the Pfam (www.sanger.ac.uk/Software/Pfam) and PROSITE (www.expasy.ch/prosite) protein family databases to identify conserved protein domains and sequence patterns (Bateman *et al.*, 2000, Hofmann *et al.*, 1999).

Multiple alignment and phylogenetic analysis of *P. salmonis* proteins

A multiple alignment of *P. salmonis* OspA and *R. prowazekii* 17 kDa antigen was constructed using the FASTA3 algorithm (Pearson, 1998). Similar (conserved) amino acids were identified by FASTA3 using the BLOSUM50 matrix because of its ability to detect weak protein similarities (Henikoff and Henikoff, 1992).

A multiple alignment of *Rhizobium etli* LipA, *P. salmonis* OspA, and *R. prowazekii* 17 kDa antigen was constructed using the ClustalW v1.8 algorithm (Thompson *et al.*, 1997). Similar (conserved) amino acids among sequences were determined using the amino acid similarity groups of the PAM250 scoring matrix which is considered to be a good matrix to use for distantly related proteins (Altschul, 1991).

Unrooted cladograms were constructed based on multiple alignments generated using ClustalX 1.8 (Thompson *et al.*, 1997). Alignments were bootstrapped using 1000 replicates and trees were generated using distances calculated by the Neighbour Joining method of Saitou and Nei (Saitou and Nei, 1987).

RESULTS

Identification of immunoreactive *P. salmonis* lambda ZAP II clones

Screening of the *P. salmonis* expression library with high titre rabbit anti-*P. salmonis* 82CR serum identified 18 strongly immunoreactive plaques. These plaques were picked and were rescreened until pure. Initial attempts to excise the pBluescript clones from the lambda phagemids using helper phage were unsuccessful. Endonuclease digests conducted on genomic lambda DNA from all 18 clones showed that 16 of the clones had 7 kb inserts, while the other two clones had inserts sizes of 6 and 5.5 kb. Restriction fragment length analysis with frequently cutting endonucleases suggested that 16 of the clones were identical and all 18 clones contained a common region of DNA (data not shown).

Successful *in vivo* excision of the pBluescript clones was achieved only when the *lac* promoter was repressed using a 1% glucose supplement in all media. Only the 5.5 kb lambda clone yielded a stable insert-containing pBluescript clone, pB12, encoding a 5 kb insert (Fig. 10A), but was very slow growing even under glucose repression.

To confirm that the insert obtained in clone pB12, was of *P. salmonis* origin and was the same insert encoded by the lambda phagemid clones, DNA dot blot analysis was used. Genomic DNA from all 18 lambda clones, *P. salmonis*, CHSE-214, and pBluescript plasmid DNA was analyzed by DNA dot blotting using insert DNA from pB12 as the probe. Hybridization revealed that the pB12 insert was of *P. salmonis* origin. The pB12 insert also hybridized with all 18 lambda clone genomic DNA samples indicating that all the inserts encoded an overlapping fragment of *P. salmonis* DNA.

Sequence analysis of clone pB12

DNA sequencing of *Exo* III/S1 nuclease deletion clones of the pB12 insert revealed that the insert was 4,983 bp long and had a adenine (A) and thymine (T) content of 59% (Fig. 10A). These sequences (Appendix 1) were submitted to the DDBJ/EMBL/GenBank databases under accession number AF184152. Coding predictions using Borodovsky's Markov method (Borodovsky *et al.*, 1995) identified 4 intact ORF's and 1 partial ORF that created a gene fusion in frame with *lacZ* (Fig. 10B). The predicted ORF's were subjected to BLAST2 (Altschul *et al.*, 1997) and FASTA3 (Pearson, 1998) analysis to determine if any similar sequences were known.

The 499 bp partial '*alr* ORF (Fig. 10A) had a 54% A+T content and was predicted to encode a 176 aa protein fused to the N-terminal fragment of LacZ. The predicted molecular weight of the LacZ-'Alr fusion is 22.2 kDa. The predicted aa sequence of the '*alr* ORF shares 49% aa identity and 65% aa similarity with the 167 aa C-terminal region of alanine racemase from *Pseudomonas aeruginosa* (GenBank AAD47082).

A 732 bp ORF (*bax*; Fig. 12) with a 61% A+T content was predicted to encode a 243 aa, 27.6 kDa protein. Both FASTA3 and BLAST2 only identified low scoring aa homology (33% identical, 49% similar) between the central 187 aa region of the *bax* ORF and a 274 aa predicted ORF, BAX, in *E. coli* K12 (GenBank AAB18547).

A 1,368 bp ORF (*radA*; Fig. 10A) with a 57% A+T content was predicted to encode a 456 aa, 49.4 kDa protein. A high degree of aa homology was found between the entire predicted aa sequence of *P. salmonis* RadA and the RadA DNA repair enzymes from a variety of bacteria. *P. salmonis* RadA is most homologous to the RadA of *P. aeruginosa*

(SwissProt P96963) with 62% aa identity and 77% aa similarity. *P. salmonis* RadA also has 59% aa identity and 75% aa similarity to *E. coli* RadA (SwissProt P24554).

A 486 bp ORF (*ospA*; Fig. 10A), immediately following *radA*, with a 51% A+T content was predicted to encode a 162 aa, 17.7 kDa protein with the 21-162 aa region having sequence similarity to the processed rickettsial 17 kDa genus common antigen (Fig. 13A). The predicted aa sequence of the *ospA* ORF was up to 39% identical and 62% similar to the 17 kDa protein antigens of *R. prowazekii* (SwissProt P16624; Fig. 13A), *Rickettsia japonica* (SwissProt Q52764), *Rickettsia rickettsii* (SwissProt P05372), and *Rickettsia typhi* (SwissProt P22882). Pfam domain analysis (Bateman *et al.*, 2000) identified that OspA contained a domain between residues 59-155 that belongs to a protein domain family (ProDom PD010874) that includes the rickettsial 17 kDa antigens and *Rhizobium* LipA lipoproteins (SwissProt O69776, Q52854) (Fig. 13B).

The final 717 bp ORF (*tnpA*; Fig. 10A) had a 66% A+T content and was predicted to encode a 239 aa, 27.7 kDa protein. The *tnpA* ORF is flanked by a perfect 288 bp direct repeat (Fig. 10A). Amino acid similarity searches returned strong matches between the predicted aa sequence of the *tnpA* ORF and a variety of transposases. The closest match was a transposase encoded in a *Porphyromonas gingivalis* insertion element, IS195 (GenBank U83995), with 47% aa identity and 65% aa similarity (Lewis and Macrina, 1998).

In vitro transcription and translation of clone pB12

Deletion clones of pB12 were used for *in vitro* transcription and translation experiments. Deletion clones were chosen on the basis of whether or not they encoded particular predicted ORF's (Fig. 10A). Clone pB12-8.7 was chosen because it only lacked

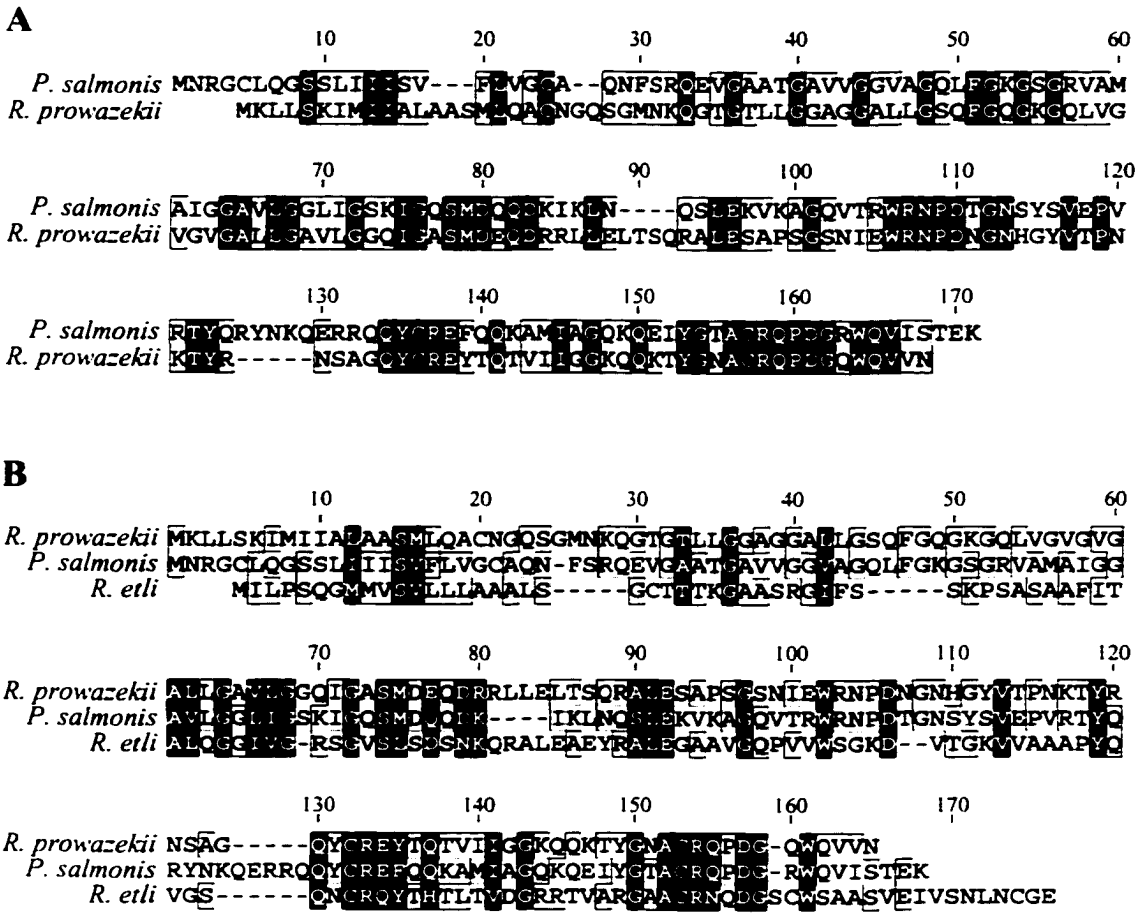

Figure 13. Multiple sequence alignment analysis of OspA. **(A)** Pairwise sequence alignment of the *P. salmonis* 17 kDa antigen, OspA, and the *R. prowazekii* 17 kDa antigen (SwissProt P16624). The pairwise alignment was generated using the FASTA3 algorithm. The *P. salmonis* 17 kDa antigen shares 39% identity (black background) and 62% similarity (outlined) with the 17 kDa antigen of *R. prowazekii*. Synthetic peptides representing the region from aa 110-129 of OspA were used to generate rabbit polyclonal serum. **(B)** Multiple alignment of the *R. prowazekii* 17 kDa antigen, OspA, and *Rhizobium etli* LipA. The three proteins share 26% identical or conserved residues (black background) and 39% of the residues are identical or conserved between *P. salmonis* and *R. etli* (outlined). Alignment was generated using the ClustalW 1.8 algorithm.

the majority of the *lacZ-'alr* construct (Fig. 10A). Clone pB12-6.3b lacked the majority of the *radA* ORF and pB12-5.2 lacked the *ospA* ORF as well (Fig. 10A). Five proteins with masses of 17, 22, 28, 28 and 49 kDa were predicted to be expressed from the predicted ORF's of clone pB12. A protein in the 49 kDa range corresponding to RadA was expressed by clones pB12 and pB12-8.7 at levels similar to β-lactamase (31 kDa) in the pBluescript control (Fig. 14). Expression of the 49 kDa product was abolished by elimination of the *radA* ORF in clones pB12-6.3b and pB12-5.2 (Fig. 14). No detectable expression of proteins corresponding to the 3 other ORF's was observed.

Northern blot analysis of clone pB12

Total RNA from clone pB12 was probed for expression of *bax* and *radA* transcripts. Total RNA from clone pB12-6.3b was selected for analysis of *ospA* and *tnpA* expression because it did not encode the *bax* and *radA* ORF's and it grew far better than pB12.

Transcriptional products for the *bax*, *radA*, and *tnpA* ORF's were not detected in clone pB12 and pB12-6.3b (Fig. 15). However, *ospA* transcripts were detected in pB12-6.3b (Fig. 15). The *ospA* probe hybridized with a broad band around 1.2 kb in size (Fig. 15). This band appears to be comprised of three unresolving bands approximately 1.5, 1.2, and 0.9 kb in size.

Expression of *ospA* under the control of the T7 promoter

The *Xba* I/*Hin*d III fragment of clone pB12-6.3b (Fig. 10A) was cloned into pBC KS to place *ospA* under the control of the T7 promoter. Only low levels of a 17 kDa protein were produced upon induction of pBC-17kDa in *E. coli* XL1-Blue.

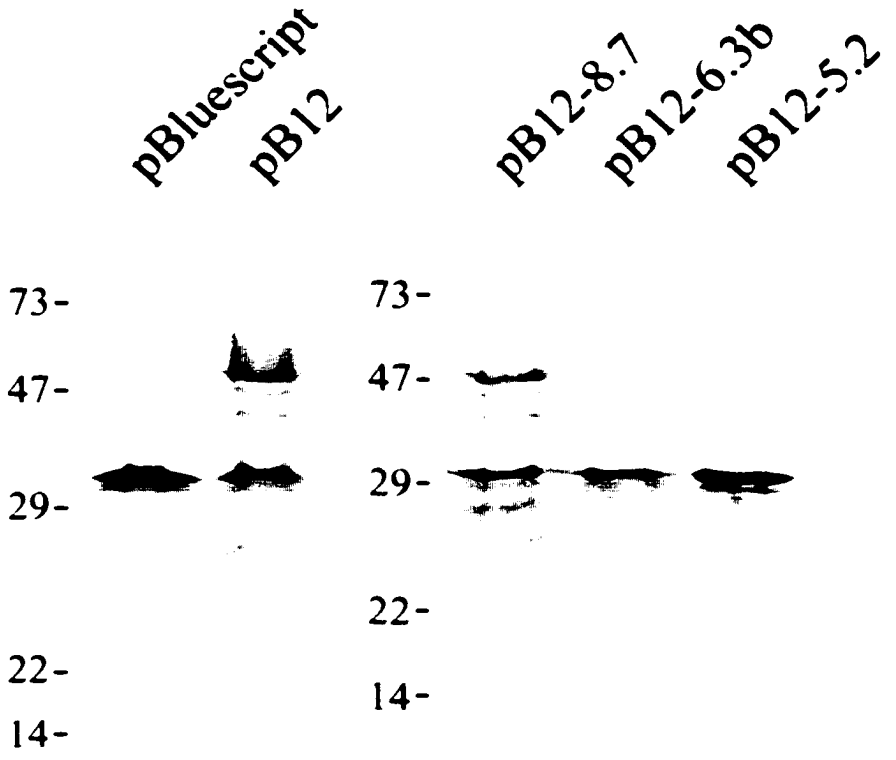

Figure 14. *in vitro* transcription and translation of pB12 using *E. coli* S30 extracts. Products of *in vitro* transcription and translation were [^{14}C]leucine labeled and analyzed by 12% SDS-PAGE. A 49 kDa protein produced by the pB12 and pB12-8.7 plasmids was not produced by deletion clones pB12-6.3b and pB12-5.2. Molecular mass is on the left in kDa.

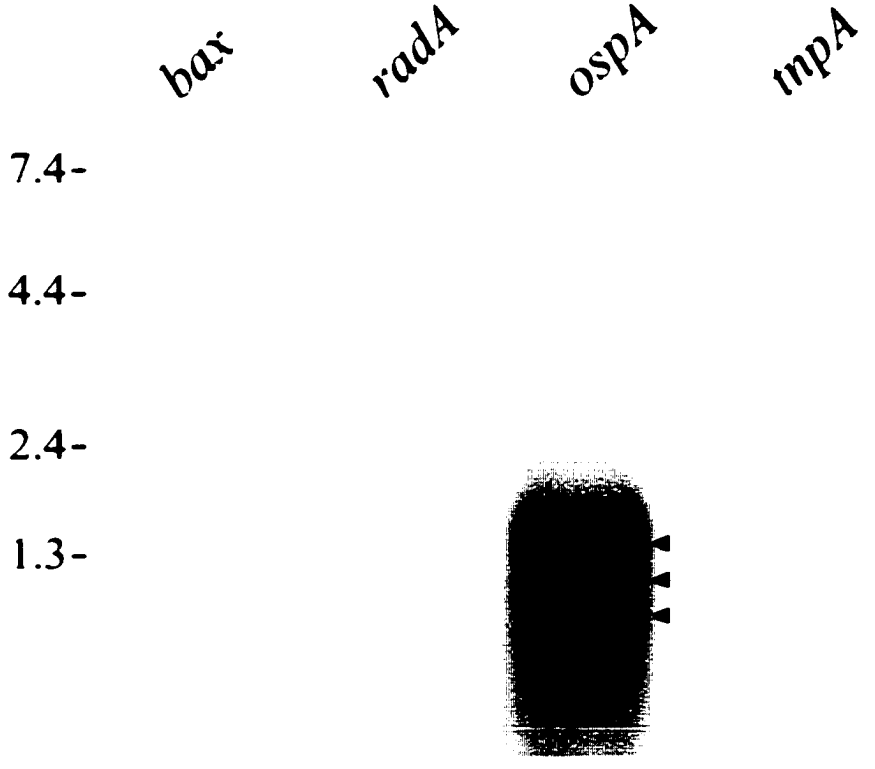

Figure 15. Northern blot analysis of pB12. Total RNA from clones pB12 and pB12-6.3b was analyzed by 1.2% formaldehyde agarose gel electrophoresis. Clone pB12 total RNA was hybridized with probes specific to the *bax* and *radA* ORF's. Clone pB12-6.3b total RNA was allowed to hybridize with *ospA* and *tnpA* probes. The *ospA* probe hybridized with 3 transcripts (1.4, 1.2, and 0.9 kb) in the pB12-6.3b sample. No transcripts specific to the *bax*, *radA*, and *tnpA* ORF's were detected. Molecular mass standards shown on the left are in kb.

PCR synthesis of a codon optimized *ospA* gene, 17E2

In an effort to obtain high level *E. coli* expression of the *P. salmonis ospA* ORF, a synthetic version of *ospA* (17E2), was constructed by PCR (Fig. 11). Analysis of the codon usage of *ospA* found that 20% of the codons were rare *E. coli* codons (Henaut and Danchin, 1996). When the 17E2 gene was synthesized the Asn-2 codon was removed and codon usage of the entire gene was optimized for high level expression in *E. coli*.

Four putative pET-17E2 clones, identified by *Nde* I/*Hin*d III excision of an appropriately sized insert, were DNA sequenced. Only one of the four 17E2 clones was confirmed to have its reading frame intact. The reading frames of the other three clones were destroyed by single base pair deletions at various positions throughout the gene. The chosen pET-17E2 clone contained two base pair substitutions that created transitions of Ala-54→Cys-54, and Arg-149→Pro-149. These base pair substitutions were attributed to the fidelity of *Taq* DNA polymerase during PCR and were considered acceptable because they did not alter the antigenic profile of OspA.

Primary structure analysis of OspA

PROSITE ProfileScan analysis of OspA identified a consensus pattern for prokaryotic membrane lipoprotein lipid attachment (PS00013) with Cys-21 (Fig. 16) as the predicted acylation site in the 11-21 aa region (Hofmann *et al.*, 1999).

SignalP calculated OspA has a 0.464 probability of having a signal peptide (Fig. 16). Replacement of the OspA signal peptide region with the signal peptides of Braun's lipoprotein and the *R. rickettsii* 17 kDa antigen improved calculated probability of having a signal peptide to 0.991 and 0.998, respectively (Fig. 16).

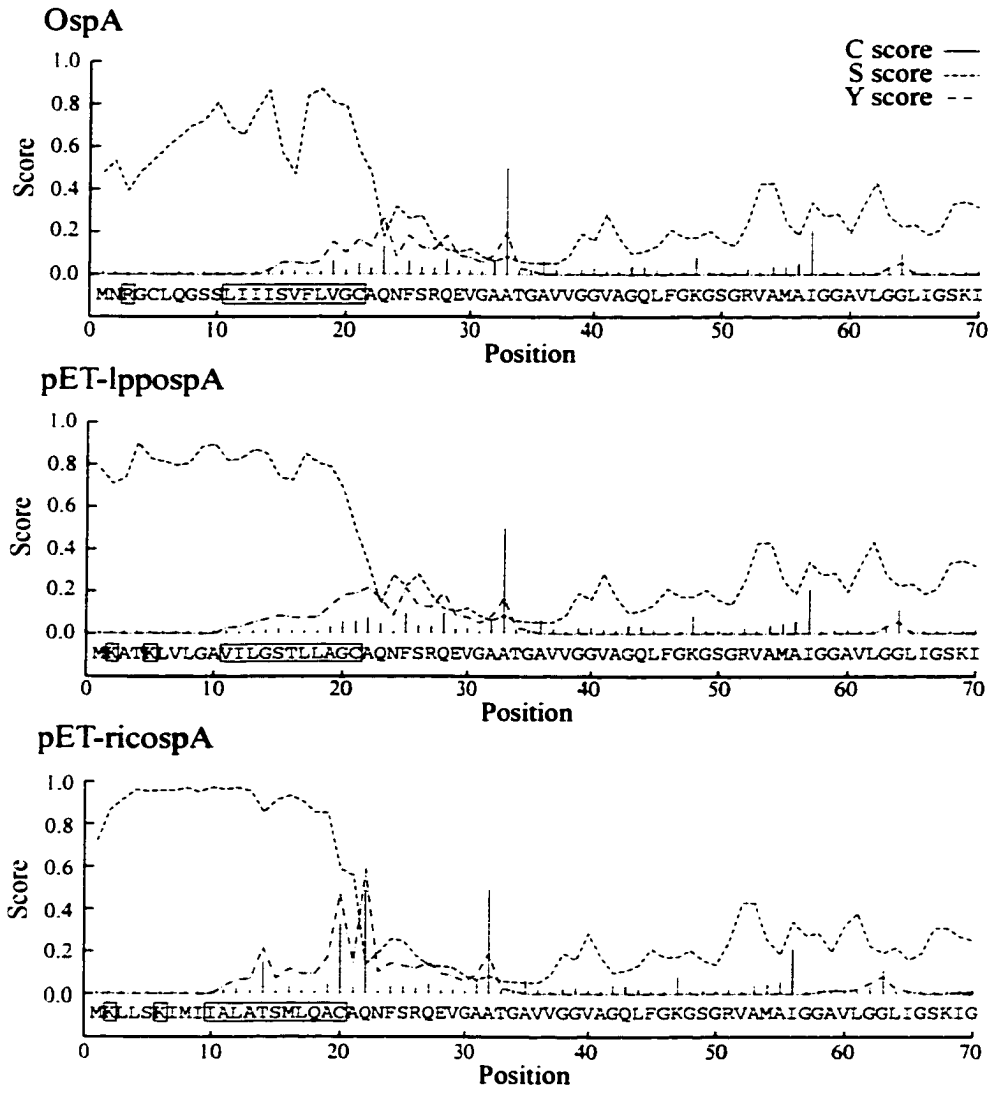

Figure 16. SignalP analysis of OspA and chimeric signal peptide constructs. The cleavage score (C-score) is output from a neural network trained to recognize cleavage sites versus other sequence positions. Ideally, the maximum C-value should occur immediately after the cleavage site. The signal peptide score (S-score) recognizes signal peptide versus non-signal-peptide positions and should be high at all positions before the cleavage site. The combined cleavage score (Y-score) is based on both the S- and C-scores. Residues surrounded by a box represent the PROSITE consensus regions for prokaryotic lipid attachment.

Removal and replacement of the OspA signal sequence

To determine if OspA had a signal peptide, and whether or not it was hampering production of OspA, the signal sequence of pET-17E2 was removed by PCR to create a truncated version of *ospA*, pET-Δ17E2. The Δ17E2 construct lacked the N-terminal 20 aa of wild type OspA (Fig. 13), and Cys-21 was replaced by a Met start codon.

The signal sequence of 17E2 was also replaced with the type II signal sequences of Braun's lipoprotein (*lpp*) and the *R. rickettsii* 17 kDa antigen (Fig. 16). The codon usage of the signal sequences were optimized for high level *E. coli* expression and added to the Δ17E2 construct by PCR (Fig. 11).

Comparative expression of OspA constructs

Induced production levels of OspA from pET-17E2 in *E. coli* BL21 were not noticeably higher than those obtained with wild type OspA from pBC-17kDa (Fig. 17). Replacement of the OspA signal peptide with signal peptides from Braun's lipoprotein and the *R. rickettsii* 17 kDa antigen signal peptides did not result in readily detectable expression levels of OspA (Fig. 17). However, removal of the OspA signal peptide allowed substantially increased production of 'OspA (13% total protein) when compared to OspA production from pBC-17kDa and pET-17E2 (Fig. 17).

Posttranslational modification of OspA

[^{14}C]palmitate incorporation was used to determine whether OspA is processed as a lipoprotein in *E. coli*. Actively growing cultures of pBC-17kDa, pET-lppospA, pET-ricospA, and pET-Δ17E2 were induced and labeled with [^{14}C]palmitate. Whole cell lysates of the clones were analyzed by 15% SDS-PAGE (Fig. 18). Wild type OspA

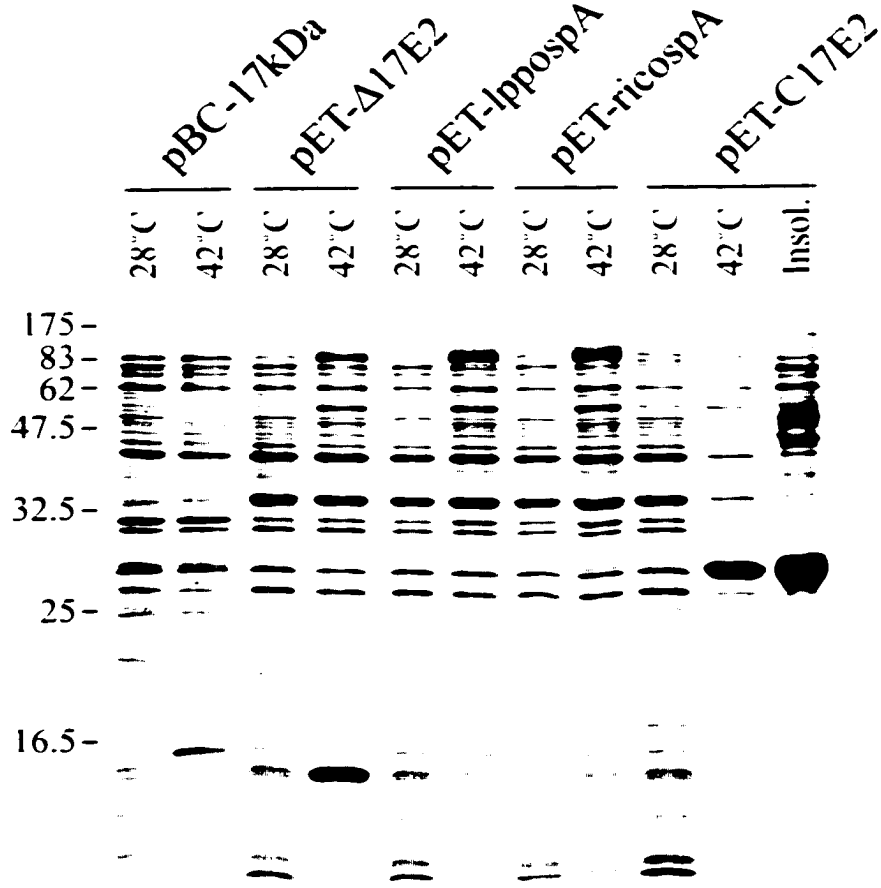

Figure 17. Comparitive expression of *ospA* constructs. Whole cell lysates of *E. coli* OspA clones were analyzed by SDS-PAGE (15% polyacrylamide). Samples of all OspA constructs at time 0 (28°C) and after 10 hr induction at 42°C were stained with GelCode. Induced production of OspA from constructs pBC-17kDa and pET-Δ17E2 were visible around 16 kDa, and at 28 kDa in the pET-C17E2 sample. The 28 kDa OspA fusion protein was localized to the insoluble (Insol.) fraction of lysed cells. Quantification of samples revealed that truncated OspA was produced as 13% of total protein and the OspA fusion was produced as 34% total protein representing 42% of the insoluble protein fraction. Molecular mass is on the left in kDa.

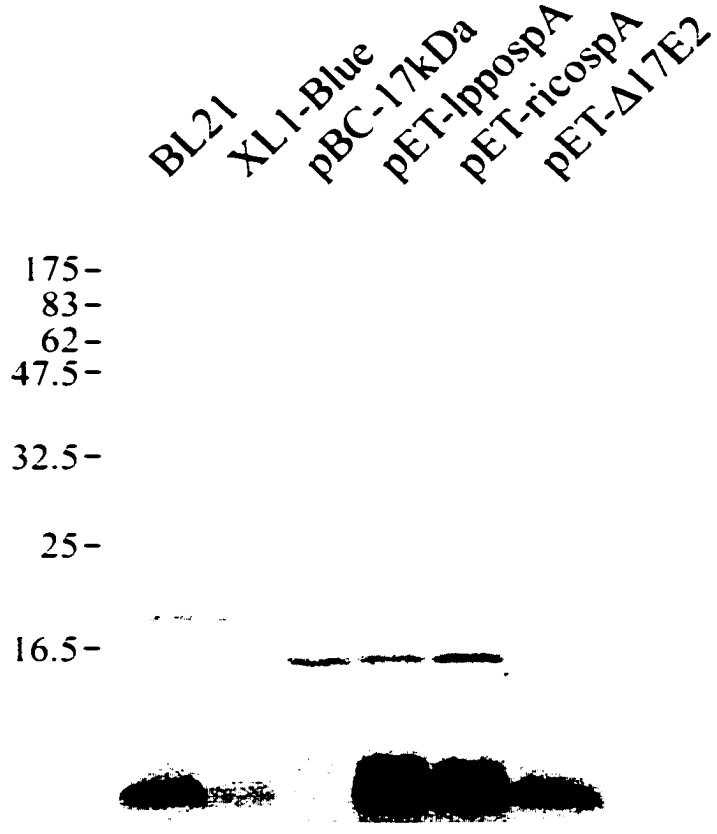

Figure 18. [^{14}C]Palmitate incorporation analysis of OspA. [^{14}C]Palmitate-labeled induced cultures of *ospA* constructs were analyzed by SDS-PAGE (15% polyacrylamide). The first two lanes contain *E. coli* negative controls that were induced under the same conditions as the OspA constructs. Note the [^{14}C]palmitate-labeled product with a relative mobility of ~16 kDa present in induced cultures of pBC-17kDa, pET-lppospA, and pET-ricospA. No labeled products, that differed from the *E. coli* BL21 control were observed in the pET-Δ17E2 sample. Molecular mass is on the left in kDa.

and the chimeric ricospA and lppospA constructs were all labeled with comparable levels of [^{14}C]palmitate (Fig. 18). Removal of the OspA signal peptide region abolished [^{14}C]palmitate incorporation (Fig. 18).

Coupling of OspA with an N-terminal fusion partner

17E2 was cloned in frame with a 91 aa N-terminal fusion partner protein in an effort to prevent recognition of the signal peptide and subsequent lipid modification in *E. coli*. Very high production of the OspA fusion construct (34% total protein), C17E2, was obtained in *E. coli* BL21. The OspA fusion was recovered as inclusion bodies comprising 42% total protein of the insoluble cellular fraction (Fig. 17).

Immunoblot analysis of OspA

Expression of the immunoreactive product responsible for the initial identification of the lambda clone was not detected in clone pB12. The full length clone pB12 grew very poorly even when the *lac* promoter was catabolite repressed. Clone pB12-6.3b, which lacked the *bax* and *radA* ORF's (Fig. 10A), grew well under standard conditions and produced a weakly immunoreactive 17 kDa product (results not shown).

To confirm expression of *ospA* in *P. salmonis*, the Jameson-Wolf method was used to identify the 110-129 aa region of OspA as having a high antigenic index (Jameson and Wolf, 1988). Rabbit antibodies were raised against a 20 aa synthetic peptide of this region. The rabbit anti-OspA peptide serum reacted with a 17 kDa product in whole cell *P. salmonis* and OspA in induced pET-17E2 samples (Fig. 19).

Convalescent serum from coho salmon, against *P. salmonis*, also strongly recognized OspA and the OspA fusion in induced samples of pET-17E2 and pET-C17E2 (Fig. 19). The

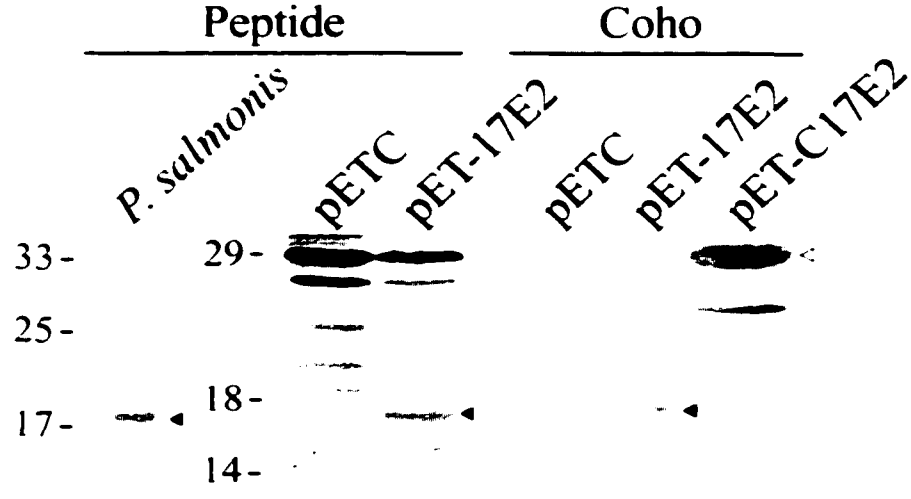

Figure 19. Immunoblot analysis of OspA. Whole cell lysates of *E. coli* clones and *P. salmonis* were analyzed by SDS-PAGE (12% polyacrylamide). Whole cell lysates of *P. salmonis*, pETC and pET-17E2 were reacted with rabbit polyclonal anti-OspA peptide serum. A 17 kDa product was recognized in both the *P. salmonis* and pET-17E2 samples. Whole cell lysates of pETC, pET-17E2, and pET-C17E2 were also reacted with convalescent serum from coho salmon. Both OspA and the OspA fusion were recognized by the salmonid serum, the OspA fusion being strongly recognized. Black arrowheads point to OspA, the hollow arrowhead points out the OspA fusion. Molecular mass is on the left in kDa.

protein C fusion partner of C17E2 was not recognized by either the anti-OspA peptide or convalescent coho serum (Fig. 19).

Phylogenetic analysis of *P. salmonis* 16S rRNA, RadA, and 'Alr

The phylogenetic relationship of *P. salmonis* to other *Proteobacteria* and more distantly related bacteria was analyzed using 16S rRNA nucleotide sequence and predicted aa sequence of RadA and alanine racemase (Fig. 20). As reported previously by Mauel *et al.* (1999), 16S rRNA sequence establishes *P. salmonis* as a member of the gamma subgroup of *Proteobacteria*, most closely related to *Coxiella burnetii* (Fig. 20A). Phylogenetic trees constructed from multiple alignments of RadA and alanine racemase enzymes revealed a placement of *P. salmonis* similar to the 16S rRNA data (Fig. 20B & 20C). *P. salmonis* repeatedly clustered within the gamma subgroup of *Proteobacteria* most closely to *P. aeruginosa* distant from *R. prowazekii*.

Discussion

Rickettsiae are notoriously slow growing bacteria and, like *C. burnetii* and members of the tribe *Ehrlichieae*, *P. salmonis* is even more difficult to grow and to separate from host cell components because of its location within cytoplasmic vacuoles. Contamination of genomic DNA preparations is a serious concern when constructing genomic libraries of obligate intracellular bacteria. Therefore, with all their imperfections PCR techniques are often preferred over genomic library screening when cloning genes from rickettsiae (Anderson, 1997) but are inadequate for detecting novel genes.

Prior to this study, no nucleotide sequence data was available from *P. salmonis* with the exception of ribosomal RNA genes (Fryer *et al.*, 1992, Mauel *et al.*, 1999). Therefore,

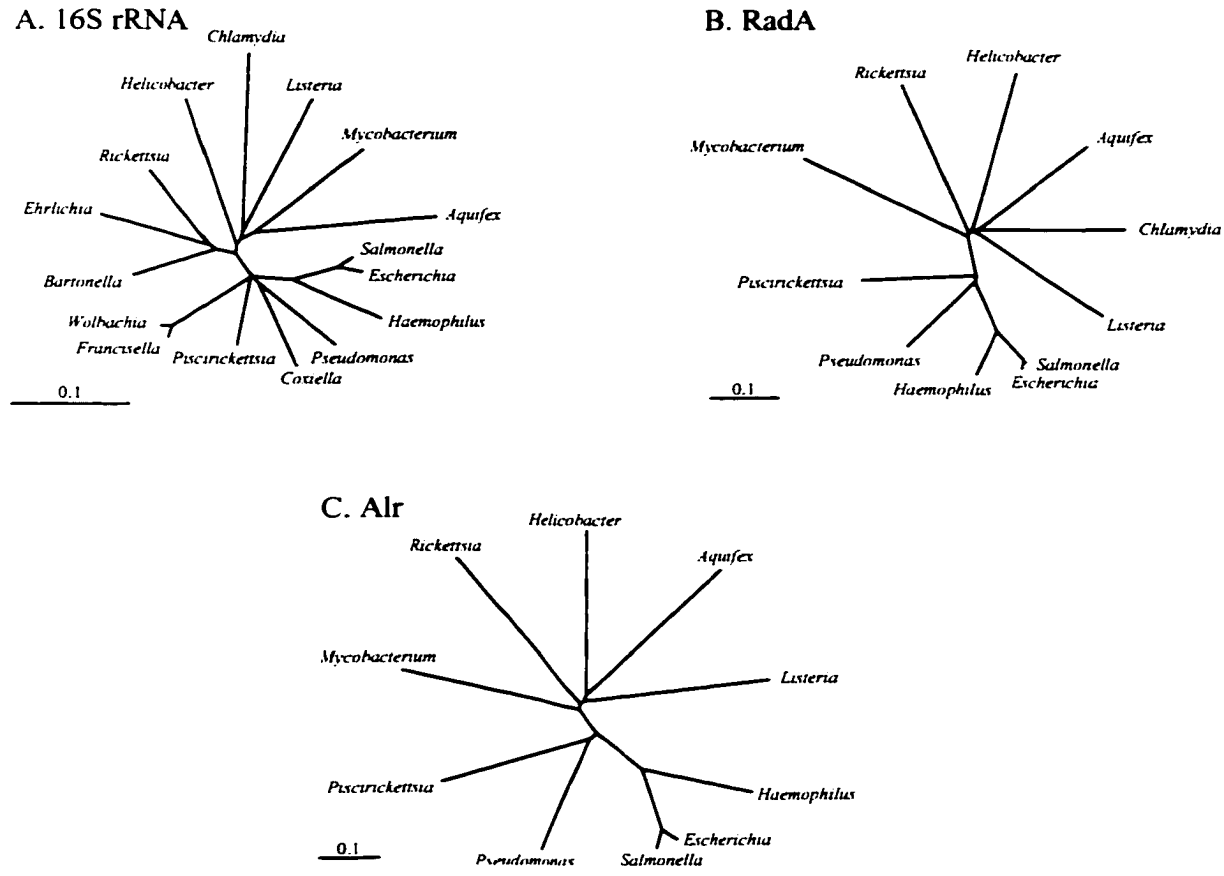

Figure 20. Phylogenetic analysis of *P. salmonis*. Unrooted trees of ClustalX multiple alignments using the Neighbour-Joining method of Saitou and Nei (1987) were constructed using nucleotide and protein sequences of 16S rRNA, RadA and 'Alr proteins among selected *Proteobacteria* and other bacteria. In all three trees, *P. salmonis* is placed distantly from the genuses Rickettsia and Ehrlichia. Gamma *Proteobacteria*: *Coxiella burnetii*, *Escherichia coli*, *Francisella tularensis*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, *Wolbachia persica*, and *Salmonella typhimurium*. Alpha *Proteobacteria*: *Bartonella quintana*, *Ehrlichia risticii*, and *Rickettsia prowazekii*. *Helicobacter pylori*, an epsilon *Proteobacteria*, *Aquifex aeolicus*, *Chlamydia trachomatis*, *Listeria monocytogenes*, and *Mycobacterium tuberculosis* were selected as distantly related bacteria. Scale is the distance of 0.1 substitutions per site.

to identify antigens with vaccine potential, a *P. salmonis* genomic DNA expression library was constructed and screened with rabbit anti-*P. salmonis* serum 82CR which predominantly recognizes surface associated antigens. All 18 isolated immunoreactive lambda clones encoded a common region of the *P. salmonis* genome. DNA sequencing of clone pB12 revealed a 4,983 bp insert with an A+T content of 59% and 4 predicted ORF's and 1 partial ORF (Fig. 10).

The *P. salmonis radA* ORF was well expressed by *E. coli* S30 lysates (Fig. 14) and seriously interfered with subcloning of the pB12 insert. *P. salmonis* RadA is apparently toxic to *E. coli* as clones that lacked the initial 26 bp of *radA* grew as well as *E. coli* DH5α. Even though *radA* was ~1.5 kbp downstream of *lacP*, catabolic repression of *lacP* was still required to obtain a stable insert in *E. coli*. *P. salmonis* RadA is closely related to that of *P. aeruginosa* and *E. coli* (≥60% identical) (Fig. 20). The RadA enzyme family are DNA repair enzymes suspected to be involved in the repair of DNA alkylation (Song and Sargentini, 1996). Even though a *radA* transcript could not be detected (Fig. 15), the presence of an intact *radA* ORF had a dramatic impact upon the growth of *E. coli*. Toxicity is presumably the result of unregulated *radA* expression.

The *ospA* gene product is a promising recombinant vaccine candidate. The aa sequence of the 17.7 kDa protein encoded by the *ospA* ORF, outer surface protein A (OspA), displayed homology to the 17 kDa rickettsial genus common antigen, an outer membrane lipoprotein (Anderson *et al.*, 1988, Anderson and Tzianabos, 1989). The rickettsial 17 kDa membrane antigen is one of only three characterized *Rickettsia* surface proteins and is highly conserved within the genus *Rickettsia* with greater than 90% aa identity (McDonald

et al., 1997). The function of the 17 kDa antigen is unknown and until now has never been identified in any bacteria outside the genus *Rickettsia* (Anderson, 1990).

Both OspA and the rickettsial homologue exhibit sequence similarity to a lipoprotein of similar mass, LipA, in *Rhizobium etli* (Fig. 13B) (Tabche *et al.*, 1998, Yeoman *et al.*, 1997). The function of the rhizobial LipA protein is also undetermined, but the *lipA* gene is located within a cluster of 6 genes whose function are required for high affinity acquisition of iron (Yeoman *et al.*, 1997).

Although *E. coli ospA* expression was not detected by *in vitro* transcription and translation, a weakly immunoreactive 17 kDa protein was observed by immunoblot analysis of pB12 deletion clones (results not shown). In an effort to increase the production level of OspA for further characterization studies and future protection studies, *ospA* was cloned under control of the phage T7 promoter (pBC-17kDa). OspA was visibly produced from pBC-17kDa after anywhere from 3-10 h of induction, but only at a low level (Fig. 17). Therefore, poor expression of the *ospA* gene did not appear to be the result of its promoter being poorly recognized in *E. coli*. It is possible that the ribosome binding site (RBS) of *ospA* is weakly recognized in *E. coli*, or *ospA* mRNA may have secondary structure in the 5' region (Makrides, 1996), or that differential use of minor codons is regulating gene expression post-transcriptionally (Saier, 1995).

A PCR strategy to synthesize the *ospA* gene using codons optimized for *E. coli* was formulated (Fig. 11). Previous studies on *E. coli* expression of low m.w. recombinant proteins demonstrated that the position 2 codon can have a significant impact on translation with Arg (CGT) codons generally being favoured (Belagaje *et al.*, 1997). Therefore, the Asn-2 codon of *ospA* was deleted in the synthetic gene, 17E2, making Arg-3 the second

codon in 17E2 (Fig. 13). The 17E2 gene was cloned into pET21a under control of the T7 promoter and with an optimal RBS. Expression of pET-17E2 and subsequent constructs were studied in protease deficient *E. coli* BL21 because degradation of low m.w. proteins in *E. coli* inherently makes their production difficult (Belagaje *et al.*, 1997). OspA production levels from codon optimized pET-17E2 were no higher than those previously obtained from pBC-17kDa (Fig. 17).

The rickettsial 17 kDa antigen is an outer membrane protein that has a signal peptide recognized by signal peptidase II. Removal of type II signal peptides is a three step process involving addition of diacylglyceryl to a Cys prior to cleavage of the signal peptide on the N-terminal side of the Cys and addition of a fatty acid to the free amide of the N-terminal Cys (Stanley *et al.*, 1998, von Heijne, 1990). A signal peptide prediction algorithm, SignalP (Nielsen *et al.*, 1997), marginally identified OspA as a possible secreted protein (Fig. 16), and the 11-21 aa region contains a consensus pattern for prokaryotic membrane lipoprotein lipid attachment (Fig. 16) with Cys-21 being the predicted acylation site (Hofmann *et al.*, 1999).

Presence of a signal peptide alone does not guarantee transport of a protein across the bacterial inner membrane (Makrides, 1996). Removal of the signal sequence of a *Borrelia burgdorferi* lipoprotein gene permitted much higher expression in *E. coli* (Dunn *et al.*, 1990). To determine the effect the signal peptide of OspA has on its expression and lipidation in *E. coli*, the signal sequence (aa 1-20) of 17E2 was removed by PCR. Oligonucleotides of type II signal peptides from Braun's lipoprotein and the *R. rickettsii* 17 kDa antigen that are well recognized by *E. coli* were synthesized and added to Δ17E2 by PCR (Fig. 12). (Anderson *et al.*, 1988, Hansson *et al.*, 1995). Addition of the *R. rickettsii*

and Braun's lipoprotein signal peptides to OspA dramatically improved SignalP prediction of OspA as a secreted protein (Fig. 16). To determine if the heterologous signal peptides improved production or lipidation of OspA, expression of *ospA* constructs pET-Δ17E2, pBC-17kDa, pET-lppospA, and pET-ricospA were compared (Fig. 17). OspA production was significantly improved by removing its putative signal peptide (Fig. 17). The addition of Braun and rickettsial signal peptides did not improve OspA production and products were not detected after 10 h of induction (Fig. 17). But, levels of OspA production similar to pBC-17kDa had been observed from pET-lppospA and pET-ricospA during shorter periods of induction.

Palmitate incorporation was conducted to determine if OspA is recognized and processed as a lipoprotein in *E. coli*. Palmitate was incorporated at similar levels into the OspA products from pBC-17kDa, pET-lppospA, and pET-ricospA (Fig. 18). Removal of the signal peptide from OspA abolished the incorporation of palmitate (Fig. 18). It appears that the inability to produce high levels of OspA in *E. coli* stemmed principally from the presence its signal peptide, similar to results obtained with *B. burgdorferi* lipoproteins (Dunn *et al.*, 1990). Effective production of secreted proteins in *E. coli* is not a trivial task; protein exporting machinery can become overloaded and cytoplasmic preproteins can be subjected to proteolysis resulting in poor production levels (Makrides, 1996).

Although the improved production of truncated OspA from pET-Δ17E2 was quite promising, we anticipated that a higher production level might be obtained by partnering OspA with an N-terminal fusion protein. By adding the fusion partner we aimed to mask the OspA type II signal peptide from secretory machinery and further protect it from proteases. Excellent production of the 28 kDa OspA fusion protein was obtained from

pET-C17E2 in *E. coli* BL21 with the fusion product isolated as inclusion bodies from the insoluble fraction of sonicated cells (Fig. 17). Localization of the OspA fusion to inclusion bodies was not detrimental to our goal of high level OspA production because neither biological activity nor proper folding of recombinantly produced OspA were required for our applications.

OspA produced in *E. coli* was never found to be strongly immunoreactive with polyclonal rabbit anti-*P. salmonis* serum that was originally used to identify clone pB12. Antibodies generated against a 20 aa synthetic peptide of OspA did react with a 17 kDa product in *P. salmonis* and weakly recognize the OspA product from pET-17E2 (Fig. 19), thereby confirming expression of *ospA* in *P. salmonis*. None of the OspA constructs were more immunoreactive than the wild type OspA produced from pBC-17kDa.

The ability of whole cell *P. salmonis* to elicit a detectable antibody response in salmon against OspA was of considerable interest. Both OspA and the OspA fusion protein reacted strongly and specifically with convalescent serum from coho salmon (Fig. 19) once again identifying OspA as a prospective recombinant vaccine candidate.

In summary, a *P. salmonis* lambda clone encoding an immunoreactive outer surface protein, OspA, was identified using polyclonal anti-*P. salmonis* rabbit serum. The 5 kbp insert was A+T rich, encoded four intact ORF's, and represents the first non-ribosomal DNA sequence data from *P. salmonis*. OspA is modified as a bacterial lipoprotein in *E. coli* and its closest homologue is a rickettsial 17 kDa surface lipoprotein previously thought limited to the genus *Rickettsia*. Both OspA and the rickettsial 17 kDa antigens exhibit intriguing sequence similarity to a 15.7 kDa *Rhizobium* lipoprotein. A codon optimized version of *ospA* was constructed and the lipoprotein nature of OspA was identified as a

limiting factor in its production. High level *E. coli* production of immunoreactive OspA targeted to inclusion bodies was achieved by partnering OspA with an N-terminal fusion protein. The OspA fusion was recognized by convalescent salmon sera indicating that during a natural infection in the salmonid host, *P. salmonis* elicits an immune response against OspA. OspA appears to be an excellent candidate for a recombinant vaccine against *P. salmonis*.

CHAPTER 4

VACCINOLOGY OF *P. SALMONIS*

Introduction

Although rickettsiae cause a number of significant human and veterinary diseases, few effective vaccines are currently available. Numerous protective antigens of rickettsiae have been identified as potential candidates for recombinant vaccines (McDonald *et al.*, 1987, Nyika *et al.*, 1998, Ohashi *et al.*, 1998, Palmer *et al.*, 1994, Seong *et al.*, 1997, Sumner *et al.*, 1995, van Vliet *et al.*, 1994), but no recombinant vaccines are available against any rickettsial diseases. Rickettsial vaccines have traditionally been based on inactivated whole cell preparations. Bacterin vaccines are available for a number of rickettsial diseases, but incomplete and variable protection has limited their usage to high risk individuals and livestock (Mahan *et al.*, 1998, Maurin and Raoult, 1999). Vaccine trials using inactivated whole cell preparations of *P. salmonis* have yielded disappointing results in field trials (Smith *et al.*, 1997). No evidence of significant protection against SRS was found in coho salmon (*Oncorhynchus kisutch*) vaccinated with *P. salmonis* bacterin, and disease was exacerbated in some vaccinated groups suggesting the presence of immunosuppressive antigens (Smith *et al.*, 1997). Control of rickettsial diseases has primarily relied upon chemoprophylaxis and control of insect and rodent vectors (Kazar and Brezina, 1991).

During the past decade, numerous studies have investigated the ability of promiscuous T cell epitopes (TCE's) incorporated into chimeric peptides and proteins to enhance the immunogenicity of other epitopes within the chimeric peptide or protein in mammalian immune systems (El Kasmi *et al.*, 2000, Kjerrulf *et al.*, 1997, O'Hern *et al.*, 1997, Partidos *et al.*, 1992, Pillai *et al.*, 1995, Valmori *et al.*, 1992). Promiscuous TCE's from measles virus fusion protein (MVF) (288-302) (Partidos and Steward, 1990) and *Clostridium tetani* tetanus toxin (tt) P2 epitope (830-844) (Panina-Bordignon *et al.*, 1989) were incorporated

into OspA fusion proteins to determine if these mammalian TCE's could enhance the immunogenicity of OspA within the salmonid immune system.

Here it is shown that recombinantly produced OspA forms an effective subunit vaccine that elicits a protective immune response in coho salmon against *P. salmonis*. Addition of xenobiotic TCE's to OspA fusion protein constructs dramatically improved the immunogenicity of OspA which was reflected by increased protection of vaccinated salmon.

Materials & Methods

Bacterial strains and media

Chapters 1 & 3 encompass descriptions of the growth of *P. salmonis*, and *E. coli* strains.

Creation of TCE OspA constructs

Chapter 3 contains a description of the construction of pET-C17E2. TCE's tt P2 (830-844 QYIKANSKFIGITEL) (GenBank X06214) and MVF (288-302 LSEIKGVIVHRLEGV) (GenBank M81903) were synthesized as oligonucleotides (Table 5) using codons optimized for high level expression in *E. coli*. The epitope coding regions of the MVF and tt oligonucleotides were flanked by *Bam*H I, *Nde* I and *Vsp* I, *Hin*d III restriction endonuclease sites and primer binding sites for subsequent PCR amplification and subcloning. The MVF and tt P2 oligonucleotides were converted to double stranded DNA and amplified by PCR using standard conditions (Giovannoni, 1991) and cloned into pBCKS-V using *Bam*H I and *Hin*d III restriction endonuclease sites to create pBC-MVF and pBC-ttP2. Vector

Table 5. Oligonucleotides of of tt P2 and MVF TCE's. Bold nucleotides represent the coding region of both epitopes.

OLIGO.	SEQUENCE
MVF	CGC CAG GGT TTT CCC AGT CAC GAC GGA TCC GTC TCA TAT G**CT GTC TGA AAT CAA AGG TGT TAT CGT TCA TCG TCT GGA** **AGG CGT G**AT TAA TTA AGC TTC GGA CCA GGA CGA GAG GAC G
tt P2	CGC CAG GGT TTT CCC AGT CAC GAC GGA TCC GTC TCA TAT G**CA GTA CAT TAA AGC AAA CTC TAA ATT CAT CGG TAT TAC** **CGA ACT G**AT TAA TTA AGC TTC GGA CCA GGA CGA GAG GAC G

pBCKS-V is a variant of pBC KS(+) (Stratagene) that lacks *Vsp* I restriction endonuclease sites at 925 and 984 bp. pBC KS(+) was digested with *Vsp* I, single stranded ends were filled in using Klenow fragment, and blunt end ligation was performed to create pBCKS-V.

The *Bam*H I and *Vsp* I fragments of pBC-MVF and pBC-ttP2 were separately subcloned into the *Bam*H I and *Nde* I sites of pET-C17E2 (Fig. 21). This subcloning step placed the TCE's in frame between *ospA* and the N-terminal fusion partner to create pET-CM17E2 and pET-CT17E2 (Fig. 21). Ligation of the *Vsp* I and *Nde* I cohesive ends destroyed the respective restriction sites while an *Nde* I site was encoded in the 5'-terminal region of the TCE insert to allow subsequent ligation of inserts in frame and upstream of the TCE using *Bam*H I and *Nde* I (Fig. 21).

A third construct encoding both TCE's was created by subcloning the *Bam*H I and *Vsp* I fragment of pBC-MVF into the *Bam*H I and *Nde* I sites of pET-CT17E2 to create pET-CMT17E2 (Fig. 21).

The sequences of all constructs were confirmed by DNA sequencing described in Chapter 3.

Expression of TCE *ospA* constructs

For induction experiments, pGP1-2 encoding T7 RNA polymerase (Tabor and Richardson, 1985) was co-electroporated with each construct into *E. coli* BL21. Transformants were selected overnight at 30°C on MacConkey media (Kn/Ap).

For expression studies of the *ospA* constructs, overnight cultures (30°C) were diluted with an equal volume of fresh TB broth and temperature was shifted to 42°C for 3 h of

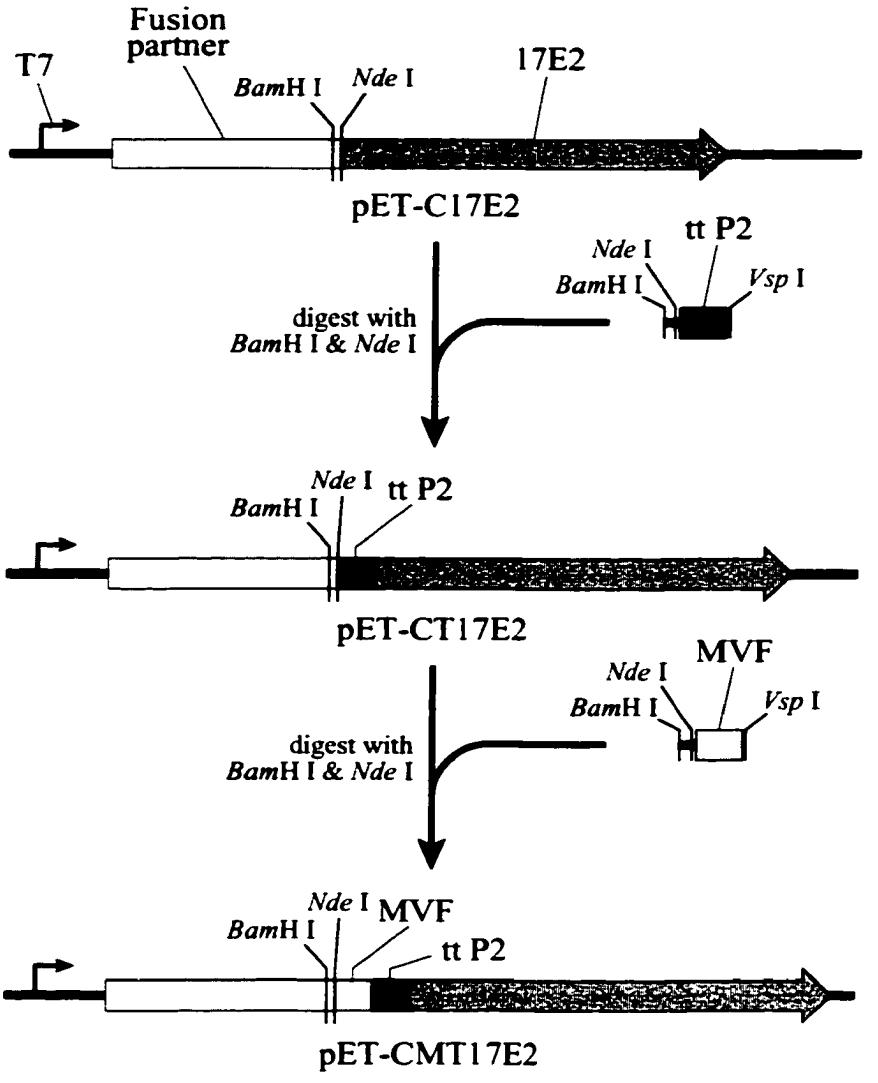

Figure 21. Incorporation of TCE's into OspA. A diagram illustrating the cloning strategy employed to create the OspA fusion protein constructs encoding promiscuous T cell epitopes. 17E2 is the synthetic *ospA* gene that was created using codons optimized for *E. coli* high level expression. MVF is the measles virus fusion protein T cell epitope (288-302). tt P2 is the tetanus toxin P2 epitope (830-843).

induction. Inclusion bodies were collected by sonicating induced cultures for 3 × 60 s followed by centrifugation at 10,000 × *g*.

Inclusion body samples were separated by SDS-PAGE, 12% polyacrylamide, using a discontinuous SDS gel system (Ames, 1974, Laemmli, 1970). Protein samples were visualized using Coomassie brilliant blue (Sambrook *et al.*, 1989) and quantified using a Gel Documentation System and AlphaEase software (Alpha Innotech Corp.).

Calculation of relative percent survival (RPS)

Calculation of RPS is an accepted method of determining the effectiveness of a vaccine (Amend, 1981). RPS is calculated as a ratio of the cumulative mortality of a test group to the cumulative mortality of an unvaccinated control group.

$$\text{RPS} = \left[1 - \left(\frac{\text{\% mortality of test group}}{\text{\% mortality of control group}}\right)\right] \times 100\%$$

Determination of *P. salmonis* 50% lethal dose

Six groups of coho salmon fry (~13 g), 20 fish per group, were IP-injected with either *P. salmonis* or MEM. Five groups each received an injection of *P. salmonis* differing in titre by a 10-fold dilution. Groups received neat *P. salmonis* ($10^{6.3}$ $TCID_{50}$/ml) through $10^{2.3}$ $TCID_{50}$/ml. The control group received an IP injection of cell culture media, MEM. Fish were held in freshwater for the extent of the challenge, and mortalities were recorded and confirmed.

The 50% lethal dose (LD_{50}) of *P. salmonis* in coho salmon fry was calculated using the protocol of Reed and Muench (1939).

Bacterin vaccine trial

Cell culture supernatants from *P. salmonis* infected CHSE-214 monolayers displaying complete cytopathic effect (~10^6 $TCID_{50}$/ml) was inactivated with formalin (0.5% v/v) for 12 h at 4°C with shaking. Inactivated *P. salmonis* antigen was diluted appropriately and mixed with Microstim™ adjuvant (20% v/v) to create 3 bacterin preparations that contained undiluted (neat) *P. salmonis* antigen, 1:10 dil. antigen, and 1:20 dil. antigen.

Coho salmon (~18 g) were anaesthetized in 1 ppm metomidate hydrochloride (Marinil; Wildlife Laboratories, Fort Collins, CO), fin clipped for group identification, and then intraperitoneally (IP) injected with 0.1 ml of each vaccine preparation with 30 fish per group. Control fish were injected with 0.1 ml of spent cell culture media from uninfected CHSE-214 monolayers formulated with adjuvant (20% v/v, oil in water emulsion). Salmon were held 10°C for 3 weeks prior to infectious *P. salmonis* challenge.

Vaccinated coho were anaesthetized (1 ppm Marinil) and challenged with a 0.1 ml IP injection of *P. salmonis* infected CHSE-214 cell culture supernatant (~$10^{4.6}$ $TCID_{50}$/ml). Following challenge, salmon were held at 13°C, mortalities were logged and confirmed by ELISA using polyclonal anti-*P. salmonis* rabbit serum and immunofluorescence microscopy of liver and kidney tissue prints as previously described (Kuzyk *et al.*, 1996).

OspA recombinant vaccine trial

OspA fusion proteins were purified as inclusion bodies from *E. coli* BL21 and protein concentrations were determined using the BCA protein assay (Pierce). The relative percentages of the OspA fusion proteins within each preparation were determined by SDS-PAGE analysis and quantification of the fusion protein bands using a Gel Documentation system and AlphaEase software. Each protein sample was fixed by the

addition of formalin (1 ml/l) and incubation with shaking at 15°C for 24 h. Each protein solution was added aseptically to Microstim™ adjuvant (20% v/v) to obtain a final target protein concentration of 250 mg/L.

Coho salmon (~15 g) were anaesthetized (1 ppm Marinil), fin clipped for group identification, and IP-injected with 0.2 ml of vaccine with 60 fish per group. There were 6 groups in total: C17E2, CT17E2, CM17E2, CMT17E2, CM17E2 plus CT17E2 (1:1), and an adjuvant control. Salmon were held for 8 weeks in freshwater at 8.5°C post-vaccination.

All vaccinated coho were anaesthetized (1 ppm Marinil) and IP-injected with 0.1 ml of *P. salmonis* infected CHSE-214 cell culture supernatant (~10^6 $TCID_{50}$/ml). Salmon were maintained in freshwater at 13°C post-challenge and mortalities were logged. External and internal observations along with PCR of kidney and central liver sections using *P. salmonis* 16S rRNA primers (Giovannoni, 1991, Marshall *et al.*, 1998) were performed for confirmation of mortality.

Results

Establishment of a *P. salmonis* challenge model

Both coho and Atlantic salmon have routinely been challenged with lethal doses of *P. salmonis* and characteristic symptoms of advanced SRS develop within 10-15 days post-infection. *P. salmonis* has been successfully reisolated in purity from infected kidney tissue on CHSE-214 monolayers. Reisolated strains of *P. salmonis* tend to grow more quickly than strains that have been passaged *in vitro* at length. We suspect that periodic *in vivo* passaging of *P. salmonis* is required for optimal growth in cell culture. After approximately

10 passages *in vitro*, *P. salmonis* titres obtained from infected monolayers decrease and *P. salmonis* eventually ceases to grow (results not shown).

In order to develop a reproducible *P. salmonis* challenge model for vaccine trials, its 50% lethal dose (LD_{50}) had to be determined. Lower infectious doses of *P. salmonis* administered to fish resulted in a delay before mortality was observed, and an overall reduction of cumulative mortality (Fig. 22). The LD_{50} of *P. salmonis* in coho salmon fry was calculated to be $10^{4.13}$ $TCID_{50}$/fish ($10^{5.13}$ $TCID_{50}$/ml adjusted for the 0.1 ml injection volume).

P. salmonis bacterin protects coho salmon

Control coho salmon injected with adjuvant reached a cumulative mortality of 66.7%, 48 days after challenge with *P. salmonis* (Fig. 23). Coho salmon vaccinated with neat *P. salmonis* bacterin were protected with a 35% RPS (Fig. 23). Salmon vaccinated with *P. salmonis* bacterin dil. 1:5 exhibited a mild protective response (17.5% RPS) and salmon vaccinated with bacterin dil. 1:25 experienced 86.7% cumulative mortality higher than that of the unvaccinated controls (Fig. 23).

OspA elicits a protective immune response against _P. salmonis_ in coho salmon

An OspA vaccine trial was conducted in coho salmon fry using the OspA fusion product of construct pET-C17E2. Mortality in the OspA vaccinated group was delayed 12 days over the control group (Fig. 24). A challenge pressure of 58% cumulative mortality was attained in adjuvant injected control salmon (Fig. 24). OspA protected vaccinated salmon with a 58.6% RPS (Fig. 24).

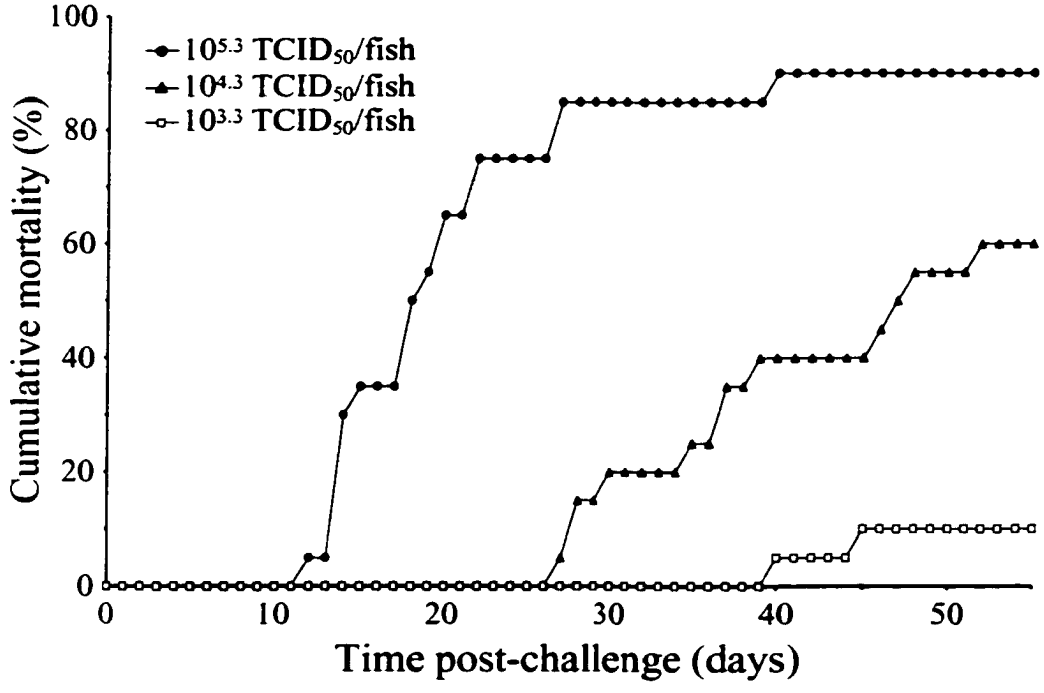

Figure 22. 50% lethal dose of *P. salmonis* in coho fry. Fish that received neat *P. salmonis* ($10^{5.3}$ $TCID_{50}$/fish) experienced 94% cumulative mortality. Subsequent groups that received $10^{4.3}$ $TCID_{50}$/fish and $10^{3.3}$ $TCID_{50}$/fish experienced 58 and 9% cumulative mortality. Note the delay in the onset of mortality in groups that received lower challenge doses.

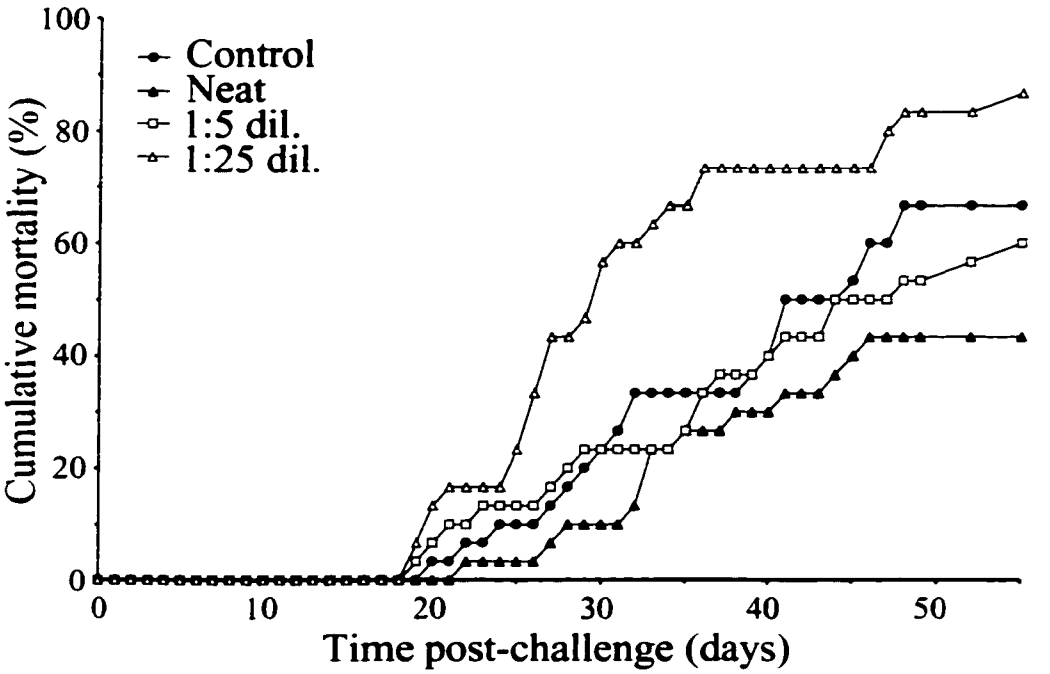

Figure 23. *P. salmonis* bacterin trial in coho salmon fry. Cumulative mortality reached 66.7% in the cell culture media-injected control group following challenge with *P. salmonis* by IP injection. Salmon vaccinated with neat bacterin experienced 43.3% cumulative mortality. The group vaccinated with 1:5 dil. bacterin reached 60% cumulative mortality and the 1:25 dil. bacterin vaccinated group had an 86.7% cumulative mortality. RPS values of *P. salmonis* bacterin administered neat, dil. 1:5, and dil 1:25 were 35.0, 17.5, and -30%, respectively.

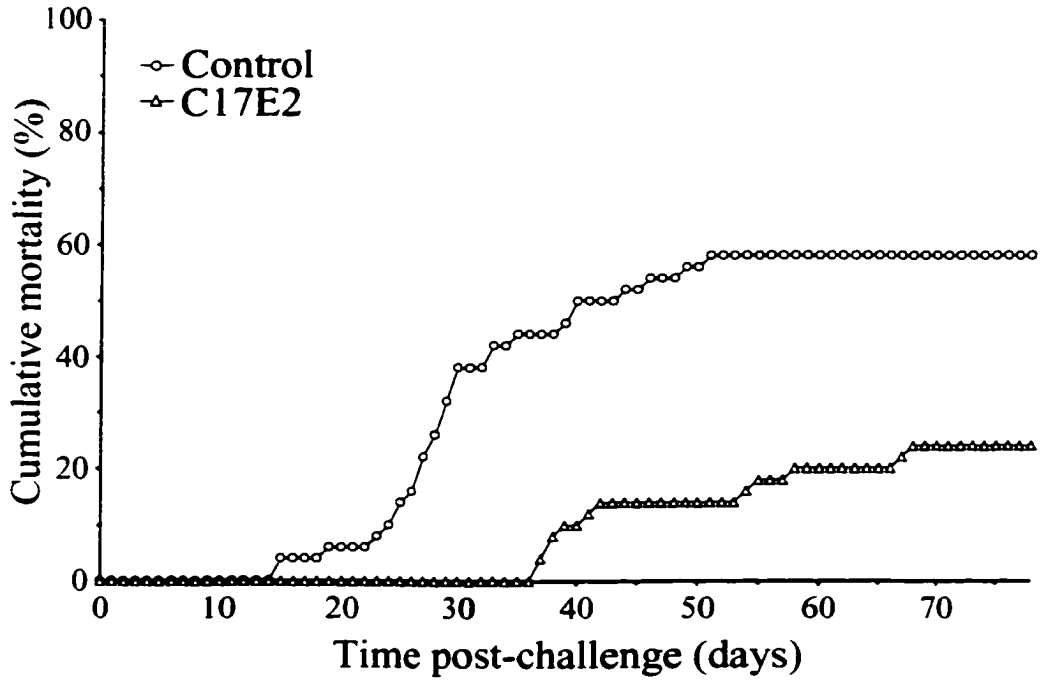

Figure 24. Recombinant OspA vaccine trial. The OspA fusion protein construct, C17E2, was used to immunize an outbred population of coho salmon. Adjuvant-injected salmon experienced a cumulative mortality of 58% when challenged with *P. salmonis* by IP injection. C17E2 vaccinated salmon reached a cumulative mortality of 24%. The RPS value of C17E2 is 58.6%.

Addition of promiscuous TCE's to OspA

Three TCE-encoding OspA fusion protein constructs, pET-CM17E2, pET-CT17E2, and pET-CMT17E2, were created from pET-C17E2 by the addition of MVF and tt P2 TCE's (Fig. 21). To determine whether addition of the TCE's altered the production level of the OspA fusion construct, inclusion body samples from induced cultures were analyzed by SDS-PAGE (Fig. 25). All the OspA fusion protein constructs were produced at levels equal to or greater than pET-C17E2 (Fig. 25) except the pET-CMT17E2 construct which was produced at a slightly lower level (20% total protein) (Fig. 25).

Vaccine trial with OspA constructs encoding xenobiotic TCE's

Vaccine formulations of OspA fusion protein constructs C17E2, CM17E2, CT17E2, and CMT17E2 were administered by IP injection to groups of 60 fish. Mortalities in the TCE OspA construct vaccinated groups began 7-10 days after the control group (Fig. 26). Challenge pressure was higher than in the previous OspA vaccine trial, with cumulative mortality reaching 85.5% in the control group (Fig. 26). The C17E2 vaccinated group reached 59.6% cumulative mortality, 30.2% RPS (Fig. 26). The CT17E2 vaccinated group reached a cumulative mortality of 35.6%, 58.4% RPS (Fig. 26). CM17E2 vaccinated salmon reached 20.0%, 76.6% RPS (Fig. 26). Salmon vaccinated with a 1:1 mixture of CM17E2 and CT17E2 reached 18.6% cumulative mortality giving a 78.2% RPS (Fig. 26). The lowest mortality was observed in the CMT17E2 vaccinated group, with only 14.5% cumulative mortality and an 83.0% RPS (Fig. 26).

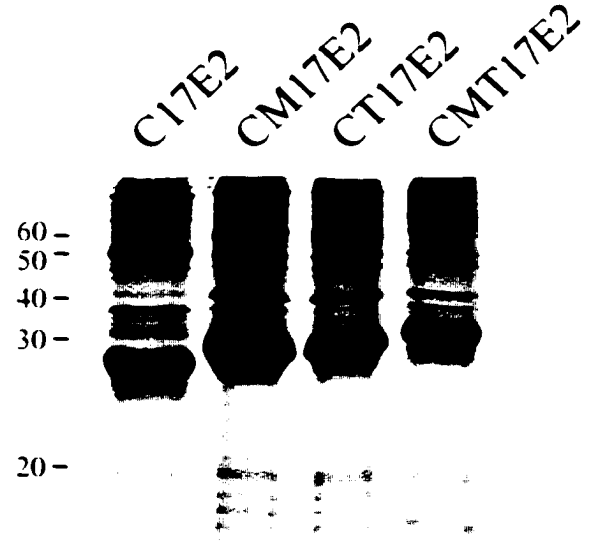

Figure 25. Analysis of TCE-encoding OspA production levels. Inclusion body samples of induced pET-C17E2, pET-CM17E2, pET-CT17E2, and pET-CMT17E2 cultures were analyzed by SDS-PAGE, 12% polyacrylamide. The C17E2, CM17E2, CT17E2, and CMT17E2 chimeric proteins have predicted molecular masses of 28.0 , 29.9, 30.0, and 31.8 kDa, respectively. The C17E2, CM17E2, CT17E2, and CMT17E2 OspA fusion proteins comprised 24, 30, 29, and 20% of the total protein loaded in each sample, respectively. Molecular mass is on the left in kDa.

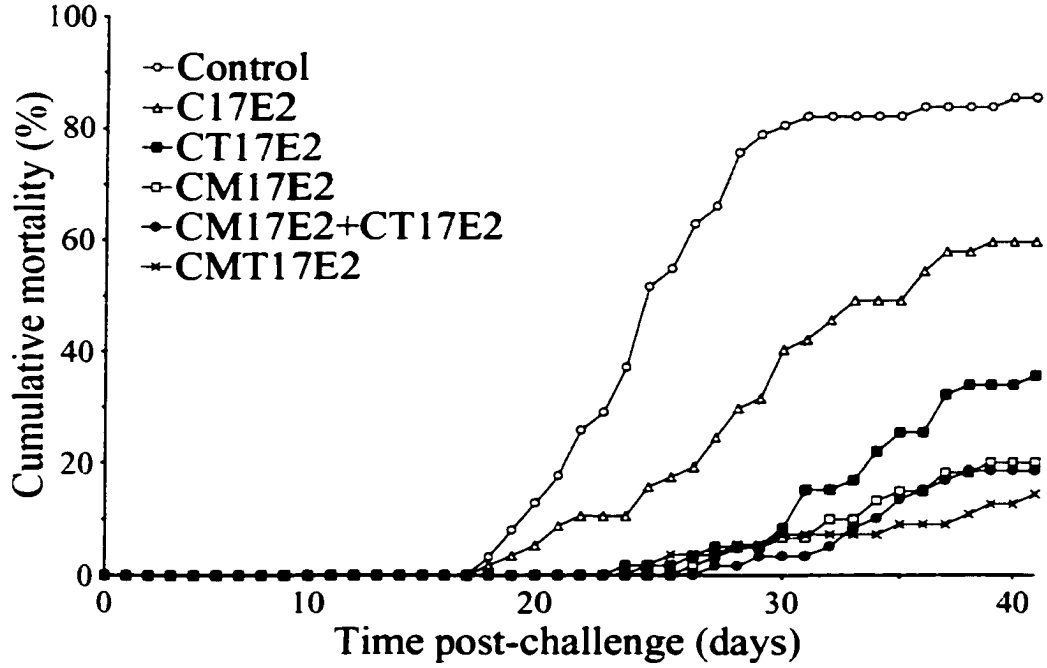

Figure 26. Vaccine trial using TCE–encoding OspA fusion proteins. Adjuvant-injected salmon experienced a cumulative mortality of 85.5% when challenged with *P. salmonis* by IP injection. C17E2 vaccinated salmon reached a cumulative mortality of 59.6%. CT17E2 vaccinated salmon experienced 35.6% cumulative mortality. CM17E2 and the CM17E2 + CT17E2 groups experienced 20 and 18.6% cumulative mortality, respectively. The CMT17E2 vaccinated group experienced only 14.5% cumulative mortality. RPS values of C17E2, CT17E2, CM17E2, CM17E2 + CT17E2, and CMT17E2 were 30.2, 58.4, 76.6, 78.2, and 83.0%, respectively.

Discussion

The infectious dose of *P. salmonis* required to challenge experimental fish has been observed to vary between batches of fish depending on their geographical origin and size. Before protection experiments can be conducted with new groups of fish, a control population from each group must be prechallenged with *P. salmonis* to determine if the *P. salmonis* challenge dose must be adjusted to achieve a statistically significant challenge. The LD_{50} of *P. salmonis* was calculated in coho salmon fry to help standardize a reproducible challenge model and provide a starting point from which an expected 50% cumulative mortality should be obtained from a *P. salmonis* challenge (Fig. 22). A *P. salmonis* dose of $10^{4.13}$ $TCID_{50}$/fish was calculated as the LD_{50} for coho salmon fry (Fig. 22). To ensure statistical significance of challenge trials, groups must contain at least 25 fish each, a cumulative mortality of at least 60% must be achieved in control groups, and cause of mortality must be determined (Amend, 1981).

Prior to the development and testing of a candidate recombinant vaccine for *P. salmonis*, we investigated whether it was possible to elicit a protective immune response in salmon against *P. salmonis* using a simple bacterin vaccine. Although the efficacy of bacterin vaccines against other rickettsiae is highly suspect (Dutta *et al.*, 1998, Mahan *et al.*, 1998, Maurin and Raoult, 1999), several are still commercially available against a variety of rickettsiae: *Ehrlichia risticii*, *Cowdria ruminantium*, and *Coxiella burnetii*. A *P. salmonis* bacterin prepared from cell culture supernatants was tested at various dilutions for efficacy in coho salmon (Fig. 23). A protective effect (35% RPS) was observed in salmon vaccinated with neat *P. salmonis* bacterin (Fig. 23). Salmon that received a 1:5 dil. of the *P. salmonis* bacterin exhibited only a minor amount of protection (17.5% RPS) with

mortalities still accumulating when the trial was terminated (Fig. 23). Interestingly, salmon vaccinated with a 1:25 dil. of the bacterin experienced a significantly higher cumulative mortality (86.7%) than the control group (66.7%) (Fig. 23). The exacerbative effect of 1:25 dil. *P. salmonis* bacterin was confirmed when the bacterin vaccine trial was repeated (data not shown). Smith *et al.* (1997) reported variable protection in *P. salmonis* bacterin vaccinated coho salmon with some vaccinated groups exhibiting higher mortality than unvaccinated control fish. Our results indicated that it was possible to elicit a protective immune response against *P. salmonis* in coho salmon and also suggested that *P. salmonis* may contain immunosuppressive components.

OspA is an N-terminally acylated lipoprotein of *P. salmonis* that is recognized by convalescent coho salmon sera, thereby identifying it as an excellent candidate for a recombinant vaccine against *P. salmonis*. A vaccine trial with recombinantly produced OspA fusion construct, C17E2, in coho salmon demonstrated that OspA is capable of eliciting a protective immune response against *P. salmonis* (Fig. 24). Although challenge pressure was only moderate with cumulative mortality reaching 58% in control adjuvant-injected fish, OspA protected vaccinated salmon with a 58.6% RPS (Fig. 24).

Highly immunogenic TCE's from tt and MVF were added to OspA fusion protein construct C17E2 (Fig. 21) to determine if they would improve the protective response elicited by OspA. Incorporation of highly immunogenic TCE's into chimeric fusion proteins is an elegant extension of the principles that underlie the immunostimulatory effect of toxoid carrier proteins on conjugated haptens (Bixler and Pillai, 1989). Toxoids provide TCE's that are required to elicit a strong T helper cell-mediated immune response against haptens (Bixler and Pillai, 1989). Incorporation of TCE's into synthetic peptide

or chimeric fusion proteins can have an immunostimulatory effect on other T cell and humoral epitopes within the peptide or protein (Hathaway *et al.*, 1995, Kjerrulf *et al.*, 1997, O'Hern *et al.*, 1997, Pillai *et al.*, 1995, Valmori *et al.*, 1992). To minimize genetic restriction of these immunostimulatory responses, promiscuous TCE's capable of binding a MHC molecules from a variety of haplotypes are used in chimeric vaccine constructs. Tandem repeats of TCE's can also often improve immunogenicity of chimeric proteins better than single TCE's (Kjerrulf *et al.*, 1997, Partidos *et al.*, 1992).

The tt P2 and MVF epitopes have been established as strong T helper cell epitopes that exhibit universal antigenicity and are highly immunogenic in human and murine models (Demotz *et al.*, 1989, Panina-Bordignon *et al.*, 1989, Partidos and Steward, 1990). Both tt P2 and MVF TCE's are MHC class II restricted and are able to bind MHC class II molecules from a wide variety of haplotypes. Genetic restriction of murine responses to malarial epitopes has been overcome by incorporation of the tt P2 epitope into synthetic peptide-based malarial vaccines (Valmori *et al.*, 1992).

Although functional presentation of antigen by MHC class I and II complexes has not been confirmed in teleosts, current evidence suggests that teleost MHC molecules play an antigen-presenting role analogous to mammalian MHC molecules (Nakanishi *et al.*, 1999, Partula *et al.*, 1996, Stet *et al.*, 1996, Warr, 1997). Immunostimulatory effects have been observed in teleosts; protection against *Aeromonas salmonicida* was increased in coho salmon vaccinated with an *A. salmonicida* bacterin that contained *Mycobacterium butyricum* cells (Olivier *et al.*, 1985). We sought to determine whether the tt P2 and MVF TCE's would have an immunostimulatory effect within the context of the salmonid immune system.

The protection conferred by TCE-encoding OspA fusion proteins was directly compared to the efficacy of OspA fusion protein, C17E2, in a second vaccine trial in coho salmon (Fig. 26). Protection of coho salmon vaccinated with OspA fusion proteins encoding tt P2 and MVF epitopes was dramatically increased when compared to salmon vaccinated with C17E2 (Fig. 26), suggesting that the universal immunogenicity of tt P2 and MVF epitopes extends to the teleost immune system. The C17E2 OspA fusion protein which protected 58.6% of vaccinates in the previous trial (Fig. 24) protected only 30.2% of vaccinates when challenged with a higher infectious dose of *P. salmonis* (Fig. 26). Addition of TCE's to OspA improved its efficacy nearly 3-fold (Fig. 26). The strongest protective effect was observed when salmon were vaccinated with the CMT17E2 OspA fusion protein containing both the tt P2 and MVF epitopes in tandem with an 83% RPS (Fig. 21, Fig. 26).

In human and murine studies using tt P2 and MVF epitopes, increases in immunogenicity were found to correlate with increased antibody titres (O'Hern *et al.*, 1997, Partidos *et al.*, 1992). We did not observe a correlation between elevated antibody titres to OspA and increased protection (data not shown). The best protection was observed when OspA vaccine preparations contained the MVF epitope which clearly had a greater stimulatory effect on the immunogenicity of OspA than the tt P2 epitope (Fig. 26). A variety of factors may account for this observed difference: the extent of the TCE's MHC restriction may differ; MVF may be more immunogenic within the salmonid immune system; and MVF may be more effective at activating the arm of the immune system required to clear *P. salmonis* infections. Taking into account the intracellular life cycle of *P. salmonis* it seems logical that the increased protection observed upon incorporation of

either TCE into OspA could be attributed to an improved effector response by cytotoxic T lymphocytes. The specific effects that the tt P2 and MVF epitopes have on the salmonid immune response should be the focus of further analysis.

In this study we have determined that vaccination of salmon with a *P. salmonis* bacterin can induce a weak protective immune response. A recombinantly produced 17 kDa lipoprotein antigen of *P. salmonis*, OspA, is capable of inducing a strong protective immune response in coho salmon. MVF and tt P2 TCE's that are highly immunogenic in human and murine models were shown to retain their immunostimulatory properties within the context of the salmonid model. Addition of TCE's to OspA, particularly MVF, dramatically augmented the protective immune response that it elicited in salmon. The strongest protection was observed when salmon were vaccinated with an OspA fusion protein construct encoding both the tt P2 and MVF epitopes.

GENERAL DISCUSSION

This research developed a growth and purification strategy for *P. salmonis* that permitted creation of molecular biology tools that facilitated the development of an efficacious vaccine against *P. salmonis*. Although *P. salmonis* was isolated in 1989 (Fryer *et al.*, 1990), it has remained largely unstudied because of its obligate intracellular nature and fastidious growth requirements. Molecular biology of rickettsiae as a whole has lagged behind comparable fields of bacterial research (Anderson, 1997). Many rickettsial studies have relied on identification and characterization of proteins using degenerate oligonucleotide probes and PCR primers based on proteins characterized in other bacteria (Anderson, 1997). But, functions of novel rickettsial proteins continue to elude researchers because of inherent limitations of the rickettsial genetic system.

P. salmonis was effectively separated from CHSE-214 host cell debris using a combination of differential sedimentation and Percoll density gradient centrifugation. Purified *P. salmonis* was suitable for genomic DNA isolation and generation of polyclonal rabbit sera. Rabbit anti-*P. salmonis* serum was shown to have a bias towards surface-associated antigens and sera collectively recognized numerous protein antigens with relative mobilities ranging from 50-80, 27, and 24 kDa and a carbohydrate antigen at 16 kDa that is likely the LOS component of LPS.

Rabbit anti-*P. salmonis* serum was chosen over salmonid convalescent sera to screen a *P. salmonis* genomic DNA expression library. At the time no reliable anti-salmon Ig monoclonal antibodies were available and salmonid Ig is generally less specific than mammalian Ig which would result in numerous false positives and a high background during screening. Rabbit serum identified a 4,983 bp *P. salmonis* clone, pB12, that encoded

1 partial and 4 intact ORF's. The first non-ribosomal nucleotide sequence data from *P. salmonis* revealed that, like other obligate intracellular bacteria, *P. salmonis* has a low molar percentage content (41%) of guanine and cytosine (Hackstadt, 1996).

Predicted aa sequences of pB12 ORF's '*alr* and *radA* are most closely related to alanine racemase and RadA of *P. aeruginosa* with >65% aa similarity. Phylogenetic placement of *P. salmonis* using 16S rRNA sequence places *P. salmonis* amongst *Coxiella*, *Legionella*, and *Francisella* spp. (Mauel *et al.*, 1999). Phylogenetic analysis using Alr and RadA proteins consistently placed *P. salmonis* distant to members of the order *Rickettsiales* and closest to *P. aeruginosa*. Unfortunately sequence data for *Coxiella*, *Legionella*, and *Francisella* spp. Alr and RadA proteins are not available to determine the extent of phylogenetic similarities with *P. salmonis*. In contrast to the phylogenetic distance and morphological differences of *P. salmonis* and *Rickettsia* spp., the *ospA* ORF of clone pB12 encodes a 17 kDa outer surface protein homologous to the *Rickettsia* genus-common 17 kDa antigen (Anderson *et al.*, 1987).

OspA and the rickettsial 17 kDa antigen are both N-terminally lipid-modified putative outer membrane proteins with unknown function. OspA recombinantly expressed as a fusion protein in *E. coli* elicits a protective immune response in coho salmon fry with an RPS that varied between 30-59% depending on the intensity of the *P. salmonis* challenge. Highly immunogenic, promiscuous TCE's, previously only characterized within mammalian immune systems, were incorporated into OspA fusion proteins. Addition of these TCE's dramatically improved efficacy of OspA, increasing the RPS nearly 3-fold. The strongest protective effect from OspA (83% RPS) was observed when two TCE's, tt P2 and MVF, were present. Having both TCE's present in OspA may improve the overall

protective immune response by increasing the repertoire of MHC haplotypes that able to respond to the immunostimulatory effect of the TCE's. Further work is necessary before the actual effect of these xenobiotic TCE's on the salmonid immune system can be fully realized. Elucidation of the specific effects of the promiscuous TCE's on the salmonid immune system may improve the overall understanding of the MHC molecule's antigen presenting role in the salmonid immune system.

The ability of a single protein, OspA, to elicit such a strong protective immune response against *P. salmonis* in salmon was unanticipated. Prevention of a *P. salmonis* infection with an strong anti-OspA immune response suggests that OspA may play a vital role in the virulence of *P. salmonis*. OspA and the rickettsial 17 kDa antigen appear to be distantly related to a lipoprotein of similar mass in *Rhizobium* spp. The rhizobial *lipA* gene is located among 6 genes that have been implicated in the high affinity acquisition of iron (Tabche *et al.*, 1998, Yeoman *et al.*, 1997). Mechanisms of iron acquisition by pathogenic bacteria are crucial for effective survival and replication while in a host (Braun and Killmann, 1999). Although it has not been determined if LipA is involved in iron acquisition by *Rhizobium* spp., functional characterization of LipA within the rhizobial system is foreseeable and may aid in the design of experiments to determine the function of OspA in *P. salmonis*. Further investigation of *P. salmonis* molecular pathogenesis should be a research priority to help better define its relationship to other bacteria and further improve the efficacy of a *P. salmonis* recombinant vaccine.

In conclusion, a reproducible growth and purification strategy for *P. salmonis* allowed the identification, cloning, and characterization of a 17 kDa lipoprotein, OspA. OspA was recognized by both rabbit and salmonid convalescent antisera and used as the basis of

a recombinantly produced subunit vaccine for *P. salmonis*. OspA protected vaccinated salmon against *P. salmonis* challenge and efficacy of the OspA vaccine was further improved by the addition of promiscuous TCE's from tetanus toxin and measles virus fusion protein. An efficacious recombinant vaccine for *P. salmonis* will provide the aquaculture industry with a viable and inexpensive strategy to control SRS.

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APPENDIX 1. Nucleotide sequence of *P. salmonis* clone pB12

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   1 AATTCTGCTG CAATTTTTAA TTTTAGCTAT GAACGGTATG ATTGGGTGCG TCCGGGGATT
  61 ATGTTGTATG GTATTTCACC ATTTGCTGAT AAAAACGGGG TGGATTTGGA GTTACAACCG
 121 GTGATGCATG TGGTTTCTCG GTTAATCAGC GTAAAGCAGT TGCGTCAAGG TGAGAGTGTG
 181 GGCTATGGGG CAACTTGGCA ATGTCCTGAA GATATGCAGG TCGGTATTCT TTCTTTAGGC
 241 TATGGCGATG GCTATCCAAG GCTTGCAGCA AGTGGTACGC CATTTTTGGT TCGAGGTCAG
 301 CGTTGTGCGT TGATTGGGCG CGTTTCTATG GATATGATTG CGATTGACTT AAGAAGGTGT
 361 CCAGATGCAG GTGTTGGAGA AGCTGTGACA GTATGGGGGC AGGATTTACC CGTTGAAGAG
 421 ATAGCGCGTC ATGTCGGAAC GATTGCTTAT GAGTTAGTTT GTAATATGCC ACTGCGTGCA
 481 CCTTATATAT GGCAAGAATA GGTTAAGTTA TTTTAGAGTC AAGACTCAAG GGATCTGGGA
 541 AGTGCTTTCT TTTGATAAAT AGCGTGTGAT AATTGTTTGA ATGAGTTCAA CGTATTTAGT
 601 TCCTTTAATA GAATAAGCAG TTAAGCCTTC TGCGAGCTTA AAGGCGGATA CATCTTGATG
 661 GTTCGCACGC ATGTGTGCAC GTAAATCACG CAGTGCTTGA TAGGCGTTAT GTGTATTTAG
 721 TGTATGTATG TAGGCACGAA CTGATGCGGT CATTGATGAA TAGTTGGCAA CTTCCCAATG
 781 GTTGTTAGCT GGGCGTGCTT TGGGAATAAT GCCACATCCT GGATAATGGC AGCGCATGCC
 841 AAAAAAATTA TTGCCTTCTA CAGCAAAGCG TGAGCGTCCC CAATTTGATT CGTTAATCGC
 901 TTGTGCGCTA ATGAGAGCGT TTGGAATAAT ATCAACACGT TTTAATAACA GGCTGAAGTC
 961 TTGTGGGTGT TTAAAGTGTA GGTTGTTAAG CTTGTATTGT TTAGCTAGAG CTTGAAGTTG
1021 AGTGATTTCC TGCTTTGATA AGCTTTTTTT CTGTATTAAG CTAAGAATAT GTTGGCGTTT
1081 TTGCAGAATA ATTTGATTAA TTGTGTTTTC GCTTTGGGTG ATTTTTATTA TAAATTCTTG
1141 CTTGATTGAT TGTATGTCTT GTGAATAGGC TGTATTTATC TGTGTTTTGG ATGTTGGTGC
1201 TGAGAATAAG GGTTGTTGAG CAAGATAGGC AGTGGTAATA ACAGCAAGGC TTAGCATGAT
1261 ATAATCGATC CTTGTGGGTA GGGTTGGTAT TTTACCATGA TTGTGGATTT TTACCTTGAT
1321 GATAATTTTA ATTTAACTAT ATAAAAAAAT AGCTTAGAAG GCCAAGCATA GTATGAGCAA
1381 GCAAAAAAAC CAATATGTTT GTAGTGATTG TGGCGGTATT GCAAAAAAAT GGTTAGGCCA
1441 GTGCCCACAT TGCCAGCAAT GGAATAGCTT AAGTGAAGTA AAAGAAGTGC TGCCAAATCG
1501 GCCAGGACGT AGTCAGCGTT TTGCAGGCTT TGCTGGTATT GATGCACCTA AAGTGAAGTG
1561 TTTAAGTGAA ATCACACCGG AACAAATCAG TCGCCAGCCT GTTGGAATTT GTGAATTTGA
1621 TCGGGTGCTT GGTGGAGGGT TAGTCCATGG GGCGGTCATT TTATTAGGGG GTGACCCTGG
1681 AATTGGTAAA TCAACGCTAT TATTACAGAC TTCTGTAAAT TGTACGCAGT TTGGTAAGGT
1741 GCTTTATGTC ACGGGTGAAG AGTCGCTTGA GCAAGTGACT TTAAGGTCAA AACGTTTGGG
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1801 ATTGTCTCAG GACGTTGATT TGAGATTGTT AGCAGAAACC CAAGTTGAAC GTATTTTGAA

1861 AGCGGCGGAA ATAGAGCAGC CAAAAGTGCT TATTGTCGAT TCGATTCAAA CAATTTTCAC

1921 TGAAAGTCTA CAGTCGGCTC CTGGAGGTGT GGCACAAGTT AGAGAAAGTG CGGCAATATT

1981 GACGCAATTT GCAAAACGTA CTGGGACGTG TTTATTTTTA GTCGGTCATG TGACTAAAGA

2041 AGGCGCGCTT GCCGGTCCTC GGGTATTAGA GCATATGGTT GATACTGTGC TTTATTTTGA

2101 AGGTGAGCAG GACAGTCGTT TTCGCTTATT ACGGGCGGTT AAAAATCGTT TTGGTGCGGC

2161 GAATGAATTG GGTATTTTTG CTATGACAGA AACTGGACTG AAGACTGTCA GTAACCCTTC

2221 AGCAATTTTT TTATCACGCT ATGAAGACTT GCAGCCAGGG AGTGTGGTGA TGGTTGCTTG

2281 GGAAGGAACA CGCCCGTTAT TAGTTGAAGT ACAGGCTTTA GTTGATGAAT CCCATTCGTC

2341 AAATCCAAGG CGAATTGCGG TTGGGTTAGA TCAGCAGCGT TTAGCTATGT TGCTGGCAGT

2401 ATTAAATCGT CATGGTGGTA TTGCCAGTTA TGACCAGGAT GTATTTATTA ATGTGGTTGG

2461 AGGGATGAAA ATTACCGAAA CTGCTGCGGA TTTAGCCTTA TTACTTGCCT GTGTTTCTAG

2521 TTTGCGAGGA AAAGCGTTGT CACAGGAGTT GATTGTTTTT GGTGAAATTG GTTTGTCTGG

2581 GGAAATTCGT CCAGTGCAAC GAGGACTAGA GCGAATTAAA GAGGCGGTCA AACATGGTTT

2641 TACACAAGCA ATTGTACCTC TTGCAAACTG TCCTAAACAA AAAGTCGATG GTATTGAAAT

2701 TGTCGGTGTT AAGCATTTGG AAGATGCTCT GGCTACACTA TAATTAATTA TAGTTTAATT

2761 AATATTTGCC TTGGATCTTG GGTTCTAGAC TTTGGGTTTT AGGTTTTTAT CTTTGCTATT

2821 AGTGAGAGAA ATAATGAACA GAGGATGTTT GCAAGGTAGT AGTCTAATTA TTATCAGTGT

2881 GTTTTTAGTT GGCTGTGCCC AGAACTTTAG TCGTCAAGAA GTCGGAGCTG CGACTGGGGC

2941 TGTTGTTGGC GGTGTTGCTG GCCAGCTGTT TGGTAAAGGT AGTGGTCGAG TTGCAATGGC

3001 CATTGGTGGT GCTGTTTTGG GTGGATTAAT TGGTTCTAAA ATCGGTCAAT CGATGGATCA

3061 GCAGGATAAA ATAAAGCTAA ACCAGAGTTT GGAAAAGGTA AAAGCAGGGC AAGTGACACG

3121 TTGGCGTAAT CCAGATACAG GCAATAGTTA TAGTGTTGAG CCAGTGCGTA CTTACCAGCG

3181 TTACAATAAG CAAGAGCGTC GCCAGCAATA TTGTCGAGAA TTTCAGCAAA AGGCGATGAT

3241 TGCAGGGCAG AAGCAAGAGA TTTACGGCAC TGCATGCCGG CAACCGGATG GTCGTTGGCA

3301 AGTCATTTCA ACAGAAAAAT AATAATTAGG TTATGGCTAA ATTATAAAAG TAGCAGTGAA

3361 TTTATTTTAA TAATATTTGC TGTAAATTGA TATTTAGAAG ACAGCAATAA TAAAAAAAAT

3421 TGAGTTTTAT GGTGAATATA TTATTTATTT TAGATGCTAA AATGGAATCG ATTCTTGAAA

3481 TAAGAGGAGT GTAACTTTAG AGGTAAAGCG TGATTGCTGA TTTATTAATT GCGCTGTTTT

3541 TTGGTGGCTA GCAGCTTACG CTGCGAGCCA TGGAGTGCTA AGCACGGGGT GAGTAGGCCG

3601 AGGTAGGTTC GGGTTTTTAG CAGAGCGGAG TTGCGAAAGG CCGTTAGGTC TGTAGCAAGC

3661 GTAACGAGTG TCTAAAAATC TATACGAACC GTAGAGTCAT GTGAGAGCAC AGTAGTGGAG

3721 TGTGCCGCTT CAAGGCACGT AACGCTGTGT GACGCGGACA GCCGAGGGTT TATAGTCGTC

3781 GCGTTTTGCT CGGCGATTTT GCATCATTGG ATGTGCAAAA TACCTACCGA GGTAGCGACG

3841 CTTACGCTAT AATCCCCCTT TTTTTTAATA GAATTTTATC AATCATTAAA ACCAGCTTAT

3901 TTTTCATGTT TTTACGAATC TTTGTGATAA GTTGAAGTCC TTTTTCATAC AATTGATCAA

3961 ATAATTTTTG TGAAATATAG CCTTTATCAC CAATAATTTT ACCTGTTAAG TTCTCGGCCA

4021 TTTTAGGTAA TACGACACGG TCATCTGTAG TAGCTTTACT CATTTTAAAG GCCATTAACT

4081 CTCCCATATC ATTCACAATA ATGTGGAGCT TAAAGCCGTA GTACCACCCC ATCGTTGACT

4141 TAGATTTTTT AGCTAATCCC TTGAAAGCTC TATTTTGAGA CGCACGTTTT TCATGACAAA

4201 CACGGAGTAT TGTCGAATCA ACAAAATAAA TCCCTGTTGC TGTTTTACCT TGGGCCGCAA

4261 TGAAAAAACA CAGAGGCAAT AAGATGCTCG GCATGAGTTC AACGAATCTG TTGTAGCTAA

4321 CGCTGTTTGG AAAGTATTTT ACCATGCTAC CTTTTATTAC ATGAAGATAA TACATCTTAA

4381 AATTTCGGTA ATTAGATTTG TGAAAATAAA TCATAATTGT CATTATTTCA CTTGTTGACA

4441 TTTGTGAAGG CTTATTACGT TTTTTATTCG TATCTTCTAG CAAAATAGCA TTCCATTGAG

4501 GTAATAACTC TTGGCAGAAA TCATCTATTA CACAAAAGAG AGAAATCAAT GTTAAGTCCA

4561 TCGCTGCGAG CCATGGAGTG CTAAGCACGG GGTGAGTAGG CCGAGGTAGG TTCGGGTTTT

4621 TAGCAGAGCG GAGTTGCGAA AGGCCGTTAG GTCTGTAGCA AGCGTAACGA GTGTCTAAAA

4681 ATCTATACGA ACCGTAGAGT CATGTGAGAG CACAGTAGTG GAGTGTGCCG CTTCAAGGCA

4741 CGTAACGCTG TGTGACGCGG ACAGCCGAGG GTTTATAGTC GTCGCGTTTT GCTCGGCGAT

4801 TTTGCATCAT TGGATGTGCA AAATACCTAC CGAGGTAGCG ACGCTTACGT TTATTGCTTC

4861 TTTAGAACTA AATTTAGACT CTATTTAGCC GCAAAACCAC TGGTTTTTCA AATACTTCTT

4921 ATGTCGAACT CACGTTGTTT AGCTAAAATA AAGATAATTG GATAAATTTG TGTAAACGAT

4981 AATCCGAT