Characterization of the Fish Pathogen *Flavobacterium psychrophilum* towards Diagnostic and Vaccine Development

by

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Abstract

Flavobacteria are a poorly understood and speciated group of commensal bacteria and opportunistic pathogens. The psychrophile, *Flavobacterium psychrophilum*, is the etiological agent of rainbow trout fry syndrome (RTFS) and bacterial cold water disease (BCWD), septicaemic diseases which heavily impact salmonids. These diseases have been controlled with limited success by chemotherapy, as no vaccine is commercially available. A comprehensive study of *F. psychrophilum* was carried out with respect to growth, speciation and antigen characterization, culminating in successful recombinant vaccines trials in rainbow trout fry.

Two verified but geographically diverse isolates were characterized phenotypically and biochemically. A growth medium was developed which improved the growth of *F. psychrophilum*, enabling large scale fermentation. A PCR-based typing system was devised which readily discriminated between closely related species and was verified against a pool of recent prospective isolates. In collaborative work, LPS O-antigen was purified and used to generate specific polyclonal rabbit antisera against *F. psychrophilum*. This antiserum was used to develop diagnostic ELISA and latex bead agglutination tests for *F. psychrophilum*.

F. psychrophilum was found to be enveloped in a loosely attached, strongly antigenic outer layer comprised of a predominant, highly immunogenic, low MW carbohydrate antigen, as well as several protein antigens. Surface exposed antigens were revealed by a combination of immuoflourescence microscopy, immunogold transmission and thin section EM They were discriminated by Western blotting using rabbit antisera, by selective extraction with EDTA/polymyxin B agarose beads as well as by extrinsic labeling of amines with sulpho-NHS-biotin and glycosyl groups with biotin hydrazide. The predominant ~16 KDa antigen was identified as low MW LPS, whereas high MW LPS containing O-antigen was not as prevalent on whole cells but was abundant in culture supernatants.

Genomic DNA was isolated from *F. psychrophilum* and used to construct an expression library in lambda ZAP II. The library was screened with rabbit anti-*F.*

psychrophilum serum. The respective DNA inserts in the immunoreactive clones were sequenced providing 15 kb of novel DNA sequence encoding 13 hypothetical proteins. Two open reading frames encoding a 91 amino acid HU-beta-like protein (FP91), and a 166 amino acid ribosomal L10-like protein (FP166), were cloned and expressed as fusion proteins in *E. coli*.

Rainbow trout convalescent antisera strongly recognized both MW classes of LPS as well as a predominant ~20 kDa protein. The 20 kDa antigen was separated by 2D gel electrophoresis, isolated and subject to proteolysis. Peptide fragments were analysed by quadrupole time-of-flight mass spectrometry. Fragmented peptide spectra were generated and peptide sequences obtained. Degenerate PCR was used to amplify a 537 bases corresponding to179 amino acids; the PCR product was cloned and expressed as a fusion protein in *E. coli*.

The recombinant proteins were tested in rainbow trout fry for their ability to confer protective immunity against *F. psychrophilum*. All proteins were shown to have some protective effect. In an attempt to boost immunity, a T cell epitope from measles virus was incorporated into the recombinant vaccines. The presence of the T cell epitope affected the protection of each protein differently, nevertheless, successful recombinant vaccine candidates were developed.

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List of Abbreviations

A	absorbance
aa	amino acid
Ap	ampicillin
ADH	adipic acid dihydrazide
ATCC	American type culture collection
bp	base pairs
BLAST	basic linear alignment search tool
BSA	bovine serum albumin
CFB	*Cytophaga-Flavobacterium-Bacteroides*
cfu	colony forming units
CLB	*Cytophaga*-like bacteria
Cm	chloramphenicol
CR	Congo red
CTAB	hexadeclytrimethylammonium bromide
dd	degree days
dH_2O	deionised water
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dPCR	degenerate polymerase chain reaction
EDTA	(ethylene diamine)tetraacetic acid
EtBr	ethidium bromide
EtOH	ethanol
ELISA	enzyme linked immunosorbent assay
EM	electron microscopy
ETP	EDTA/TEA/polymyxin B
FITC	fluorescein isothiocyanate
g	grams
x g	gravitational force
IFAT	immunofluorescence antibody technique
IgG	immunoglobulin G
i.p.	intraperitoneally
IPTG	isopropyl β-D-thiogalactoside

kb	kilobase(s)
kDa	kilodalton
KDO	2-keto-3-deoxyoctonate
KLH	keyhole limpet hemocyanin
Kn	kanamycin
kV	kilovolt(s)
l	litre(s)
LB	Luria broth (1 % NaCl, 1 % tryptone, 0.5% yeast extract, pH 7.0)
LPS	lipopolysaccharide
mA	milliamps
mAb	monoclonal antibody
MAOB	modified Anacker and Ordal broth, (0.5% tryptone, 0.05% yeast extract, 0.02% NaCOOH and 0.02% beef extract)
MALDI-TOF	matrix-assisted laser desorption / ionization time-of-flight
MAT	1% maltose, 0.02% Na acetate, TYES
mg	milligram(s)
ml	milliliter(s)
mM	millimolar
MS	mass spectrometry
MV	measles virus
MW	molecular weight
m/z	mass / charge
ng	nanogram(s)
nm	nanometer(s)
OPS	O-polysaccharide (O-antigen)
ORF	open reading frame
pAb	polyclonal antibodies
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline (1mM KH_2PO_4, 10 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl pH 7.4)
PCR	polymerase chain reaction
pfu	plaque forming units
pI	isoelectric point
PK	Proteinase K

PTA	phosphotungstic acid
pmol	picomole(s)
Q-TOF MS	quadrupole time-of-flight mass spectrometry
RAPD	random amplification of polymorphic DNA
rRNA	ribosomal ribonucleic acid
rpm	rotations per minute
RT	room temperature
RTFS	rainbow trout fry syndrome
s.c.	subcutaneously
SDS	sodium dodecyl sulfate
sdH_2O	sterile deionised water
TBS	tris buffered saline (10 mM Tris-HCl (ph 7.5), 0.9% NaCl)
TE	10 mM Tris, 1 mM EDTA pH 8
Tet	tetracycline
TEA	triethanolamine
TEM	transmission electron microscopy
TFB	terrific broth
Tm	melting temperature
TYES	tryptone-yeast extract-salts medium (0.4% tryptone, 0.04% yeast extract, 0.05% $CaCl_2$, 0.05% $MgSO_4$)
TYE	tryptone-yeast extract medium (0.4% tryptone, 0.04% yeast extract)
U	units
UV	ultra violet
V	volts
µg	microgram(s)
µl	microlitre(s)
v/v	volume per volume
w/v	weight per volume
X-gal	5-Bromo-4-Chloro-3-indoyl-β-D-galactopyranoside
YPB	yellow-pigmented bacteria

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Dedication

In memory of

Dr Julian C. Thornton

You found the fun in everyday life and science

May your joyful spirit live on to inspire us all

General Introduction

Flavobacterium psychrophilum (syn. *Cytophaga psychrophila,* syn. *Flexibacter psychrophilus)* is a psychrophilic, yellow-pigmented, filamentous gram-negative bacterium belonging to the family *Flavobacteriacea.* First isolated by Borg in 1960 (30), *F. psychrophilum* (*Cytophaga psychrophila*) was named as the aetiological agent of bacterial cold water disease (BCWD) in fish in the Pacific Northwest, USA (51), and later rainbow trout fry syndrome (RTFS) in Europe (22); diseases which cause significant losses in aquaculture. Following the recent growth in the aquaculture industry, *F. psychrophilum* was found to a common agent of fish disease worldwide, mainly affecting salmonid species. As yet, no vaccine is commercially available to protect against *F. psychrophilum.*

A major difficulty in understanding and controlling the diseases caused by *F. psychrophilum* has been the reliable detection of the aetiological agent. The taxonomic group to which the causative agent of BCWD was originally assigned, the *Cytophagaceae*, was a heterogeneous group containing several species only very distantly related. Characterization of organisms in this poorly understood group has been difficult, awaiting reorganization of the whole taxonomic branch.

The objectives of this study were: 1) to develop methods whereby *F. psychrophilum* could be rapidly and easily identified; 2) to characterize the major antigens of *F. psychrophilum* and 3) to identify molecules as potential vaccine candidates, in hopes of developing an efficacious vaccine against RTFS.

TAXONOMY

The complex history and heterogeneity of the genera *Flavobacterium, Cytophaga* and *Flexibacter* is well documented. Due to the confusion surrounding the taxonomy and identification of these filamentous, yellow-pigmented bacteria, much of the recent research has focused on their taxonomy (16, 26, 27, 24, 135, 193) and identification (123, 184, 185). Ribosomal RNA sequence data (16, 68, 134, 212) has recently shown the three genera to belong to one of the main phylogenetic branches of bacteria. This phylogenetic branch has been given several names including the "*Flavobacter-Bacteroides*" phylum (68), "*Cytophaga-Flavobacter-Bacteroides*" (CFB) group (68), "*Flavobacterium-Cytophaga-Flexibacter* complex" (16), the *Flavobacterium-Cytophaga* complex (134), the *Cytophaga-Flavobacterium-Bacteroides* phylum (26, 77) and the *Bacteroidetes* phylum, according to the National Center for Biotechnology Information (NCBI). For the purpose of this text, the name *Cytophaga-Flavobacterium-Bacteroides* (CFB) will be used to describe this phylum.

The CFB phylum belongs to the CFB/*Chlorobi* superphylum. The *Chlorobi* (green sulphur bacteria) are predominately aquatic bacteria that grow photosynthetically under anoxic conditions (66). The relationship between the Flavobacteria and the green sulphur bacteria is a strong one (211). Interestingly, relationship between the two phyla, identified on the basis of 23S rRNA and 16S rRNA data, links a lineage of presumed photosynthetic ancestry to non-photosynthetic bacteria (211).

Much confusion surrounds the taxonomy of the CFB group due to the past reliance on phenotypic characteristics to define bacterial genera. Defining bacteria based on characteristics such as pigmentation, gliding motility and enzymatic activity (21, 79)

has proved unreliable and led to the creation of very heterogeneous genera. The advancement of molecular techniques in recent decades has allowed the comparison of bacterial strains at the genomic level and provided grounds to reclassify and identify related organisms. However, only recently have these techniques been adopted to study important fish pathogens.

The Family Flavobacteriaceae

The family *Flavobacteriaceae* (class *Flavobacteria,* order *Flavobacteriales*) was first proposed by Reichenbach in the order *Cytophagales* (154) and contains many environmental species that have been isolated from soil and aquatic environments. *Flavobacteriaceae* infect a wide range of hosts, including mammals (85, 170), birds (194) and fish (87). In humans, *Flavobacterium* sp. cause neonatal meningitis, catheter–associated bacteremia and pneumonia and have also been associated with some cases of advanced HIV disease (121, 170).

Several bacterial species belonging to the family *Flavobacteriaceae* are considered pathogenic for fish: *Flavobacterium psychrophilum, F. columnare, F. branchiophilum, F. johnsoniae, F. scophthalmum, Tenacibaculum maritimus* and *T. ovolyticus.* Amoung the *Flavobacterium* sp., *F. columnare* (syn. *Cytophaga columnaris,* syn. *Flexibacter columnaris*) was the first described fish pathogen of the CFB group. *F. columnare* was identified in 1922 by Davis as the causative agent of a disease which became known as columnaris disease (27, 50). Columnaris disease affects many species of freshwater fish, and occurs at comparatively high temperatures (200), generally over 15 °C. *Flavobacterium branchiophilum* is the causative agent of bacterial

gill disease, a disease which affects freshwater fish worldwide (146, 199, 203). Other *Flavobacterium* species, although not shown to be pathogenic, have been isolated from diseased fish (*F. hydatis*, *F. succinicans*) (27).

A new genus, called *Tenaicibaculum,* has recently been added to the family *Flavobacteriaceae.* This new genus was created to classify marine *Flexibacter* species that were distantly related to the type species of their genus (*Flexibacter flexilis*) and phylogenetically belong to the family *Flavobacteriaceae.* The *Flexibacter* species transferred to the new genus were marine fish pathogenic species *Flexibacter maritimus* (201) and *Flexibacter ovolyticus* (71), reclassified as *Tenacibaculum maritimum* and *T. ovolyticum* respectively (179). *Tenacibaculum maritimum* (syn. *Flexibacter maritimus,* syn. *Cytophaga marina*), causes black patch necrosis and mouth rot (23, 144, 179). *T. ovolyticum* (*Flexibacter ovolyticus*) has been found to be a pathogen of eggs and larvae of Atlantic halibut (71).

Chryseobacterium scophthalmum (syn. *Flavobacterium scophthalmum, Scophthalmus maximus*) causes haemorrhagic septicaemia in farmed turbot in Scotland (132, 195). The best known species in the genus is the human pathogen *Chryseobacterium* meningosepticum (syn. *Flavobacterium meningosepticum*), which is associated with a sometimes fatal meningitis of infants (79, 195).

Historically, yellow-pigmented, filamentous, gram-negative bacteria associated with fish disease have been classified as "myxobacteria", which included *Cytophaga psychrophila* (*Flavobacterium psychrophilum*) and *Cytophaga columnaris* (*Flavobacterium columnare*) until the reclassification of *Cytophaga* sp. to the order *Cyophagales* (105). *Cytophaga*-like bacteria (CLB) and the term yellow-pigmented

bacteria (YPB) have been widely used to describe the heterogeneous group organisms, including *Cytophaga*, *Flavobacterium* and *Flexibacter* species associated with disease in fish and the latter will be used in this text to describe such unknown species.

The genus Flavobacterium

In 1996, Bernardet *et al* (27) emended the description of the genus *Flavobacterium* to have the following main characteristics: gram-negative aerobic rods, 2-5 μm long, 0.3-0.5 μm wide, with rounded or tapered ends that are motile by gliding, yellow (cream to orange) colonies on agar, decompose several polysaccharides but not cellulose, G+C contents of 32 - 37 %, and are widely distributed in soil and freshwater habitats. The type species is *F. aquatile*. *Flavobacterium* sp. are isolated from freshwater and marine environments, soil, foods and clinical specimens such as blood, urine and infected wounds (79).

Flavobacterium psychrophilum

In 1989, Bernardet and Grimont renamed the causative agent of BCWD, *Cytophaga psychrophila*, as *Flexibacter psychrophilus,* pending further reorganization of the whole phylogenetic branch (24). More recently, Bernardet *et al* (26, 27) amended the classification and description of the family *Flavobacteriaceae* and the genus *Flavobacterium*, which included *Flavobacterium psychrophilum* (syn. *Cytophaga psychrophila, Flexibacter psychrophilus*).

A psychrophilic organism, *F. psychrophilum* grows well between 4 and 23 °C, with optimal growth at 15 °C, and no growth over 25 °C. *F. psychrophilum* is a strict

aerobe and tolerates 0.8 % but not 2 % NaCl. Colonies take approximately 2-4 days to appear at 15 °C on MAT agar, are 1-3 mm in diameter and are bright orange due to the presence of flexirubin-like pigments. The colonies are entire or have a thin spreading margin (Figure 1A). Cells are long flexible, slender rods 0.4 - 0.5 μm wide, 1.5 - 7.5 μm long, although filaments up to 40 and 70 μm have been reported (30, 82), with rounded or tapered ends, as seen in Figure 1B. The DNA base content is 33 % GC (25).

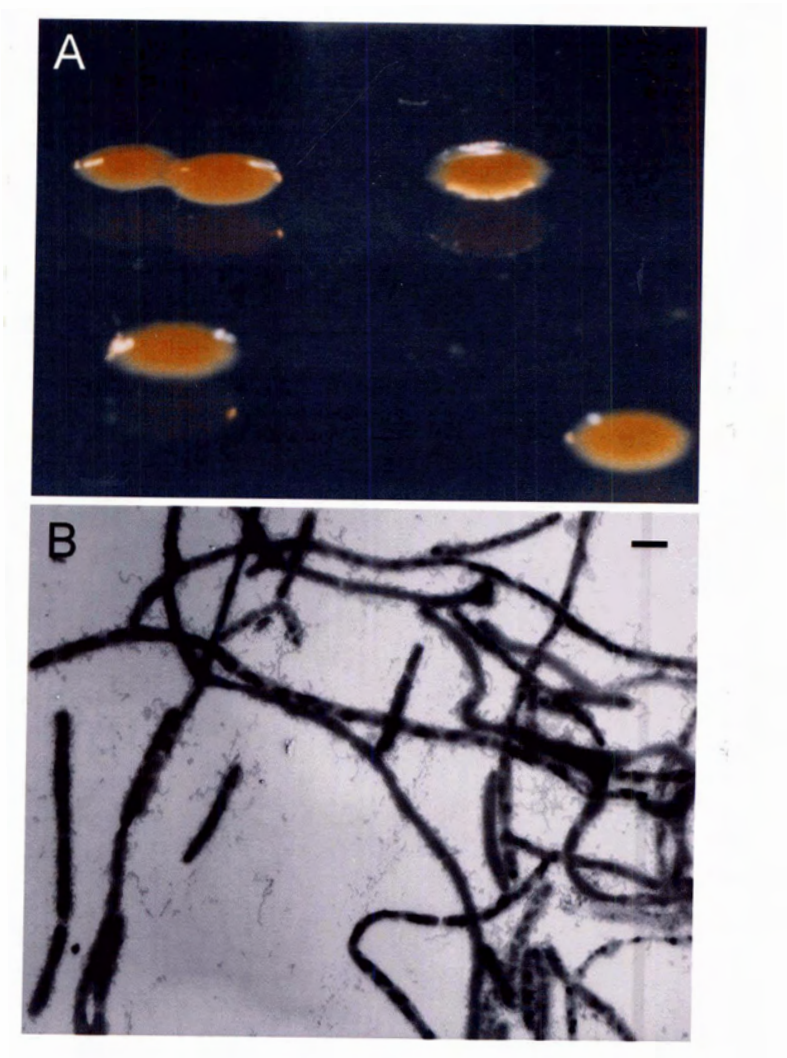

Figure 1. ***F. psychrophilum.*** A) Colonies of *F. psychrophilum* strain PBS9701 growing on MAOA (Approx. 30X), courtesy of David Bos. B) Electron micrograph of negatively stained *F. psychrophilum* cells (Bar = 1 μm).

AQUACULTURE AND DISEASE

Aquaculture, the cultivation of aquatic plants and animals, has grown significantly in recent years. Worldwide aquaculture production has more than doubled from 16 million metric tons (mt) in 1990 to 42 million mt in 1999 (63). In Canada, aquaculture production has mirrored world trends (Figure 2) and in 1999 Canada produced 113,600 mt, worth an estimated $360 million US (63). Currently, fish produced from farming activities accounts for over one quarter of all fish directly consumed by humans (136), a figure which seems set to rise given the recent trends and expanding human population. Asia accounts for approximately 90 % of world aquaculture production (63). Europe, North America and Japan collectively produce just over 10 % world aquaculture production but consume the bulk of farmed seafood traded internationally (136).

Today, more than 220 species of fish are farmed (63). Ownership of stock and the deliberate intervention in fish life cycles (husbandry) distinguish fish farming from capture fisheries. Farmed fish are typically enclosed in an environment where they can thrive on a plentiful food supply, away from predators. Disease, however, poses a significant threat. Fish are susceptible to a wide range of bacterial, viral, fungal and parasitic diseases, which are exacerbated under the conditions of intensive rearing. The major diseases affecting aquaculture today are listed in Table 1.

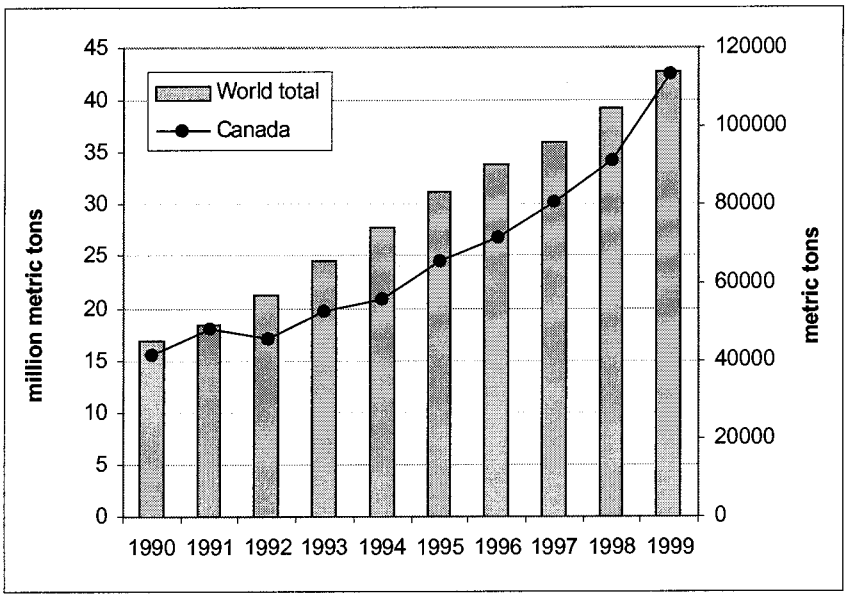

Figure 2. World aquaculture production 1990 - 1999, figures from (63).

Table 1. Major diseases of fish affecting aquaculture.

Bacterial Disease	Agent
Bacterial cold water disease (BCWD) / rainbow trout fry syndrome (RTFS)	*Flavobacterium psychrophilum*
Columnaris disease	*Flavobacterium columnare*
Bacterial gill disease	*Flavobacterium branchiophilum*
Flexibacteriosis / salt water columnaris / mouth rot / Black patch necrosis	*Tenacibaculum maritimum* (*Flexibacter maritimus*)
Edwardsiella septicaemia	*Edwardsiella tarda, E. ictaluri*
Enteric redmouth disease (ERM)	*Yersinia ruckeri*
Vibriosis	*Vibrio anguillarum, V. ordalii, V. salmonicida, V. vulnificus*
Furunculosis	*Aeromonas salmonicida*
Motile Aeromonad septicaemia	*Aeromonas hydrophila, A. caviae, A. sobria*
Pasteurellosis	*Pasteurella piscicida*
Pseudomonas infection	*Pseudomonal anguilliseptica, P. fluorescens*
Alteromonas infection	*Alteromonas putrefaciens*
Bacterial Kidney Disease (BKD)	*Renibacterium salmoninarum*
Streptococcal infections	*Streptococcus iniae, S. facecalis, S. faecium*
Clostridial infections	*Clostridium botulinum*
Mycobacteriosis	*Mycobacterium marinum, M. fortuitum, M. chelonae*
Salmonid Rickettsial Septicaemia (SRS)	*Piscirickettsia salmonis*
Viral Diseases	
Infectious salmon anaemia (ISA)	Orthomyxovirus
Infectious hematopoietic necrosis virus	Rhabdovirus
Infectious pancreatic necrosis virus (IPNV)	Birnavirus
Viral Hemorrhagic septicaemia (VHS)	Rhabdovirus
Lymphocystis	Iridovirus
Parasitic Diseases	
Sea lice	*Caligus elongates, Lepeophtheirus salmonis*
Proliferative kidney disease /PKX	*Tetracapsula byrosalmonae*
White spot disease	*Ichthyophthirius multifiliis*
Myxosporean diseases	*Henneguya sp., Kudoa thyrsites*
Fungal Diseases	
Ichthiosporidiosis	*Ichthyophonus hoferi*

DISEASES CAUSED BY *F. psychrophilum*

Bacterial Cold Water Disease (BCWD)

In 1946, H. Davis described a fatal disease of juvenile rainbow trout in which a characteristic lesion appeared on, or near, the peduncle (tail), giving rise to the name peduncle disease (51). Borg (29) named the disease "low temperature disease" because most occurrences were found when the water temperature was 4-10 °C. The disease later became known as cold-water disease (CWD) or bacterial cold-water disease (BCWD). Borg (30) first isolated the aetiological agent from external lesions of infected juvenile coho salmon (*Oncorhynchus kisutch*) in the state of Washington, USA and successfully infected healthy coho salmon with organisms isolated from BCWD affected fish. After phenotypic and biochemical characterization of the aetiological agent of BCWD, Borg classified the bacterium in the genus *Cytophaga* and named it *Cytophaga psychrophila* for its low optimum temperature of 15 - 20 °C (30).

BCWD is a serious septicaemic infection of hatchery reared salmonids. In the Pacific Northwest of the United States, losses of 30-50 % have occurred in certain hatcheries (213). BCWD was observed in up to 10 % of under yearling coho salmon, rainbow trout and steelhead trout in several fisheries in Washington and Oregon, USA (98); the affected fish did not recover. In Japan, the occurrence of BCWD in wild freshwater ayu (*Plecoglossus alltivelis*) was 16 % (86).

The symptoms of BCWD depend on the size of the infected fish. In alveins, or sac fry, external signs are limited to erosion of the skin covering the yolk (82). In fingerlings, darkening and erosion of the peduncle area or loss of the tail is a common finding. In severe epidemics, many fish die with only a marked darkening of the

peduncle area (213). If BCWD does not occur until several weeks after fish begin to feed, skin and muscle lesions may appear on other areas of the body, i.e. lower jaw, anterior to dorsal fin (82).

Rainbow trout fry syndrome (RTFS)

Disease caused by *F. psychrophilum* was only reported in North America (BCWD) until the late 1980s. Since that time, *F. psychrophilum* has emerged as a causative agent of severe rainbow trout fry mortality throughout Europe (RTFS) (19, 22, 33, 112, 162, 163, 182) and is now known to affect salmonids worldwide (145, 167, 202, 209). The host-range, previously believed to be limited to salmonids (173), appears to have broadened with several more non-salmonid fish species being affected, such as eel, cyprinids and ayu (86, 106). The most serious losses occur in fry of approximately 0.2 - 2 g, where 30 - 90 % cumulative mortality can result (111). In fish of this size, the signs of disease are internal, in contrast to most observations of BCWD and so Baudin-Laurencin *et al* (19) proposed the term "visceral form of cold water disease" in 1989. In fry, RTFS causes acute septicaemia, anaemia, indicated by pale gills and an enlarged spleen (162). In larger fish, external, convex lesions appear (33) as described in BCWD.

Transmission

F. psychrophilum is an opportunistic pathogen. The natural reservoir of *F. psychrophilum* is not certain, however many Flavobacteria are found in aquatic environments and are part of the normal microflora of salmonid skin (44, 84), gills (140, 187) and intestine (14, 157), although *F. psychrophilum* has not been specifically

identified. Pacha and Ordal (147) postulated that the bacterium maintains itself in a vegetative state throughout the year, although it is possible that adult fish may serve as carriers. Wild ayu and pale chub have recently been found to be infected in Japan (86) and Baltic salmon (*Salmo salar*) brood fish were shown to be carriers in Sweden (57). Vertical transmission of *F. psychrophilum* has been supported by several studies. Ekman *et al* (57) found *F. psychrophilum* in eggs, ovarian fluid and milt of Baltic salmon (*Salmo salar*) brood fish. Holt (80) reported the bacterium in ovarian fluid, milt and skin mucus of sexually mature chinook salmon (*Oncorhynchus tshawytscha*). Brown *et al* (32) isolated the bacterium from ovarian fluid of and from inside newly fertilized eggs, eyed eggs and newly hatched alevins.

A recent study demonstrated the age related resistance to *F. psychrophilum,* using 1g (age 10 weeks), 25 g (age 20 weeks) and 300 g (age 15 months) rainbow trout. Decostere *et al* (52) reported survival of *F. psychrophilum* in rainbow trout fry phagocytes *in vivo* following intraperitoneal injection. The number of phagocytes containing *F. psychrophilum,* as well as the number of *F. psychrophilum* cells within phagocytes, increased from 12 h to 3 d post infection. Interestingly, in larger fish tested (25 g and 300 g), intraperitoneal injection with *F. psychrophilum* did not induce phagocytosis. Only the fry displayed clinical signs of disease and suffered mortality.

Control of BCWD/RTFS

In the absence of a vaccine to protect against *F. psychrophilum* infection, previous studies concentrated on the control and prevention of disease through better husbandry and chemotherapy. Better husbandry can improve the health of the fish and improve

their chances of surviving infection. Fish kept at high density require a higher flow rate to oxygenate the water. Wood (213) found less severe BCWD in fry kept in shallow troughs with a low flow rate compared to fry kept in deeper troughs with a higher flow rate. The higher flow rate may have abraded the fry making them more susceptible to infection. Experimental infection studies carried out at different temperatures showed a dramatic decrease in *F. psychrophilum* infection from 96 % mortality at 9 °C to just 8 % when fish were held at 21 °C, and zero mortality at 23 °C (81). However, raising the holding temperature of fish is not considered seriously in the field due to high costs and the risk of facilitating other bacterial infections. Diet had also been shown to influence the development of RTFS (49).

One method which seemed promising was the prophylactic treatment of eggs, since eggs have been shown to be contaminated with *F. psychrophilum* (32, 82). Although organic iodine compounds have been shown to kill *F. psychrophilum* (6, 111) the prophylactic treatment of eggs with organic iodine (iodophor) was found ineffective in preventing BCWD in fry (82). Bath treatments with surface disinfectants or oxytetracycline have been found ineffective in the treatment of BCWD in coho salmon because the disease is primarily systemic (5). However, by incorporating the drug in to the diet, oxytetracycline (Terramycin) was found to be effective in controlling the disease (172, 213). As a therapy for RTFS / BCWD, the most widely used has been an oxytetracycline supplemented feed (111). However, studies performed in Denmark in the early 1990s have shown an increasing number of oxytetracycline resistant isolates (111).

Over time and with increased use, antibiotic resistance is inevitable. The only hope for the effective, long lasting control of *F. psychrophilum* is in the development of

an inexpensive, efficacious vaccine. As well as protecting young fish from infection and death, carrier states in adult fish may also be eliminated, thus preventing shedding of bacteria into the local environment; an outcome with positive implications for surrounding wild populations.

VACCINOLOGY OF FISH

In aquaculture, as in other areas of intense stock rearing, e.g. pigs, poultry and cattle, antimicrobial agents have been widely to treat disease and consequently promote growth. However, the increased use of antimicrobials has led to the emergence of drug resistant bacteria and health concerns regarding toxic or allergic effect on humans of antimicrobial breakdown products. A recent study in Denmark demonstrated high levels of individual and multiple antimicrobial resistances among collected *Flavobacteria* and *Aeromonads* from four Danish fish farms (166). With ensuing tighter government restrictions on drug use, research into vaccines has increased, which is hoped will provide longer lasting disease control with fewer side-effects than extended chemotherapy.

In 1942, the first study to show that antibody production in fish corresponded to a protective immune response was published by Duff *et al* (56), working with *Bacterium salmonicida* (*Aeromonas salmonicida*). Not until the mid-1970s and early 1980s however did the field of fish vaccinology begin to emerge. The slow onset of fish vaccinology research has been attributed in part to the popular use of antimicrobials (reviewed in (61)). However, the high cost and short-term benefit of chemotherapy, as well as the emergence of antibiotic resistant strains and the potential for deleterious effects on humans and the environment, have led to increased research in fish vaccinology.

The impact of vaccine research on the use of antimicrobials was clearly demonstrated in Norwegian studies. Markestad and Grave (124) investigated the correlation between the introduction of vaccines and the use of antibacterial drugs in farmed fish. Their findings demonstrated that the introduction of oil-adjuvanted vaccines has been the single most important cause of the substantial reduction in use of antibacterial drugs in Norwegian fish farming. The amount of antimicrobial drugs prescribed for use in farmed fish in Norway fell from 24,063 Kg in 1991 to just 983 Kg in 1996 (69).

The ideal vaccine for aquaculture must be effective in preventing death, be inexpensive to produce and license, provide long-term immunity and be easily administered (107). Today, commercially available vaccines are available for several bacterial and some viral fish diseases. Important bacterial fish pathogens for which no vaccines are commercially available include *F. psychrophilum* (27, 51), *F. columnare* (27, 50), *Flexibacter maritimus,* syn. *Tenacibaculum maritimum* (179, 201) and *F. branchiophilum* (199, 203).

Fish can be vaccinated by several routes: injection, immersion, spray or oral, each method with its own advantages and disadvantages (reviewed in (138)). Injection, either intraperitoneally (i.p.), intramuscularly (i.m.) or subcutaneously (s.c.), had proved to be highly effective in conferring immunity and allows for the addition of adjuvant but requires handing of the fish individually which is stressfull to the fish and is labour intensive, making it expensive. Immersion, where fish are dipped or briefly held in a bath, is less stressful than injection and results in high protection levels with some bacteria. However, it is still somewhat labour intensive and does not allow for adjuvant

delivery. Spraying fish sometimes provides high levels of protection but requires handling and specialized machinery. Oral immunization can be achieved by formulating vaccine into the feed. The delivery of oral vaccines requires no handling or specialized machinery, however, protection has been variable and not as effcetive as other methods.

There are several types of vaccines in current use, such as live attenuated strains, whole killed cells (eg bacterin), purified subunits, recombinant proteins produced from cloned genes and more recently, DNA vaccines. Live attenuated vaccines offer several advantages. If the vaccine strain is shed by fish, effective dissemination of the vaccine would take place over time, also, due to multiplication in the host, a low dose would be required (70). Marsden *et al* compared the response to live vaccine to an inactivated vaccine of the same microorganism and found both B- and T-cell populations from fish given the live vaccine showed higher proliferative responses (125). Generally, live vaccines provide very high levels of protection but have not succeeded commercially due to concerns of strains reverting to pathogenic states and the requirement for a suitable marker technology, which has prevented the licensing of live vaccines in most European and North American markets (128). Inactivated, or killed, vaccines are usually simple bacterins prepared by inactivating bacterial cells, usually by formalin treatment. Therefore, inactivated vaccines cannot replicate and thus are non-infectious. As a result they tend to provide less protection than live vaccines and booster injections and the addition of adjuvant are often needed to improve immunogenicity (108).

In cases where whole killed vaccines or purified subunit vaccines provide unsatisfactory protection, or where large scale production is prohibitively expensive, recombinant vaccines may provide a cost effective alternative. Recombinant DNA

technology has provided the means to produce sufficient quantities of vaccines at low cost. Recombinant protein vaccines include preparations of antigenic proteins, produced from cloned genes in a variety of expression systems, or the chemical synthesis of peptides corresponding to known epitopes. Recombinant DNA technology has also allowed the production of multivalent vaccines which elicit immunity to two or more pathogens simultaneously, as well as the incorporation of adjuvants or targeting components (115). Formulating complex mixtures of antigens can however lead to complications arising from antigenic competition, in which the immune response to one antigen is suppressed by the response to a second, unrelated antigen (34).

A new field of vaccinology has emerged in the last decade, namely DNA vaccination. DNA vaccines involve the direct introduction of naked DNA, in the form of a plasmid, which contains a gene of interest under the control of a strong promoter recognized by the mammalian host. DNA vaccination was pioneered by Liu *et al* in 1993 (191), who induced protective immunity against influenza in mice by injection of a gene encoding a viral protein. The immune responses to DNA vaccines can be enhanced by the DNA acting as its own adjuvant (108), by virtue of immunostimulatory sequences (ISS) (165).

A number of safety concerns surround the field of DNA vaccines, including possibility of the DNA becoming integrated into the host genome and activating a protooncogene or deactivating a suppressor gene, thus inducing cancer (108). The possibility of inducing anti-DNA antibodies is another safety concern for DNA vaccines. Kanellos *et al* (95) tested the safety and longevity of DNA vaccines administered to fish.

They were able to induce long-term humoral and cell-mediated immunity without autoimmunity or integration in goldfish.

Recent reports of experimental DNA immunization in fish have shown very high levels of protection. Relative percent survival (RPS) rates of up to 97% against haemorrhagic septicaemia virus (VHSV) have been achieved in young (13 g) rainbow trout (116). Against infectious hematopoietic necrosis virus (IHNV), 100 % RPS has been achieved in salmon (57g) (186) and rainbow trout fry (1.8 g) (46) injected with DNA encoding the IHNV glycoprotein. In addition to high efficacy, DNA vaccines have been shown to provide significant protection in as little as 4 days post-immunization (104).

Although still in its infancy, fish vaccinology has significantly impacted the aquaculture industry, providing protection against several bacterial and viral pathogens. Advancements in the field will rely on the further investigation and understanding of the host immune system.

THE IMMUNE SYSTEM OF TELEOST FISH

The immune system can be divided into two components: the innate and the acquired response. Innate, or non-specific, immunity refers to the hosts basic resistance to disease that are present before exposure to a pathogen. Defense mechanisms of innate immunity include anatomical barriers such as mucous membranes, physiological barriers such as pH, phagocytic cells and the inflammatory response. The innate immune response provides the first line of defense against an invading pathogen and is initiated in the first few hours after infection. Many molecular structures recognized by the innate immune response are shared by large groups of organisms. These structures are called

pathogen-associated molecular patterns (PAMPs) (126), such as LPS of gram-negative bacteria. The immune response generated to these common molecules requires no memory component.

Acquired, or specific, immunity usually takes several days to develop following exposure to a pathogen and is based on the specific recognition of pathogen-associated molecules. With each subsequent exposure, the response increases in speed and magnitude. The hallmarks of acquired immunity, being specificity, memory, diversity and self/nonself recognition. Acquired immunity is mediated by lymphocytes and their products. Lymphocytes possess the genetic mechanisms to create tremendous variety in their antigen receptors. The two main types of lymphocyte are T cells and B cells. T cells, via the T cell receptor, recognize antigen presented by the major histocompatability complex (MHC) proteins which are present on most cell types. B cells produce antibodies which are either bound as membrane receptors or secreted.

Vaccination strategies target and rely on the acquired immune response. By priming the immune system, through prior exposure to infectious agents, potentially lethal microorganisms can be efficiently and rapidly eliminated from the body. The acquired immune response does not act independently of the innate response, instead the two types of response act synergistically to protect the host.

The innate and acquired immune responses can be divided into humoral and cell-mediated responses. The term humoral pertains to extracellular fluid, including plasma and lymph, and is derived from the latin *humor*, meaning body fluid (102). Fish posses a variety of specific and non-specific humoral and cell-mediated mechanisms of defense against microorganisms. With the rapid growth in aquaculture, research on fish

immunity has largely focused on salmonids (*Oncorhynchus* and *Salmo*), catfish (*Ictalurus)* and carp (*Cyprinus*).

I shall focus this introduction to fish immunity on the differences in the acquired immunity between fish and higher vertebrates and on the ontogeny of fish immunity as it pertains to the disease of immature fish (fry).

Cells and Organs of the Teleost Immune System

The major lymphoid organs in teleost fish are the thymus, kidney and spleen. The kidney is an important primary lymphoid organ, considered to be the bone marrow equivalent in teleost fish. The kidney consists of two distinct segments: the anterior, or head kidney and the trunk, or posterior kidney (217). Both regions exhibit hematopoietic capacity whilst renal function is only in the posterior kidney. The thymus in most teleosts is remarkable for its location near the gill cavity and permanent continuity with the pharangeal epthelium (217). Despite the striking morphological differences, evidence suggests that the teleost thymus functions as the main source of T cells, as it does in higher vertebrates. Secondary lymphoid organs of teleost fish, involved in trapping antigen, are the spleen and the gut-associated lymphoid tissue (GALT).

It has been well established that fish possess lymphocytes analogous to mammalian B and T lymphocytes (42). One method used to separate fish lymphocytes has used monoclonal antibodies (mAb) raised against serum immunoglobulin (Ig). This technique separates the surface Ig positive (sIg^+) cells, presumptive B cells, from the sIg^- cells, or putative T cells. Using mAb specific for rainbow trout Ig, the sIg^+ cells were shown to differentiate into antibody producing cells, which predominantly responded to

LPS (but not ConA), and did not respond in mixed leukocyte reactions (MLRs). In contrast, the sIg^- cells were the predominant responders to the T-cell mitogen concanavalin A (ConA) and responded in MLRs (reviewed in (42)).

Innate Immune Response

Nonspecific Humoral Response

The humoral response is mediated by serum proteins. Non-specific humoral defense mechanisms depend on a range of proteins that act mainly to inhibit replication of microorganisms (inhibitors) or lyse foreign cells (lysins). Inhibitors of bacterial growth include: iron-binding plasma proteins, such as transferrin, that limit the availability of the essential element for bacteria; antiproteases and lectins. Although the biological role of lectins in fish remains unclear, they have been shown to inhibit growth of pathogenic bacteria (215). Another inhibitor, interferon, is produced which inhibits viral replication. Lysins work to disrupt bacterial cells, they include antibacterial peptides, lysozyme, C-reactive protein and complement. Antibacterial peptides have been isolated from skin secretion of a number of fish species (60). These are low MW cationic peptides that come together to form a pore in bacterial membranes and induce apoptosis. Lysozyme specifically hydrolyses components of peptidoglycan layer in bacterial cell walls has been found in fish mucus, serum and tissues rich in leukocytes (60). C-reactive protein (CRP) binds phosphorylcholine, a widely occurring surface component of invading bacteria, fungi and parasites. CRP is able to activate complement thereby activating lytic and phagocytic defense mechanisms. Complement is comprised of about 35 individual proteins (40).

Nonspecific cell-mediated response

The non-specific, cell-mediated response in fish involves a variety of white blood cells (leukocytes) including monocytes, macrophages, granulocytes and non-specific cytotoxic cells (NCC) (168). The non-specific response provides a rapid mobilization of a large number of cells, however, there is no memory component. Methods of nonspecific cellular defense include phagocytosis, nonspecific cytotoxicity and inflammation.

Acquired Immune Response

Specific Humoral Response

The specific arm of the humoral response is mediated by antibody, or immunoglobulin. The specific humoral response in fish shares basic features with that of mammals, including: the basic immunoglobulin structure and the role played by antibodies in neutralization, complement fixation and opsonization (94). In teleosts, the antibody molecules appear largely to be tetramers (1), however, monomers, dimers and trimers have also been described (160). Other characteristics of fish antibodies include their low affinity for the individual binding sites (intrinsic affinity), the apparent lack of ability for serum antibodies to increase in affinity over time (affinity maturation) and the limited amount of antibody binding site heterogeneity (reviewed in (94)). The lack of intrinsic affinity is compensated for by having numerous binding sites per molecule. As with the mammalian pentameric IgM, which also exhibits low intrinsic affinity, the multiple binding sites increase the affinity of the entire molecule, therefore the overall avidity of the molecule is high.

Initially, only tetrameric forms of antibody were isolated from fish serum (1), which led to the supposition that fish only possess one, IgM-like, isotype (94). More rigorous procedures have shown there to be serologically defined isotypic differences in fish antibodies in rainbow trout (161) and Atlantic salmon, (83). In catfish, four distinct heavy chains have been reported (109) and two different light chain classes (110).

Analysis of heavy chain gene of catfish showed a 24 % similarity with mouse μ chains (67). Catfish also showed an unusual arrangement of cysteines which is thought to give rise to several hypothetical disulphide linkages resulting in the observed immunoglobulin dimers, trimers and tetramers (67). This is unlike mammalian IgM, which requires stringent cross-linking of five monomers.

Pre-existing paradigms for immunological memory are based on higher vertebrates (93). In mammalian systems, immunological memory is characterized by antibody class (isotype) switching and an increase in monomeric (IgG) antibody concentration and affinity maturation. These specific phenomena either do not occur in fish or occur to a much lesser degree (94). Aspects of mammalian memory considered evolutionarily sophisticated may not be required by fish. Recently it was proposed that a memory response is simply one which is distinctive in its form and function from that of a primary response (93). In fish, an increased sensitivity to antigen upon secondary exposure, as well as enhanced antibody production, has been demonstrated in trout (13).

Specific cell mediated response

The specific cell mediated response is independent of antibody and is characterized by the ability to transfer the antigen-specific response from one individual to another by means of live cells. Most information on specific cell mediated immunity

in fish has been demonstrated by transplantation experiments. Transplantation of skin tissue or scales from another individual of the same species (allograft) are rejected whereas autograft (from the same individual) transplants are not. Immunological specificity and memory is demonstrated by the accelerated rejection time of second-set allografts (repeat grafting from same donor). The faster response is possible due to the clonal expansion of specific lymphocytes recognizing the foreign tissue. In addition to allograft rejection, teleost fish have been shown to display a wide variety of specific cell-mediated immune functions including graft-versus-host reactions, delayed-type hypersensitivity reactions (DTH) and mixed leukocyte reactions (MLR) (reviewed in (122)).

Major histocompatability receptors in fish

The cell surface structures involved in specific recognition of antigens are the major histocompatability complex (MHC) molecules and T cell receptors (TCRs). Initially characterized in mice, the MHC encodes two classes (I and II) of surface proteins that present antigenic peptides to different subsets of T cells. In teleosts, class I and II genes have been identified, as well as the gene for β-2-microglobulin which is associated with class I molecules (reviewed in (122)).

Recent studies on MHC genes in fish have shown that unlike MHC genes in humans and mice, which are tightly linked in a long continuous stretch on a single chromosome, MHC genes are not linked in bony fish (euteleostei) (164). Therefore, the term "complex" has been dropped from their name (54). Non-classical class I MHC genes have also been described in salmonids (169) and cyprinids, which show similarity

to CD1 sequences in terms of hydrophobicity and glycosylation patterns (reviewed in (176)).

T-cell receptors

T-cell receptor (TCR) genes have recently been reported in teleost fish. Partula *et al* have reported TCR α- and β-chains in rainbow trout (148, 149). The TCR cannot function without additional accessory molecules which impart selectivity and play a role in signal transduction (204). The first accessory molecule in fish was recently reported by Hansen *et al* (72) who sequenced the TCR coreceptor, CD8α, in rainbow trout.

Cytokines

An intrinsic part of the immune response is the role of cytokines in cell-cell communication. Cytokines have a regulatory role, acting in the local vicinity of the producing cell. Although a variety of cells can secrete cytokines, the two principal producers of cytokines are the T helper cells and macrophages (102). Several cytokines have now been identified in fish with similar activities to mammalian cytokines (reviewed in (122)).

Factors affecting fish immunity

One of the most important factors affecting fish immunity is temperature. The poikilothermic nature of fish means that they are subject to the temperature of their local environment. It has long been known that temperature effects immunity in cold-blooded vertebrates (28). The immunosuppressant effect of cold temperature on fish has been well documented (42, 129). However, most studies on the effect of temperature have been short term. Recently, Alcorn *et al* (3) performed a long term study on the effect of

temperature on the immune functions of sockeye salmon over their entire life cycle. Their findings supported that of other studies and showed that at the cooler temperature (8°C), fish had a greater percentage of macrophages and higher complement activity. In contrast, fish reared at 12 °C possessed more lymphocytes and produced a greater antibody response. Their findings suggest that at lower temperatures, fish rely more heavily on their innate immunity.

An intriguing phenomenon of fish immunity is the observed seasonal variation in the immune response. Even at a constant temperature, seasonal variations occur in fish humoral immune responses (219). This poorly understood effect has been observed in fish kept at a constant temperature throughout the year; lower antibody titres were observed in rainbow trout immunized in Autumn, compared to fish immunized in Spring (reviewed in (180)).

As in other physiological systems, poor health brought on by stress, pollutants and malnutrition can severely compromise the immune response. Aspects of fish farming which cause stress include high population density, handling, transport and anaesthesia (59). Various pollutants, including pesticides and heavy metals in the environment are also known to suppress the immune response of fish (59). The importance of diet for fish health was demonstrated in a recent study a linking elevated presence of oxidized lipid in feed and the development of rainbow trout fry syndrome (49).

Ontogeny of response to vaccination and challenge

Diseases of young fish pose additional difficulties regarding not only delivery of vaccine but the level of immune competence. In diseases of fry, it is important to

establish the age at which fish can be successfully vaccinated. Fish immunocompetence is determined by the functioning of lymphocytes rather than the early histological development of primary (thymus) secondary and peripheral (kidney, spleen, GALT) lymphoid organs or the mere identification of lymphoid cells (218).

Studies on the early onset and duration of immunity in salmonid fry by Johnson *et al* (90, 91) found that immunity was a function of size and not age. The minimum size at which protective immunity occurred was between 1 and 2.5 g. Generally, immunity was longer lasting in bigger fish. When fish were vaccinated at 1 g, immunity lasted 120 days, 180 days in 2 g fry and over a year in 4 g fry (90). Tatner and Horne (181) tested the ability of rainbow trout to mount an immune response against *Vibrio anguillarum* from 2 weeks post hatch (0.14 g) onwards. Protection of 100 % was achieved in 0.5 g fry for intraperitoneal vaccination and 50 % for immersion vaccination. Further understanding of the ontogeny of the fish immune response will aid in the development of vaccination strategies better suited for fry.

In summary, the main features of the adaptive response in teleosts in may ways mirror that of higher vertebrates. However, the poikilothermic nature of fish means they are affected in unique ways by the temperature of their surroundings. There remain unexplained differences, such as the observed seasonal effect. We have much to learn about the evolution of immune response through the study of piscine immunology. As more is learned about fish immunology, perhaps a different view on the evolution of the immune system will be accepted. Instead of viewing fish immunity according to the mammalian paradigms, leading to descriptions such as unsophisticated and primitive, fish may be seen to possess an immune system equally well adapted to their niche.

Materials and Methods

BACTERIAL STRAINS, GROWTH AND CHARACTERIZATION

Table 2. Wild type bacterial strains used in this study

Species	Strain	Source
Flavobacterium psychrophilum	259-93[*1]	Rainbow trout, Idaho, USA
Flavobacterium psychrophilum	UP96/017[*2]	Rainbow trout, Weymouth, UK
Flavobacterium psychrophilum	ATCC 49418	American Type Culture Collection
Flavobacterium psychrophilum	911209-2[*3]	Rainbow trout, Denmark
Flavobacterium psychrophilum	911209-1[*3]	Rainbow trout, Denmark
Flavobacterium psychrophilum	910619-1[*3]	Rainbow trout, Denmark
Flavobacterium psychrophilum	910614-2[*3]	Rainbow trout, Denmark
Flavobacterium psychrophilum	911126-2[*3]	Rainbow trout, Denmark
Flavobacterium psychrophilum	911126-3[*3]	Rainbow trout, Denmark
Flavobacterium psychrophilum	910614-3[*3]	Rainbow trout, Denmark
Flavobacterium psychrophilum	910614-5[*3]	Rainbow trout, Denmark
Flavobacterium psychrophilum	970522-1[*4]	Rainbow trout, Denmark
Flavobacterium psychrophilum	960104-1[*4]	Rainbow trout, Denmark
Flavobacterium psychrophilum	950824-1[*4]	Rainbow trout, Denmark
Flavobacterium psychrophilum	950920-1[*4]	Rainbow trout, Denmark
Flavobacterium psychrophilum	951027-1[*4]	Rainbow trout, Denmark
Flavobacterium columnare	ATCC 43622	American Type Culture Collection
Flavobacterium aquatile	ATCC 11947	American Type Culture Collection
Flavobacterium johnsoniae	ATCC 29585	American Type Culture Collection
Tenacibaculum maritimum	ATCC 43397	American Type Culture Collection
YPB	PBS9701[*5]	Coho salmon, Vancouver Island, Canada
YPB	PBS9702[*5]	Coho salmon, Vancouver Island, Canada
YPB	PBS9703[*5]	Coho salmon, Vancouver Island, Canada
YPB	PBS9704[*5]	Coho salmon, Vancouver Island, Canada
YPB	PBS9705[*5]	Coho salmon, Vancouver Island, Canada
YPB	PBS9706[*5]	Cutthroat trout, Vancouver Island, Canada
YPB	PBS9707[*5]	Cutthroat trout, Vancouver Island, Canada
YPB	PBS9708[*5]	Coho salmon, Vancouver Island, Canada
YPB	PBS9709[*5]	Coho salmon, Vancouver Island, Canada

YPB	PBS9710[*5]	Coho salmon, Vancouver Island, Canada
YPB	PBS9711[*5]	Chinook salmon, Vancouver Island, Canada
YPB	PBS9712[*5]	Chinook salmon, Vancouver Island, Canada
YPB	PBS9713[*5]	Chinook salmon, Vancouver Island, Canada
YPB	PBS9714[*5]	Coho salmon, Vancouver Island, Canada
YPB	PBS9715[*5]	Coho salmon, Vancouver Island, Canada
YPB	PBS9716[*5]	Coho salmon, Vancouver Island, Canada

YPB, Yellow-pigmented bacteria

[*1] Kindly provided by Dr. Scott LaPatra, Clear Springs Food, Buhl, Idaho, USA.

[*2] Kindly provided by Dr. David Alderman, CEFAS, Weymouth, UK.

[*3] Kindly provided by Dr. Ellen Lorenzen, Danish Veterinary Laboratory, Aarhus, Denmark.

[*4] Kindly provided by Dr. Inger Dalsgaard, Royal Veterinary and Agricultural University, Copenhagen, Denmark.

[*5] Kindly provided by Dr. Dorothy Keiser, Pacific Biological Station, Nanaimo, BC, Canada.

Table 3: *Eschericia coli* strains used in this study

Strain	Description and relevant genotype
E. coli BL21(DE3)	Expression strain lacking Lon and ompT proteases, chromosomal T7 RNA polymerase, Tet ^{R}
E. coli SOLR	Phagemid excision strain, does not allow replication of ExAssist helper phage, Kan^{R}, λ^{R}, Su^{-}.
E. coli XL1-Blue	Cloning strain, blue-white screening, Tet ^{R}
E. coli XL1-Blue MRF'	Restriction-minus strain, enhanced blue-white colour selection

All *E. coli* strains from Stratagene, La Jolla, CA, USA

Growth conditions

Eschericia coli BL21(DE3), SOLR, XL1-Blue and XL1-Blue MRF' (Stratagene, La Jolla, CA, USA) were grown in LB or terrific broth. When required, supplements were added at the following concentrations: ampicillin (Ap) 50 or 100 μg/ml, kanamycin (Kn) 50 μg/ml, tetracycline (Tet) 12.5 μg/ml, IPTG 1 mM, X-gal.

F. psychrophilum and *F. columnare*, were routinely grown on MAT or TYES medium (Table 4) at 15 °C. *F. aquatile* was grown on M5 medium (205) at 26 °C. *F. johnsoniae* was grown on TYE medium at 26 °C. *Tenacibaculum maritimum* was grown on TYES medium, using Instant Ocean (Aquarium Systems, Mentor, OH, USA) in place of deionised water, at 26 °C.

Table 4 Growth media for *Flavobacteria*

Medium	Composition
MAOB(25)	0.5% tryptone, 0.05% yeast extract, 0.02% sodium acetate and 0.02% beef extract
TYES (80)	0.4% tryptone, 0.04% yeast extract, 0.05% $CaCl_2$ and 0.05% $MgSO_4$
TYE (64)	0.4% tryptone and 0.04% yeast extract
MAT (this study)	0.4% tryptone, 0.04% yeast extract, 0.05% $CaCl_2$, 0.05% $MgSO_4$, 0.02% sodium acetate, 1% maltose
M5 (205)	0.2 % Na caseinate, 0.1 % peptone, 0.05 % yeat extract, 0.05 % K_2HPO_4

Agar was added to 1.5% when required, pH 7.2 - 7.4.

Carbohydrate metabolism

To test whether *F. psychrophilum* fermented various carbohydrates, sugar was added to broth medium to a final concentration of 1 % (w/v) in the presence of the pH indicator dye phenol red (0.025 g/l). Fermentation of sugar was indicated by the lowering of pH, apparent as a colour change from red to yellow.

Large scale growth

Large scale growth was initially tested in a 3 l Chemap fermenter (Chemap AG, Volketswil, SWI) containing 2.5 l MAT broth (Table 4) or MATH (MAT plus 5 μg/ml

hemin) at 15 °C, stirring at 300 rpm, aeration 15 l/min. Large scale growth was achieved in 35 l Chemap fermenter (Chemap AG, Volketswil, SWI) containing 28 l MAT broth (Table 4), inoculated with 280 ml overnight culture (1/100 dilution), 15 °C, stirring at 300 rpm with aeration at 20 l/min until the A_{600} peaked at ~1.4-1.6. During exponential growth, cultures were fed with either maltose (1 % v/v), yeast extract (0.08 %) or half the original amount of all MAT medium components.

Biochemical and physiological characterization of isolates.

The presence of characteristic flexirubin-type pigments in the bacterial cell wall was tested by the method of Reichenbach (155): 20% KOH was added directly onto the MAOA culture; a positive reaction was indicated by a change in colour from orange to brown. To test whether *F. psychrophilum* could absorb the aromatic sulfonated diazo dye, heme analogue, Congo red, cells were grown on MAOA supplemented with 30 µg/ml Congo red. The presence of oxidase was tested with 1 % tetramethyl-*p*-phenylenediamine dihydrochloride (101). The presence of catalase was tested with 3 % H_2O_2 on a glass slide as previously described (171). Elastase production was tested as follows: 0.1 % (w/v) elastin (Sigma, St. Louis, MO., USA) was added to MAT agar and holes (~5 mm diameter) cored out of the agar with a sterile pasteur pipette. 50 µl culture was added to the wells and the plates incubated at 15 °C for 7 days. Clearing of the surrounding elastin in the agar was indicative of elastase production.

PROTEIN ANALYSIS

SDS PAGE

Protein analyses of whole cell lysates were carried out by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) according to the method of Laemmli (103) as modified by Ames (7). Samples were resolved in 12 % polyacrylamide separating gels with 5 % stacking gels. Molecular weight (MW) was estimated according to the apparent MW of prestained protein markers.

Gel staining

SDS PAGE gels were washed for 15 min in dH_2O and stained with GelCode (Pierce, Rockford, IL, USA), a Coomassie based stain.

Proteinase K treatment

Digestion of material with proteinase K was carried out at a final concentration of 1mg/ml proteinase K at 65 °C overnight.

Acetone precipitation

Ten volumes of cold acetone was added to the sample which was vortexed briefly and incubated at -20 °C overnight. Precipitated protein mixture was centrifuged at 4 °C, 17,400 *x g* 15 min, the supernatant discarded and the pellet air dried.

[^{14}C] Palmitate labeling of F. psychrophilum lipoproteins

F. psychrophilum cells were grown up in 1 ml MAT broth 15 °C, 72 h containing 1μCi /ml [1-^{14}C] palmitic acid (Amersham Biosciences Corp. Piscataway, NJ, USA). The culture was centrifuged in pre-weighed tubes and the resulting pellets were washed once in PBS. The [^{14}C] metabolically labeled cells were then extracted with Triton X-114. The [^{14}C] labeled samples were resolved by SDS PAGE and visualized using a storage phosphor screen imaging system.

Biotinylation of cell-surface proteins.

Whole cells of *F. psychrophilum* were surface labeled using the extrinsic labeling reagent, sulpho-NHS-biotin (Pierce, Rockford, IL, USA). Cells were harvested from TYES broth, washed and resuspended in PBS to 10 mg/ml (wet wt). For cell extract controls, cells were lysed by sonication at 40 W for 4 x 15 s intervals. Sulpho-NHS-biotin (4 μl of 1 mg/ml in dimethylsulphoxide (DMSO)) was added to 100 μl aliquots of the cells and the samples incubated at RT for 1 min. The reaction was stopped with a 1000 fold excess of glycine (pH 7.4). SDS PAGE sample buffer was added and the mixture boiled for 10 min. After electrophoresis, proteins were immobilized onto nitrocellulose membranes which were subsequently blocked in PBS-1% gelatin (1 h at RT). Biotin was detected by incubating the membranes with Streptavidin-biotinylated alkaline phosphatase (0.5 mg/ml biotin, 1/5000 dilution) (Caltag Labs, San Francisco, CA, USA) in PBS-1% gelatin (1 h at RT). The biotinylated proteins were detected with NBT and BCIP as outlined above.

Triton X-114 phase partitioning of F. psychrophilum.

Wet cell pellets were resuspended in Triton® X-114 (Calbiochem corp., La Jolla, CA , USA and Sigma, St. Louis, MO, USA) solubilization buffer (1% Triton X-114 / 10mM Tris pH 8 / 5mM EDTA pH8) to 20 mg / ml. Cells were solubilized by rotating the tube at 4 °C for 2 - 3 hours. The solution was centrifuged for 15 min, 12,100 *x g*, 4 °C in a Beckman J-21C centrifuge, JA-20 rotor. The clear supernatant was transferred to a new tube (Triton soluble fraction), leaving behind the 'cold pellet'. The Triton soluble fraction was then incubated at 37 °C for 15 min to precipitate the detergent and then centrifuged at room temperature for 15 min, 12,100 *x g*. This resulted in a top aqueous phase, a lower detergent phase and a clear 'warm pellet'. The aqueous and Triton phases were transferred to new tubes and washed three times each as follows: To the aqueous phase, Triton X-114 was added to a final concentration of 1 % and to the Triton phase, an equal volume of TE (10 mM Tris / 5 mM EDTA pH8) was added. Both Triton X-114 suspensions were dissolved by incubating on ice for 10 min then transferred to 37 °C for 10 min for phase partitioning prior to centrifugation to separate the phases. The washed Triton and aqueous phases were transferred to new tubes. Acetone precipitations were performed on the samples in order to remove the detergent from the Triton phase and to concentrate proteins in the aqueous phase.

Alternatively, instead of acetone precipitation to remove Triton X-114, proteins were precipitated with chloroform and methanol as described by Wessel and Flugge (207). Briefly, 400 µL of methanol was added to 100 µL of protein sample. The resulting mixture was vortexed followed by brief centrifugation (9,000 *x g*, 10 s). 100 µL of chloroform was then added and the sample was again vortexed and centrifuged. Phase

separation was achieved by the addition of 300 µL of distilled water, vortexing and centrifugation (9,000 *x g*, 1 min). The upper aqueous phase was carefully removed, leaving behind the lower organic phase and the protein containing interface. The precipitated proteins were pelleted by centrifugation (9,000 *x g*, 2 min) following the addition of 300 µL of methanol and vortexing. The supernatant was removed and discarded. The precipitated proteins were dried in a SpeedVac SC100 (Thermo Savant, Holbrook, NY, USA) for 10 min.

Two dimensional gel electrophoresis and mass spectrometry

High-resolution two-dimensional SDS-PAGE was performed using the ISO-DALT multiple 2-D system (10, 11). Protein samples were solubilized in 30 µl of urea mix (9 M urea, 4 % NP-40 (v/v), 2% Pharmalyte 3-10 ampholines (v/v), 2 % DTT (w/v)) equivalent to A_{600} 8. Samples were subsequently loaded onto pre-focused tube gels containing pH range 3-10 ampholines (Pharmalyte 3-10, Amersham Pharmacia, Upsala, SWE). First dimension isoelectric focusing was conducted at 800 V for 18 h (14,400 Vh). Following electrophoresis the tube gels were equilibrated for 15 min at room temperature in equilibration buffer and immediately mounted onto 10-16.5 % gradient SDS-PAGE slab gels with the acidic end positioned to the left. Electrophoresis was performed at 4 °C at 1 Amp until the dye front was about 1 cm from the bottom of the gel (~ 5 h). After electrophoresis the gels were either fixed and then stained with colloidal Coomassie Brilliant Blue G-250 or electroblotted onto nitrocellulose membranes.

Alternatively, protein was submitted to the University of Victoria Genome BC Proteomic Centre for 2D gel electrophoresis. At the Proteomic Centre, protein samples

were suspended in 465 µl of Sample Rehydration Buffer (8 M Urea, 2 % CHAPS, 0.5 % pH 3-10 ZOOM Carrier Ampholytes (Invitrogen, Carlsbad, CA, USA), 20 mM DTT and 0.002 % bromophenol blue). Insoluble debris was removed by brief centrifugation and the supernatant applied to rehydrate non-linear, pH 3-10 ZOOM immobilized pH gradient (IPG) strips (Invitrogen, Carlsbad, CA, USA) overnight at room temperature. The rehydrated IPG strips were placed in the ZOOM IPGRunner (Invitrogen, Carlsbad, CA, USA) and focused at 200 V for 20 min, 450 V for 15 min, 750 V for 15 min and finally 2000 V for 30 min. After focusing, the IPG strips were removed and equilibrated in 1X NuPAGE® LDS Sample Buffer with Sample Reducing Agent (Invitrogen, Carlsbad CA, USA) for 15 min at RT. Following equilibration, the IPG strips were loaded into the IPG well of a NuPAGE® Novex 4-12 % Bis-Tris ZOOM® Gel and overlaid with 0.5 % agarose in MOPS running buffer (50 mM MOPS, 50 mM Tris, 0.1 % SDS, 1 mM EDTA pH 7.7). The gel cassettes were placed in the XCell *SureLock*. Mini-Cell (Invitrogen, Carlsbad, CA, USA) and electrophoresed for 45 min at 200 V. After electrophoresis the gels were either fixed and then stained with colloidal Coomassie Brilliant Blue G-250 or electroblotted onto nitrocellulose membrane.

Staining of 2D gels with Sypro Ruby

Gels were fixed in 10 % methanol / 6 % acetic and stained with Sypro Ruby (Molecular Probes, Eugene, OR,USA) overnight. Gels were destained in 2 x 1 h washes in 10 % methanol / 6 % acetic acid. The Sypro Ruby stained gel was visualized under UV light and recorded using a ChemiImager™ 4000 equipped with AlphaEase™ image processing and analysis software (Alpha Innotech Corporation, San Leandro, CA, USA).

Staining of 2D gels with Coomassie Brilliant Blue G-250

Gels were agitated gently in fixative (50 % (v/v) ethanol, 3 % (v/v) ortho phosphoric acid) for 1-4 days at RT, washed three x 30 min in distilled water and allowed to equilibrate in Neuhoff's solution (16 % (w/v) ammonium sulphate, 25 % (v/v) methanol, 5 % (v/v) orthophosphoric acid (137) for one hour with gentle agitation. One gram of CBB G-250 (EM Science, Gibbstown, NJ, USA) was sprinkled into the Neuhoff's solution and staining continued for 3-5 days. Once well-stained protein spots were visible, gels were either scanned and protein spots cored or the intact gels were transferred into a 20 % (w/v) ammonium sulphate solution for storage at 4 °C (137).

Digital images of both 2-D CBB stained gels were captured by scanning at 300 dpi using a colour scanner (UMAX Astra 3400, Fremont, CA) after briefly rinsing the gels in dH_2O.

Reduction, alkylation and tryptic digests of 2D gel spots

Protein spots of interest were cored using a 4 mm plastic straw and either transferred to 1.5 ml Eppendorf microcentrifuge tubes (previously autoclaved and rinsed with 50 % methanol to remove any contaminants) for digestion or to 96 well sterile tissue culture plates (one spot per well in 10 µl of 20 % (w/v) ammonium sulphate (137) for storage at -20 °C. For analysis by mass spectrometry, 2-D protein spots were destained (50 % (v/v) methanol / 5 % (v/v) acetic acid), reduced with 10 mM DTT and alkylated with 100 mM iodoacetamide as described previously (99). The carboxyamidomethylated protein spots were digested overnight at 37 °C with 20 ng/µl modified, sequence grade,

porcine trypsin according to the manufacturer's directions (Promega, Madison, WI). Peptides were extracted from the gel pieces using a series of elutions with 50 % (v/v) acetonitrile / 5 % (v/v) formic acid. The resulting pooled eluates were each reduced to a final volume of 20 µl in a Speed Vac Concentrator (Savant, Hicksville, NY, USA) and processed for mass spectrometry.

MALDI-TOF mass spectrometry

Peptides from each trypsin-digested sample were desalted using a ZipTip (C18 resin; P10, Millipore Corporation, Bedford, MA, USA). For each sample, 1.0 µl of the desalted peptide mixture was mixed (1:1) with the matrix, alpha-cyano-4-hydroxycinnamic acid (Sigma, St. Louis, MO, USA), bradykinin, fragment 2-9 (FW 904.4681) and adrenocorticotropic hormone, fragment 18-39 (FW 2465.1989) and spotted onto a Voyager, 100 position, stainless steel MALDI plate (Applied Biosystems, Foster City, CA, USA). An Applied Biosystems Voyager DE-STR mass spectrometer (Applied Biosystems, Foster City, CA, USA) running in delayed extraction, reflectron mode was used to acquire MALDI-TOF data. MALDI spectra were processed and analysed using Data Explorer software (Applied Biosystems, Foster City, CA, USA). Selected peptide masses were submitted to MS-Fit (Protein Prospector software package; San Francisco, CA, USA: http://prospector.ucsf.edu/) and Mascot (Matrix Science, London, UK: http://www.matrixscience.com/) for database searching and determination of peptide mass maps.

Nanospray tandem mass spectrometry (MS/MS) and peptide sequencing

Tryptic peptides were desalted using glass capillary needles (Protana Inc., Staermosegaardsvej, DK) packed with C18 resin (Perceptive POROS R2, 50 μm bead) and were extracted into sample needles using 1.0 μl 50 % (v/v) methanol / 1 % (v/v) formic acid. Nanospray electrospray ionisation (ESI) was used to introduce ions into a PE-SCIEX Q-STR*i* quadrupole time-of-flight mass spectrometer (Q-TOF) (Applied Biosystems, Foster City, CA, USA). Data were managed with Bioanalyst Software (PE-SCIEX, Boston, MA, USA) and peptides sequenced by following Kinter's nine step strategy for interpretation of product ion spectra (99). Peptide fragmentation data searching was performed using the Mascot MS/MS Ions Search algorithm (Matrix Science; London, UK: http://www.matrixscience.com/).

Virtual mass mapping

F. psychrophilum DNA sequence (~8.5 kb) was searched for open reading frames (ORFs) using NCBI's ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The ORFs were then translated into amino acid sequence using the same programme. The translated amino acid sequence was then subjected to a virtual tryptic digest using the online program MS-Digest (http://prospector.ucsf.edu/), allowing for 2 missed cleavages. Theoretical peptide fingerprints, estimated MWs and pIs were generated for each of the translated ORFs and compared to the observed peptide fingerprints resulting from the same cloned and expressed DNA sequence.

Electroblotting of 2D gels onto PVDF membrane for N-terminal sequencing

Gels were blotted onto nitrocellulose or Sequi-Blot PVDF (Bio-Rad Laboratories, Hercules, CA, USA) using NuPAGE Transfer buffer (Invitrogen, Carlsbad, CA, USA) with 20 % methanol and 100 V for 1 h in the Bio-Rad Mini-TransBlot Electophoretic Transfer Cell (Bio-Rad Laboratories, Hercules, CA, USA). The nitrocellulose blot was stained with Sypro Ruby Blot Stain (Molecular Probes, Eugene, OR, USA) according to the manufacturer's instructions. Sypro Ruby stained blots were visualized under UV light and recorded using a ChemiImager™ 4000 equipped with AlphaEase™ image processing and analysis software (Alpha Innotech Corporation, San Leandro, CA, USA). The PVDF membrane was then stained with 0.025 % Coomassie Brilliant Blue R250 (Sigma) in 40 % methanol and destained with 50 % methanol. Proteins were excised from the PVDF membrane and submitted to the University of Victoria Genome BC Proteomics Centre.

Protein expression in E. coli (of pETC clones)

Induction experiments were carried out in *E. coli* BL21 DE3. Overnight, 37 °C cultures in LB_{Ap} or TFB_{Ap} were used to inoculate fresh LB_{Ap} media. Cultures were grown at 37 °C to an A_{600} of 0.6-0.8, at which point they were induced by the addition of 1 mM IPTG for 2 h.

Isolation and quantification of inclusion bodies

Inclusion bodies were isolated from cells by sonication as follows: induced culture was centrifuged at 10,000 *x g* for 10 min and resuspended in dH_2O. The cell

suspension was sonicated on ice for 10 x 15 sec using an ultrasonic processor (model W-385, Heat Systems Ultrasonics, Farmingdale, NY, USA) and centrifuged at 10,000 *x g* for 15 min. Pellets were resuspended in dH_2O and the procedure repeated. The final inclusion body samples were resuspended in dH_2O and stored at -20 °C.

Quantification of expressed protein concentration

Coomassie stained gel images were captured on a Chemi Imager 4000 low light imaging system (Alpha Innotech Corp., San Leandro, CA, USA) and quantified using Alpha Ease software (Alpha Innotech Corp., San Leandro, CA, USA) and compared to a lysozyme standard curve.

DNA ANALYSIS

Plasmids used in this study

Table 5. Plasmids used in this study

Plasmid	Description / Relevant genotype	Source
pGEM-T	A-T cloning vector, Ap^r	Promega.
pGEM-T Easy	A-T cloning vector, Ap^r	Promega
pBluescript SK	f1, lacZ, Ap^R	Stratagene
pP2	pBluescript SK containing *F. p.* insert P2	This study
pP3	pBluescript SK containing *F. p.* insert P3	This study
pP4	pBluescript SK containing *F. p.* insert P4	This study
pP5	pBluescript SK containing *F. p.* insert P5	This study
pP6	pBluescript SK containing *F. p.* insert P6	This study
pP7	pBluescript SK containing *F. p.* insert P7	This study
pP8	pBluescript SK containing *F. p.* insert P8	This study
pP9	pBluescript SK containing *F. p.* insert P9	This study

pETC	pET21 derived, C protein fusion partner, T7 promoter, Ap^R	J. Burian, Microtek Intl. Ltd.
pETC7-166	pETC containing 501 b ORF from *F. p.*insert P7	This study
pETC8-91	pETC containing 276 b ORF from *F. p.*insert P8	This study
pETC8-92	pETC containing 279 b ORF from *F. p.* insert P8	This study
pETCTX20'	pETC containing partial ORF for 20 kDa *F.p.* antigen TX20	This study
pETCM	pETC with additional measles epitope	J. Burian, Microtek Intl. Ltd.
pETCM7-166	pETC7-166 with additional measles epitope	This study
pETCM8-91	pETC8-91 with additional measles epitope	This study
pETCM8-92	pETC8-92 with additional measles epitope	This study
pETCMTX20'	pETCTX20' with additional measles epitope	This study

Purification of chromosomal DNA from F. psychrophilum

F. psychrophilum chromosomal DNA was purified by a method particularly useful for Gram-negative bacteria that produce large amounts of contaminating extracellular polysaccharides, described in Current Protocols in Molecular Biology (15). This method uses the detergent CTAB (cetyltrimethylammoniumbromide) which complexes with both proteins and polysaccharides. Using this method, 250 mg (wet wt) *F. psychrophilum* cells were resuspended in 5.67 ml TE buffer in a 25 ml glass Corex tube. 300 µl 10 % SDS and 30 µl PK 20 mg/ml were added and the cells incubated at 37 °C for 1 h. 1 ml 5 M NaCl was added and the solution mixed gently by inversion. 800 µl of the CTAB / NaCl solution (10 % CTAB in 0.7 M NaCl) was added, the solution mixed gently and incubated at 65 °C for 10 min. An equal volume of chloroform / isoamylalcohol (24:1) was then added followed by gentle mixing and centrifugation at RT for 5 min, 1,000 *x g*. Using a wide-bore blue tip, the top aqueous layer was

transferred to a new tube and an equal volume of phenol / chloroform / isoamylalcohol (25:24:1) was added. The solution was mixed by inversion and then centrifuged at RT, 10 min, 1,000 *x g*. The phenol / chloroform / isoamylalcohol extraction was repeated three times followed by one extraction with chloroform to remove residual phenol. A 0.6 volume of isopropanol at RT was added and the tube shaken until white DNA precipitate was visible. Precipitated DNA was spooled with a capillary tube and washed once by immersion in 70 % ethanol and then left to air dry. Chromosomal DNA was rehydrated in 500 µl TE buffer at 4 °C overnight.

Routine isolation of F. psychrophilum chromosomal DNA for PCR

Genomic DNA was isolated from *F. psychrophilum* using Chelex 100 resin (BioRad, Hercules, CA, USA) (personal communication, D. Machander, Microtek Intl. Ltd. Saanichton, B.C., CAN). Colonies were picked and resuspended in 100 µl Chelex bead suspension (5 % Chelex in dH_20), boiled for 15 min and centrifuged (30 s at 14,000 *x g*). DNA in 50 µl of supernatant was spectrophotometrically quantified (A_{260}) and adjusted to 50 ng/µl.

Isolation of plasmid DNA

Plasmids were routinely purified using standard alkaline lysis plasmid preps (159). Alternatively, plasmid purification was performed using a QIAprep Spin MiniPrep Kit or Qiagen Plasmid Maxi Kit (Qiagen Inc., Mississauga, ON) according to the manufacturers' instructions.

Restriction enzyme digests

DNA was digested using restriction enzymes from NEB (New England Biolabs, Beverly, MA, USA), according to the manufacturer's suggested protocol. Complete digests were routinely performed at 37 °C for 3 h. In partial digestion reactions, digestion was prematurely terminated by removing the samples from the optimal digestion temperature sooner than the length of time required to give complete digestion.

Agarose gel electrophoresis

Unless otherwise indicated, 1 % agarose gels were used. High strength analytical grade agarose (BioRad, Hercules, CA, USA) was dissolved in Tris-acetate/EDTA (TAE) electrophoresis buffer (20 mM Tris acetate, 0.5 mM EDTA pH 8.5) to prepare the gel. Gels were stained in ethidium bromide (EtBr) solution and destained in tap water. The EtBr labeled DNA was visualized and recorded using a ChemiImager™ 4000 equipped with AlphaEase™ image processing and analysis software (Alpha Innotech Corporation, San Leandro, CA, USA). The images obtained were saved digitally in TIFF format.

Isolation of DNA from agarose gels

Qiagen method

DNA was isolated from agarose gels using either the QIAEX II Gel Extraction Kit (Qiagen Inc., Mississauga, ON, CAN) according to the manufacturer's instructions or by the freeze/thaw method.

Freeze/thaw method

Extraction of large DNA fragments (~ 10 kbp) was performed by a freeze-thaw method. Gel pieces were placed in 1.5 ml polypropylene tubes, incubated at -20 °C for 15 min followed by incubation at 37 °C for 15 min. The freeze-thaw cycle was repeated 3 times and the liquid containing the eluted DNA fragments aspirated into a new tube.

DNA ligations

DNA ligations were performed using T4 DNA ligase (Life Technologies, Burlington, ON, CAN) according to the manufacturer's suggested protocol.

Ethanol precipitation

To a dilute DNA solution, 10 % (v/v) of 3 M sodium acetate pH 5.5 and three volumes of cold 95 % EtOH were added. The mixture was vortexed briefly and incubated at –70 °C for at least 30 min. The DNA was pelleted by centrifugation at 12,000 *x g* for 15 min. The pellet was washed twice with 500 µl 70 % EtOH, centrifuging as above. The washed pellet was then dried in a SpeedVac SC100 (Thermo Savant, Holbrook, NY, USA) and finally resuspended in TE buffer.

Preparation of electrocompetent cells

Electrocompetent *E. coli* cells were prepared according to the BioRad Gene Pulser manual as follows: One liter of cells were grown at 37 °C to an A_{600} of 0.5 – 1.0. The cells were then chilled by placing the flask on ice for 30 min and centrifuged in a cold rotor at 4,000 *x g* for 15 min. Pellets were gently resuspended in 1 l of cold sdH_20

and centrifuged as above. The pellets were then resuspended in 0.5 l cold sd$H_2$0 and centrifuged as above. The pellet was resuspended in 20 ml 10 % glycerol, centrifuged and resuspended in a final volume of 2-3 ml 10 % glycerol. The cell suspension was frozen in aliquots on dry ice and stored at -70 °C.

Electroporation

Electroporation of *E. coli* was performed using a Gene Pulser II (BioRad, Hercules, CA, USA.) according to the manufacturer's instructions. Prior to electroporation, 50 µl aliquots of electrocompetent *E. coli* cells were removed from -70 °C and thawed on ice. Plasmid DNA or ligation mixtures were mixed with the cells and the cell/DNA mixture transferred into an ice cold 0.2 cm diameter cuvette (BTX, San Diego, CA, USA). The cuvette was placed into the holder and the Gene Pulser II set at a resistance of 200 Ohms, a capacitance of 25 µF. DNA was transferred into the cells by applying a pulse of 2.5 kV. Cells were recovered in 1 ml SOC broth for 1 h at 37 °C after which cells were plated onto LA containing the appropriate antibiotics.

Automated DNA sequencing

Plasmids submitted for sequencing were purified using a Qiaprep Spin MiniPrep Kit (Qiagen Inc., Mississauga, ON, CAN) according to the manufacturers' instructions. Pure double-stranded plasmids were submitted to the sequencing facility in The Centre for Biomedical Research, University of Victoria, Victoria, BC and automated dideoxynucleotide sequencing was performed using a NEN Global IR2 DNA Sequencer (LI-COR, Inc., Lincoln, NB, USA) using dye labelled primers. Alternatively, DNA

inserts greater than 1.5 kb were sequenced by primer walking using the dye terminator method at the University of Victoria sequencing facility using the Applied Biosystems Model 377 DNA Sequencing System and DYEnamic ET terminator cycle sequencing kit (Amersham Biosciences Corp. Piscataway, NJ, USA). The *F. psychrophilum* inserts P2, P3, P7 and P8 were sequenced with a redundancy of at least 4x coverage.

DNA sequence analysis

DNA traces were visually examined for errors and ambiguous regions and aligned using Seqman II vs. 5.03 sequence alignment software suite from DNASTAR, Inc. (Madison, WI, USA) or ContigExpress from Vector NTi Suite 7 (InforMax Inc, Bethesda, MD, USA) for derivation of the final consensus sequence. Coding predictions were made to identify open reading frames (ORFs) using the National Center for Biotechnology Information (NCBI) programme, ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Sequences were subjected to BLAST2 (4) and FASTA3 (150) analysis to determine if any similar sequences were known. Multiple sequence alignments were performed using AlignX from Vector NTi Suite 7 (InforMax Inc, Bethesda, MD, USA).

Primers used to sequence F. psychrophilum DNA from genomic library

Wherever possible, sequencing primers were selected to have an overall GC content of at least 40 %, with GC clamps and 18-30 b in length. Primer sequence analysis was performed using Vector NTi (InforMax Inc, Bethesda, MD, USA) to check

for likelihood of dimer and hairpin formation. All primers were ordered from AlphaDNA (Sherbrook, ON, CAN).

PCR protocols

Custom oligonucleotides for PCR were ordered from AlphaDNA, (Sherbrook, ON, CAN) and rehydrated in sdH_2O. Thermocycling was performed in a PTC-100 programmable thermal cycler (MJ Research Inc., Waltham, MA, USA). PCR products were separated on a 1 - 2 % agarose gels at 70 V, stained with EtBr and photographed under UV light.

RAPD-PCR for the detection of F. psychrophilum.

Each 50 µl reaction contained the following: 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM deoxyribonucleoside, 20 pmol of primer, 1.65 U of Taq DNA polymerase (Roche, Laval, QC, CAN) and 50 ng template of DNA. The primers used were: 5'-TTCGCAGATCCCAACAACAA-3' and 5'-CTAAGTACCGCCCCG ATC-3'. Amplification was performed as follows: 1 cycle at 94 °C for 3 min; 2 cycles at 94 °C for 1 min, 47 °C for 1 min for 72 °C 1 min; 41 cycles at 94 °C 1 for min, 55 °C for 1 min, 72 °C for 3 min; 1 cycle at 72 °C for 10 min.

Amplification of ORFs and insertion of restriction sites.

Primers used to amplify ORFs and insert restriction sites for cloning purposes are listed in Table 6. The forward primers began CGACGGATCCGTCTCATATG-, encoding *Bam*HI and *Nde*I resriction sites respectively (underlined). The reverse primers encoded *Hin*dIII and *Vsp*I restriction sites respectively (underlined), using the tag

TCCGAAGCTTAATTAAT-. Each 50 µl reaction contained the following: 20 mM Tris HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM deoxyribonucleoside, 20 pmol of primer 1 U of Taq DNA polymerase (Roche, Laval, QC, CAN) and 50 ng template of DNA. Amplification was performed as follows: 1 cycle at 94 °C for 2 min, 30 cycles of 94°C for 30 s, 52°C for 30 s and 72°C for 30 s followed by 1 cycle at 72 °C for 5 min. The annealing temperature, described here as 52 °C, was adjusted as necessary depending on the Tm of the primers, keeping it within 5°C of the lowest Tm of each primer pair.

Table 6. Primers used to amplify ORFs

Clone/ORF	Primer sequence 5' → 3'
pP3 ORF	Forward - CGACGGATCCGTCTCATATGAACTTCCAAGTACAATCAGATTACAAACC
	Reverse - TCCGAAGCTTAATTAATGATTTTTTCTTGCCAAATCTTTAATTTCATCTCGC
pP7 ORF166	Forward - CGACGGATCCGTCTCATATGACAAGAGAAGAAAAATCAATCGCTATTGG
	Reverse - TCCGAAGCTTAATTAATTTCCGCTACCTCACCTTTAGATTCTGC
pP8 ORF91	Forward - CGACGGATCCGTCTCATATGAACAAATCAGATTTAATCGATGC
	Reverse - TCCGAAGCTTAATTAATTTTATTTACTGCTATTTCTAATTCAGCACC
pP8 ORF92	Forward - CGACGGATCCGTCTCATATGCTAAAAGAAGGGGTTTTATATAC
	Reverse - TCCGAAGCTTAATTAATGCCTAATCTAATTTTTGCATTTAACTTTG
TX20'	Forward - CGACGGATCCGTCTCATATGAACGTGGCAGGTACAGTGGGTTTTAACTC
	Reverse -TCCGAAGCTTAATTAATGAAATTTTTAGAGATACCAACGCTTACTTGTTTTCCG

Degenerate PCR (dPCR).

Each 50 µl reaction contained the following: 20 mM Tris HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM deoxyribonucleoside, 50 pmol of each degenerate primer, 1 U of Taq DNA polymerase (Roche, Laval, QC, CAN) and 500 ng template of DNA. Each of three peptide sequences were used to design complementary degenerate PCR

primers. The three peptide and their related dPCR primers are lised in Table 7. Degenerate PCR primers. The primers were named according the first amino acid of the peptide.

Table 7. Degenerate PCR primers

Peptide	Forward primer (+)	Reverse primer (-)	Degeneracy
AANDFE	TCRAARTCRTTNGCNGC	GCNGCNAAYGAYTTYGA	128
GVDMK	GGNGTNGAYATGAA	YTTCATRTCNACNCC	32
NVAGTVG	AAYGTDGCHGGWACHGTDGG	CCHACDGTWCCDGCHACRTT	324

The final product eventually cloned and sequenced was amplified by primers N+ and A- as follows: 2 min 94 °C, 30 cycles of 94 °C, 30 s; 52 °C, 30 s; 72 °C, 1 min followed by 5 min at 72 °C.

Uneven PCR

Uneven PCR was carried out according to the method of Chen *et al* (37) with minor modifications. Each 50 µl reaction contained the following: 20 mM Tris HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM deoxyribonucleoside, 12.5 pmol of specific primer, 2.5 pmol of decamer random primer, 1 U of Taq DNA polymerase (Roche, Laval, QC, CAN). The template in the first set of cycles (round 1) was 50 ng genomic DNA, amplified with primer 1 and the random primer. 1 µl of the final amplified reaction mix from round 1 was used as the template for round 2 of reactions, using a nested primer (primer 2) and the same random primer. The cycling reactions were performed as described by Chen *et al* (37) with changes in the specific primer annealing temperatures. Annealing temperatures in round 1 were cycled between 52/54 °C and 50/52 °C Round 2 annealing temperatures tested were 51 °C and 52 °C

Table 8. Primers used in Uneven PCR.

Primer	Sequence 5'→ 3'
Un1	GCTACCTTCAATACCTACTGTCATG
Un2	GCCAACCAACTTTTGGAGAAAAATCGAAACC
Random 2	GGTGCGGGAA
Random 3	GTAGACCCGT
Random 4	AAGAGCCCGT
Random 5	AACGCGAACC

A-T Cloning of PCR products

Amplified PCR products were gel purified using a QIAEX II Gel Extraction Kit (Qiagen Inc., Mississauga, ON) and ligated into pGEM-T® or pGEM-T® Easy (Promega Corp., Madison, WI, USA) according to the manufacturers' instructions.

CARBOHYDRATE ANALYSIS

Small scale extraction of surface polysaccharide

Polysaccharide samples suitable for SDS-PAGE were extracted using a modification of the method outlined by Valverde *et al* (192) using EDTA/TEA/polymyxin B (ETP). Approximately 20 mg of wet cells were gently resuspended in 50 µl 100 mM EDTA (titrated with TEA to pH 7.0), incubated at RT for 15 min and centrifuged (2 min at 10,000 *x g*). 50 µl of a 10% polymyxin B resin (Sigma, St. Louis, MO, USA.) suspension in dH_2O was added to the supernatant, incubated for 15 min at RT and centrifuged (2 min at 10,000 *x g*). The pellet was resuspended in 100 µl wash solution (100 mM KH_2PO_4/150 mM NaCl, pH 7), centrifuged once more (2 min at 10,000 *x g*) and finally resuspended in 50 µl Laemmli sample buffer (103). Culture supernatant was collected from a 2 ml MAT broth, (15 °C, 6 days, 150 rpm) culture

after centrifugation of 1 ml (1 min at 14,000 *x g*). Samples were boiled in Laemmli sample buffer and separated by SDS-PAGE.

Silver staining

SDS PAGE gels were silver stained to visualize LPS according to a modified method of Tsai and Frasch (189). Gels were fixed in 40 % ethanol / 5 % acetic acid for 30 min. Gels were oxidized with 0.7 % periodic acid in 40 % ethanol / 5% acetic acid for 5 min. and then washed 6 times in dH_2O over 30 min. Gels were stained in freshly made staining reagent made as follows: 1 ml concentrated NH_4OH was added to 14 ml 0.1 M NaOH. 2.5 ml 20 % $AgNO_3$ was added with agitation to dissolve the transient brown precipitate before adding 58 ml dH_2O. Gels were stained for 10 min and then washed 3 times over 30 min. Gels were developed with a formaldehyde developer containing 0.1 ml 37 % formaldehyde in 100 ml 0.01 % (w/v) tripotassium citrate. The reaction was stopped with 5 % acetic acid.

KDO determination

Dry *F. psychrophilum* cells were resuspended in 0.02 N H_2SO_4 and boiled for 20 min. The suspension was centrifuged (2 min, 13,000 *x g*) and the supernatant tested for the presence of 2-keto-3-deoxyoctonate (KDO). Alternatively, cells were resuspended in 3 % SDS and 1 mg/ml proteinase K added when necessary, digested overnight at 65 °C and centrifuged (5 min, 13,000 *x g*). The presencnce of KDO was determined by the method of Weissbach and Hurwitz (206) as modified by Osborn (143) and Vincent and Cameron (196). Absorbance was scanned between 500 and 600 nm and read at 548 nm

(Ultrospec 3000, Pharmacia Biotech). Under these conditions, 1 mM KDO (Sigma, St. Louis, MO, USA) was calculated to give an A_{548} of 21.

Purification of LPS

LPS was purified from 250 g (wet wt) *F. psychrophilum* cells by the aqueous phenol extraction procedure according to the method of Johnson *et al* (92).

Biotin labeling of glycosyl groups

Biotinylation of 1,2 glycols and/or hydroxy carbonyl glycosides was carried out using the method of Doig *et al* (1996) (55). *F. psychrophilum* whole cells were boiled in Laemmli sample buffer, separated by SDS-PAGE and transferred on to nitrocellulose membrane as described above. The membrane was washed in PBS for 10 min at RT. The blot was oxidized with 10 mM sodium *meta*-periodate in 50 mM sodium acetate buffer (pH 5.5) for 30 min at RT and then washed 3 times in PBS. Oxidized carbohydrate was reacted with 5 mM biotin hydrazide (Sigma, St. Louis, MO, USA.) in 50 mM sodium acetate buffer (pH 5.5) for 1 h at RT. The membrane was washed three times with TBS, blocked with 1 % BSA in TBS for 30 min and washed a further three times in TBS. The blot was incubated for 1 h at RT with streptavidin conjugated alkaline phosphatase diluted in TBS. After washing three times in TBS the blot was developed as described above.

Extrinsic labeling of whole cells was via a modification of the method of Aragon *et al* (1996) (12). *F. psychrophilum* cells were suspended in 50 mM sodium acetate buffer pH 5.5/15 mM sodium *meta*-periodate (RT, 30 min). The oxidation reaction was

stopped with 0.5 volume of 80 mM sodium sulphite (RT, 15 min). Labeling was initiated by adding 0.5 volume of 15 mM biotin hydrazide (Sigma, St. Louis, MO, USA.) in 100 mM sodium acetate buffer pH 5.5 (RT, 1 h). Cells were washed twice in PBS, separated by SDS PAGE and transferred on to nitrocellulose membrane as described above. The presence of biotin was detected with streptavidin conjugated alkaline phosphatase as described above.

Conjugation of O-polysaccharide to protein (BSA/KLH)

Purified O-polysaccharide from *F. psychrophilum* was conjugated to KLH and BSA according to the method of Conlan *et al* (45). O-polysaccharide (5 mg/ml in saline) on ice was adjusted to pH 10.5-11.0 with 0.1 M NaOH. An equal weight of cyanogen bromide (Sigma, St. Louis, MO., USA) (1 g/ml in acetonitrile) was added and the mixture incubated on ice for 6 min. An equal volume of 0.8 M adipic acid dihydrazide (ADH, Sigma, St. Louis, MO., USA) in 0.5 M $NaHCO_3$, the pH adjusted to 8.5, and the mixture stirred overnight at 4 °C. The mixture was dialyzed against saline for 24 h and then against dH_2O for 48 h, lyophilized and weighed.

ADH-activated O-polysaccharide was dissolved in saline to 5 mg/ml, an equal weight of BSA or KLH added and the pH adjusted to 5.1-5.5. 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (EDAC, Sigma, St. Louis, MO., USA) was added to a final concentration of 0.05 M on ice and the pH maintained at 5.1-5.5 for 4 h. The mixture was dialyzed against saline for 48 h and lyophilized.

IMMUNOLOGICAL METHODS

Generation of antisera

Antisera were generated in New Zealand white rabbits injected subcutaneously (SC) and intramuscularly (IM) with a total of 200 μg protein. Whole cells were formalin fixed (5 % formalin, overnight, 4 °C), washed in PBS and emulsified in Freund's complete adjuvant or Emulsigen (MVP Inc., Ralston, NE, USA). Pre-immune serum was collected prior to the primary immunization. Rabbits was boosted twice with a further 200 μg protein in Freund's incomplete adjuvant or Emulsigen, beginning 2-3 weeks after the first injection, at 2 week intervals. The titer of the immune serum was determined by an enzyme-linked immunosorbent assay (ELISA) as described by Collinson *et al* (43).

Rainbow trout convalescent serum was obtained from rainbow trout that survived challenge with *F. psychrophilum* 259-93. Rainbow trout (15 g) were injected with live *F. psychrophilum* (2.4×10^4 cells) from a 24 h MAT broth culture. Pooled Rainbow trout serum from 45 surviving fish was obtained 5 weeks post challenge. Sera were also obtained from unexposed fish injected with saline for use as a negative control.

Enzyme linked immunosorbent assay (ELISA)

96 well plates (Corning, Acton, MA, USA) were coated with antigen by adding 50 μl PBS containing 1 μg or 10 μg antigen per well and drying plates overnight at 37 °C. Antigen coated plates were blocked for 1 h at 37 °C in blocking buffer (TBS/0.05 % Tween 20/5 % skim milk). Wells were rinsed three times with TBS/ 0.05 % Tween. Primary antibody (rabbit) was diluted in 1 % skim milk/TBS (1/1000 dilution) and

incubated for 1 h at 37 °C. Primary antibody was detected with goat anti-rabbit IgG conjugated to alkaline phosphatase (1/3000 dilution) (Caltag Labs, San Francisco, CA, USA) and washed as before. To each well was added 100 µl substrate solution (10 mg *p*-nitrophenylphosphate (Sigma, St. Louis, MO., USA) in 10 ml diethanolamine buffer, consisting of 9.7 % v/v diethanolamine, 0.01 % $MgCl_2$, 0.02 % NaN_3, pH 9.8). The plates were incubated in the dark for 30 min at 37 °C. The results were read on an ELISA plate reader (ELx808, Bio-Tek Instruments Inc., Winooski, VT, USA) at 405 - 490 nm.

ELISAs carried out with rainbow trout primary antibody were performed at 1/40 dilution of primary antibody and incubated overnight at 4 °C. Fish primary antibody was detected using rabbit anti-salmon Ig (1/3000 dilution, 1 h RT) (Immuno Precise Antibodies Inc., Victoria, BC, CAN) followed by goat anti-rabbit IgG conjugated to alkaline phosphatase as above.

Immunofluorescence microscopy.

F. psychrophilum cells were incubated in rabbit anti-*F. psychrophilum* primary antibody (1 h, RT) diluted 1/50 in PBS/5 % fetal calf serum (FCS). Cells were washed twice with PBS/5% FCS, resuspended in goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (FITC) (Caltag Labs, San Francisco, CA, USA) diluted 1/40 in PBS/5 % FCS and incubated (1 h, RT) in the dark. Cells were then resuspended in PBS and observed under a fluorescent microscope (Axioscop, Carl Zeiss MicroImaging Inc, Thornwood, NY, USA) by using barrier filters for FITC.

Immunogold electron microscopy

W*hole cells:* Cells were harvested from a 3 day 15 °C culture in TYES, resuspended in wash solution (10 mM Tris, 150 mM NaCl, pH 7.4) and mounted on formvar coated copper grids by floating the coated copper grids on drops of bacterial suspension for 1 min. Grids were floated on droplets of blocking buffer (Tris/NaCl/1% skim milk, 0.02 % NaN_3) for 40 min, washed three times and incubated for 45 min with primary antibody diluted 1/1000 in blocking buffer then washed once more. Grids were then incubated for 30 min with 15 nm gold-labeled Protein A (Amersham Biosciences Corp. Piscataway, NJ, USA) which was diluted 1/50 in blocking buffer. Grids were washed as before and stained for 30 s with 0.1 % phosphotungstic acid (PTA) and viewed with a Hitatchi 7000 TEM at an accelerating voltage of 75 kV.

Thin sections: Cells were harvested from a 48 h 15 °C culture in TYES, washed once in PBS and centrifuged (10 s at 3000 *x g*). Primary fixation (4 % paraformaldehyde, 1 % glutaraldehyde, 150 mM NaCl and 0.2 M Millonig's phosphate buffer pH 7.4 (130) was carried out for 1 h on ice. Cells were then washed three times in PBS before postfixation (1 % osmium tetroxide, 150 mM NaCl and Millonig's phosphate buffer), for 1 h at 4°C. After washing once in PBS, cells were sequentially dehydrated in 95 % ethanol, absolute ethanol and then propylene oxide, prior to embedding in an Epon/Araldite epoxy resin mix. Thin sections were cut using glass knives on an ultramicrotome and mounted on formvar coated nickel grids. The labeling procedure was as follows: sections were washed on drops of dH_2O (10 min), treated with 1 % sodium *meta*-periodate (30 min), washed with dH_2O (5 min), blocked with fetal calf serum (FCS) (15 min) and diluted 1/20 with blocking buffer (0.5 % BSA and 0.05 % Tween-20 in

PBS). The sections were then reacted with primary antibody (rabbit anti-*F. psychrophilum* polyclonal antiserum or pre-immune serum), diluted 1/1000 in blocking buffer for 1 h and then washed (3 x 5 min) with blocking buffer. Secondary antibody, 5 nm gold conjugated goat anti-rabbit IgG (Cedar Lane Labs Ltd., Hornby, ON, CAN.), diluted 1/50 with blocking buffer was added and incubated for 1 hr. The sections were subsequently washed (3 x 5 min) with blocking buffer followed by another wash (2 x 5 min) with PBS, postfixed using the primary fixative (15 min), rinsed on dH_2O (5 min), stained with 2 % aqueous uranyl acetate (30 min) and viewed with a Hitatchi 7000 TEM at an accelerating voltage of 75 kV.

Culture supernatant: Culture supernatants were collected and concentrated as indicated above. Formvar coated copper grids were floated on concentrated culture supernatant (2 min) prior to labeling with immunogold as described above for whole cells.

Western blotting

Bacterial cell proteins separated by SDS-PAGE were transferred onto nitrocellulose membranes by electroblotting at 50 mA/gel for 1 h in a semidry transblot apparatus (LKB Multiphor II, Pharmacia, Uppsala, SWE) as described by Towbin *et al* (183). The membrane-immobilized immunogenic proteins were detected using either rabbit or rainbow trout serum raised against *F. psychrophilum*. The primary rabbit antibody (1/1000 dilution) was detected using goat anti-rabbit IgG (1/4000 dilution) conjugated to alkaline phosphatase (Caltag Labs, San Francisco, CA, USA.) as outlined previously (43). Primary fish antibody (1/40 dilution) was incubated with the membrane

overnight at 4 °C and detected using a mAb raised against purified rainbow trout Ig (1/100 dilution) (kindly donated by R. Beecroft, Immuno-Precise Antibodies Ltd, Victoria, B.C., CAN) followed by goat anti-mouse IgG1 conjugated to alkaline phosphatase (1/2000 dilution) (Caltag Labs, San Francisco, CA, USA.). Alternatively, the primary fish antibody was detected by rabbit anti-salmon Ig polyclonal antibody (R. Beecroft, Immuno-Precise Antibodies Ltd, Victoria, B.C., CAN) followed by a goat anti-rabbit IgG as above. Negative controls were carried out using pre-immune sera as the primary antibody. The immunoreactive bands were visualized using 5-bromo-4-chloro-3-indoylphosphate (BCIP) and 4-Nitro Blue Tetrazolium chloride (NBT) (Sigma, St. Louis, MO., USA) as previously described (133).

Acetone powder preparation and cross adsorption of antiserum

To 6 ml of amplified λ phage particles (10^9 pfu/ml), 3 volumes of cold acetone was added in a 25 ml glass centrifuge tube. The mixture was vortexed and incubated overnight at -20 °C. The white precipitate was collected by centrifugation (9,000 *x g*, 10 min, 4 °C), and the pellet air dried. To cross-adsorb antiserum, 100 µl serum was placed in a 1.5 ml polypropylene tube and acetone powder was added to antiserum to 1 % w/v and incubated at 4°C for 40 min and centrifuged (1 min, 12,000 *x g*) and the supernatant transferred to a new tube.

Antibody purification from whole serum

Antibodies were purified from serum by caprylic acid precipitation according to SOP # 056-120601, Microtek Intl. Ltd., Saanichton, BC, CAN.

Adsorption of antibodies onto latex beads

Purified anti-*F. psychrophilum* OPS antibodies were adsorbed onto latex beads (Polysciences Inc., Los Angeles, CA, USA) according to the protocol provided by the manufacturer. 0.5 ml of manufacturer's latex bead suspension (2.5 %) was added to 1 ml borate buffer (0.1 M boric acid, adjusted to pH 8.5 with 1 M NaOH) and mixed. The beads solution was centrifuged and the bead pellet washed a further two times in 1 ml borate buffer before finally resuspending in 1 ml borate buffer. Approximately 400 µg of Ig 10 mM Tris pH 7.4 was added and incubated for 2.5 h at RT with constant mixing. The beads were the centrifuged, and blocked by resuspending in 1 ml borate buffer containing 10 mg/ml BSA and incubated at RT for 30 min, centrifuging the beads and repeating the blocking procedure. The beads were finally resuspended in 1 ml storage buffer (PBS containing 10 mg/ml BSA, 5 % glycerol and 0.1 % NaN_3).

Latex bead agglutination assay for F. psychrophilum

Bacterial cells to be tested were suspended in PBS and heated at 65 °C for 10 min prior to testing to prevent non-specific agglutination of bacterial cells to latex beads. An equal volume of cell suspension (1 colony/ 100 µl PBS or > OD 0.3 – 3.0) was mixed with anti-*F. psychrophilum* OPS Ig-adsorbed latex beads and agglutination was observed ≤ 2 min.

GENOMIC DNA LIBRARY

Construction of an F. psychrophilum genomic library

A genomic library of *F. psychrophilum* was constructed using the Lambda Zap® II Predigested Vector Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. *F. psychrophilum* DNA was partially digested with *Eco*RI to yield fragment sizes of ~10 kbp. Gel purified DNA fragments were then ligated into predigested Lambda ZAP®II arms with T4 DNA ligase (Roche, Laval, QC, CAN).

Immunological screening of a F. psychrophilum genomic library

Approximately 36,000 plaques of the *F. psychrophilum* expression library were immunologically screened. Plaques were grown on 150 mm Petri dishes at a density of ~ 4,000 plaques per dish. Plaque lifts were performed in duplicate using nitrocellulose (BioRad, Hercules, CA, USA.) cut into 145 mm discs and impregnated with 10 mM IPTG. Immunopositive plaques were detected with rabbit anti-*F. psychrophilum* serum that had been cross-adsorbed with an acetone powder of λ phage particles. Immunopositive plaques were picked and re-screened until pure phage cultures were obtained.

Excision of pBluescript clones from the λZAP II vector.

In vivo excision and recircularisation of the pBluescript phagemid clones was achieved using the helper phage ExAssist® according to the manufacturer's protocol. The excised phagemids packaged as filamentous phage particles were then used to infect

E. coli SOLR. Transformants were selected by overnight growth on LA_{Ap} plates. Purified phagemid DNA was digested with *Eco*RI to release the insert DNA. Immunopositive clones confirmed to contain insert DNA by were then sequenced by primer walking.

FISH INFECTION AND VACCINOLOGY

Infection studies

Passaging bacteria

Juvenile rainbow trout (~15 g) were experimentally infected by i.p. injection (0.1 ml) of *F. psychrophilum* UP96/017 or 259-93 grown on MAOA and resuspended in sterile phosphate buffered saline, pH 7.4 (PBS). *F. psychrophilum* was subsequently reisolated from the kidney and spleen tissue of the infected fish post mortem.

Injection challenge model

Fish were anaesthetized with 1 ppm Marinil (Metomidate, Syndel Intl. Inc., Vancouver, BC, CAN). Injection challenges were performed byi.p. injection of 50 µl live *F. psychrophilum* cells. Rainbow trout fry weighing on average 1.5 g were used for the development of a challenge model. *F. psychrophilum* was grown up onvernight in MAT, 15 °C to an A_{600} 0.3-0.4. 50 µl cell culture was injected either neat, diluted 1/10 in PBS or concentrated 10X by centrifugation (1,000 *x g*, 15 °C, 30 min) and resuspension in 1/10 volume supernatant.

Immersion challenge

As an immersion challenge, 50 1-2 g rainbow trout fry were exposed for 1 h to either a ½ dilution and a 1/10 dilution of overnight culture (A_{600} 0.37, ~ 1 x 10^8 cfu / ml) in a total volume of 1l of saline (0.85 % NaCl) at 12-13 °C. After 1 h exposure, the flow was then turned on to 0.2 l/min allowing the 1 l to be diluted slowly to a final 40 l volume. After 1 h immersion, fish were returned to fresh-water tanks and mortalities monitored for 21 days.

Vaccine trials

All vaccinations were performed with 0.5-1.0 g rainbow trout fry. Fish were anaesthetized with 1 ppm Marinil (Metomidate, Syndel Intl. Inc., Vancouver, BC, CAN) and injected 50 µl i.p. dose of adjuvant formulated vaccine or adjuvant and saline control. Rainbow trout fry were held at 12-13 °C. Each group contained approximately 50 fish at the time of challenge. Vaccinated fish were challenged by i.p. injection of 50 µl *F. psychrophilum* cells (~ 7 x 10^7 cells). Rainbow trout fry were maintained at 12-13 °C post-challenge. Mortalities were logged for 21 days and the presence of *F. psychrophilum* confirmed by re-isolation from kidney tissue that had been streaked onto MAT agar and incubated at 15 °C for 3-4 days.

Bacterin vaccines

F. psychrophilum cells were grown in MAT to a an A_{600} of 0.3-0.4. The culture was then inactivated with formaldehyde (5 %) overnight at 4 °C. The cells were washed three times with PBS and formulated with an oil-in-water adjuvant, Emulsigen (MVP Laboratories, Ralston, NE, USA) (20 % v/v) to give a concentration of 125 µg total protein per 50 µl dose.

E.coli BL21 DE3 cells expressing C-protein fusions were grown in LB containing 100 µg/ml ampicillin. Cells were induced with IPTG for 2 h, inactivated with formalin (7 ml / l) (approx. 0.25 % formaldehyde) overnight at 4 °C and washed three times with PBS. Cells were formulated with Emulsigen adjuvant (20 % v/v) at a concentration of 10 µg fusion protein per 50 µl dose. For *E. coli* controls, the same amount of cells, as measure by total protein concentration, were administered as required to deliver 10 µg C891 fusion protein (125 µg total protein).

Recombinant vaccines

C-protein fusions were isolated as inclusion bodies from *E. coli* BL21 DE3 that had been induced with IPTG. Inclusion body samples were formulated in 0.05 % thymol, 5 mM EDTA and 20 % v/v Emulsigen adjuvant to a final concentration of 10 µg fusion protein per 50 µl dose.

Protection data analysis

During vaccine trials, fish mortality was recorded over 21 days and cumulative mortality curves were plotted. Specific mortality due to *F. psychrophilum* was confirmed by aseptically removing kidney tissue, streaking onto MAT agar and incubating for 4 days. Mortalities found negative for *F. psychrophilum* and post injection (within 24 h)

mortalities were excluded from the trial. The percentage cumulative mortality was considered using chi-squared tests in Microsoft Excel.

The relative percent survival (RPS) is an accepted method of determining effectiveness of a vaccine. RPS is a ratio of the cumulative mortalities of a test group to the cumulative mortalities of an unvaccinated control group.

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

Chapter 1

Growth, Characterization and Speciation of *F. psychrophilum*

INTRODUCTION

Paramount in any comprehensive study of an organism, is the ability to cultivate it reproducibly. A primary goal in this study was to optimize and standardize growth conditions for this fastidious, slow growing, psychrotrophic microorganism. The aim was to achieve reproducible, laboratory scale growth in a fermentor for a possible vaccine or detailed molecular characterization.

F. psychrophilum belongs to a heterogeneous group of yellow-pigmented bacteria (YPB) called the *Cytophaga-Flexibacter-Bacteroides* (CFB) phylum or CFB group. Many CFB group members exist in aquatic environments and at least seven members of this group are now known to cause disease in fish and some in compromised humans. The group is poorly understood and speciation previously relied on laborious biochemical characterization. An important goal of our initial studies was to readily and reliably differentiate *F. psychrophilum* from related filamentous bacteria associated with diseased fish.

This chapter describes optimization of growth media, biochemical characterization of *F. psychrophilum*, and methods by which *F. psychrophilum* could be distinguished from related bacteria.

RESULTS

BIOCHEMICAL CHARACTERIZATION

Strain characterization.

Isolates of *F. psychrophilum* were obtained from diseased and moribund fish, displaying clinical symptoms of RTFS. The isolates were first characterized by a variety of standard tests and chosen as reference strains based on their detailed phenotypic and biochemical characteristics. Reference strains grew from 4 - 25 °C, but not at temperatures over 25 °C. Optimal growth was achieved at 15 °C. Growth was obtained on MAOA containing 0.2 - 0.5 % NaCl and was inhibited at 1.0 % NaCl. Colonies were yellow with thin spreading margins. Growth on MAOA was enhanced by the inclusion of 3% fish or horse blood but was strongly inhibited in the presence of 30 μg/ml Congo red. Additional tests confirmed that the reference strains were gram-negative, oxidase negative, catalase positive rods and possessed flexirubin-type pigments. Casein, gelatin and elastin were readily hydrolyzed whereas agar and cellulose were not. Based on these criteria, two isolates of *F. psychrophilum,* one from the U.K. (UP96/017) and one from the U.S.A. (259-93) were chosen as reference strains.

GROWTH

Comparison of different media for F. psychrophilum.

Two commonly cited growth media for *F. psychrophilum* are MAOB and TYES. *F. psychrophilum* grown in the two media exhibited strikingly different growth characteristics. Cells grown in MAOB had a much longer lag phase than cells grown in TYES (Figure 3A). The extended lag period in MAOB varied from 1 to 5 days

depending on the inoculum. Faster growth of *F. psychrophilum* was always obtained in TYES broth (Figure 3A).

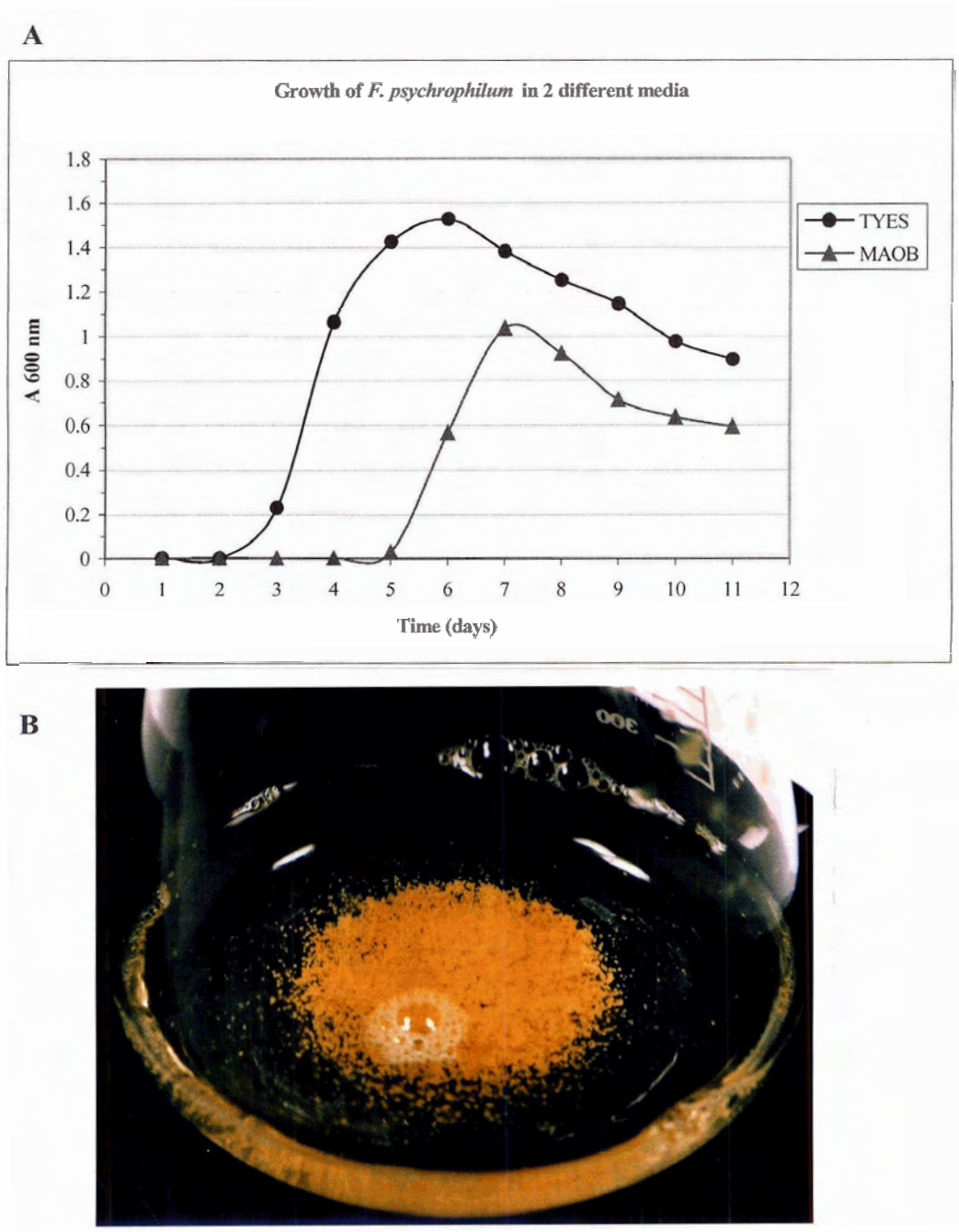

Figure 3. Comparison of growth characteristics in 2 different media. A) Growth in MAOB and TYES over time. B) Clumping of *F. psychrophilum* in TYES medium. The photograph shows a 200 ml culture after 48 h growth at 15 °C, shaking at 200 rpm. The clumps are shown here settled to the bottom of the flask.

During propagation, the cultures were visibly different. Cells grown in MAOB formed a typical uniform suspension, whereas TYES grown cells aggregated to form multicellular spheres (Figure 3B). The size of the clumps depended on the rate of shaking; cultures shaken slowly produced spheres of approximately 3 mm in diameter whereas rapid shaking produced fine, sand-like particles. By systematically omitting medium components, it was found that the presence of 0.05 % $CaCl_2$ in TYES resulted in cell aggregation. Clumps first appear when the culture reaches an absorbance greater than A_{600} 0.4, typically reached after 24 h at at 15 °C, 200 rpm in MAT medium (1/100 inoculum), and disaggregate after approximately 3 days under these conditions. To measure the absorbance of TYES-grown culture, flasks were swirled to create an even suspension while taking samples. The cell aggregates could then be temporarily disrupted by pipetting and vortexing before measuring the absorbance.

Development of a growth medium for F. psychrophilum.

In an attempt to boost growth of *F. psychrophilum*, various carbon sources were added to TYE medium containing the pH indicator dye phenol red. Any metabolism of the carbon sources, which would result in a lowering of pH, was visible by a colour change from red to yellow. Sugars were added to 5 ml TYE/phenol red medium in duplicate to a final concentration of 1 %. Broth was inoculated with 100 µl cell suspension, incubated at 15 °C without shaking and the colour monitored for 13 days. Growth was evident in every tube. The most dramatic colour change was achieved by supplementing TYE medium with maltose (Table 9). A weaker colour change was achieved by the addition of D-ribose. Maltose appeared to be the most readily fermented

carbon source of those tested and was selected for addition to TYES to test whether there would be any affect on growth.

Table 9. Fermentation of sugars.

Sugar	Fermentation (colour change)	Sugar	Fermentation (colour change)
D-glucose	-	D-ribose	+
D-mannose	-	glycerol	-
D-galactose	-	sucrose	-
D-gluconate	-	lactose	-
D-arabinose	-	maltose	+++

Growth of *F. psychrophilum* in various media was compared by measuring the A_{600} of the culture over time (Figure 4). TYES was shown to support much faster growth than MAOB. Supplementing TYES with 1 % maltose led to an increase in growth rate, which decreased the time for the culture to reach an A_{600} of 0.6 by approximately 10 hours. Other medium components tested for their ability to increase growth and overall cell yield were inactivated calf serum (5 %) and fish peptone (FP). When added to MAOB, the calf serum resulted in a shorter lag period but a lower culture density. Replacement of tryptone in TYES broth with fish peptone (FPYES) led to pellicle formation on the sides of the flask and so accurate culture density could not be established.

In this study, the medium established for routine growth of *F. psychrophilum* was based on TYES. Additional 0.2 % sodium acetate, as present in MAOB and 1 % maltose were added. The medium is referred to in the text as MAT (maltose/acetate/TYES). MAT-blood agar plates were prepared by adding 3 % fish or horse blood. Colonies

grown on blood agar were brown instead of bright orange. The change in appearance was thought to be due to the cells binding heme from the blood.

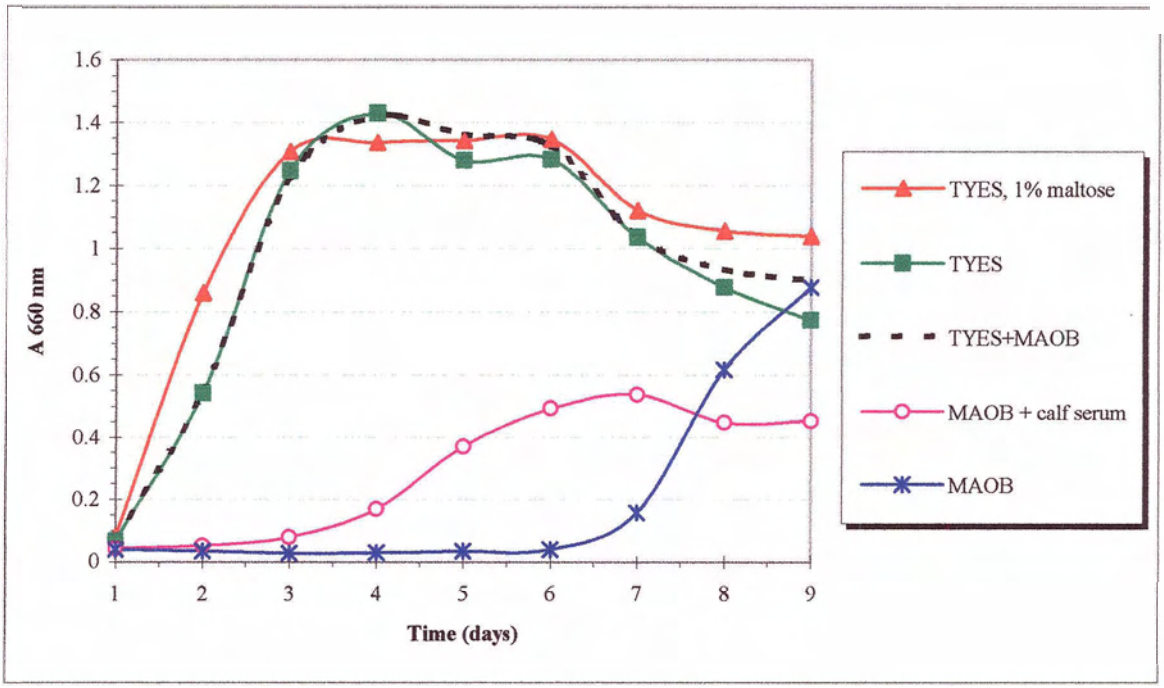

Figure 4. Growth of *F. psychrophilum* in various media. Duplicate flasks containing 50 ml various media were inoculated with MAOB-grown *F. psychrophilum* and incubated at 15 °C, 200 rpm. The absorbance of each culture was measured and the averages plotted over time.

Effect of Congo red on growth

Following from the observation that *F. psychrophilum* colonies were brown on blood agar plates, cells were streaked onto agar containing the heme analogue and diazo dye Congo red (CR, 100 μg/ml). Interestingly, growth was completely inhibited by the presence of CR in the media. Sixteen isolates of *F. psychrophilum* (Table 2) from the UK, USA and Denmark were then tested for CR sensitivity and all were found to be inhibited by CR. Broth cultures (2 ml) were set up containing dilutions of CR to investigate the minimum inhibitory concentration (MIC), which was found to be 5 μg/ml. To test whether *F. psychrophilum* cells bind Congo red, colonies were flooded with a 0.01 % aqueous solution. In this case, the edges of the colonies were seen to bind CR, and underwent a distinct colour change from orange to red.

To test whether CR inhibited growth of other related species, *F. columnare*, *T. maritimum*, *F. johnsoniae* and *F. aquatile* were streaked onto TYE agar with and without Congo red; all grew on TYE/CR medium. In all cases, colonies bound the dye and changed colour, becoming pink or bright red in appearance. A collection of yellow pigmented bacteria (YPB) isolated from diseased fish (strains PBS9701 - PBS9716) were also tested on TYE and TYE/CR agar and 6 of the 16 YPB strains (PBS9701, -02, -03, -08, -09, 10) were inhibited by Congo red (Figure 5), just as all the *F. psychrophilum* strains tested.

Different heme analogues were also tested for their ability to inhibit *F. psychrophilum* growth. To measure inhibition, 7 mm wells were cut out of spread plates of *F. psychrophilum* and 5 μl sterile solutions of either hemin or heme analogues added to the wells. The plates were incubated at 15 °C and the diameter of each clear zone,

excluding the 7 mm well, was measured after 4 days of growth (Table 10). The results show that inhibition of growth by Congo red was alleviated by the presence of hemin and analogues hematin and protoporphyrin IX.

Table 10. Inhibition of growth by heme analogues

Heme analogue	Zone of inhibition*
Hemin	-
Hematin	-
Protoporphyrin IX	1
Benzopurpurine	4
Congo Red	11
Congo red + hemin	6
Congo red + Hematin	7
Congo red + Protoporphyrin IX	8
Solvent control (DMSO)	1

*well diameter 7 mm, - signifies no inhibition

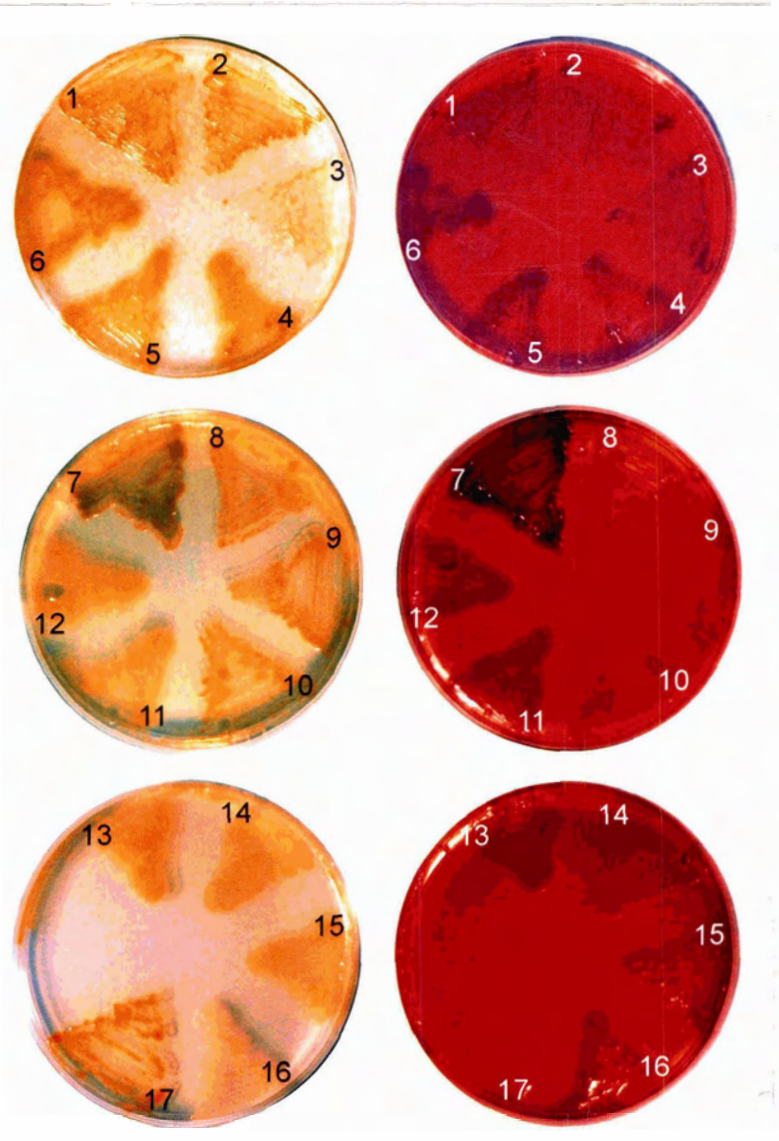

Figure 5. Growth of yellow pigmented bacteria (YPB) from diseased fish on CR agar. YPB isolated from diseased fish (strains PBS9701 – PBS9716, labelled 1-16 respectively) and *F. psychrophilum* 259-93 (labelled 17), were streaked out onto TYE and TYE/CR agar (red plates) and incubated at 15 °C for 4 days. Inhibition by Congo red is evident by comparing growth on TYE versus TYE/CR agar.

Large scale growth

Large scale growth was initially tested in a 3.5 l fermentor containing 3 l MAT medium at 15 °C. High levels of aeration and stirring led to excessive foam production, resulting in loss of cells through the condensing apparatus at the top of the fermentor. Polypropylene glycol (PPG) was added drop-wise to subsequent cultures to disperse the foam and prevent cell loss. The characteristics of the 3 l culture appeared to mimic flask culture, with the density peaking at approximately A_{600} 1.6 (Figure 6A). Large scale growth was achieved in a 35 l fermentor containing 28 l MATH broth (MAT supplemented with hemin (5 μg/ml)). Again, a similar growth curve to MAT broth flask culture was achieved (Figure 3B). Attempts to increase the yield were made by feeding the culture and by adding NaOH to maintain the pH, however, no improvements in yield were made (Figure 6B).

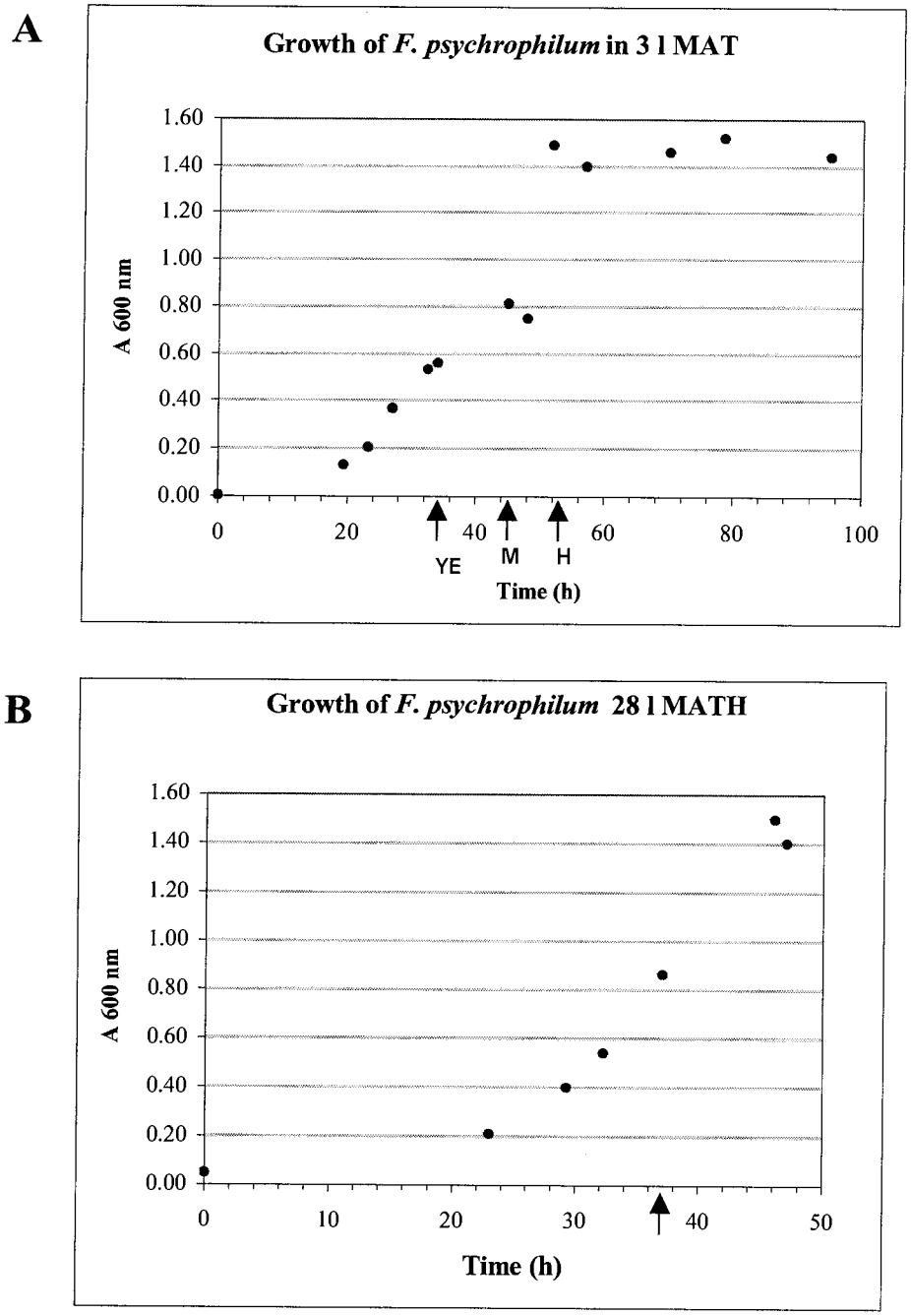

Figure 6. Large scale growth of *F. psychrophilum*. A) Growth in 3.5 l fermenter containing 3 l MAT broth. The culture was fed with, yeast extract (YE, 0.08 %), maltose (M, 1 %) and hemin (H, 5 μg/ml), as indicated by arrows. B) Growth in 28 l MAT+ hemin broth in 35 l fermenter. The culture was fed once (arrow) with half amount of original medium components.

SPECIATION

Strain typing by RAPD-PCR

The aim of the RAPD PCR was to find a method whereby *F. psychrophilum* isolates could be readily distinguished from related organisms isolated from diseased fish. RAPD-PCR "fingerprints" were generated for four strains of *F. psychrophilum*, including the type strain ATCC 49418. Initially, three sets of primers were used to generate the fingerprints. Each primer set produced identical RAPD-PCR profiles for both *F. psychrophilum* reference strains. The primer set chosen for further studies produced a single 600 bp band for all *F. psychrophilum* strains (Figure 7, arrow), which was important given the diverse geographical origins of these strains. RAPD-PCR was then performed using DNA from two related fish pathogens, *Flavobacterium columnare* and *Tenacibaculum maritimum* as well as from sixteen isolates of yellow pigmented bacteria from diseased coho and chinook salmon (isolates PBS9701 - PBS9716). The fish had presented symptoms of BCWD and originated from four, well separated local hatcheries. The RAPD-PCR profiles of these sixteen putative *F. psychrophilum* isolates were compared with profiles of the reference strains of *F. psychrophilum* from the USA and UK. Six of the putative *F. psychrophilum* isolates (PBS9701, PBS9702, PBS9703, PBS9708, PBS9709 and PBS9710) had the same RAPD-PCR profile as the reference strains. The same six isolates were all characteristically unable to grow on 1 % tryptone agar containing Congo red (30 μg/ml) (Figure 5). Figure 7 shows examples of the characteristic RAPD PCR profile identifying *F. psychrophilum* as well as typical non-*F. psychrophilum* reactions. Based on the RAPD-PCR fingerprints, six of the isolates were

identified as *F. psychrophilum*, a finding confirmed by the previous phenotypic characteristics.

Immunochemical strain typing

To further aid in the differentiation between *F. psychrophilum* isolates and related organisms recovered from diseased fish, Western blot analysis of four *F. psychrophilum* strains was carried out using polyclonal rabbit serum raised against *F. psychrophilum* UP96/017. The four geographically diverse *F. psychrophilum* strains had almost identical Western blot profiles (Figure 8). To determine whether the sixteen hatchery isolates possessed cellular antigens similar or cross reactive with those of the *F. psychrophilum* strains, whole cell lysates of the sixteen isolates (PBS9701-PBS9716) were prepared containing an equal concentration of cells. All isolates, of equal concentration, were resolved by Western blotting using rabbit polyclonal antiserum raised against *F. psychrophilum* UP96/017 (Figure 8). When compared to the *F. psychrophilum* known strains, the isolates were subsequently grouped based on their characteristic Western blot patterns. Six of the sixteen isolates possessed characteristically similar patterns with particularly common prominent bands from ~16 kDa - ~80 kDa, which were also present in previously characterized *F. psychrophilum* strains but were not common to the other 10 isolates (Figure 8, arrows). The six isolates were PBS9701, PBS9702, PBS9703, PBS9708, PBS9709 and PBS9710. The same six isolates were also identified as *F. psychrophilum* following RAPD-PCR analysis. Although the same amount of material was loaded in each lane, some variation in band intensity was seen particularly in the ~16 kDa band. A 5 fold dilution of *F.*

psychrophilum strain 259-93 shows a doublet band at ~ 16kDa, as seen for strains PBS9702 and PBS9703. The Western blot profiles of *F. columnare* and *T. maritimum* show considerable cross-reactivity with the anti-*F. psychrophilum* serum. No bands were observed when *F. psychrophilum* was reacted with pre-immune serum (data not shown).

The grouping of the sixteen, yellow-pigmented bacterial isolates from diseased fish was also supported by their similar growth phenotypes: incubation time for colony formation, colonial morphologies and inhibited growth in the presence of Congo red. These criteria could be consistently and reproducibly used for the presumptive identification of *F. psychrophilum,* but when combined with RAPD-PCR and immunochemical tests provided a reliable identification strategy.

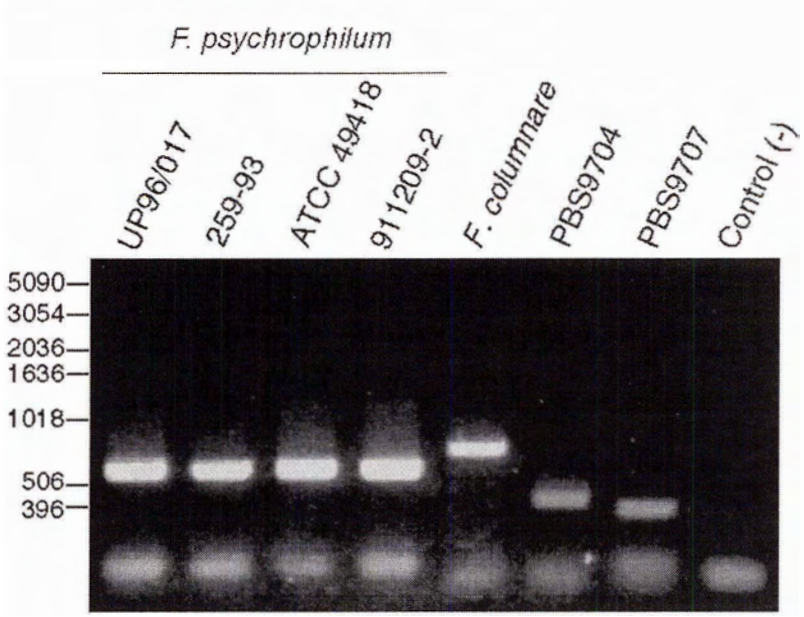

Figure 7: RAPD-PCR analysis of *F. psychrophilum* and related bacteria. Four geographically diverse *F. psychrophilum* strains, *Flavobacterium columnare* and two yellow pigmented bacteria isolated from diseased salmonids. The PCR products were separated by electrophoresis through 1.5% agarose and stained with EtBr. The negative control reaction contained no template DNA. MW standards (bp) are indicated on the left.

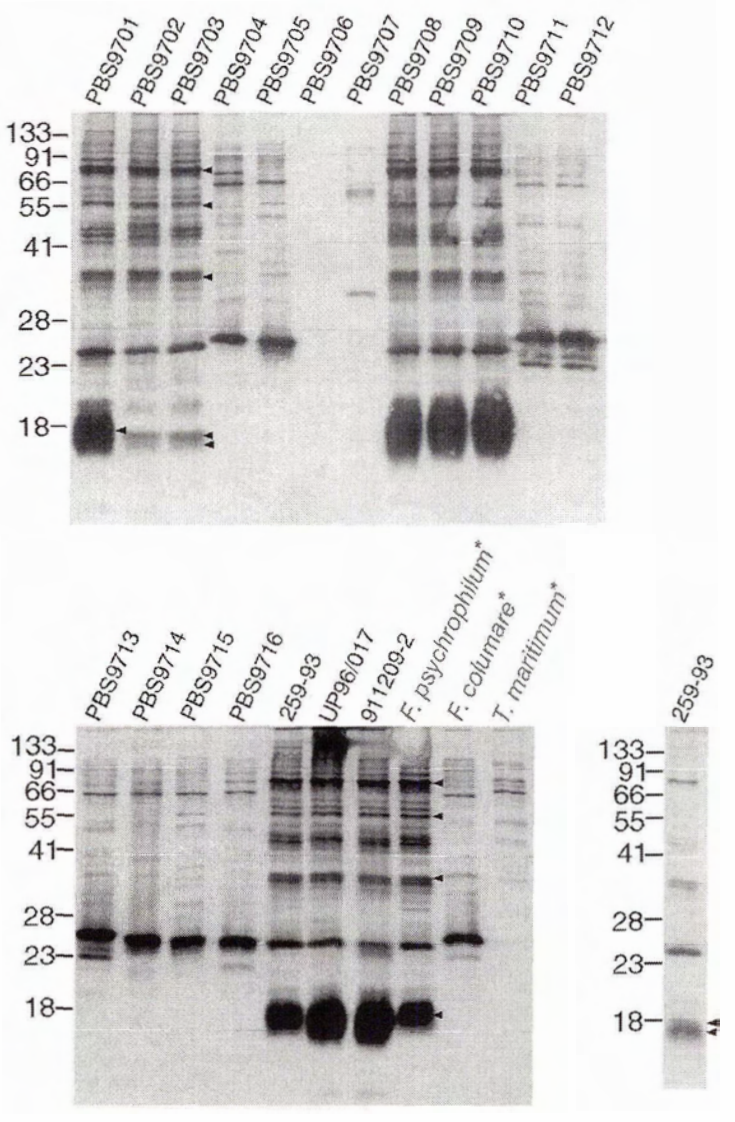

Figure 8. Western blot analysis of various yellow pigmented bacteria (YPB). YPB isolated from diseased salmonids (PBS9701 - PBS9716), *F. psychrophilum* strains and type strains of related bacterial fish pathogens. All strains analyzed were grown in MAT broth. Whole cell lysates were separated by SDS-PAGE, blotted onto nitrocellulose and reacted with anti-*F. psychrophilum* rabbit serum followed by immunochemical staining. All strains were reacted with rabbit pre-immune serum as a control which resulted in no bands (data not shown). Arrows indicate antigens common to *F. psychrophilum* and field isolates. Asterisks indicate ATCC type strains. *F. Flavobacterium; T. Tenacibaculum.* The MW standards (kDa) are indicated at the left of each blot.

DISCUSSION

The general characteristics of the two type strains of *F. psychrophilum* selected and characterized here (UP96/017 and 259-93) are largely in agreement with published phenotypic characteristics of *F. psychrophilum* strains (25, 106, 117, 167) and differ significantly from other related strains (117).

One of the primary focus points during this work was the optimization of growth of *F. psychrophilum*. Commonly cited media include a modification of Anacker and Ordal medium (8, 25), also referred to as '*Cytophaga* medium', referred to as modified Anacker and Ordal broth or agar (MAOB/A) and tryptone-yeast extract-salts medium (TYES) (82). The striking difference in growth characteristics and antigenic profile observed when *F. psychrophilum* was grown in two commonly used media was caused, in part, by the presence of calcium which caused the cells to aggregate. Conceivably, divalent cations facilitate aggregation by bridging acidic polysaccharides or proteins. The tendency of *F. psychrophilum* cells to aggregate in TYES broth may also be a result of increased levels of polysaccharide on the surface of these bacteria.

Although it has been reported that *F. psychrophilum* does not use carbohydrates (127), we found that *F. psychrophilum* fermented maltose and to a lesser extent, ribose. Supplementing TYES broth with maltose resulted in faster growth and was adopted as component of MAT broth. MAT medium was chosen for its ability to support faster growth. The aggregative effect was overcome by routinely measuring cell amount by wet weight instead of absorbance. When viable cells were required, *F. psychrophilum* was grown to an A_{600} of 0.3-0.4, a point at which they are pre-aggregative.

The substitution of fish peptone for tryptone in the growth medium resulted in pellicle formation on the side of the culture flask. This might suggest that a component of fish peptone signals a requirement of cell adhesion or attachment. The surface ulcers seen in bacterial cold water disease are indicative of film formation on the surface of the fish, ultimately leading to invasion. Further work is needed to identify the factors involved in increased adherance of *F. psychrophilum* cells and its possible role in pathogenesis.

The apparent binding of hemin on blood plates, which was implied by the change in colour of the colonies from yellow/orange to brown, suggested that the cells may bind the heme analogue and dye, Congo red (CR). For strains that bind Congo red, growth on CR-containing agar results in red colonies. Interestingly, we found that Congo red inhibited growth of *F. psychrophilum* at concentrations over 5 μg/ml. We hypothesized that the inhibition may be due to CR binding to receptors involved in iron acquisition and preventing the uptake of iron, leading to cessation of growth. The observed reduction of CR inhibition, in the presence of heme, supported this hypothesis to some degree, highlighting the dependence of iron acquisition for bacterial growth and survival in the host. CR binding is associated with virulent strains of *Shigella flexneri* (48), enteroinvasive *E. coli* (177) and the fish pathogen *Aeromonas salmonicida* (88). The binding of CR by *S. flexneri* and *E. coli* grown on CR agar is associated with a 101 kDa heme-binding protein (177). CR agar (30 μg/ml CR) is used as a differential medium as an indicator of virulence for the fish pathogen *Aeromonas salmonicida* (88). At high concentrations (> 5 μg/ml) binding of CR by *Aeromonas salmonicida* is attributed to non-specific and hydrophobic interactions with the surface A-layer which is required for

virulence (97). However, CR binding to *A. salmonicida* has been shown to be inhibited by the presence of porphyrins and conversely, CR has been reported to inhibit hemin binding by *A. salmonicida* (97). *F. psychrophilum* has been shown to bind CR by flooding colonies with a solution of CR. We found the inhibitory effect of CR useful as a facile way to test for *F. psychrophilum*. In all cases tested, *F. psychrophilum* was the only isolate from diseased salmonid fish that was inhibited by CR.

The current confusion over the taxonomy of *Flavobacterium* sp. mandated the development of reliable speciation tools. In this study, RAPD-PCR fingerprinting, Western blotting as well as specific growth characteristics provided a means of readily distinguishing *F. psychrophilum* from the myriad of other closely related bacteria found in diseased salmonid fish. Recently, other PCR techniques have been described which also differentiate *F. psychrophilum* from related bacteria (36, 184, 210). The Western blot profiles of the different *Flavobacterium* species studied here highlighted the immunological similarities between these species, given that they all reacted strongly with the anti-*F. psychrophilum* serum. However, *F. psychrophilum* -specific antigens were also observed, including a major antigen at ~16 kDa, showing that in Western blot analysis, they could be differentiated using anti-*F. psychrophilum* polyclonal antiserum. The combination of these techniques provides a facile and foolproof method of readily identifying *F. psychrophilum*.

Chapter 2

Localization of *F. psychrophilum* Antigens

INTRODUCTION

Antigenic characterization of *F. psychrophilum* was carried out in order to identify immunogenic cell surface molecules that may be involved in pathogenesis and that are potential vaccine targets. Using rabbit immune serum raised against *F. psychrophilum*, several antigens of interest were identified. Immune serum from rainbow trout, the natural host, was also used to identify antigens as potential vaccine targets. Using a variety of labeling techniques, we were able to partially characterize the major antigens of *F. psychrophilum*, which is a necessary prelude to rational vaccine development.

RESULTS

Immunogold electron microscopy.

Immunogold TEM showed rabbit anti-*F. psychrophilum* antibodies to be predominantly localized at the cell surface. TYES-grown *F. psychrophilum* was shown to possess a prodigious outer layer surrounding these cells, heavily labeled with immuno-gold particles (Figure 9A). Similar immunochemical labeling of thin sections confirmed that the gold particles were primarily localized at or near the bacterial surface (Figure 9B).

Western blotting analysis

To discriminate the antigens of *F. psychrophilum* further, Western blotting was carried out using rabbit anti-*F. psychrophilum* serum. A major antigen with an apparent MW of ~16 kDa (Figure 10 A, lanes 1 and 3) as well as other lesser immunoreactive bands with apparent MWs of ~22, 24, 35, 47, 60, 74 and ~100 kDa were readily identified. Lysed whole cells were then extensively digested with proteinase K to reveal those remaining antigens principally comprised of carbohydrate; two immunoreactive bands were clearly seen, a large one at ~16 kDa and a smaller one at ~25 kDa (Figure 10A, lanes 2 and 4, arrow). High MW, protein-free material (~70 kDa - ~200 kDa) seen in lane 4 was apparent as a fine banding ladder, indicative of repeating units, presumably of high MW, LPS O-antigen. Rabbit pre-immune serum as a negative control was not reactive (data not shown).

The gel profile of immunoreactive species detected in either whole cell lysates or proteinase K digested samples could be altered somewhat depending on which growth

medium was used. Western blots of TYES grown cells had an extra band at ~24 kDa which reacted strongly with rabbit anti-*F. psychrophilum* serum but was not present in MAOB grown cells. As well, the two different media gave rise to strikingly different growth characteristics of *F. psychrophilum,* as seen in Chapter 1.

To discover which antigens promoted a humoral response in the salmonid host, Western blots of *F. psychrophilum* cell lysates were carried out using convalescent rainbow trout anti-*F. psychrophilum* serum (Figure 10B). Major immunoreactive proteins were seen with apparent MWs of ~20 kDa and ~75 and ~83 kDa. An immunoreactive, proteinase K-resistant band was seen with an apparent MW of ~17 kDa. The negative control carried out with sera from healthy fish showed numerous but faint protein bands. These results suggest that the major proteinase K-resistant antigen recognized by rabbit anti-*F. psychrophilum* sera also reacts with convalescent rainbow trout anti-*F. psychrophilum* sera (Table 11).

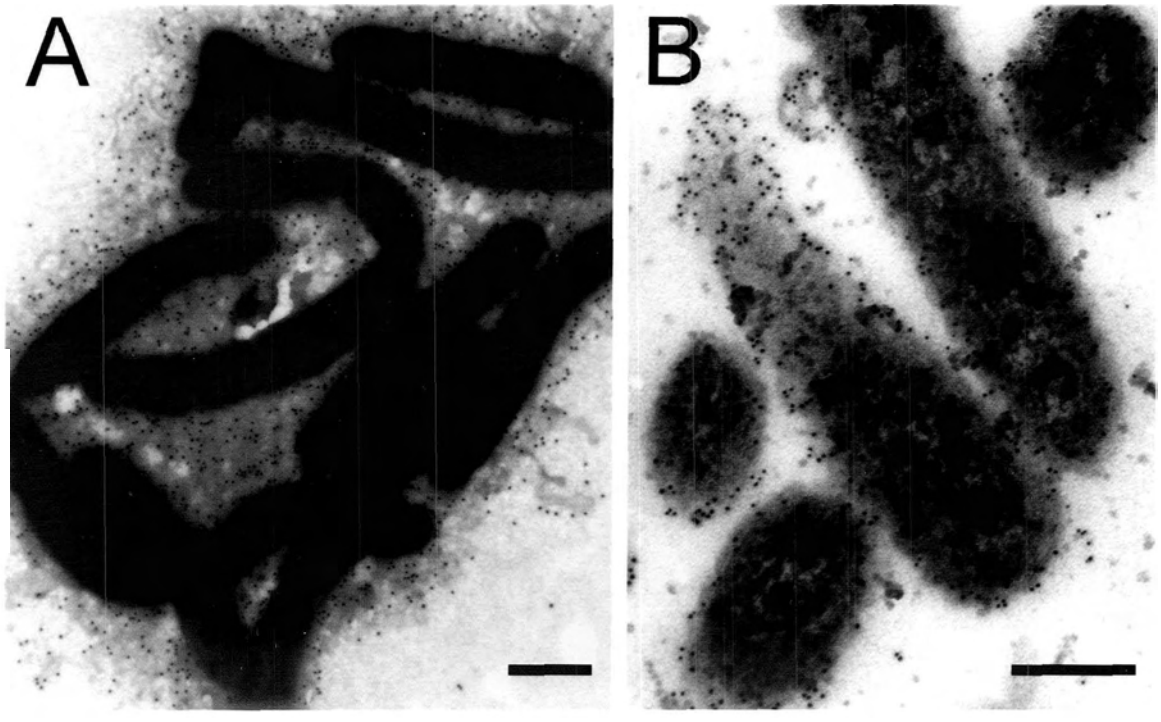

Figure 9. Immunogold labeling of *F. psychrophilum*. A) Cells grown in TYES and labeled directly with protein A-gold (15 nm particles) after pre-incubation with rabbit anti-*F. psychrophilum* serum. Bar = 0.5 μm. B) Thin sections were labeled with goat anti-rabbit IgG conjugated to 5 nm gold particles after pre-incubation with rabbit anti-*F. psychrophilum* serum. Bar = 0.2 μm.

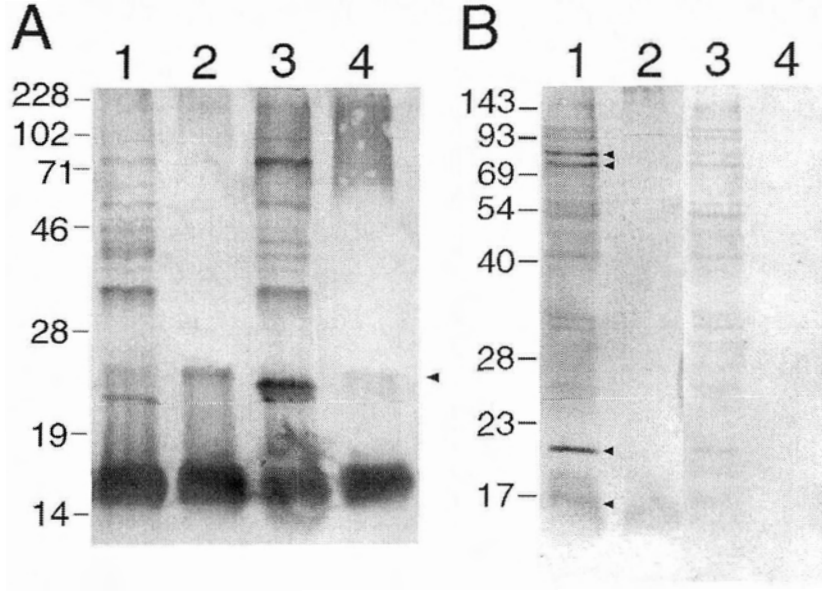

Figure 10. Western blot analysis of *F. psychrophilum*. A) *F. psychrophilum* was grown in MAOB (lanes 1 and 2) or TYES (lanes 3 and 4). In lanes 1 and 3 are whole cell lysates; in lanes 2 and 4 are proteinase K digests of intact cells. The samples were separated by SDS-PAGE (12%) and reacted with rabbit anti-*F. psychrophilum* serum Whole cells did not react with rabbit pre-immune sera. B) *F. psychrophilum* was grown in TYES and reacted with pooled rainbow trout convalescent anti-*F. psychrophilum* sera (lanes 1 and 2) and naïve rainbow trout sera (lanes 3 and 4). In lanes 1 and 3 are whole cell lysates, in lanes 2 and 4 are proteinase K digests of intact cells. Arrows indicate 4 antigens strongly identified by convalescent serum, compared to the naïve serum controls. MW markers (kDa) are indicted on the left of each blot.

Metabolic labeling of cells with [^{14}C]-palmitate and fractionation of cells with TX114

To determine whether any *F. psychrophilum* antigens comigrated with lipoproteins, cells were grown in the presence of [^{14}C]-palmitate. Incorporation of [^{14}C]-palmitate by the cells results in radio labeled lipoproteins. Following growth in the presence of [^{14}C]-palmitate, the *F. psychrophilum* cells were fractionated with Triton X-114 and the detergent (hydrophobic) and aqueous phases analyzed by SDS PAGE. Lipoproteins in the gels were visualized using a storage phosphor imaging screen. 5 major lipoproteins were detected at ~20, 24, 36, 45 and 55 kDa as well as a faint smear at ~14-17 kDa, believed to be LPS (Figure 11).

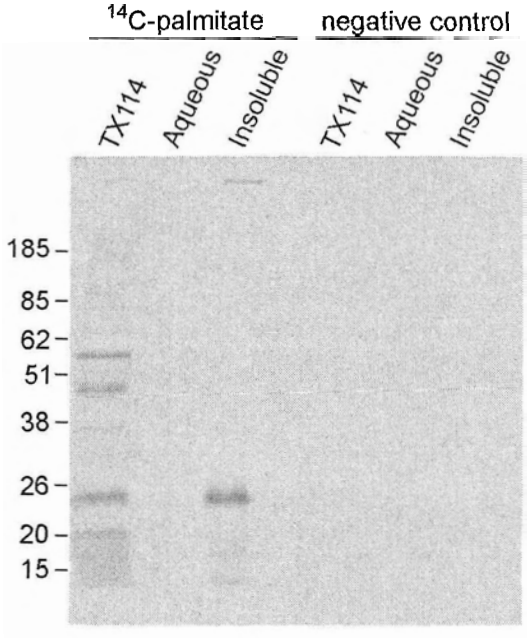

Figure 11. [^{14}C]-palmitate labeled *F. psychrophilum* cells. *F. psychrophilum* cells were grown in the presence of [^{14}C]-palmitate and fractionated with Triton X-114 into aqueous, detergent soluble and insoluble phases. The detergent phase shows the presence of at least 5 major lipoproteins whereas no lipoproteins are seen in the aqueous phase. The insoluble phase showed only one lipoprotein at ~24 kDa. Cells grown without [^{14}C]-palmitate were used as a negative control. MW markers (kDa) are indicated on the left.

Western blotting analysis of Triton-X114 fractions.

To further characterize the 20 kDa antigen recognized by fish serum, cells were fractionated with Triton X-114, and the phases separated by SDS PAGE. Western blotting revealed a 20 kDa antigen which is presumably hydrophobic, being found only in the detergent phase (Figure 12).

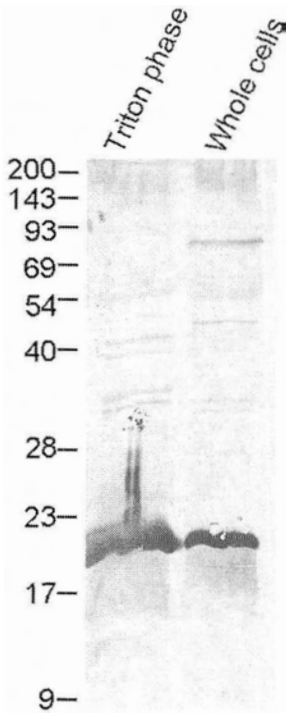

Figure 12. Western blot analysis of whole and fractionated *F. psychrophilum* cells with convalescent rainbow trout serum. The major ~20 kDa antigen in whole cell lysates recognized by fish serum was seen to partition into the hydrophobic (Triton) phase, no trace was found in the aqueous phase. MW markers (kDa) are indicated on the left.

Biotin labeling of surface proteins

Cell surface exposed proteins of *F. psychrophilum*, accessible to the anionic extrinsic probe, sulpho-NHS-biotin, were separated by SDS-PAGE, labeled and detected enzymatically after reaction with streptavidin-alkaline phosphatase (Figure 13). As a control, sonicated cells were also labeled to show the differences between intact and disrupted cells. The extra material labeled in the sonicated sample representing internal proteins or those surface proteins which were not accessible to the probe in intact cells. Since labeling with biotin over extended periods can result in some internalization of the probe, a set of timed labeling reactions was carried out. Labeling for 5 min resulted in a profile more similar to that of sonicated cells, consequently, cells labeled for 1 min were chosen to more closely represent accessible surface proteins of *F. psychrophilum.* The major protein accessible to this probe had an apparent MW of 24 kDa. Other less prominent bands appear at ~27 and 33 kDa. No bands were detected following proteinase K digestion, thus confirming that the extrinsically labeled bands were indeed proteinaceous.

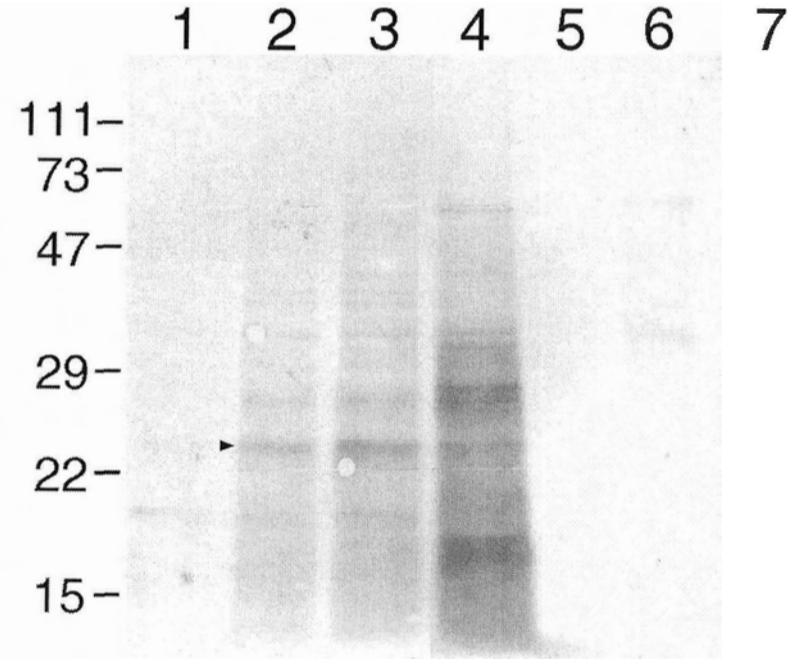

Figure 13. Biotin-labeled surface proteins of *F. psychrophilum*. Whole cells were reacted with sulpho-NHS-biotin, cell lysates separated by SDS-PAGE (12%) and proteins detected by streptavidin-conjugated alkaline phosphatase. Lanes 1-3 show labeling at 0 s, 30 s and 1 min respectively. Lane 4 shows sonicated cells labeled for 1 min. Negative controls were carried out without cells (lane 5), without reagent (lane 6), as well as with proteinase K treated cells (lane 7). The reagent control showed 2 faint bands with apparent MWs of 35 and 65 kDa. No labeling was apparent in the no cell control. Proteinase K treated cells show a faint band at ~15 kDa. MW markers (kDa) are indicted on the left. The arrow shows the major protein accessible to this probe.

Extraction of the immunoreactive outer layer of F. psychrophilum.

In order to selectively isolate some cell surface antigens, a non-lytic LPS extraction method was employed using polymyxin B (192). Western blot analysis of polymyxin B bound polysaccharides, first detached by EDTA from cells grown in MAOB or TYES, revealed antigenic bands with apparent MWs of approximately 35, 50, 67 and 95 kDa with major bands at ~16 and 23 – 25 kDa (Figure 14A, lanes 1 and 3). However, after digestion with proteinase K only the major band at ~16 kDa and a fainter band at ~22 kDa (difficult to see) remained visible on Western blots (Figure 14A, lanes 2 and 4). The effect of different growth media on the EDTA-extractable, polymyxin B bound surface antigens of *F. psychrophilum* is shown in Figure 14, lanes 1 (MAOB) and 3 (TYES). The main ~16 kDa, polymyxin B-extractable component that reacted with anti-*F. psychrophilum* serum was entirely resistant to proteinase K digestion and equally present in cells grown on either media. An extra protein band (~22 kDa) was seen in MAOB cells (Figure 14A, lane 1) and a higher MW (~70 kDa) protein band in TYES cells (Figure 14A, lane 3), presumably representing anionic proteins binding to the polycationic beads.

Western blot analysis of culture supernatant

The culture supernatant was also found to contain antigens of similar MW to those discovered by ETP LPS extraction (Figure 14B). Once again, a major antigenic band was seen at ~16 kDa in both samples. The supernatant showed several protein bands at ~24 kDa and between 45 – 75 kDa (Figure 14B, lane 1) which were not present in the proteinase K treated sample (Figure 14B lane 2). A faint band at ~22 kDa was

present both before and after proteinase K treatment. Western blot analysis of the proteinase K treated culture supernatant revealed a banding pattern consistent with a LPS-like profile, comprised of a major band at ~16 kDa and high MW material (Figure 14B, lane 2), characteristic of LPS O-antigen oligomers.

Immunogold analysis of culture supernatant revealed large aggregates of amorphous material which were labeled extensively with 15 nm protein-A gold, as well as smaller pieces of labeled antigenic material ~10-20 nm (data not shown). No antibody labeling was evident in the culture supernatant samples incubated with pre-immune serum. Following proteinase K digestion of the concentrated supernatant, large aggregates were absent but small fragments that remained were associated with single gold particles (data not shown).

Biotin hydrazide labeling of glycosyl groups

The presence of carbohydrate material of *F. psychrophilum* was investigated further by labeling periodate treated cells with biotin hydrazide. Labeling was performed on fresh intact cells (extrinsic labeling) and for comparison on cellular material first immobilized on a nitrocellulose membrane prior to labeling (non-extrinsic). The methods of labeling employed here gave strikingly different results. Whole cells which were labeled extrinsically prior to SDS PAGE showed the majority of labeling occurred in high MW material (Figure 15, lanes 3 and 4). Major protein bands (those sensitive to PK digestion) had apparent MWs of 24, 53 and 72 kDa. Following PK digestion, the MW of the remaining, poorly resolving material was >45 kDa, likely high MW LPS. However, cells which were labeled following transfer on to nitrocellulose showed labeling only in

low MW material in the range of ~16-18 kDa (Figure 15, lanes 1 and 2). Inexplicably, this band ran marginally lower following PK digestion (Figure 15, lanes 1, 2, 5 and 6). Western blot analysis of the biotin hydrazide labeled material revealed that several of the prominent bands comigrate with major antigens, for example at ~16, 24 and ~73 kDa.

Table 11. Major *F. psychrophilum* antigens recognised by rabbit and fish immune sera.

Antigen	Rabbit antisera		Fish antisera
	MAOB	TYES	TYES
L-LPS (16 kDa)	+	+	+
H-LPS	+	+	+
20 kDa	+	+	+
~ 24 kDa	-	+	-
~22 kDa	+	+	-

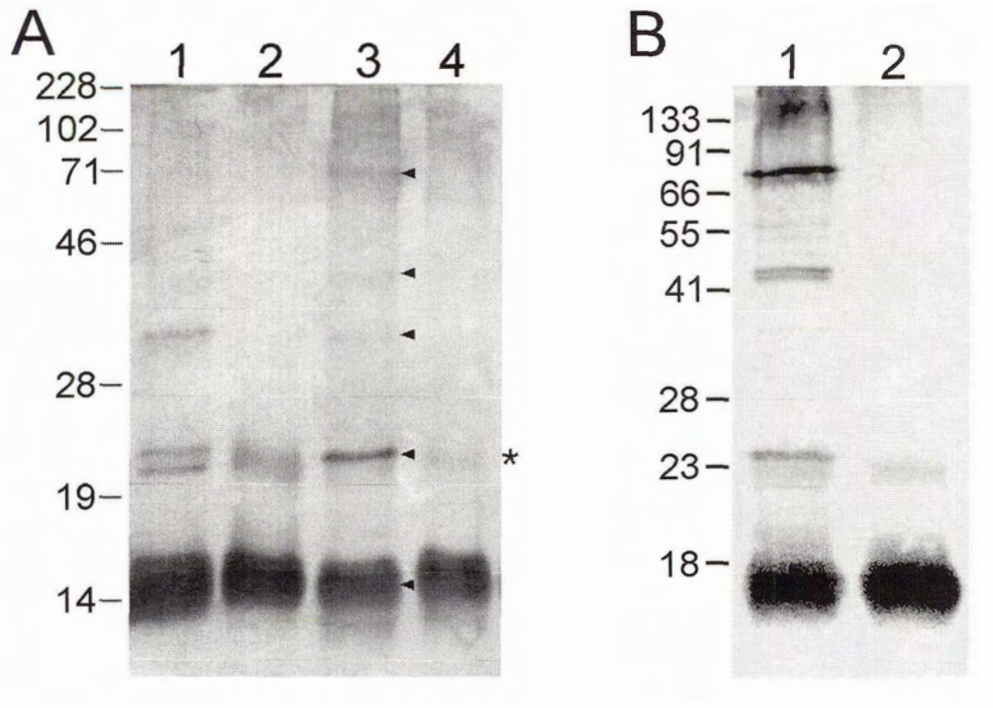

Figure 14. Western blot analysis of surface material from *F. psychrophilum*. **A)** Cell surface material was derived from cells grown in MAOB (lanes 1 and 2) and TYES (lanes 3 and 4) by incubation with EDTA/TEA to dissociate LPS and by adsorption to polymyxin B resin, followed by digestion with proteinase K (lanes 2 and 4). The extracts were separated by SDS-PAGE (12 %) and reacted with rabbit anti-*F. psychrophilum* serum. MW markers (kDa) are indicted on the left. Arrows indicate the major antigens. The asterisk indicates a proteinase resistant antigen. **B)** Western blot characterization of *F. psychrophilum* culture supernatant. Lane 1) culture supernatant. Lane 2) an identical sample after proteinase K digestion. MW markers (kDa) are indicted on the left.

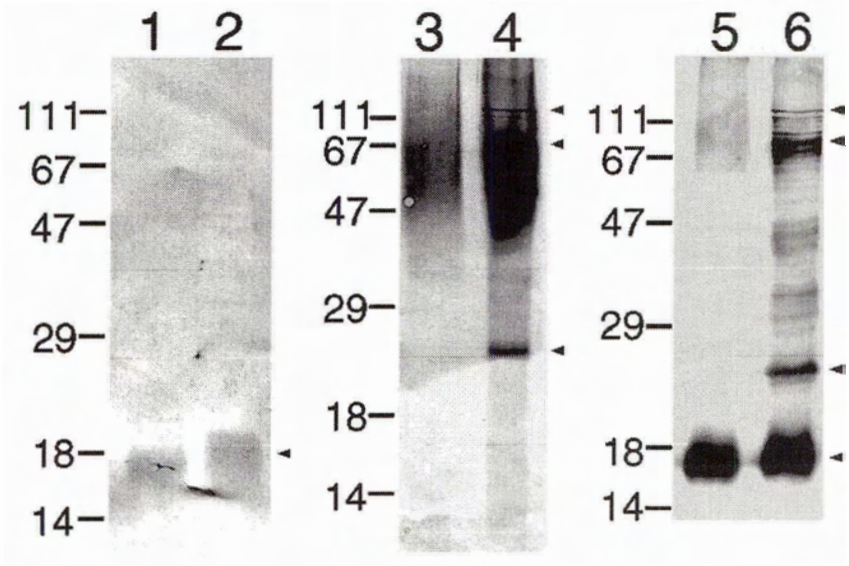

Figure 15. Biotin hydrazide labeling of periodate oxidized *F. psychrophilum*. Biotin hydrazide labeling of the transblot (intrinsic labeling) of SDS PAGE of *F. psychrophilum* cells following proteinase K digestion (lane 1) and undigested cells (lane 2). Transblot of *F. psychrophilum* cells extrinsically labeled with biotin hydrazide following proteinase K digestion (lane 3) and undigested cells (lane 4). Lanes 5 and 6 show Western blots of the unlabeled proteinase K digested and undigested cells respectively. The arrows indicate biotin-hydrazide-labeled bands (lanes 1-4) which comigrated with major antigens (lanes 5 and 6). MW markers (kDa) are indicted on the left.

DISCUSSION

As a prelude to vaccine development, characterization of the surface antigens of *F. psychrophilum,* using both rabbit and trout antiserum, was carried out. Electron microscopy has revealed that the major antigens are localized to the cells surface, mainly to a thick outer layer resembling a slime layer outside the cell. Western blot analysis with rabbit anti-*F. psychrophilum* serum revealed ~9 predominant antigens. The humoral response stimulated in fish was considerably weaker than in the rabbit with only four antigens eliciting a strong humoral response, the strongest being a 20 kDa hydrophobic protein. The major antigen recognized by rabbit serum was a proteinase K resistant molecule that migrated with an apparent MW of ~16 kDa. The major ~16 kDa antigen, presumably LPS, is likely to be the heavily immunogold labeled extracellular material seen in the electron micrographs.

The extrinsic primary amine labeling reagent, sulpho-NHS-biotin, revealed several surface proteins accessible to this probe, including a major surface protein with apparent MW of 24 kDa which comigrated with a band also visualized by Western blotting. At least two other higher MW bands were seen, one of which was immunogenic.

Metabolic labeling of cells with [^{14}C]-palmitate resulted in the labeling of 5 lipoproteins. All the [^{14}C]-labeled proteins were located in the detergent (hydrophobic) phase following extraction with the non-ionic detergent Triton X-114. One of the labeled proteins was also present in the insoluble pellet. The antigen recognized by fish serum also partitioned to the detergent phase.

To identify which antigens seen in the whole cell preparations could be isolated from the cell surface, a gentle and non-lytic procedure (192) was adopted. Thus, EDTA/polymyxin B extracted material was analyzed by Western blotting. Polymyxin B is a cationic, antibacterial peptide which avidly binds acidic polysaccharides, especially bacterial LPS, by forming a stable complex with the lipid-A moiety (131). The high-titre antiserum generated against *F. psychrophilum* appears to be biased toward surface molecules based on thin section immunogold EM. The highly immuno-reactive SDS-PAGE band at ~16 kDa is likely the predominant component of the unusually thick slime layer seen on the surface of these bacteria because it is also found in abundance sloughed off in the culture medium and could also be obtained by this non-lytic LPS extraction method. LPS is typically released from gram negative bacteria when cell surface Ca^{2+}/Mg^{2+} is chelated. Western blots of culture supernatant (Figure 14B), particularly after proteinase K treatment, show a high MW ladder, typical of repeating O-antigen units of LPS. Thus *F. psychrophilum* exhibits an LPS comprised of both low MW oligosaccharide and higher MW O-antigen containing polymers, confirmed by the positive reaction for KDO with both whole and proteinase K treated cells.

Following the labeling of periodate-treated intact cells with biotin hydrazide, only high MW PK resistant material and a PK sensitive band at 24 kDa was accessible to the probe. Labeling of cells after SDS-PAGE and immobilization on nitrocellulose however appeared to be far less sensitive and resulted in only a single band at ~16 kDa. The revealed 24 kDa, apparently glycosylated band comigrated with a protein antigen seen on Western blots.

Antigenic characterization was carried out as a prelude to rational vaccine and diagnostic design, in order to identify major antigens and potentially unique antigens. The major antigen of *F. psychrophilum* is a carbohydrate, which migrates with an apparent MW of ~16 kDa during SDS PAGE and appears to be unique to *F. psychrophilum*. Further characterization of the carbohydrate antigen is described in Chapter 3. Other major antigens were identified as surface proteins. A major antigen recognized by fish serum was found to be a hydrophobic protein with an apparent MW of 20 kDa, further characterization of this antigen is described in Chapter 5.

Chapter 3

Carbohydrate Antigen Characterization

INTRODUCTION

The outer membrane of gram-negative bacteria consists of phospholipids, proteins and the unique molecule, lipopolysaccharide (LPS). LPS, also referred to as endotoxin, is a major component of the outer membrane where it is anchored by hydrophobic lipid A molecules (Figure 16) (reviewed in (151)). The central core oligosaccharide region is covalently attached to lipid A and links lipid A to O-polysaccharide (OPS), also called O-antigen. The O-antigen is made up of a repeating units, consisting of 1 to 8 sugars (35). Some gram-negative organisms lack O-antigen, such as *Chlamidia trachomatis* (31). The terms "rough" and "smooth" are often used to describe LPS and are derived from the colony morphology of LPS mutants lacking O-antigen, which were rough in appearance (reviewed in (78)). The O-antigen region is highly variable compared to the core region and lipid A. The type and number of sugars within a unit, as well as the nature of the linkages and the number of repeating units, all contribute to the enormous structural diversity of O-antigens. It is this diversity that provides the basis for serotyping species or strains of bacteria. In addition to LPS, may bacteria possess extracellular polysaccharides (EPS). The EPS can form a morphological entity termed the capsule (capsular polysaccharide, CPS), which is often covalently bound (208). EPS can also exist on the cell surface with little or no cell association, referred to as slime polysaccharides (208).

The aim of this chapter was to further characterize the major carbohydrate antigen of *F. psychrophilum.* As described in Chapter 2, the major antigen of *F. psychrophilum* is a carbohydrate molecule that migrates with an apparent MW of ~16 kDa in SDS

PAGE. Although rabbit anti-*F. psychrophilum* serum has been shown to cross-react extensively with related bacteria associated with diseased fish, the ~16 kDa antigen appears to be unique to *F. psychrophilum* amoung the strains tested in Chapter 1, which were from geographically diverse locations. This chapter describes the purification of *F. psychrophilum* LPS, analysis by SDS PAGE and the elucidated O-antigen structure obtained in collaboration with Dr. Malcolm Perry, at the National Research Council, Ottawa.

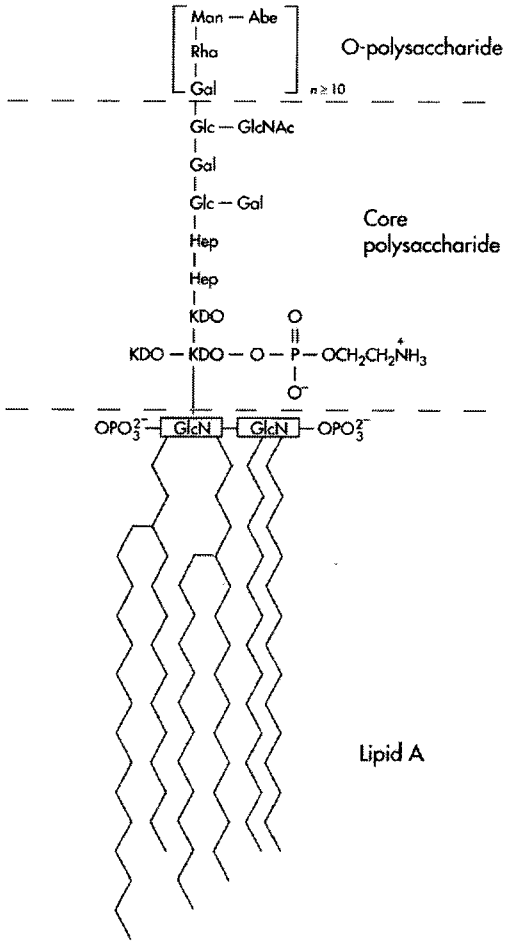

Figure 16. Schematic representation of LPS from *Salmonella typhimurium*.
Adapted from (96)

RESULTS

Isolation of LPS

Large scale growth of *F. psychrophilum* for LPS extraction was achieved in a 35 l fermentor. 70 g (wet wt) *F. psychrophilum* cells were used to obtain LPS. LPS was isolated by aqueous phenol extraction resulted in a yield of ~10 % of wet wt. Both the aqueous and phenol phases contained LPS. Upon examination of the two phases, the LPS in both phases was found to be identical (Dr. M.B. Perry, National Research Centre, Ottawa, personal communication).

Visualisation of LPS

LPS was purified by the hot phenol/water method, and both aqueous phase and phenol phase purified LPS were separated by SDS PAGE. LPS was visualized by silver stain (Figure 17A) and Western blotting using rabbit anti-*F. psychrophilum* serum and convalescent rainbow trout sera (Figure 17A). The silver stain shows a major band at ~17 kDa, corresponding to core polysaccharide and faint bands are present between 60 and 80 kDa, indicating the presence of O-chain. Western blot analysis of purified LPS was performed with both rabbit anti-*F. psychrophilum* serum and convalescent rainbow trout serum. Both rabbit and fish serum reacted in a similar fashion, labeling a major low MW band at ~16 -18 kDa as well as faint bands over 60 kDa (Figure 17A).

To visualize LPS without prior purification, *F. psychrophilum* cells were digested with proteinase K, separated by SDS PAGE and visualized by Western blotting using rabbit anti-*F. psychrophilum* serum (Figure 17B). The high MW banding pattern and a

low MW band which are clearly indicative of LPS-containing O-chain and core region lipooligosaccharide respectively.

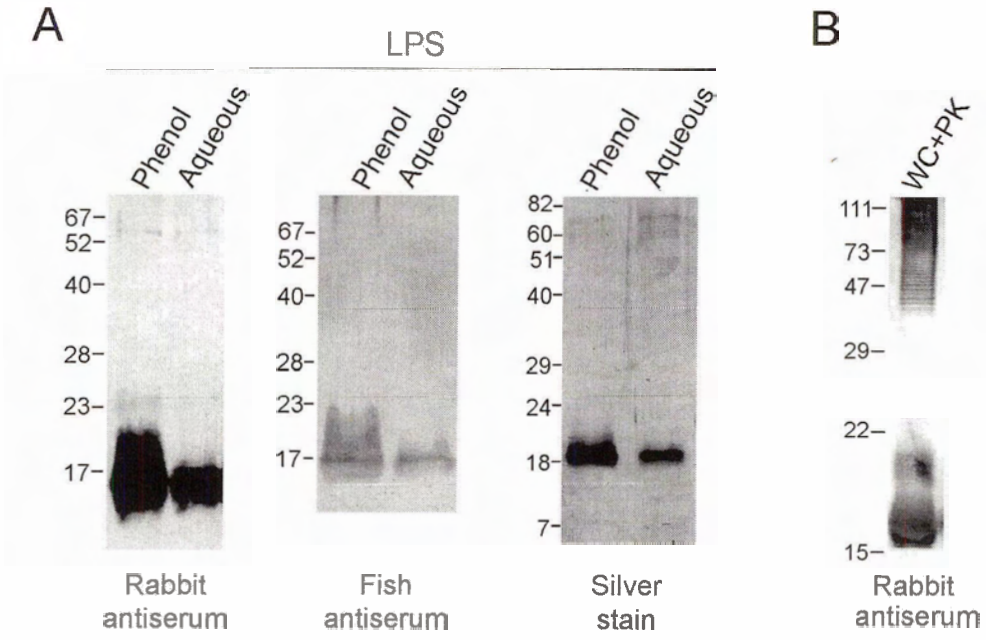

Figure 17. SDS PAGE analysis of *F. psychrophilum* LPS. LPS was purified from both the phenol phase and aqueous phase using the hot phenol/water extraction method. LPS purified from both the phenol phase and the aqueous phase was separated by SDS PAGE and visualized by **A)** Western blot analysis, using rabbit anti-*F. psychrophilum* serum and convalescent rainbow trout serum and silver staining **B)** *F. psychrophilum* cells were digested with proteinase K, separated by SDS PAGE and visualized by Western blotting using rabbit anti-*F. psychrophilum* serum. The high MW banding pattern is indicative of smooth LPS with various lengths of O-antigen repeats. MW markers (kDa) are indicated on the left.

Structure of O-antigen

The structure of the LPS O-antigen of *F. psychrophilum* was determined by Dr. Malcolm Perry, National Research Council, Ottawa, using analytical NMR spectroscopy, mass spectrometry, glycose and methylation analysis, and partial hydrolysis degradations (Figure 18). The OPS was purified from both the phenol and aqueous phase and found to be identical.

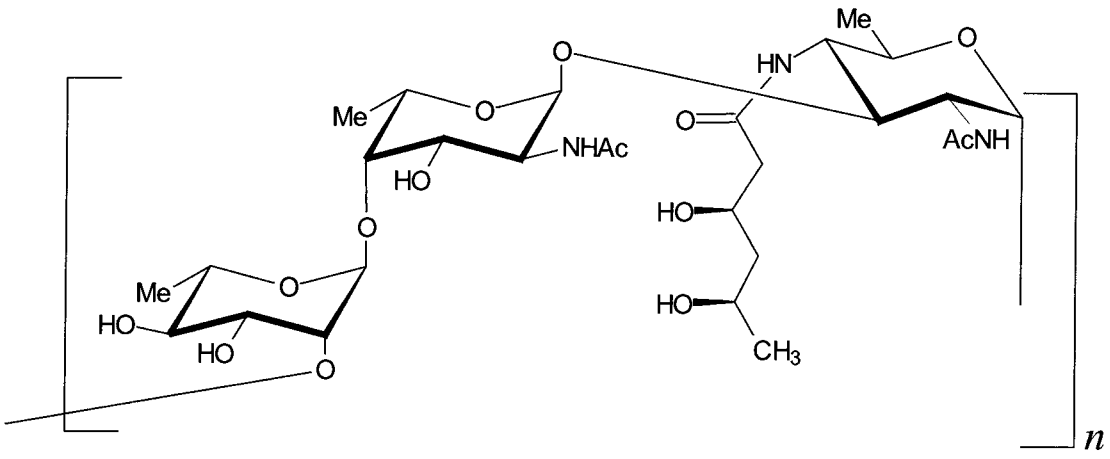

Figure 18. Structure of antigenic O-polysaccharide from *F. psychrophilum* LPS. The structure of the antigenic O-polysaccharide contained an unbranched polymer of trisaccharide repeating units composed of L-rhamnose (L-Rha*p*), 2-acetamido-2-deoxy-L-fucose (L-Fuc*p*N*A*c) and 2-acetamido-4-((3S,5S)-3,5-dihydroxyhexanamido)-2,4-dideoxy-D-quinovose (D-Qui*p*2*N*Ac4*N*R, 2-*N*-acetyl-4-*N*- ((3S,5S)-3,5-dihydroxy-hexanoyl) -D-bacillosamine) (1 : 1 : 1) and having the structure: →4)-α-L-Fuc*p*N*A*c-(1→ 3)-α-D-Qui*p*2*N*Ac4*N*R-(1→2)-α-L-Rha*p*-(1 →, where R is (3S,5S)-CH_3 $CH(OH)CH_2$ $CH(OH)CH_2$ CO-.

DISCUSSION

F. psychrophilum LPS was purified from both the phenol and aqueous phases during extraction. The extraction method is based on the fact that at temperatures above 68 °C, water and phenol are miscible. Bacteria in a 45 % (w/v) solution of phenol at 69 °C yield a monophasic suspension (92). Upon cooling, the homogeneous mixture separates into a lower phenol phase containing mainly protein, an upper aqueous phase containing LPS, polysaccharide and nucleic acid and an interphase containing cell wall material (92). As in this case, however, not all LPS molecules partition into the aqueous phase, suggesting the presence of less hydrophilic substituents. Phenol-soluble LPS molecules have been reported in another important fish pathogen, *Vibrio anguillarum* (18), as well as the plant pathogen *Xanthomonas campestris* (76), and the enteriobacterium *Citrobacter freundii* (152), reflecting their hydrophobic nature. The phenol soluble LPS molecules *V. anguillarum, X. campestris* and *C. freundii*, as well as *F. psychrophilum* have all been shown to contain acetylated amino sugars (18, 76, 118, 152).

LPS purified from both the phenol phase and aqueous phase was separated by SDS PAGE and visualized by Western blotting and silver stain. Western blotting of LPS showed a major band that migrates with an apparent MW of ~16 kDa that is recognized by both convalescent rainbow trout serum and rabbit anti-*F. psychrophilum* serum (Figure 17B). The high MW ladder, characteristic of smooth LPS, was best seen in preparations of proteinase K treated *F. psychrophilum* cells (Figure 17C). The high MW banding pattern seen here is indicative of LPS with varying lengths of O-antigen. The

silver stain of *F. psychrophilum* LPS shows a similar pattern to that seen in Western blotting, with a major band at ~16 kDa and faint, higher MW bands. Multiple bands in high MW LPS indicate the heterogeneity in the length of the polymerized chain, suggestive of some slippage in the rate of polymerization versus translocation to the cell surface (151). Silver staining did not result in dark staining, and only very faint bands were seen with a high MW. Dark silver staining was not expected because the O-antigen contains only one pair of vicinal diols on the rhanmose residue. The heavier staining of the low MW band is likely due to the oxidation of the core sugars.

Structural analysis of the O-antigen revealed an unbranched, repeating trisaccharide composed of L-rhamnose, 2-acetamido-2-deoxy-L-fucose and 2-acetamido-4-((3S,5S)-3,5-dihydroxyhexanamido)-2,4-dideoxy-D-quinovose (Figure 18). *N*-acyl derivatives of 2,4-diamino-2,4-dideoxy-D-quinovose (bacillosamine) are relatively rare and have been reported in O-antigens of *Vibrio cholerae* O:3 and O:5 (38, 74), *Pseudomonas aeruginosa* (100) and *Fusobacterium necrophorum* (75) .

The major ~16 kDa *F. psychrophilum* antigen recognized by both the natural host and rabbit anti-*F. psychrophilum* serum was shown to be LPS. Results from Chapter 1 showed this major antigen to be common to all *F. psychrophilum* strains tested from geographically distinct regions.

Chapter 4

Protein Antigen Characterization I: Construction and Immunological Screening of a DNA Expression Library

INTRODUCTION

No vaccine is commercially available to protect salmonids against RTFS. As a rational approach to vaccine design, we set out to identify, clone and characterize *F. psychrophilum* antigens which may be useful as vaccines against RTFS. Prior to this study, the only known genes of *F. psychrophilum* were the 16S rRNA gene and a partial sequence for gyrase B (*gyrB*) for use in phylogenetic analysis. The only *F. psychrophilum* protein sequence, therefore, being attributed to the partial DNA gyrase B subunit.

This chapter describes the cloning, sequencing and expression of *F. psychrophilum* protein antigen genes. *F. psychrophilum* DNA was partially digested and used to construct an expression DNA library. The library was screened with rabbit anti-*F. psychrophilum* serum to identify antigenic clones. Four unique antigenic clones were identified and the insert DNA sequenced. Following Western blot analysis of the initial clones, two were selected as putative vaccine candidates, encoding a ~10 and ~17 kDa antigen . The predicted open reading frames (ORFs) were analysed and those found to encode the two *F. psychrophilum* antigens were cloned into an expression vector encoding an N-terminal protein fusion partner. High level expression of both fusion products were then obtained in *E. coli*. Only one of the *F. psychrophilum* proteins was found to be immunoreactive following fusion and high level expression in *E. coli*. Both rabbit anti-*F. psychrophilum* serum and convalescent rainbow trout serum reacted strongly with this fusion product. In addition, rabbit antisera raised against *F. columnare* and *T. maritimum* also reacted strongly, suggesting the possibility of a vaccine that may confer immunity to more than one CFB-group fish pathogen.

RESULTS

Identification of antigenic F. psychrophilum λ ZAPII clones.

Screening of the *F. psychrophilum* expression library with rabbit anti-*F. psychrophilum* serum revealed eight immunopositive plaques. The plaques were purified and the eight pBluescript clones excised from the λ phagemids using the ExAssist helper phage. The eight pBluescript clones were digested with *Eco*RI in order to release the inserts. Four unique profiles were obtained from the eight clones, indicating that some inserts were identical. The four different profiles were represented in clones pP2, pP3, pP7 and pP8 and were estimated to be 2.5 kb, 2.2 kb, 8.5 kb and 2.7 kb respectively (Figure 19).

Western blot analysis of E. coli SOLR clones

E. coli SOLR harbouring the eight pBluescript clones were grown in the presence of the *lac* inducer IPTG. Whole cells lysates were then resolved by SDS-PAGE and immunopositive proteins visualized by Western blotting using rabbit anti-*F. psychrophilum* serum. From the initial eight immunopositive plaques, three unique Western blot profiles were obtained, indicating again that some clones were identical. Clone pP2 did not show any specific reaction with anti-*F. psychrophilum* serum. Clones representing the three antigenic profiles observed were pP3 (68 kDa, plus putative breakdown products), pP7 (~15 kDa) and pP8 (~10 kDa) (Figure 20). Western blotting with both rabbit anti-*F. columnare* serum showed cross-reactivity with the 10 kDa antigen from clone pP8 as well as the 15 kDa pP7 antigen and a ~30 kDa pP7 product not identified with anti-*F. psychrophilum* serum (Figure 20). Rabbit anti-*T. maritimum*

serum did not specifically react with the any of the clones (data not shown). Control Western blots were routinely carried out with pre-immune serum from each rabbit. Both the anti-*F. psychrophilum* serum as well as pre-immune serum from the same rabbit reacted strongly with the 68 kDa pP3 antigen and what appeared to be its breakdown products. No other pre-immune rabbit serum tested reacted with the pP3 antigen. Serum from a second rabbit used to generate anti-*F. psychrophilum* serum was tested against the pP3 clone; neither the pre-immune nor the immune serum reacted with clone pP3. Western blot analysis of the SOLR clones is summarized in Table 12.

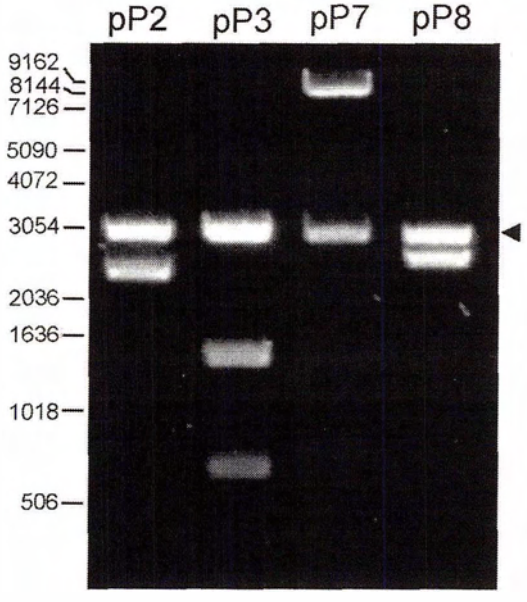

Figure 19. Agarose gel electrophoresis of *Eco*RI digested pBluscript clones. pBluescript clones containing four unique *F. psychrophilum* inserts were digested with *Eco*RI. The digested products were separated by electrophoresis through a 1 % agarose gel and stained with EtBr. Size (bp) markers are indicated on the left. The pBluescript vector (2.9 kb) is indicated by the arrow. Clone pP3 was cut twice, indicating the presence of an *Eco*RI site within the insert DNA. The sizes of the inserts were estimated to be approximately: 2.5 kb (pP2), 2.2 kb (pP3), 8.5 kb (pP7) and 2.7 kb (pP8).

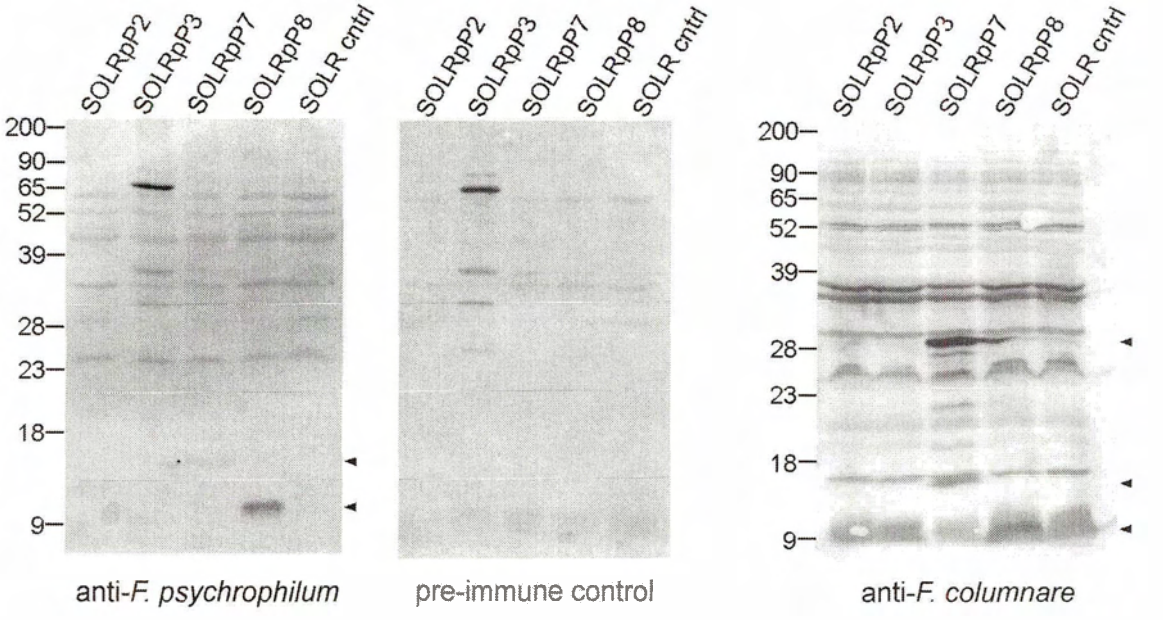

Figure 20. Western blot analysis of SOLR clones. *E.coli* SOLR cells containing pBluescript plasmids carrying the four unique inserts were grown in the presence of IPTG. Whole cell lysates were resolved by 12 % SDS-PAGE, electroblotted onto nitrocellulose membranes and visualized using six different rabbit sera: anti-*F. psychrophilum*, anti-*F. columnare*, anti-*T. maritimum* and the respective pre-immune control serum from each rabbit. Of the six rabbit sera tested, only three reacted specifically with cloned products and are shown here: anti-*F. psychrophilum*, anti-*F. psychrophilum* pre-immune control and anti-*F. columnare*. The remaining three sera, the anti-*F. columnare* rabbit pre-immune control, anti-*T. maritimum* and anti -*T. maritimum* pre-immune control sera did not specifically label any cloned proteins and thus are not shown. MW standards (kDa) are indicated on the left.

The results from Western blotting and restriction digests were in agreement. Grouping of the clones according to either inset size or antigenic profile yielded the same results; i.e. the clones with identical antigenic profiles also had the same size insert DNA. The grouping of the clones was later confirmed following sequencing both ends of each insert. The unique pBluescript clone DNA (pP2, pP3, pP7 and pP8) was sequenced by primer walking along the insert DNA and is shown in the Appendix.

Table 12 Antigens observed in the four representitive pBluescript clones.

Clone	Insert size	Observed antigens following reaction with various rabbit sera			
		anti-*F. psych.*	anti-*F. col.*	anti-*T. marit.*	pre-immune
pP2	2378 b	-	-	-	-
pP3	2117 b	68 kDa			68 kDa
pP7	8341 b	15 kDa	15 kDa, 30 kDa	-	-
pP8	2659 b	10 kDa	10 kDa	10 kDa	-

Clones pP7 and pP8 were chosen for further investigation. The consensus sequences were analysed to identify predicted open reading frames (ORFs). The aim was to identify and clone the ORFs that encoded the observed *F. psychrophilum* antigens. In doing so, the goal was to clone and express the proteins in *E. coli* in order to test their ability to confer immunity against *F. psychrophilum* in rainbow trout fry.

CLONE pP8

Open reading frame analysis of clone pP8

Analysis of *F. psychrophilum* insert P8 (2659 bp) for predicted open reading frames (ORFs) revealed four complete ORFs of 675 bp, 561 bp, 279 bp and 276 bp

(Figure 21). The two smaller open reading frames identified in insert P8 (276 bp and 279 bp), encode hypothetical proteins of 91 aa and 92 aa with theoretical masses of 9.46 kDa and 10.26 kDa respectively. Both of these ORFs corresponded in size to the 10 kDa antigen observed in the pP8 clone (Figure 20). Therefore, to identify the correct ORF, both were selected for expression experiments in *E. coli*, so that they could be distinguished by Western blotting.

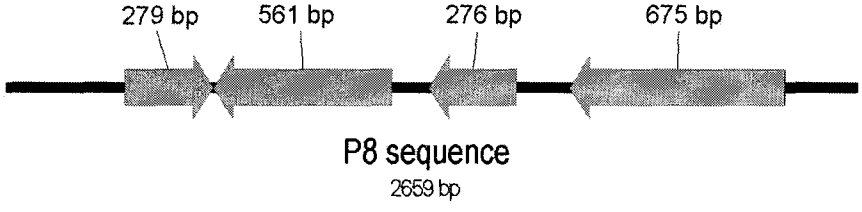

Figure 21. ORF map of 2659 bp *F. psychrophilum* pP8 insert. Arrows indicate predicted ORFs and their coding direction.

Construction and expression of F. psychrophilum protein fusions.

PCR primers were designed to amplify the 2 ORFs with flanking DNA restriction enzyme cleavage sites. The amplified ORFs were gel purified, digested with *Bam*HI and *Hin*dIII and ligated into the pET-derived expression vector pETC, encoding a 10 kDa N-terminal fusion protein, protein C (Figure 22). Plasmid constructs containing the ORFs for 91 aa or 92 aa proteins, pETC8-91 and pETC8-92, were confirmed by restriction digest and sequence analysis. *E. coli* BL21(DE3) was electroporated with either pETC-91, pETC-92 or pETC. Transformants were selected overnight on LB_{Ap} at 37 °C. For

expression experiments, overnight LB_{Ap} 37 °C cultures were used to inoculate fresh LB_{Ap}. Once cultures had reached an A_{600} of 0.6-0.8, they were treated with 1mM IPTG for 2 h to induce expression of the cloned ORF. Whole cell lysates were then separated by SDS PAGE and visualized by Coomassie staining and Western blotting.

Coomassie stained gels showed that the two ORFs were expressed as C-protein fusions, termed C8-91 and C8-92, with apparent MWs of ~22 and 24 kDa respectively (Figure 23 A). Western blotting with rabbit anti-*F. psychrophilum* serum showed C8-91 to be highly antigenic, whereas C8-92 did not react (Figure 23 B). Further Western blot analysis of with rabbit anti-*F. columnare*, anti-*T. maritimum,* revealed strong cross-reactivity with C8-91 but not with the C-protein control (Figure 24). C8-91 was also shown to react with convalescent rainbow trout serum (Figure 24). C8-92 did not react with any of the rabbit antisera tested nor with convalescent rainbow trout serum (data not shown). Therefore, the 276 bp ORF (91 aa) was found to encode the ~10 kDa antigen observed in clone pP8.

To estimate the amount of fusion protein being expressed, SDS PAGE gels of protein samples were Coomassie stained and gel images digitally captured. The relative intensity of induced protein bands were compared to lysozyme standards on the same gel. In duplicate experiments, C8-91 was found to constitute 11 % and 9 % of total cell protein.

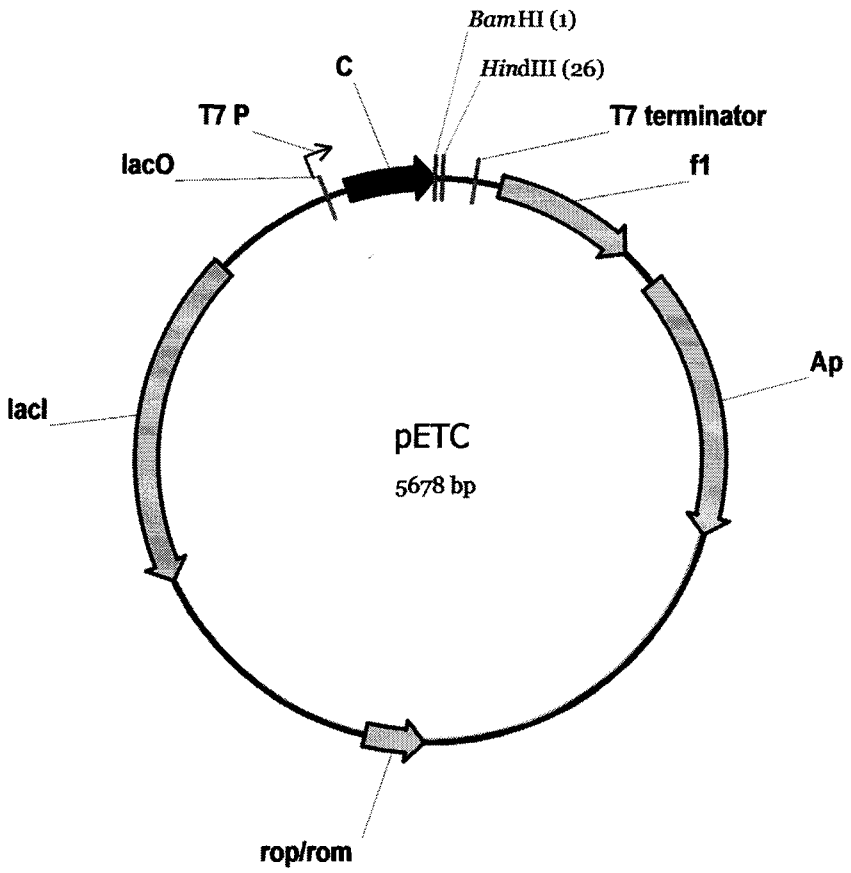

Figure 22. Schematic illustration of pETC. pETC is a pET21 derived expression vector encoding a 10 kDa N-terminal fusion protein, termed protein C (Microtek Intl. Ltd, Saanichton, BC). C-protein fusions readily form inclusion bodies to aid isolation of the expressed product.

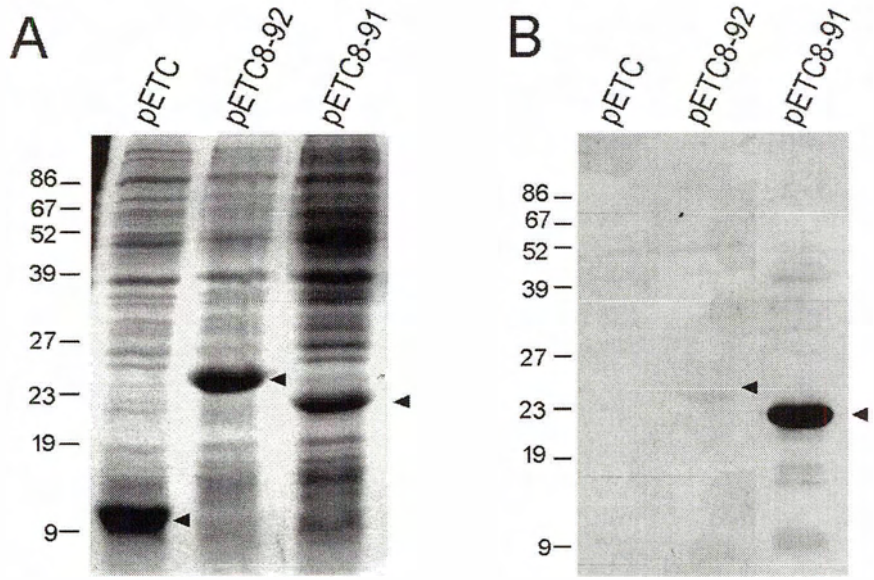

Figure 23. SDS PAGE and Western blot analysis of C8-91 and C8-92 fusion proteins. *E.coli* BL21(DE3) cells harbouring the plasmids pETC8-91, pETC8-92 or pETC were induced with IPTG and the whole cells analysed by SDS PAGE. Protein fusions were visualised by A) Coomassie stain and B) Western blotting with rabbit anti-*F. psychrophilum* serum. Arrows indicate C protein and C-protein fusions C8-91 and C8-92. MW markers (kDa) are indicated on the left.

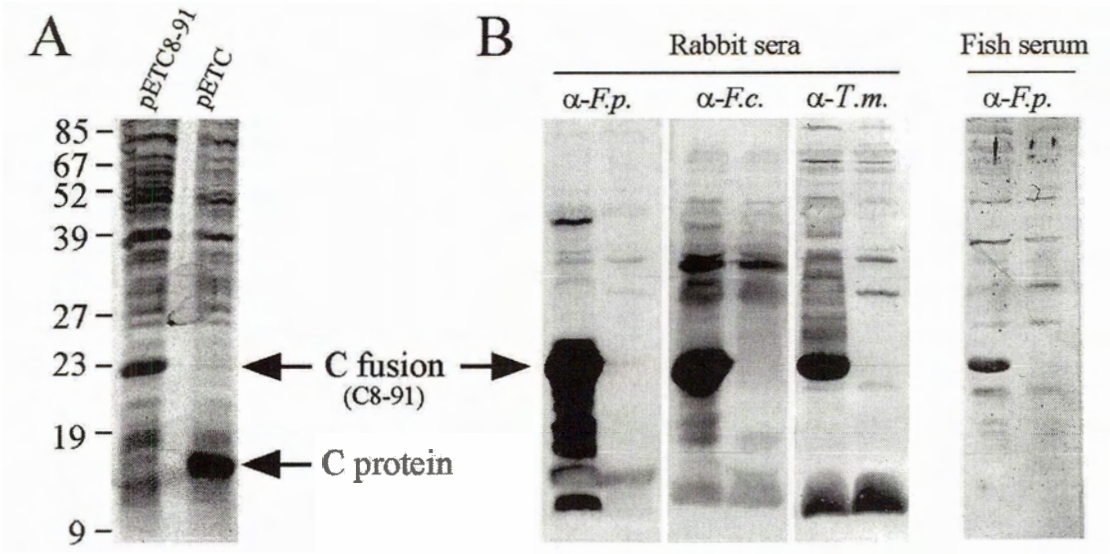

Figure 24. SDS PAGE and Western blot analysis of C8-91 fusion proteins with a variety of antisera. *E. coli* BL21(DE3) cells harbouring the plasmids pETC8-91 or pETC were induced with IPTG and whole cell lysates analyzed by SDS PAGE. Protein fusions were visualised by A) Coomassie stain and B) Western blotting with rabbit anti-*F. psychrophilum* serum (α-*F.p.*), anti-*F. columnare* serum (α-*F.c.*) anti-*T. maritimum* (α-*T.m.)* as well as convalescent rainbow trout serum. Arrows indicate C protein and C-protein fusions C8-91. MW markers (kDa) are indicated on the left.

Sequence analysis of F. psychrophilum insert P8.

The predicted ORF sequences were subjected to BLAST2 and FASTA3 analysis to determine if any sequences were known. Only two ORFs were found to be similar to known genes. The largest ORF (675 bp) encoded a 224 amino acid protein with 60 % similarity to methionyl-tRNA formyltransferase from *Staphylococcus aureus* (216 aa). The 276 bp ORF, encoding a 91 aa protein, showed 54 % homology and 76 % similarity to Hu-beta, a 90 aa histone-like protein from *Bordetella pertussis*.

A multiple sequence alignment was performed with the cloned 91 aa protein (termed FP91) sequence and the two closest matches from database searching (Figure 25). The alignment shows a high degree of similarity (95 %) and 37 % homology between FP91, HU-beta from *Bordetella pertussis* and *Eschericia coli* and a 90 aa hypothetical protein from *Cytophaga hutchinsonii* (Figure 25).

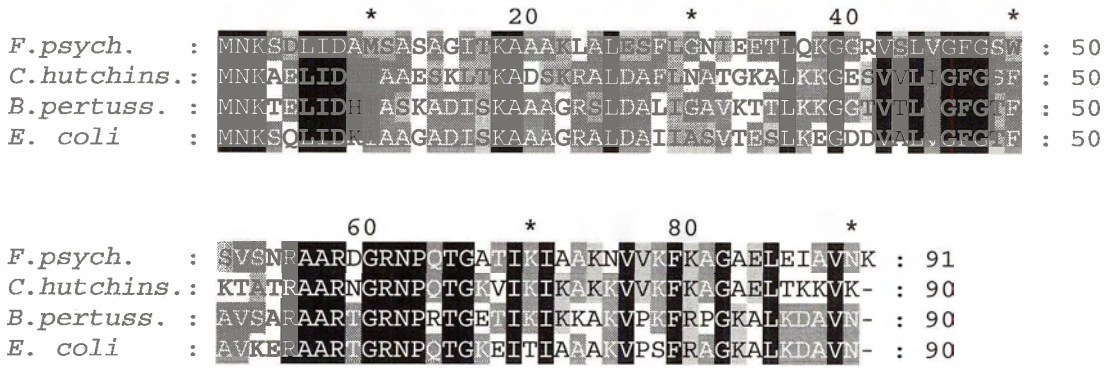

Figure 25. Multiple sequence alignment of FP91. Multiple sequence alignment of a 91 aa protein from *F. psychrophilum* (FP91), a hypothetical protein from *C. hutchinsonii* and HU-beta from *B. pertussis* and *E. coli*. Black shading indicates identity, dark grey indicates conserved residues and light grey indicates similar residues. The four sequences were found to be 95 % similar, 37 % identical. Alignments were constructed using Alignx.

CLONE pP7

Sequence analysis of clone pP7

Analysis of the P7 insert (8341 bp) revealed five complete ORFs (3813 bp, 690 bp, 501 bp, and 207 bp) and one partial ORF (2031 bp) (Figure 26). The ORF sequences were subjected to BLAST2 and FASTA3 analysis to determine if any sequences were known. The most significant matches for all *F. psychrophilum* ORFs were found with hypothetical proteins of *Cytophaga hutchinsonii,* a member of a different class (*Sphingobacteria*) in the CFB phylum. With one exception (501 bp ORF, Figure 26), all ORFs were between 66 % and 78 % identical (79 % - 84 % similar) to *C. hutchinsonii* hypothetical proteins The most significant matches to known genes were found with *Chlorobium tepidum,* which belongs to the same superphylum as *F. psychrophilum* and is the closest relative to have its genome fully sequenced. The large 3813 bp ORF showed 71% similarity to *rpoB* encoding RNA polymerase β subunit. The partial 2031 bp ORF was found to be 75 % similar to *rpoC* encoding the β' subunit of RNA polymerase. The four smaller ORFs showed between 53 % and 76 % similarity to genes *rplL, rplJ, rplA* and *rplK* encoding ribosomal protein L7/L12, L10, L1 and L11 respectively (Figure 26).

In order to identify the ORF encoding the observed ~15 kDa antigen from such a large DNA insert, a proteomics approach was taken. By analysing a peptide fingerprint from a protein of interest, comparisons can be made to the ORF sequences to identify the correct gene.

2D gel electrophoresis of E. coli SOLRpP7 and host control

Induced, whole cell lysates of *E. coli* SOLRpP7 and *E. coli* SOLR (host control) were separated by 2D gel electrophoresis. The aim was to identify a protein of the observed antigen size (~15 kDa) expressed in the pP7 clone that was absent in the host control. Samples were first separated on a tube gel with a pH gradient of 3-10. The second dimension was run on 10-16.5 % gradient slab gels. After staining the gels with Coomassie G-250, the gels were compared by eye to identify unique spots expressed in the pP7 clone, particularly with MWs less than 20 kDa. Two unique protein spots were identified with low MWs (<20 kDa) at ~11 kDa (Spot 1) and ~15 kDa (Spot 2) (Figure 27A, arrows), the latter being the approximate size of the observed pP7 antigen (Figure 20). The two protein spots were excised from the gel for processing and mass spectrometry analysis. Subsequent analysis of the gel images with software revealed a faint spot at ~15 kDa that was not visible by eye, that was more apparent in the SOLRpP7 gel (Figure 27B and C, circle).

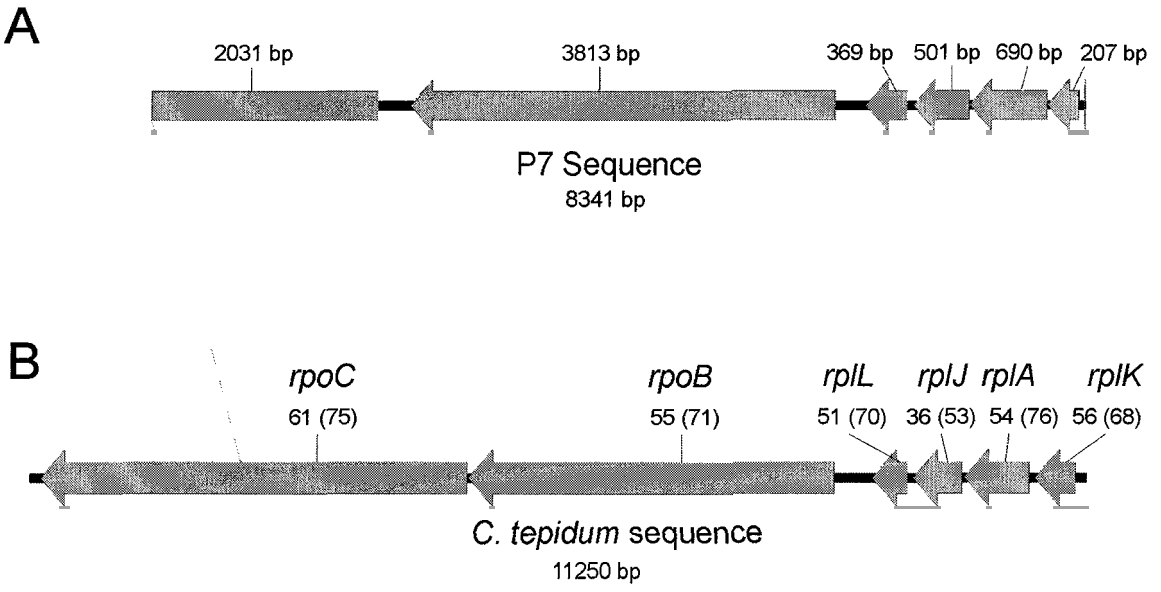

Figure 26. ORF map of clone pP7 and relation to the *Chlorobium tepidum* sequence. A) Insert P7 predicted ORFs. Arrows depict predicted ORFs and their coding direction. B) ORF map of *C. tepidum* sequence showing genes for RNA polymerase β' (*rpoC)* and β (*rpoB)* subunits; ribosomal proteins L7/L12 (*rplL*), L10 (*rplJ*), L1(*rplA*) and L11 (*rplK*). Numbers beneath gene names indicate percentage identity, and in brackets, percentage similarity, to corresponding ORFs in insert P7.

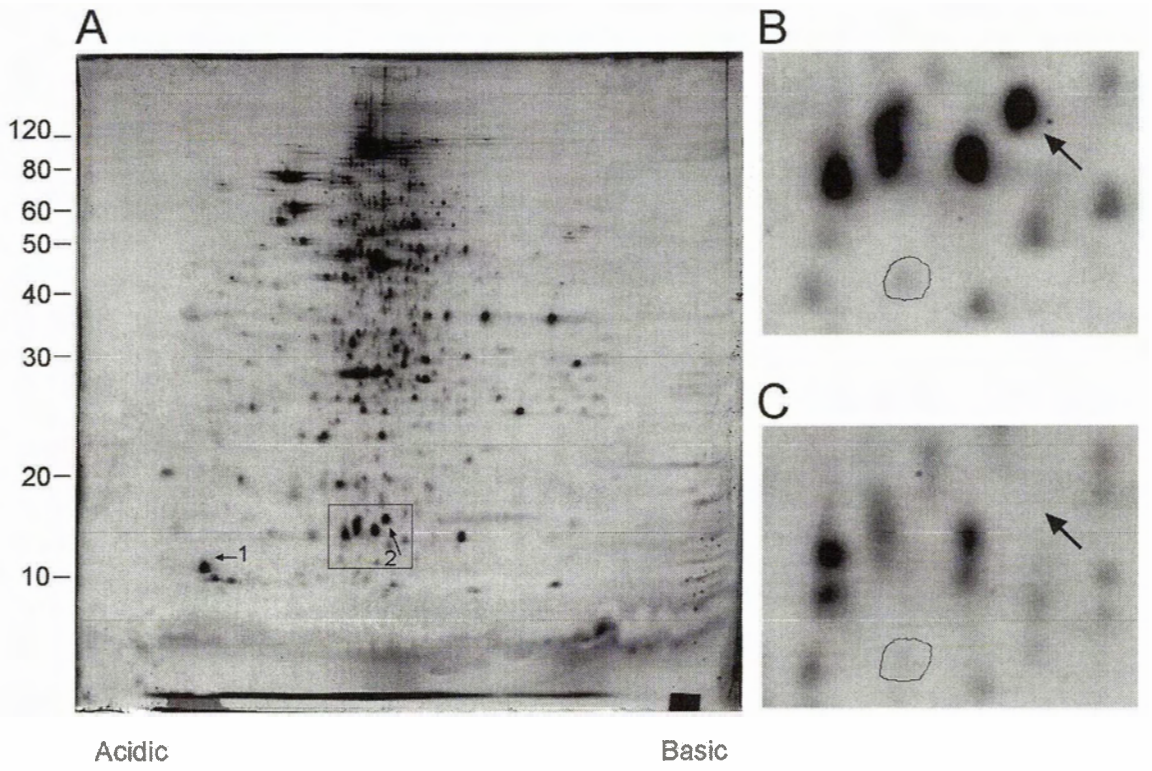

Figure 27. 2-dimensional gel electrophoresis of *E. coli* SOLR pP7 and *E. coli* host control. Whole cell lysates were first separated on a tube gel with a pH gradient of 3 - 10. The second dimension was run on a 10-16.5 % gradient slab gel. Proteins were visualized by Coomassie staining. A) 2D gel of *E. coli* SOLR pP7. Arrows indicate the 2 unique spots <20 kDa that were unmatched on the control gel. MW markers are indicated on the left. B) boxed area of gel A. C) equivalent boxed area of *E. coli* SOLR host control gel, showing position of ~15 kDa spot picked (arrow) and a faint spot not visible when the gel was examined by eye (encircled).

MALDI-TOF mass spectrometry analysis of pP7 proteins

Spots 1 and 2 (Figure 27) were picked from the gel and proteolytically cleaved. The resulting peptides were analysed by matrix-assisted laser desorption / ionisation - time of flight (MALDI-TOF) mass spectrometry to generate a peptide fingerprint of each protein. The peptide fingerprints were submitted to Mascot and MS-Fit for database searching, however, no significant matches were found for either protein.

In an attempt to identify the ORF that encoded the ~15 kDa protein spot picked, the peptide mass map of the spot was compared to the pP7 ORF sequences. ORF sequences from clone pP7 were translated into amino acid sequence and subjected to a virtual tryptic digest using the online program MS-Digest (http://prospector.ucsf.edu/). Peptide fingerprints were generated for each of the putative proteins, as well as values for their estimated MWs and pIs. The theoretical peptide fingerprints from "virtual digests" of the translated ORFs were then compared with the peptide fingerprint of ~15 kDa protein (spot 2, Figure 27). MS-Digest generated 70 peptides with m/z values ranging from 388 - 4342. Only 41 peptides were within the range of data collected for the ~15 kDa protein (m/z 844 - 3001). Six peptides from the virtual digest of the 501 bp (166 aa) ORF, (6/41 peptides, 15% match) matched peptides from the ~15 kDa protein (Table 13). No such matches were found when comparing peptide fingerprints from other translated ORFs. MS-Digest predicted that the 501 bp ORF (ORF 501) encoded a protein with a mass of 17949.9 Da and a pI of 5.68, which was in agreement with the pP7 protein picked, estimated to have a mass of ~ 15 kDa and a pH < 7. No matches were found for ~11 kDa spot 1 with any of the pP7 ORFs.

Table 13. Matching theoretical and observed peptide masses.

Virtual digest	~15 kDa spot 2
1046.5734	1046.6071
1118.6098	1118.5956
1681.9376	1681.9564
1866.9159	1866.9162
1925.0708	1925.0989
2506.3153	2506.3627

Based on peptide matches, estimated size and pI, as well as a lack of significant matches in the database, the ~15 kDa protein spot was believed to have originated from the cloned *F. psychrophilum* DNA, ORF 501, and not *E. coil.* Therefore, pP7 ORF 501 was selected for further expression experiments.

Construction and expression of ORF 501-fusion protein

In order to clone and express ORF 501, PCR primers were designed to amplify the ORF with flanking DNA restriction enzyme cleavage sites. The amplified ORF was gel purified, digested with *Bam*HI and *Hin*dIII and ligated into pETC (Figure 22). The plasmid construct pETC7-166 was confirmed by restriction digest and sequence analysis.

E. coli BL21(DE3) was electroporated with pETC7-166 or pETC. Transformants were selected overnight on LB_{Ap} at 37 °C. For expression experiments, overnight LB_{Ap} 37 °C cultures were used to inoculate fresh LB_{Ap}. Once cultures had reached an A_{600} of 0.6-0.8, they were treated with 1 mM IPTG for 2 h to induce expression of the cloned

DNA. Whole cell lysates were then resolved by SDS PAGE and visualized by Coommassie staining and Western blotting.

SDS PAGE analysis of F. psychrophilum protein fusion C7-166.

The product of C protein fused to the ORF 501 protein, C7-166, is clearly visible in Coomassie stained gels as is as the C-protein control (Figure 28A, arrows). The ~28 kDa fusion product reacted with convalescent rainbow trout serum (Figure 28B, arrow). However, neither rabbit anti-*F. psychrophilum,* anti-*F. columnare* nor anti-*T. maritimum* sera reacted with C7-166 (data not shown). In duplicate experiments, the fusion protein C7-166 was found to constitute, on average, 6.5 % of total cell protein when compared to lysozyme standards.

Amino acid sequence analysis of cloned 166 aa F. psychrophilum protein (FP166).

A multiple sequence alignment was performed with the cloned 166 aa protein (termed FP166) sequence and the two closest matches from database searching (Figure 29). The alignment shows a high degree of similarity (77 %) and 19 % homology between FP166, ribosomal protein L10 from *C. tepidum* and a 167 aa hypothetical protein from *Cytophaga hutchinsonii* (Figure 29).

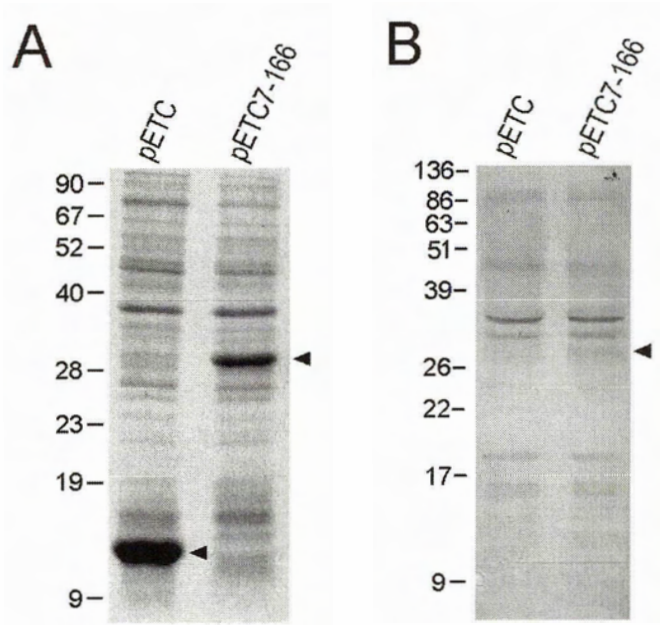

Figure 28. SDS PAGE and Western blot analysis of C7-166. *E. coli* BL21(DE3) cells harbouring the plasmid pETC7-166 were induced to express the fusion protein C7-166. Whole cell lysates were separated by SDS PAGE and visualized by A) Coomassie stain and B) Western blotting with convalescent rainbow trout serum. MW markers (kDa) are indicated on the left.

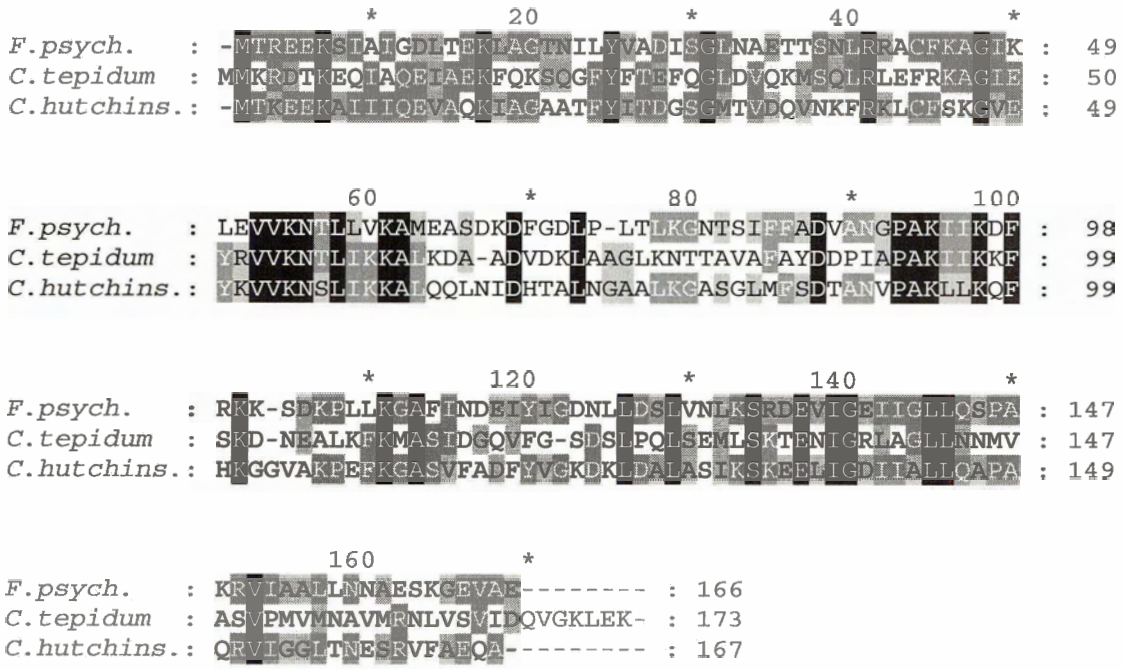

Figure 29. Multiple sequence alignment of FP166. Multiple sequence alignment between 166 aa *F. psychrophilum* protein (FP166), ribosomal protein L10 from *Chlorobium tepidum* and a hypothetical protein from *Cytophaga hutchinsonii.* Black shading indicates identity, dark grey indicates conserved residues and light grey indicates similar residues. The three sequences were found to be 77 % similar, 19 % identical. Alignments were constructed using Alignx.

Incorporation of measles virus epitope into C-protein fusion products

A 51 bp insert encoding a T cell epitope from measles virus (MV) (Microtek Intl. Ltd, Saanichton, BC, CAN) was incorporated in to *Bam*HI and *Nde*I digested pETC7-166 and pETC8-91. The presence of the MV epitope was confirmed by DNA sequencing. The C-protein plus MV epitope fusions (CM fusions), termed C7M and C8M, were expressed as described for the C-protein fusions for use in protection studies in fish (Chapter 6).

CLONE pP2

Sequence analysis of clone pP2

The ORF map of *F. psychrophilum* insert P2 (2378 bp) shows 3 complete and 2 partial ORFs (Figure 30). Database searching resulted in significant matches for only one complete ORF and one partial ORF. The 555 bp ORF encoding a 184 aa protein was found to be 58 % identical and 76 % similar to a 184 amino acid DNA repair enzyme (DNA-3-methyladenine glycosidase I) from *Lactococcus lactis*. The 222 bp partial ORF was found to be 87 % identical and 94 % similar to the carboxy-terminal portion of TruB from *Flavobacterium johnsoniae,* a 241 aa pseudouridine synthase.

Since no antigen was detected in Western blots of *E. coli* SOLRpP2 (Figure 20), this clone was not selected for further investigation.

CLONE pP3

Sequence analysis of clone pP3

A search for ORFs in *F. psychrophilum* insert P3 (2117 b) revealed a single ORF of 1992 bp (Figure 30), encoding a 663 aa protein. Database searches revealed this to be 56 % identical and 75 % similar to a UvrB, a 661 aa DNA repair enzyme from *Bacillus subtilis.* The antigenic profile of clone pP3 showed a major ~68 kDa band, sometimes seen with numerous other bands and presumed to be breakdown products. The theoretical mass of the protein cloned was found to be 76222.0 Da, which is in the range of the observed apparent molecular weight of the pP3 antigen.

Western blot analysis of clone pP3

As seen in Figure 20, the pP3 antigen reacted strongly with the pre-immune serum from the rabbit used to generate anti-*F. psychrophilum* serum. No other rabbit pre-immune serum tested reacted with the pP3 antigen. Therefore, Western blot analysis of *E. coli* SOLRpP3 was carried out with anti-*F. psychrophilum* serum generated in a second rabbit; neither the immune serum nor the pre-immune serum reacted with the pP3 clone (data not shown). Therefore, clone pP3 was not selected for further investigation.

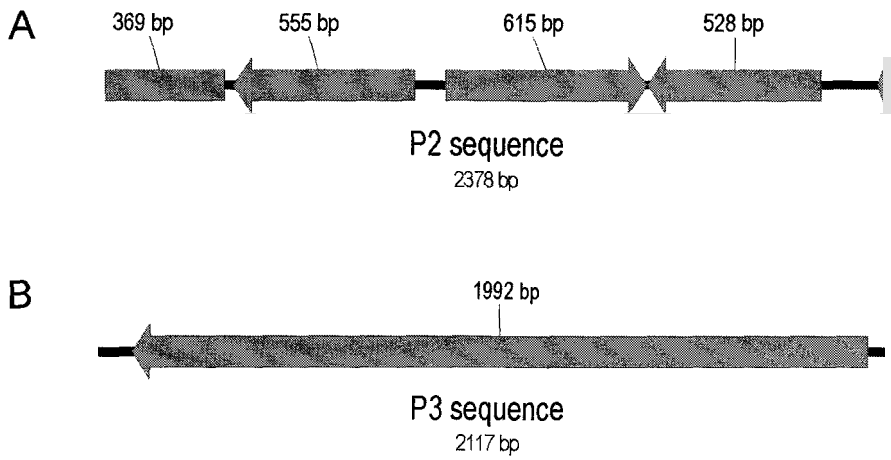

Figure 30. ORF maps of *F. psychrophilum* inserts P2 and P3. A) 2768 bp *F. psychrophilum* insert from clone pP2 showing three complete and two partial ORFs. B) 2117 bp insert from clone pP3 showing one ORF. Arrows indicate predicted ORFs and their coding direction.

Summary

Two *F. psychrophilum* proteins were cloned and expressed in *E. coli* with the aim of testing them as vaccine candidates against RTFS. The proteins cloned are summarized in Table 14.

Table 14. *F. psychrophilum* proteins cloned for used in future protection studies.

	FP91	**FP166**
ORF Size	276 bp	501 bp
Protein size	91 aa	166 aa
Homology	histone-like protein Hu-beta	ribosomal protein L10
Fusion protein	C8-91	C7-166
Rabbit anti-*F. p.* serum	+	
Rabbit anti-*F.c.* serum	+	
Rabbit anti-*T. m.* serum	+	
Convalescent rainbow trout serum	+	(+)

(+) indicates a weakly positive reaction

DISCUSSION

The goal of the work was to clone antigenic proteins from *F. psychrophilum* in order to test them as potential vaccine candidates in protection studies against RTFS. In an attempt to identify, sequence and clone protein antigens, a genomic library was constructed and screen with polyclonal rabbit anti-*F. psychrophilum* serum. Four unique clones were identified and sequenced, resulting in 15.5 kb of novel *F. psychrophilum* DNA sequence.

Prior to this study, no DNA sequence was available for *F. psychrophilum*, other than the 16 S rRNA gene and partial a partial sequence for gyrase B for phylogenetic analysis. The class *Flavobacteria* is little studied and within the phylum *Bacterioidetes,* very little sequence data exists for comparison. As yet, no genomes have been fully sequenced in entire CFB phylum, to which *F. psychrophilum* belongs. However, an unfinished genome shotgun sequence is available for the soil bacterium *Cytophaga hutchinsonii* (class *Sphingobacteria*, CFB phylum) provided close matches with hypothetical proteins. The closest relative to be fully sequenced to date is *Chlorobium tepidum,* a green sulphur bacterium belonging to the phylum *Chlorobi* (green sulphur bacteria), superphylum CFB/*Chlorobi.*

Western blot analysis of the four clones showed that products of two of the clones, pP7 and pP8, were expressed in *E. coli* and specifically reacted with both rabbit anti-*F. psychrophilum* and anti-*F. columnare* sera. The cross-reactive nature of these antigens may in turn lead to the development of a vaccine that protects against both fish pathogenic *Flavobacterium* sp.

The pP7 antigen expressed by *E. coli* SOLRpP7 migrated with an apparent MW of ~15 kDa in one dimensional SDS PAGE. A proteomics approach was taken in an attempt to identify the ORF encoding the ~15 kDa protein. 2D gel electrophoresis of *E. coli* SOLRpP7 and SOLR (host control) revealed a unique spot in the clone that migrated with an apparent MW of ~15 kDa, which was the size of the observed pP7 antigen. The spot was proteolytically cleaved and the peptides analysed by MALDI-TOF mass spectrometry. Database searching resulted in no significant matches for the peptide fingerprints. These are relatively early days in the field of proteomics and the size of available databases can be limiting, especially with regard to little studied organisms, such as *F. psychrophilum*.

The 501 bp ORF encoding the 166 aa, ~15 kDa protein was identified using software to perform a virtual tryptic digest of the translated ORF sequences and comparing theoretical and observed peptide fingerprints. The 501 bp ORF was amplified by PCR, cloned in to the expression vector pETC and expressed as a C-protein fusion. The fusion product (C7-166) did not react with rabbit anti-*F. psychrophilum* nor anti-*F. columnare* serum, which had both reacted with a cloned 15 kDa *F. psychrophilum* protein expressed in *E. coli* (Figure 20). Loss of immunoreactivity may have resulted due to different folding of the protein after fusion to the 10 kDa C-protein. Alternatively, the apparent loss of antigenicity of the protein may signify that the antigenic protein seen in Western blots of 1D gels was not the same protein picked from the 2D gels. The intensity of the pP7 antigen in 1D-Western blots was weak and attempts made to identify the antigen by Western blotting large 2D slab gels were unsuccessful. It is possible that when analyzing the 2D gel, the obvious choice was incorrect. Computer software that

was later available was used to reanalyze the 2D gels. A very faint spot, not visible by eye, was identified at ~ 13 kDa (Figure 27).

DNA sequence analysis of the 8431 bp pP7 insert revealed five complete open reading frames (ORFs) and one partial ORF. The ORF map of that matched in size, order and orientation to a region of the *C. tepidum* genome encoding RNA polymerase subunits β and β' and ribosomal proteins L1, L7/L12, L10 and L11. The 501 bp ORF cloned was 36 % identical, 53 % similar to ribosomal protein *C. tepidum* L10. Ribosomal proteins have been described as prominent antigens during human leishmaniasis. Ribosomal protein LiP2a of *Leishmania infantum* has been shown to stimulate cell proliferation *in vitro* and a humoral response by mice *in vivo* (174). Ribosomal protein L7/L12 of *Brucella abortis* has been shown to elicit cell mediated immunity and confer protective immunity in mice (142). The L7/L12 antigen is being targeted for potential use in oral vaccines against brucellosis which causes abortion and infertility in cattle (156).

The gene encoding the 10 kDa antigen observed in clone pP8 was found by expressing the two ORFs of corresponding size (276 bp and 279 bp) as C-protein fusions and identifying the correct clone by Western blotting. The results were conclusive, showing one protein, C8-91, to be highly antigenic and the other non-reactive. Interestingly, the C8-91 fusion protein also reacted strongly with immune sera from rainbow trout as well as rabbit anti-*F. columnare* and anti-*T. maritimum* sera. The positive reaction with convalescent fish serum suggested that this protein may be a promising vaccine candidate. The cross-reactivity of the antigen with sera raised against two related fish pathogens may provide the basis for a vaccine to protect against more than one fish disease caused by *Flavobacteria*. The observed cross-reactivity may be due

to the antigen being a highly conserved protein. The protein sequence was 54 % identical and 76% similar to histone-like protein HU-β, from *Bordetella pertussis.* The heterodimeric histone-like protein HU is made up of HU-α and HU-β subunits. HU is a small, basic, DNA-binding protein present at approximately 30,000 dimers per bacterial cell (41). The amino acid composition of HU has been found to resemble eukaryotic histone H2B (158).

Western blot analysis showed that clone pP2 was not immunoreactive. The lack of immuno-reactivity of clone pP2 may mean that the product is not being expressed by *E. coli*, or that the product was broken down. Further expression studies with this clone could be done in a more suitable strain for expression, such as *E. coli* BL21 to look for expression of the antigen. The ~68 kDa antigen expressed from clone pP3 on the other hand was highly immunoreactive with both pre-immune and immune sera from the rabbit used to generate anti-*F. psychrophilum* serum. The fact that three other rabbit pre-immune sera tested did not react with the antigen suggests that the rabbit may have been previously exposed to a similar organism. The protein cloned matches a highly conserved DNA repair enzyme, UvrB. It is conceivable that such a response may follow exposure to *E. coli* or *S. aureus* which rabbits in caged environments may encounter through fecal or human contact.

In summary, this chapter describes the identification, cloning and expression of two protein antigens from *F. psychrophilum* using a combination of proteomics and recombinant DNA technologies. The two *F. psychrophilum* proteins, termed FP91 and FP166, were similar to HU-β and ribosomal protein L10 respectively. FP91 and FP166 were expressed as C-protein fusions, which form inclusion bodies and aid protein

isolation. High level expression of recombinant proteins was sought with the aim testing their ability for confer protective immunity against *F. psychrophilum* in rainbow trout fry. The results of protection trials are discussed in Chapter 6.

Chapter 5

Protein Antigen Characterization II: Identification of an Antigen Recognized by Fish Serum.

INTRODUCTION

The aim of this chapter was to identify, clone and express an antigen which was strongly recognized by the host. In Chapter 2, Western blot analysis of *F. psychrophilum* cells with convalescent rainbow trout serum revealed one very strong band at ~20 kDa. The availability of convalescent rainbow trout serum was limiting and sufficient quantities required to screen an expression library were not available. Therefore, a proteomics approach was adopted to identify the specific 20 kDa antigen. Enriched protein samples were separated by two-dimensional (2-D) gel electrophoresis and the protein of interest excised, proteolytically cleaved and analyzed by mass spectrometry (MS). Quadrupole-time-of-flight (Q-TOF) MS was used to generate a fragmented peptide spectrum for sequencing purposes. Peptide sequences were then used to design degenerate PCR (dPCR) primers which, in turn, were used to amplify a portion of the gene of interest. The amplified gene fragment was cloned into an expression vector and the protein expressed as a C fusion for the purpose of amplification and testing it in future protection studies in fish.

RESULTS

2D gel electrophoresis and Western blotting analysis of TX114-partitioned F.psychrophilum proteins using convalescent rainbow trout antiserum.

To further identify the ~20 kDa antigen recognized by fish serum (TX20) seen in Figure 10 and Figure 12, 2D gels of TX114-partitioned *F. psychrophilum* cells were stained with Coomassie and electroblotted onto nitrocellulose for Western blot analysis (Figure 31). To minimize protein load on the gels and aid resolution, cells were first fractionated with Triton X-114 to enrich the hydrophobic components and the Triton phase was loaded onto the 2D gels instead of whole cell preparations. The specificity of the fish antiserum has proved to be an excellent tool, since only one protein reacts strongly with the fish antiserum, and the corresponding protein spot on a Coomassie stained 2D gel was easy to identify. The Western blot of the 2D gel revealed a long smear at 20 kDa instead of a spot (Figure 31B), as did the Coomassie stained gel (Figure 31A). Protein was excised from the gel in the center of the streak (spot y) as well as the spot formed at then end of the streak (spot x) (Figure 31A, arrows) for mass spectrometry analysis.

Mass Spectrometry analysis of 20 kDa antigen (TX20)

By comparing the Coommassie stained 2D gel of Triton phase *F. psychrophilum* material to a Western blot, a protein smear, corresponding to the ~20 kDa antigen, was identified. Two samples (termed x and y) along the smear were excised and proteolytically cleaved (Figure 31, arrows). The two samples, TX20 x and y, were analysed by Q-TOF MS. The initial time of flight mass spectrometry (TOFMS) survey

scans showed that the peaks occurred at the same m/z values, although the peak intensities were lower for spot x than spot y (Figure 32). Therefore, two samples were shown to be the same. The peptide ions generated from nanospray ionisation of each sample were analyzed for doubly charged ions by looking for peaks that differed by half a mass unit (Figure 32, asterisks). The doubly charged peptide ions obtained from spots x and y were then fragmented to produce a spectrum of collision induced product ions from which the peptide sequences were determined (Table 12). The peptide fingerprints obtained for the two samples were used to search a protein database, however no significant matches were found.

Peptide sequencing of TX20

Product ion spectra from doubly charged peptide ions were analysed using the program BioAnalyst and the peptides sequenced by following Kinter's nine step strategy for interpretation of product ion spectra (99). The product ions sequenced from each spot are listed in Table 12.

Table 15. Product ions sequenced from protein TX20. Underlined amino acids were not found in the translated dPCR sequence. Spots x and y refer to Figure 31. Numbers in brackets denote masses of unknown amino acid sequences flanking known sequence.

Product ion (m/z)	Peptide sequence	Spot
923.4	(278.20) GFYLGVTV (712.21)	y
923.5	(278.11) GFYLGVTV (712.35)	x
923.5	(1183.55) LGVDMK	x
692.3	(158.06 FADLGVM (345.09)	y
486.2	SSGFDFSPK	x
1022.5	(342.20) QFSENMTVGLE (447.14)	y
1030.5	(243.08) VQFSENFTVGLEGSFR	y
985.5	(286.21) NVAGTVGFDST (615.29)	x
613.3	AANDFELNFGK	x

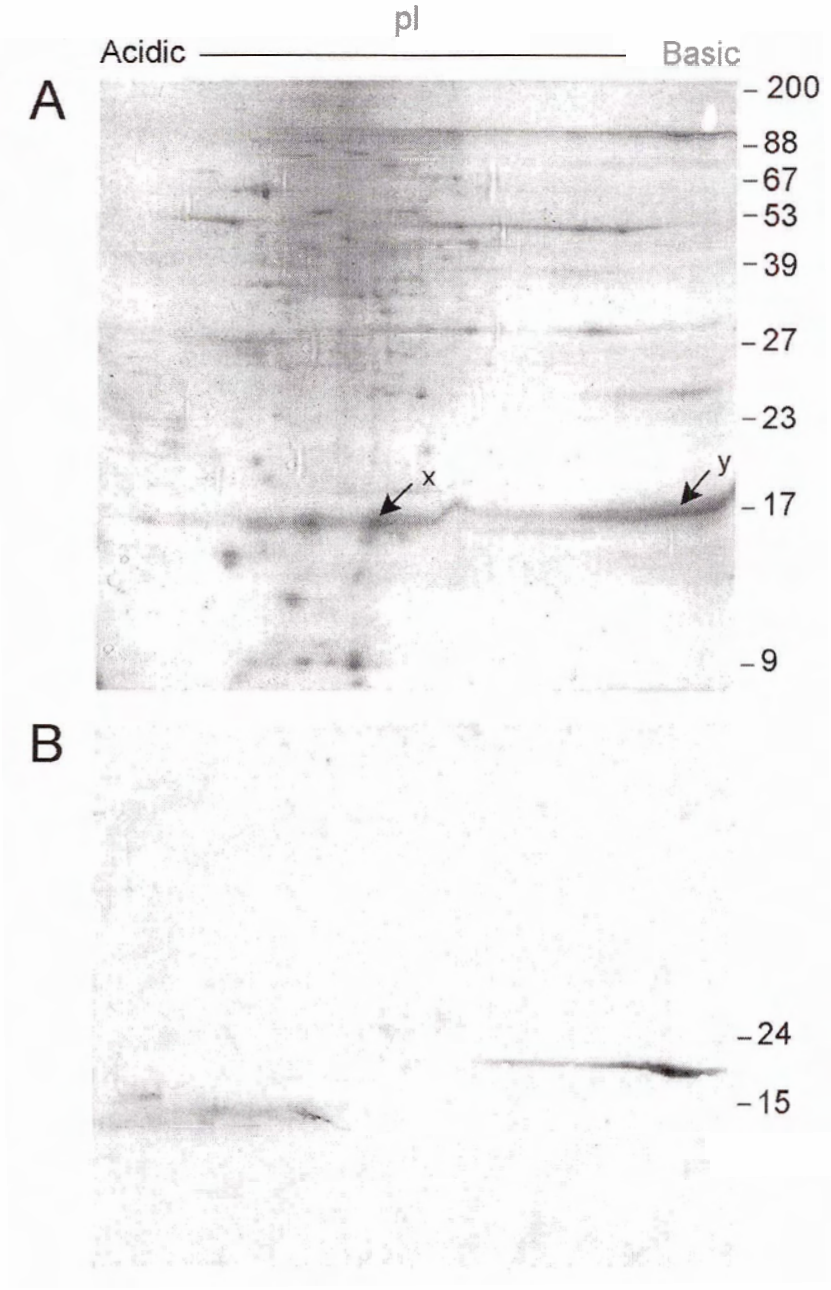

Figure 31. 2D gel electrophoresis and Western blot analysis of *F. psychrophilum*. *F. psychrophilum* cells were fractionated with Triton X-114 and the detergent-soluble, "warm pellet" fraction was separated by 2D gel electrophoresis and either stained with Coommassie (A), or electroblotted onto nitrocellulose and reacted with convalescent rainbow trout serum followed by rabbit anti-salmon Ig conjugated alkaline phosphatase (B). Arrows x and y indicate areas where protein samples were excised from the gel. MW markers are indicated on the right.

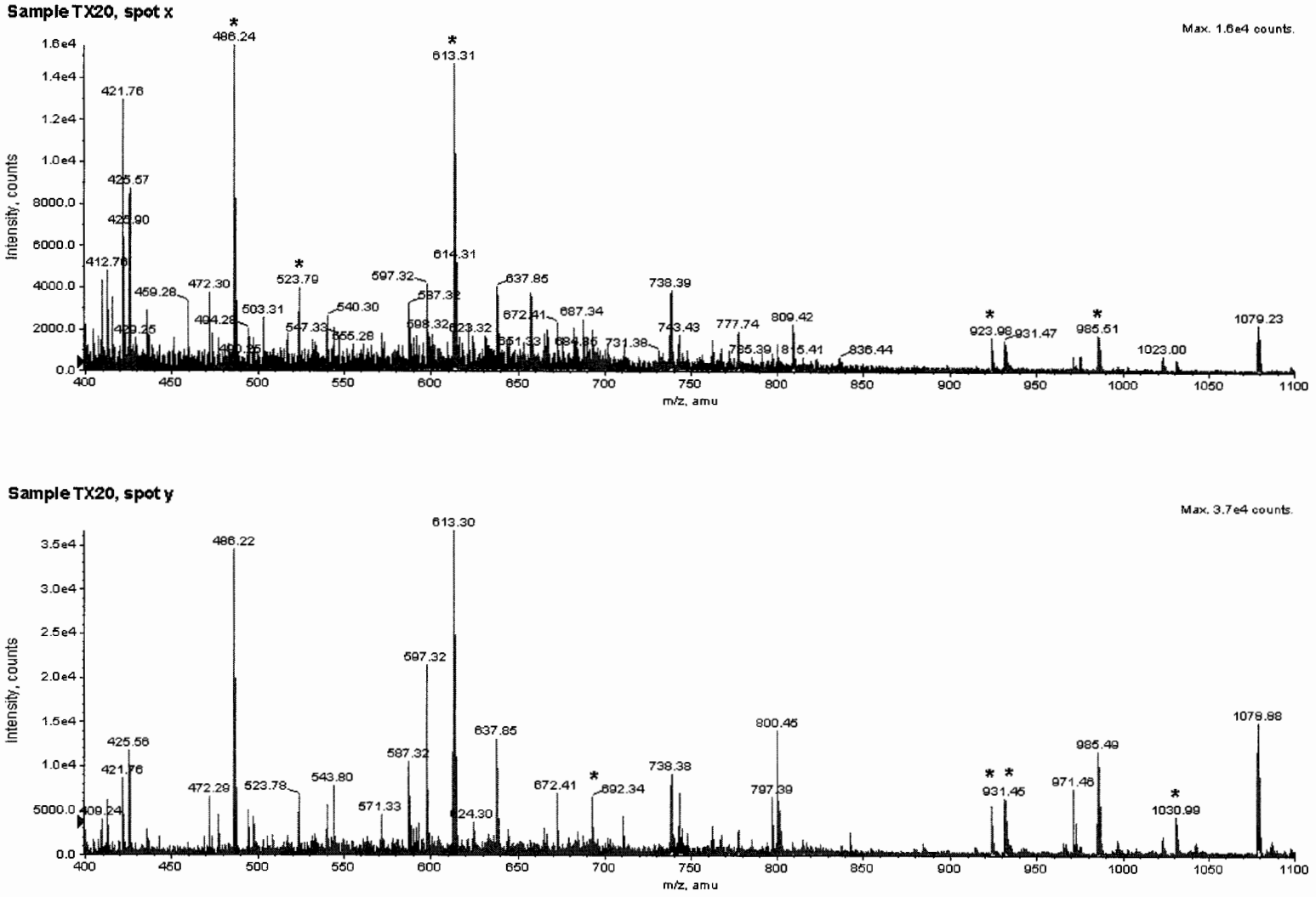

Figure 32. Survey scans of sample TX20, spots x and y. Peaks marked with an asterisk were analysed further by tandem MS and sequenced.

Identification of TX20 gene fragment

To identify gene sequence encoding TX20, a pair of complementary degenerate PCR primers were designed from three different peptide sequences. The sense (+) and antisense (-) strand primers were designated N, A and G for the first amino acid of the peptide from which they were derived. Degenerate PCR (dPCR) was then performed using each of the sense primers with alternate anti-sense primers. One set of primers (N+A-) resulted in the amplification of a reproducible 852 bp product. The dPCR product was cloned into pGEM-T and electroporated into *E. coli* XL1blue. Transformants were selected by resistance to ampicillin and blue/white screening. Three positive clones were confirmed by restriction digest analysis and sequenced. The consensus sequence (852 bp) was translated and searched for the presence of the peptide sequences obtained from TX20. All the peptide sequences (Table 12) were found within the translated dPCR product (Figure 33), thus confirming that the dPCR product encoded the protein of interest excised form the 2D gel.

DNA sequence of TX20 gene fragment

The dPCR consensus sequence was initially puzzling in that the size of the incomplete gene sequence (852 bp) was considerably larger than expected for a 20 kDa protein, as estimated from SDS PAGE. Analysis of the translated sequence revealed 10 stop codons in the C-terminal portion, starting from position 538 bp (179 aa) (Figure 33). All of the peptides sequenced were found upstream of the first stop codon, including the peptide used to design the antisense primer (AANDE). Closer inspection of the dPCR sequence showed that the 3' end was compatible with the degenerate sense strand primer

(termed N+). Fortunately, another form of the N+ primer, which had a 324 fold degeneracy, performed as an anti-sense primer which resulted in sequencing beyond the C-terminal of the protein.

The protein from position 1 to the first stop codon (538 bp, 179 aa) was found to have a theoretical mass of 18.8 kDa and pI of 8.70, which is in close agreement with the observed mass and pI from 2D gel electrophoresis (~20 kDa, pI >7), suggesting that the vast majority of the gene sequence had been obtained by dPCR. The amino acid composition was found to comprise 35 % hydrophobic, 15 % nonpolar, 32 % polar and 18 % charged amino acids.

Database searching for similar sequences resulted in significant matches to two hypothetical proteins from *Flavobacterium johnsoniae* (Figure 34). The two neighbouring *F. johnsoniae* genes were 72 % identical and encoded hypothetical proteins of 193 aa and 194 aa in length and are arbitrarily named *F.j.*1 and *F.j.*2 respectively. The TX20 DNA sequence was found to be 54 % homologous to the *F. johnsoniae* gene sequences. At the protein level, the TX20 was found to be 45 % and 49 % similar, and 37 % and 38 % identical to *F.j.*1 and *F.j.*2 respectively. Alignment of the translated amino acid sequences of the hypothetical proteins and TX20 showed overall 80 % similarity and 31 % homology between the three amino acid sequences (Figure 34).

Alignment of the three proteins showed that the *F. johnsoniae* proteins extend 23 aa upstream of the N-terminal of TX20' (Figure 34). It is likely therefore that the full length TX20 is similar in size. The two *F. johnsoniae* proteins and TX20' were analyzed using software to predict N-terminal signal sequences (iPSORT, (17)) and signal peptide cleavage sites (SignalP (139)). *F.j.*1 and *F.j.*2 were found to have putative signal

sequences with predicted cut sites between position 18 and 19 (ANA▼QK), 5 aa upstream of the TX20' sequence. No signal sequences were predicted for the truncated TX20' sequence.

N-terminal sequencing

Protein TX20' was separated by 2D gel electrophoresis, blotted onto nitrocellulose and visualized by staining with Coomassie Brilliant Blue G250. The protein spot was cut out and submitted to the University of Victoria Genome BC Proteomic Centre for sequential rounds of Edman degradation in order to sequence the N-terminal amino acids. No sequence data was obtained, thus indicating that the N-terminal was blocked. Further attempts to expand the known gene sequence over the amino terminal portion of the protein were made using Uneven PCR, the results however were also unsuccessful.

```
 N   V   A   G   T   V   G   F   N   S   T   S   Q   G   D   T   K   S      18
AAC GTG GCA GGT ACA GTG GGT TTT AAC TCT ACA TCA CAA GGT GAT ACA AAA AGT      54

 S   G   F   D   F   S   P   K   V   G   W   Q   F   S   E   N   M   T      36
TCT GGT TTC GAT TTT TCT CCA AAA GTT GGT TGG CAA TTT TCT GAA AAC ATG ACA     108

 V   G   I   E   G   S   F   R   N   N   T   E   T   S   T   T   G   S      54
GTA GGT ATT GAA GGT AGC TTT AGA AAT AAT ACT GAA ACC AGT ACT ACT GGT AGT     162

 I   S   F   G   P   I   T   L   P   G   I   S   Q   E   T   V   H   T      72
ATT AGT TTT GGT CCA ATT ACA CTT CCT GGA ATA TCT CAA GAA ACA GTA CAT ACT     216

 N   T   K   I   G   A   F   F   R   Y   A   Q   P   L   A   G   V   F      90
AAT ACT AAA ATT GGT GCT TTC TTT CGT TAT GCA CAA CCA TTA GCT GGT GTT TTC     270

 S   A   F   A   D   L   G   V   G   M   Q   S   G   K   T   S   V   N     108
TCT GCT TTT GCT GAT TTA GGT GTT GGT ATG CAA TCT GGA AAA ACA TCA GTT AAC     324

 R   P   G   S   T   D   L   K   Y   N   G   F   Y   I   G   V   T   P     126
AGA CCA GGA TCA ACA GAT TTA AAA TAT AAC GGT TTT TAC ATT GGT GTA ACA CCT     378

 A   I   G   V   D   M   K   K   G   F   C   L   N   F   A   I   G   G     144
GCT ATT GGG GTT GAT ATG AAA AAA GGA TTT TGT CTT AAC TTC GCT ATC GGA GGT     432

 L   G   Y   N   T   M   K   A   D   A   D   G   A   K   A   A   N   N     162
TTA GGA TAT AAT ACT ATG AAA GCT GAT GCT GAT GGT GCA AAA GCT GCA AAC AAT     486

 F   E   L   N   F   G   K   Q   V   S   V   G   I   S   K   N   F   *     180
TTC GAA TTG AAT TTC GGA AAA CAA GTA AGC GTT GGT ATC TCT AAA AAT TTC TAA     540

 Y   S   K   K   L   L   F   K   K   S   R   F   V   I   T   R   R   D     198
TAT TCC AAA AAA TTA CTT TTT AAG AAA TCC CGT TTC GTA ATT ACG AGA CGG GAT     594

 F   F   M   P   K   I   F   V   T   L   P   *   *   I   G   M   V   I     216
TTT TTT ATG CCC AAA ATT TTC GTT ACT TTG CCC TAA TGA ATT GGA ATG GTT ATA     648

 *   I   V   L   N   P   T   *   K   *   N   E   D   C   L   K   I   L     234
TAA ATA GTT TTA AAT CCT ACT TAA AAA TAG AAC GAG GAT TGT CTA AAA ATT CTG     702

 W   L   I   M   C   S   T   S   R   N   *   I   I   I   C   Q   T   I     252
TGG CTA ATT ATG TGC TCG ACA TCG AGA AAT TAA ATC ATT ATT TGT CAG ACA ATA     756

 I   F   M   F   R   P   *   I   *   M   S   K   R   F   S   N   L   F     270
ATA TTC ATG TTT CGC CCG TAA ATA TAA ATG AGC AAA CGA TTC AGC AAT TTA TTT     810

 T   K   Y   L   K   A   *   T   H   G   S   C   N   V                     284
ACG AAA TAT CTA AAA GCG TAA ACC CAC GGT TCC TGC AAC GTT                     852
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Figure 33. Degenerate PCR (dPCR) sequence. The translated amino acid sequence is represented in single letter code. Peptide sequences obtained by Q-TOF MS are highlighted. Underlined amino acid sequences were used to design the dPCR primers. The double underlined sequence was found to be compatible with the dPCR sense strand primer. Asterisks denote stop codons.

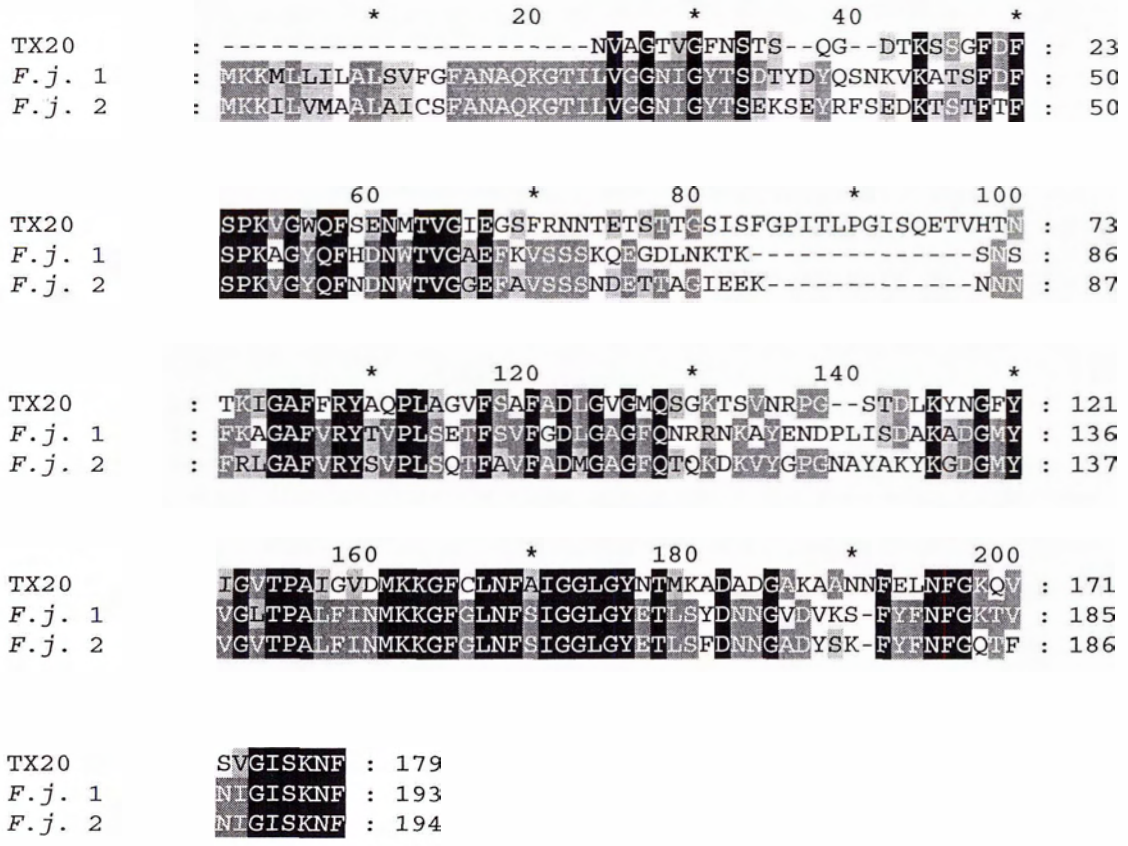

Figure 34. Multiple sequence alignment of translated TX20 gene fragment. The 179 aa TX20 fragment (TX20') from *F. psychrophilum* is shown here with two hypothetical proteins from *F. johnsoniae,* arbitrarily labeled *F.j.*1 and *F.j.*2. Black shading indicates identity between the 3 sequences, dark grey indicates identity in 2 sequences and light grey indicates similar residues. The three sequences are 80 % similar, 31 % homologous. Multiple sequence alignments were constructed using AlignX (Vector NTi).

Construction and expression of a fusion protein, CTX20

PCR primers were designed to amplify the 537 bp (179 aa) portion of the protein antigen TX20 sequenced by dPCR (N terminal amino acid to first stop codon, termed TX20'), with flanking restriction enzyme sites. The amplified gene segment was gel purified, digested with *Bam*HI and *Hin*dIII and ligated into pETC (Figure 22). Plasmid constructs were confirmed by restriction digest and sequence analysis. *E. coli* BL21(DE3) was electroporated with either pETC-TX20', or pETC. Transformants were selected overnight on LB_{Ap} at 37 °C. For expression experiments, overnight LB_{Ap} 37 °C cultures were used to inoculate fresh LB_{Ap}. Once cultures had reached an A_{600} of 0.6-0.8, they were induced with 1mM IPTG for 2 h. Induced whole cell lysates were then separated by SDS PAGE and visualized by Coomassie staining and Western blotting

Coomassie stained gels showed that the TX20' sequence was expressed as a C-protein fusion, termed CTX20', with an apparent MW of ~30 kDa (Figure 35A). Western blotting with convalescent rainbow trout serum showed CTX20' to be antigenic, whereas C protein alone did not react (Figure 35B). Further Western blot analysis of with rabbit anti-*F. psychrophilum*, anti-*F. columnare*, anti-*T. maritimum,* showed the protein to be unreactive with the three rabbit antisera (data not shown).

DNA (51 bp) encoding a T cell epitope from measles virus (Microtek Intl. Ltd, Saanichton, BC, CAN) was incorporated into *Bam*HI and *Nde*I digested pETCTX20'. The C-protein plus measles epitope fusion (CMTX20') was expressed as described for the C-protein fusion, for use in protection studies in fish (Chapter 6).

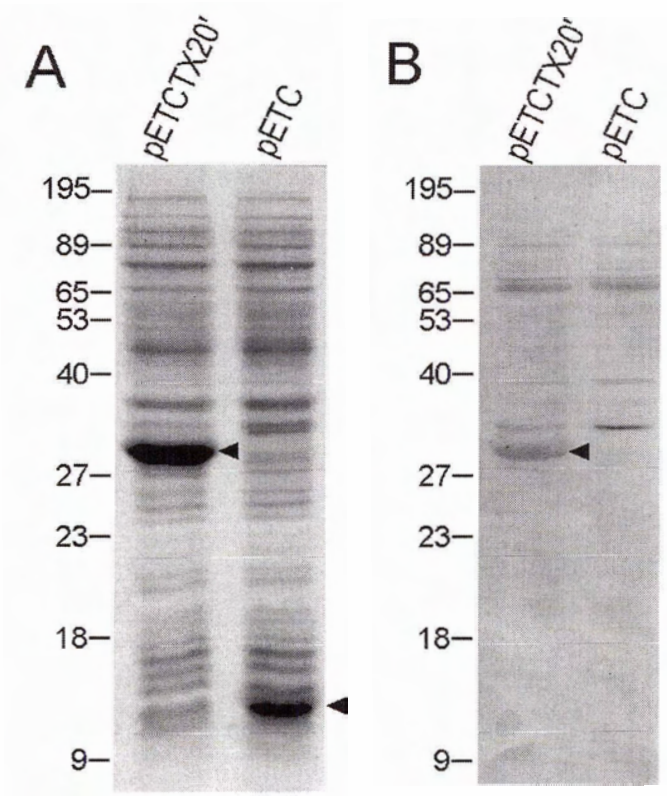

Figure 35. SDS PAGE and Western blot analysis of CTX20' fusion protein. *E. coli* BL21(DE3) cells harbouring the plasmids pETCTX20' or pETC were treated with 1mM IPTG and whole cell lysates separated by SDS PAGE. Protein fusions were visualised by A) Coomassie stain and B) Western blotting with convalescent rainbow trout serum. Arrows indicate C-protein and the C-protein fusion CTX20'. MW markers (kDa) are indicated on the left.

DISCUSSION

For routine antigenic analysis rabbit serum is relatively easy to obtain and provides insight as to the potential antigenic nature of *F. psychrophilum* in the host. Rabbit antisera has been successfully employed in the past to identify protective antigens in the production of vaccines for aquaculture. However, rainbow trout are evolutionarily far removed from mammals and so the more refined goal was to identify antigens recognized by the host. Juvenile rainbow trout were injected with sub-lethal concentrations of live *F. psychrophilum* cells in order to raise convalescent rainbow trout serum against *F. psychrophilum*. Western blot analysis showed that the fish serum did not appear to bind as many antigens as rabbit serum. One protein in particular was of interest because it bound convalescent rainbow trout serum very strongly. Although the naïve fish serum recognized much of what the convalescent serum did, the 20 kDa protein of interest had a visibly stronger reaction with the convalescent serum. The nature of fish immunoglobulin, being high avidity, low affinity, may account for the observed binding of naïve serum (Chapter 2, Figure 10).

The 20 kDa protein of interest (termed TX20) partitioned into the detergent phase following extraction with Triton X-114, indicating that the protein is, at least in part, hydrophobic and perhaps membrane bound. The Triton-extracted protein, TX20, runs as a wavy band in SDS PAGE. The wavy band may indicate heterogeneous forms or modifications that affect SDS binding, resulting in sub-populations that migrate slightly differently in SDS PAGE, or simply indicate the presence of trace amounts of Triton X-114. In 2D gel electrophoresis, the 20 kDa antigen always smeared across the second

dimension slab gel, which is indicative of a partially insoluble protein, protein overloading, multiple isoelectric forms or some combination of these factors. The smear appeared to resolve in to a protein spot and so samples were taken from both the smear and the resolved spot for mass spectrometry analysis. The results from Q-TOF MS indicated that the two samples were from the same protein. Using peptide sequence obtained from Q-TOF MS, degenerate PCR primers were designed to amplify a portion of the gene. To confirm the identity of the dPCR sequence it was translated into amino acid sequence and compared against all the peptide sequences obtained from Q-TOF MS analysis. All of the peptide sequences, from both samples along the protein smear, were found within the translated dPCR sequence, providing further evidence that the two samples extracted from the gel were from the same protein.

The results obtained from dPCR were initially surprising, being an apparent gene fragment over 50 % larger than the expected size for the observed protein. These results could be explained by one of the degenerate primers acting as both a sense and anti-sense primer. This fortunate match led to the sequencing of the protein's C-terminal, leaving only the N-terminal sequence unknown.

Several attempts were made to sequence the N-terminal portion of the protein. A PCR method called uneven PCR (37) failed to produce specific sequence. Difficulties in amplifying regions of *F. psychrophilum* DNA, with a % GC content of 33 %, are encountered when the region contains large AT rich segments, as found in *F. psychrophilum* DNA. Further attempts to PCR the N-terminal region were made by making primers to the N-terminal portion of the *F. johnsoniae* genes, in hopes they were similar enough the the *F. psychrophilum* sequence, and using them in conjunction with a

specific primer made to the C-terminal region, however no product was obtained. A third approach taken was that of simply N-terminal protein sequencing. Protein was separated by 2D gel electrophoresis and electroblotted on to PVDF membrane, excised and submitted for sequential rounds of Edman degradation. The lack of results suggested that the N-terminal was blocked, indicating that the N-terminal was post-translationally modified. Further work to elucidate the N-terminal sequence of this protein may involve the screening of the DNA library with a DNA probe, based on sequence obtained from dPCR.

The hydrophobic nature of the protein could be explained by its amino acid composition. Only 18 % of the amino acids in TX20' are charged. Hydrophobic and nonpolar amino acids accounted for 50% of the residues. It is possible that TX20 is a lipoprotein. In Chapter 2, a [^{14}C]-labelled lipoprotein of ~20 kDa was found to partition into the detergent phase during Triton X-114 fractionation (Figure 11). Lipid may attach to proteins internally via internal oxyesters to serine or thioesters to cysteine residues, or by N-terminal, or internal amine linkage to lysine residues (175). Proteins sorted to the bacterial outer membrane are lipidated at their N-terminal amino acid (175). The N-terminal sequence of TX20 is unknown and may be the site of lipid attachment via an amide linkage. Analysis of the known sequence (180 aa) of TX20 therefore did not reveal the liporotein modification consensus site, ie L-A/S-G/A-C , which is the site of processing, the cysteine residue becoming the first amino acid (+ 1 position) in the mature lipoprotein (73).

Database searching with the 180 aa partial TX20 sequence resulted in only two significant matches. The only two similar sequences found were hypothetical proteins

from *F. johnsoniae*. The two proteins were 193 and 194 aa in length and are encoded by consecutive genes on the *F. johnsoniae* chromosome (2). Database searching revealed that the 194 aa *F. johnsoniae* protein is 27 % identical and 46 % similar to Ail from *Yersinia pseudotuberculosis* (214). Ail is a 17 kDa outermembrane protein that mediates adhesion to mammalian cells and contributes to serum resistance. The Ail sequence contains the prolipoprotein consensus sequence LIAC within the first 20 amino acids (214). Similarly, the 194 aa hypothetical protein from *F. johnsoniae* contains the sequence LAIC in the N-terminal region (2). Although the majority of consensus sequences do not contain isoleucine (I) in the -1 position, they have been reported in gram-positive prolipoprotein consensus sequences (178). If TX20 is indeed a lipoprotein, as might be implied by its partitioning into the detergent phase and the comigration of a radio-labelled lipoprotein in Chapter 2 (Figure 11), then the blocked N-terminal may well be due to an amide linked lipid molecule.

In summary, a 20 kDa antigen recognized strongly by convalescent rainbow trout serum was identified by 2D-gel electrophoresis, mass spectrometry and degenerate PCR. The deduced partial gene sequence, encoding 179 amino acids, was expressed in *E. coli* for use in protection studies which are described in Chapter 6.

Chapter 6

Infection Studies and Vaccinology of

F. psychrophilum

INTRODUCTION

Following on from the identification and cloning of *F. psychrophilum* protein antigens, the goal was to test their ability to confer protective immunity against *F. psychrophilum* in rainbow trout fry (Figure 36). The production of efficacious vaccine is a complex task, requiring the consideration of several variables including the effect of route of administration, vaccine dose, formulation and maturity of fish. Testing all of these variables is far beyond the scope of this study. The goal of this study was to identify molecules worthy of further investigation as vaccine candidates, by assessing their ability to protect fry against lethal challenge with *F. psychrophilum*.

Paramount to the testing of vaccine efficacy is the establishment of a reproducible challenge model for the disease. In order to test a vaccine, the challenge must result in significant mortality, ideally 60-80 %. A higher mortality rate would indicate that the subjects were overwhelmed by the challenge and therefore prophylactic vaccination unlikely to succeed. A lower mortality rate would result in weak protection data. The primary goal of work therefore was to develop a challenge model for *F. psychrophilum* in rainbow trout fry using both injection and immersion modes of infection.

Figure 36. Rainbow trout fry. Rainbow trout fry used in vaccine trials were 0.50-1 g at time of vaccination, measuring approximately 4 cm. Actual size: _____

RESULTS

INFECTION STUDIES

Injection Challenge

F. psychrophilum used for the challenge model was freshly passaged through rainbow trout and reisolated from infected kidney tissue. For vaccine trials, a challenge dose resulting in 70 % mortality was desired. Rainbow trout fry, weighing 1-2 g, were successfully challenged with different infectious doses of *F. psychrophilum* grown to an A_{600} of 0.30 - 0.40. Three injection doses of 1/10 fold dilution (0.1 x), neat (1 x), and a 10 fold concentration (10 x) were administered in a 50 µl final volume into 50 rainbow

trout each and the cumulative mortalities monitored over a three week period (Figure 37). The two injection challenges were in close agreement, as seen Figure 37, showing that the disease model was reproducible over the range of doses tested. An A_{600} of 0.30 was found to correlate to 1.67 x 10^8 cfu / ml of *F. psychrophilum*. Therefore, a 50 µl dose of neat culture consists of ~ 8.35 x 10^6 cfu, and the 10 fold concentrated dose ~8.35 x 10^7 cfu, which resulted in a mortality rate of 90 %. Previous attempts at obtaining a reproducible injection challenge had failed when either fewer cells were injected or had when cells reached a culture density over A_{600} 0.9.

Immersion Challenge

As an immersion challenge, 50 1-2 g fish were exposed for 1 h to either a ½ dilution and a 1/10 dilution of overnight culture (A_{600} 0.37) in a total volume of 1 l saline (0.85 % NaCl). The cumulative mortalities for the immersion challenges were negligible at 4 % and 6 % and not further pursued.

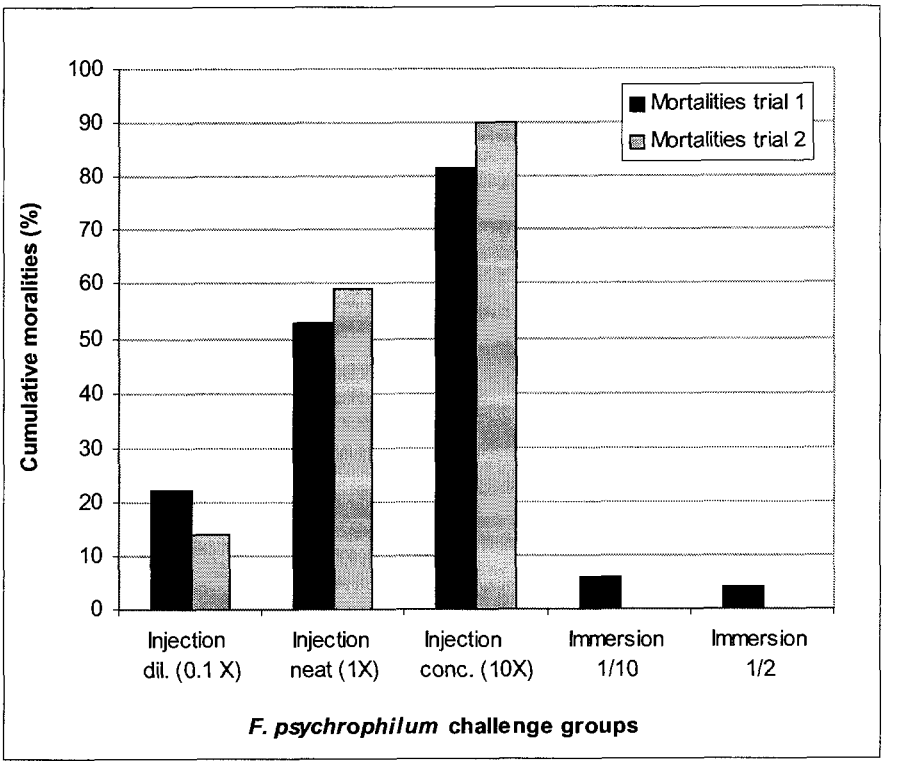

Figure 37. ***F. psychrophilum*** **killing of rainbow trout fry.** Rainbow trout fry were experimentally infected with *F. psychrophilum* by injecting fry with 50 µl bacterial culture. Alternatively, fish were exposed to a dilution, either 1/10 or ½, of bacterial culture (A_{600} 0.37) for 1 h. Cumulative mortalities were monitored for 21 days.

VACCINE TRIALS

Three vaccine trials were undertaken using out-bred populations of rainbow trout fry, weighing 0.5 - 1 g at vaccination. Fish were kept at a constant temperature of 13 °C. After 400 dd (31 days at 13 °C), fish were challenged by i.p. injection of 50 µl live cell suspension (~ 10^7 cells). Mortalities were recorded for a total of 21 days post-challenge. Necropsies were carried out on in order to determine whether *F. psychrophilum* could be isolated from kidney tissue. Only mortalities where *F. psychrophilum* was positively identified were included in the protection data.

The recombinant protein vaccines tested were C7, C7M, C8, C8M, CTX20' and CTX20'M, described in Chapters 4 and 5. The recombinant proteins were expressed in *E. coli,* in which they formed inclusion bodies. Fish were either injected with an inclusion body preparation or with killed whole cells of *E. coli* expressing the recombinant protein. In each case, 10 µg of the protein was administered by i.p. injection with adjuvant. As an *E. coli* control, the same amount of cells, measured by total protein concentration, were administered as were required for the expression of 10 µg recombinant protein C8.

Killed whole cells of *F. psychrophilum* were used to vaccinate rainbow trout fry. 50 µl of a killed cell suspension was injected (i.p.). Fish were injected with 125 µg of *F. psychrophilum*, equivalent to the amount of whole *E. coli* cells administered in the controls.

Vaccine trial 1

The goals of trial 1 were to: 1) assess the effectiveness of recombinant proteins C7 and C8 to protect fish against subsequent challenge with *F. psychrophilum*; 2) compare the difference between administering the recombinant proteins in whole cells versus inclusion body preparations; 3) test the effectiveness of an *F. psychrophilum* bacterin administered at the same concentration as *E. coli* cells. All vaccines were compared against an unvaccinated group injected with saline.

Seven groups of 65 rainbow trout fry were injected with vaccines listed in Table 16. One group of 50 fish was injected with a saline solution as a control. The challenge dose for the first trial was 50 µl of a 6 fold concentration of overnight culture, A_{600} 0.35 which was hoped to give a mortality fate of ~70 %. The average size of the fry was 1 g at vaccination, 1.5 g at the end of the trial. The mortality in the unvaccinated, saline control group was 64 %. This value was used to calculate relative percent survival (RPS) of the vaccinated groups. The results of vaccine trial 1 are summarized in Table 16. The cumulative mortalities over time are shown in Figure 38.

Although each group initially contained 65 fish, losses of approximately 40% occurred in each group following vaccination, during the 31 days (400 dd) prior to challenge. The dramatic loss in fish was in large part due to canabalism. It is thought that this behaviour was brought on by attempting to hold the youg fry at a relatively constant weight on a standard 1 % body weight feeding regime.

All of the adjuvenated injections in vaccine trial 1 resulted in a significant level ($p < 0.001$) of protection, compared to the unvaccinated, saline controls. The best

protection was provided by killed whole cells of both *F. psychrophilum* (84 % RPS) and *E. coli* expressing C protein (88 % RPS).

There was no apparent difference in the level of protection provided by the protein fusions in inclusion body preparations versus whole cells. In each case, the same cumulative mortality was was attained: 18 % mortality for C8 vaccines, 23 % mortality for C7 vaccines (Table 16). The C7+C8 inclusion body mixture resulted in a 21 % mortality.

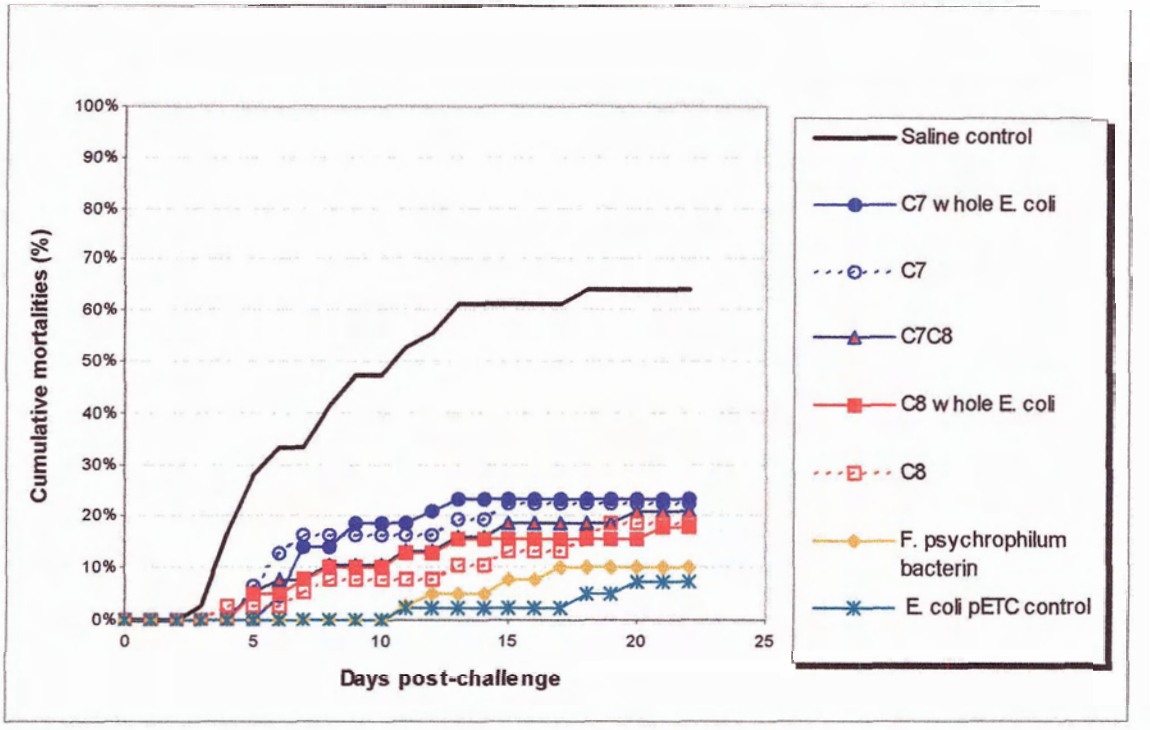

Figure 38. Vaccine trial 1 - cumulative mortalities. Groups of 65, 1 g rainbow trout fry were injected (i.p.) with various vaccine preparations or saline as a control. After 400 dd fish were challenged (i.p.) with ~ 10^7 *F. psychrophilum* cells.

Table 16. Vaccine trial 1 summary

Fish size at vaccination	1.0 g	
Fish size at end of trial	1.5 g	
Challenge dose / 50 µl	6X conc. A_{600} 0.323, ~ 8.0 x 10^7 cfu*	
Vaccine or Control	**Cumulative mortality**	**RPS (%)**
C8	7/38 (18 %)	71
C7	7/31 (23 %)	65
C8+C7	8/38 (21%)	67
E. coli C8	7/39 (18%)	72
E. coli C7	10/43 (23%)	64
F. psychrophilum bacterin	4/39 (10%)	84
E. coli + plasmid control	**3/40 (8%)**	**88**
Saline control	23/36 (64%)	0

* approximate value based on previous results

Vaccine trial 2

The goal of trial 2 was to repeat the vaccinations in trial 1 with an additional *E. coli* control group that did not contain pETC plasmid. In addition, a slightly higher challenge dose was attempted. Eight groups of 75 rainbow trout fry were injected with vaccines listed in Table 17. One group of 75 fry was injected with a saline solution as a control. The challenge dose for the first trial was 50 µl of a 7 fold concentration of overnight culture, A_{600} 0.32 which was hoped to give a mortality rate of ~75 %. The average size of the fry was 0.5 g at vaccination, 1.1 g the end of the trial. The mortality in the unvaccinated, saline control group was 67 %. This value was used to calculate relative percent survival (RPS) of the vaccinated groups. The results of vaccine trial 2 are summarized in Table 17. The cumulative mortalities over time are shown in Figure 39.

Due to the significant losses observed in trial 1 in all groups post-vaccination, the fry were fed more frequently during trial 2. This resulted in a much reduced loss in fish over the same period. For protection studies, 50 fish per group were vaccinated.

The results of trial 2 resulted in only one vaccine, giving significant protection, the *F. psychrophilum* bacterin, compared to the unvaccinated, saline control group ($P = 0.001$). The bacterin resulted in a 58 % RPS. Unlike trial 1, killed *E. coli* did not protect.

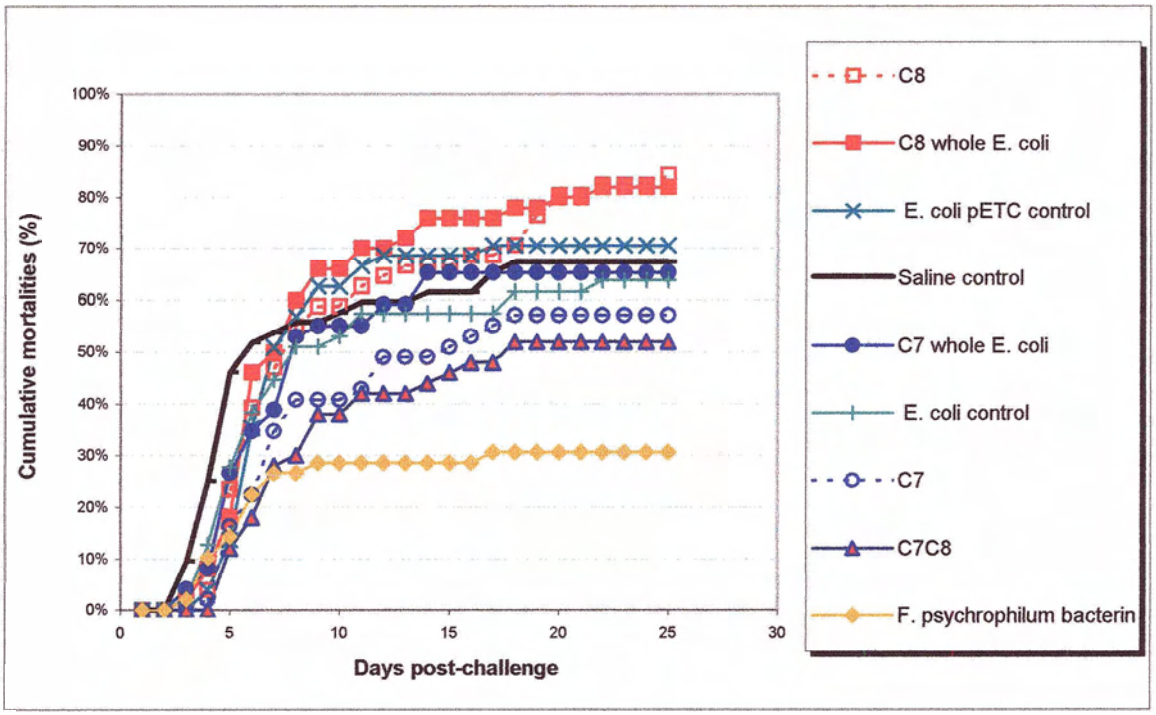

Figure 39. Vaccine trial 2 cumulative mortalities. Groups of 50, 0.5 g rainbow trout were injected (i.p.) with various vaccine preparations or saline as a control. After 400 dd, fish were challenged (i.p.) with ~5 x 10^6 *F. psychrophilum* cells.

Table 17. Vaccine trial 2 summary

Fish size at vaccination	0.50g	
Fish size at end of trial	**1.12g**	
Challenge dose (50 µl)	7X conc. A_{600} 0.349, 5.3 x 10^6 cfu	
Vaccine / Control	**Cumulative mortality**	**RPS (%)**
C8	39/47 (83 %)	-26 %
C7	27/48 (56 %)	15 %
C8+C7	24/48 (50 %)	24 %
E. coli C8	40/49 (82 %)	-24 %
E. coli C7	31/48 (65 %)	2 %
F. psychrophilum bacterin	13/47 (28 %)	58 %
E. coli + plasmid control	33/48 (69%)	0 %
E. coli control	30/47 (64 %)	0 %
Saline control	33/50 (66 %)	0 %

Vaccine trial 3

The aims of trial 3 were to; 1) test the effectivemess of incorporating a measles virus epitope in to the recombinant vaccines C7 and C8; 2) test the CM fusion of antigen recognized by the host, TX20'; 3) test the effectiveness of one vaccine, C8M in larger, 3 g rainbow trout fry; 4) repeat bacterin vaccine trials with *F. psychrophilum* and *E. coli* whole cells; 5) investigate the effect of adjuvant.

Eleven groups of 75, 0.65 g rainbow trout fry and two groups of ~ 35, 3 g rainbow trout fry were used in vaccine trial 3, summarized in Table 18. One group from each size was injected with a saline solution as a control. The challenge dose for the 0.65 g fry was 7.77×10^7 cfu/50 µl dose. The mortality in the unvaccinated, saline control group was 85 %. The challenge dose for the larger, 3 g fry was 1.54×10^8 cfu/100 µl dose which resulted in 87 % mortality in the unvaccinated saline control group. These values was used to calculate relative percent survival (RPS) of the vaccinated groups. The cumulative mortalities over time for the 0.65 g and 3 g fry and shown in Figure 40 and Figure 41 respectively. Of note was that the fry obtained for vaccine trial 3 were more uniform in size, in comparison with fry used in the two previous trials.

The best protection was offered by the C8M recombinant protein and the *F. psychrophilum* bacterin, both of which resulted in a RPS fo 89 %. In larger, 3 g fry the same vaccine and dose resulted in a cumulative mortality of 87 %, resulting in an RPS of 63 % (Figure 41).

Adjuvant effect

The graph of cumulative mortalities shown in Figure 40 showed that all the adjuvenated injections had some protective effect, including the fusion protein controls.

chi-squared analysis on the protection data revealed that only 5 of the 10 adjuvenated injections provided significant protection over the adjuvant control ($p = 0.001$). These samples were the *F. psychrophilum* bacterin, C8M, C protein, C7 and C7M.

Effect of incorporating measles epitope

The addition of an epitope from measles virus (MV) resulted in the vast improvement in the protection of C8, which had an RPS of 39 %, which increased to 89 % for C8M. However, the addition of the MV epitope reduced the level of protection offered by C7 (82 % to 74 % RPS) and TX20' (63 % to 49 % RPS), although in both cases the differences were found to be insignificant ($p > 0.2$).

C protein control

C-protein alone provided significant protection over the adjuvant control ($p = 0.001$), resulting in an RPS of 86 %. However, although this control contained the same overall protein concentration as the other vaccines, the molar equivalent of C-protein in the control was double that of C8, and three times the amount in C7. The increased dose may have led to the increased protective effect of C-protein alone. The CM protein control resulted in a RPS of 72 %.

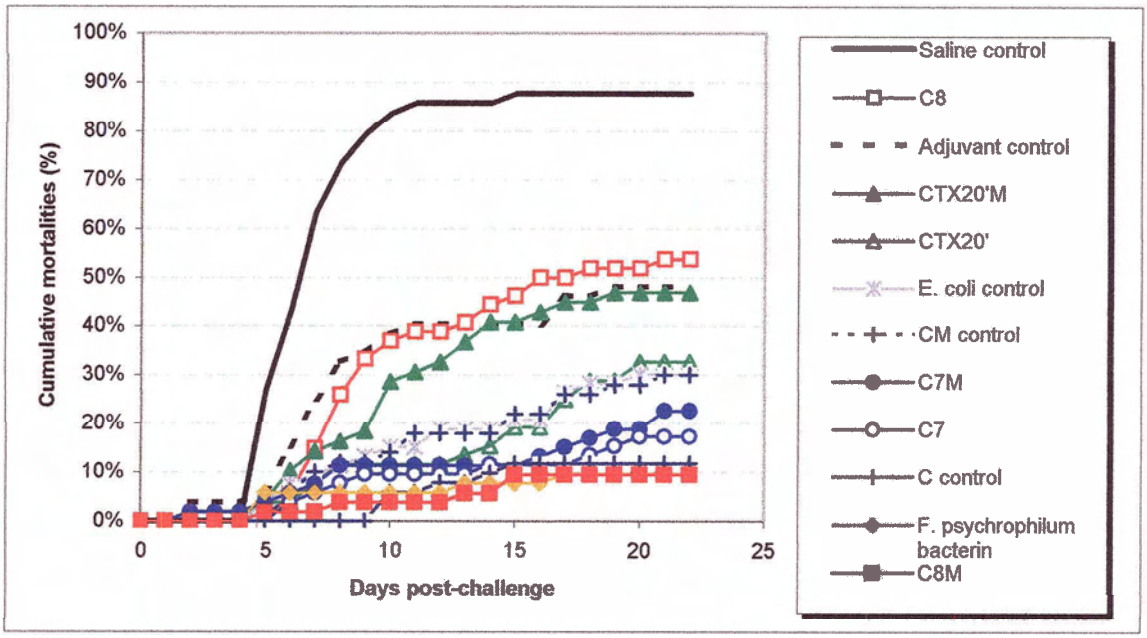

Figure 40. Vaccine trial 3a cumulative mortalities. Groups of 50, 0.65 g rainbow trout were injected (i.p.) with various vaccine preparations or saline as a control. After 400 dd fish were challenged (i.p.) with ~7 x 10^7 *F. psychrophilum* cells.

Table 18. Vaccine trial 3a summary.

Fish size at vaccination	0.65g	
Fish size at end of trial	1.10g	
Challenge dose	5X conc. A_{600} 0.304, 7.7 x 10^7 **cfu**	
Vaccine / Control	**Cumulative mortality**	**RPS (%)**
C8	27/52 (52 %)	39 %
CM8	5/53 (9 %)	89 %
C7	8/51 (16 %)	82 %
CM7	11/50 (22 %)	74 %
CTX20'	16/51 (31 %)	63 %
CMTX20'	20/46 (43 %)	49 %
F. psychrophilum bacterin	5/52 (10 %)	89 %
C protein control	6/51 (12 %)	86 %
CM protein control	11/46 (24 %)	72 %
E. coli control	15/52 (29 %)	67 %
Adjuvant control	22/49 (45 %)	47 %
Saline control	35/41 (85 %)	0 %

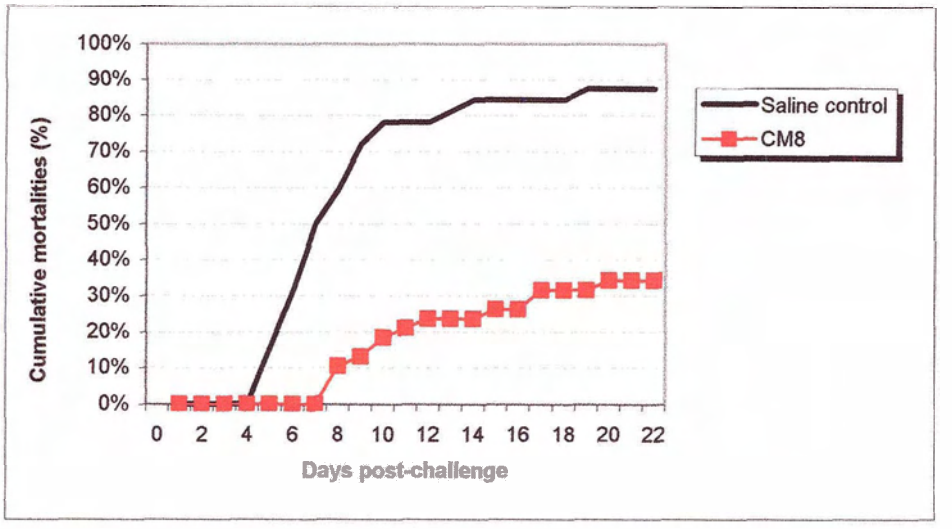

Figure 41. Vaccine trial 3b cumulative mortalities in 3 g rainbow trout. Two groups of ~35, 3 g rainbow trout were injected (i.p.) with either saline or vaccine C8M. After 400 dd, fish were challenged (i.p.) with ~1.5 x 10^8 *F. psychrophilum* cells.

Table 19. Vaccine trial 3b summary

Fish size at vaccination	3 g	
Fish size at challenge	3 g	
Challenge dose / 100 µl	1.54 x 10^8 cfu	
Vaccine / Control	**Cumulative mortality**	**RPS (%)**
CM8	12/37 (32 %)	63 %
Saline control	27/31 (87 %)	0 %

DISCUSSION

The goal of this study was to identify molecules worthy of further investigation as vaccine candidates. The evaluation of potential vaccines is highly dependent on having a reproducible disease model. In this study, a reproducible injection challenge model was developed for 0.7-1 g rainbow trout fry. Previous attempts had provided variable results, until the point along the growth curve at which cells were collected was standardized. Once standardized, challenges were reproducible, enabling the reliable testing of various vaccine candidates.

A high dose of *F. psychrophilum* cells was required in the injection challenge model. The high dose required for significant mortality in rainbow trout fry has been observed elsewhere: Madsen *et al* injected 4.5 x 10^5 cfu into 1.5 g rainbow trout fry to achieve 71 % cumulative mortality (120). In rainbow trout averaging 3-6 g, Garcia *et al* obtained ~40 % cumulative mortality following i.p. injection of 4.62 x 10^6 cfu (65). In smaller, 0.35 g rainbow trout, 7 x 10^5 cells resulted in 78 % cumulative mortality (141). The high doses required for significant mortality highlight the lack of similarity with the disease progression in the wild. However, the establishment of a reliable and reproducible injection challenge model, despite the high dose, it pivotal to testing vaccine efficacy.

It stands to reason that the agent of bacterial cold water disease (BCWD) may not kill effectively at the temperature tested here. Fish in the aquatic facility at the University of Victoria are held at 12-13 °C. The optimal temperature for BCWD to occur is between 4 and 10 °C (147). However, in experimental infections, Holt was able to kill between 80

- 90 % of 3.5 g rainbow trout at temperatures ranging between 3 and 12 °C upon s.c. injection of 4 x 10^6 cells; mortality dropped to 38 % at 18 °C (80). Generally speaking, low temperatures have been observed to have an immunosuppressant effect on fish. In addition to this effect, the bacterium may also be more virulent at certain temperatures. For example, protease production by *F. psychrophilum* measured over a range of 2 to 25 °C and found to be highest between approximately 9 and 15 °C (190). It is conceivable that other pathogenic mechanisms are expressed at low temperatures.

The results of the immersion challenge may reflect the opportunistic nature of *F. psychrophilum* killing. Environmental conditions that weaken the natural defenses, such as stress, overcrowding, diet and temperature, can lead to invasion by opportunistic pathogens. Rainbow trout fry held for one hour in approximately 10^7 cfu / ml suffered only 4 % mortality. Similarly, Decostere *et al* were unable to reproduce the disease using immersion challenge (53). Madsen *et al* (120) were able to induce 20 % mortality over the same time period in 1 g rainbow trout fry by immersion in 10^7 cfu / ml. By increasing the dose and stressing the fish with formalin treatment, mortality was increased to ~55 % after 21 days (120). Wounding the skin has been shown to greatly increase (27 % to 90 %) mortality in immersion challenge of 0.4 g fry (119).

The vaccine trials conducted in this study resulted in significant protection of rainbow trout fry against *F. psychrophilum*. Over the three trials, the most consistent protection was provided by the bacterin vaccine. However, in trial 3, recombinant protein C8M gave the highest protection, with an RPS of 89 % (Table 18). In trials 1 and 3, all adjuvenated injections resulted in protection of rainbow trout fry against *F. psychrophilum*. The adjuvant control in trial 3 resulted in 45 % mortality compared to 85

% in the saline injected group (Figure 40). In addition to their role in enhancing the specific immune response, adjuvants are also believed to elevate non-specific responses, since most are active even when given alone (reviewed in (9)). The incorporation of a measles virus epitope in the recombinant protein vaccines resulted in a great improvement in the protection of one vaccine, C8, whilst reducing the protection of C7 or TX20'.

The lack of overall protection in trial 2 compared to trials 1 and 3 was likely due to the smaller average size of fish vaccinated, being on average 0.5 g, with fish as small as 0.25 g. Obach *et al* also found no protective effect when vaccinating rainbow trout fry under 0.5 g (141). In trial 2, the lack of protection was not attributed to an overwhelming challenge because the saline injected group resulted in 67 % mortality.

The results of these trials also serve to highlight the difficulty in conducting reproducible vaccine trials in such immature fish. Unlike mammalian mouse models, these fish were from out-bred populations and therefore their responses to various vaccines can vary. Also, it is difficult to maintain fish at a constant weight during what would normally be an aggressive growth stage. By attempting to keep the fish at a relatively stable weight, canabalism ensued, reducing the number of fish in trial 1 by 40 % between vaccination and challenge. With fish of this size, uniformity in size is important in any given batch, but is difficult to achieve. Apparently minor variances in size may render some fish incompetent at mounting an effective immune response, while not others. Of note was that fish used in trial 3 were the most uniform in size.

A striking feature of the combined results was the protection conferred by control vaccines, including killed *E. coli* and C-protein. These results imply that the vaccination

of fry had an overall immunostimulatory effect. This may be a function of age. Salmonid fish are not thought to be fully immunocompetent until they reach about 4 g in size (58), and research suggests that younger fish are believed to rely more heavily on innate immune responses (3). To investigate the stimulation of the innate response, future experiments may involve short term trials where protection is tested only days after vaccination. The administration of whole *E. coli* cells in a recombinant vaccine may provide additional non-specific protective immunity. The immunostimulatory effect of LPS was investigated and prior LPS exposure was shown to increase survival or yolk sac larvae (47). In order to protect fish from *F. psychrophilum* disease, fry may be helped by stimulators of innate immunity in addition to a specific vaccine which would benefit them as they mature.

In summary, a reproducible challenge injection model for *F. psychrophilum* was established in rainbow trout fry. Non-specific immune stimulation was observed in fish receiving control vaccines. In addition, injection of the adjuvant alone resulted in a significant drop in mortality due to *F. psychrophilum* challenge. A cross-reactive antigen expressed with a measles virus epitope, C8M, resulted in the highest level of protection against *F. psychrophilum*, with an RPS of 89 %. The C8M recombinant protein expressed in *E. coli* shows promise as a future vaccine. Firstly, the use of *E. coli* for the production of recombinant vaccines would be less expensive than bacterin production, since *E. coli* can be grown to at least fifty times the density of *F. psychrophilum*. This becomes particularly important when oral vaccines are considered. Secondly, C8 is a common antigen to *F. psychrophilum*, *F. columnare* and *T. maritimum,* therefore, broad spectrum protection is expected.

Chapter 7

Development of an Antibody-Based Diagnostic Method for *F. psychrophilum*

INTRODUCTION

Following the establishment of a reliable PCR method to identify *F. psychrophilum*, as described in Chapter 1, our goal was to develop an even simpler and more rapid antibody-based test to distinguish *F. psychrophilum* from related organisms associated with diseased fish. The potential use of polyclonal serum raised against whole cells of *F. psychrophilum* was assessed. Due to the high level of cross reactivity between the species concerned, it became important to find an antigen unique to *F. psychrophilum* for diagnostic purposes. A unique antigen from *F. psychrophilum* was sought to use as the basis of a specific antibody test. For this purpose, the LPS O-antigen of *F. psychrophilum* was purified and polyclonal rabbit antiserum raised. This chapter describes the development of a latex bead agglutination test for the specific identification of *F. psychrophilum*.

RESULTS

Immunofluorescence microscopy of F. psychrophilum F. columnare and T. maritimum.

To test the potential use of polyclonal antisera in discriminating between three fish pathogens, *F. psychrophilum*, *F. columnare* and *T. maritimum* cells were reacted with rabbit antiserum raised against each of the three organisms, labeled with goat anti-rabbit fluorescein isothiocyanate (FITC)-conjugated antibodies and viewed under a fluorescence microscope (Figure 42). Not surprisingly, the most intense labeling in each case was obtained using serum raised against each respective organism. However, bright labeling was also achieved with cross-reactive serum. The results show the high level of cross-reactivity of the three antisera with the three species tested. Negative controls were carried out using the pre-immune serum of each respective rabbit and showed cells that were barely labeled, if at all (data not shown). Any potential use of serum raised against whole cells would therefore require significant cross-absorbing of antisera against all possible cross-reactive species encountered.

Development of F. psychrophilum- specific polyclonal antiserum.

In order to develop a specific antibody-based diagnostic tool for *F. psychrophilum*, antiserum was raised against the LPS O-polysaccharide (OPS) of *F. psychrophilum*. Purified OPS was conjugated to KLH for antibody production and to BSA for assaying the titer of the antiserum. High titre antiserum was obtained and tested for the ability to specifically and exclusively identify *F. psychrophilum*.

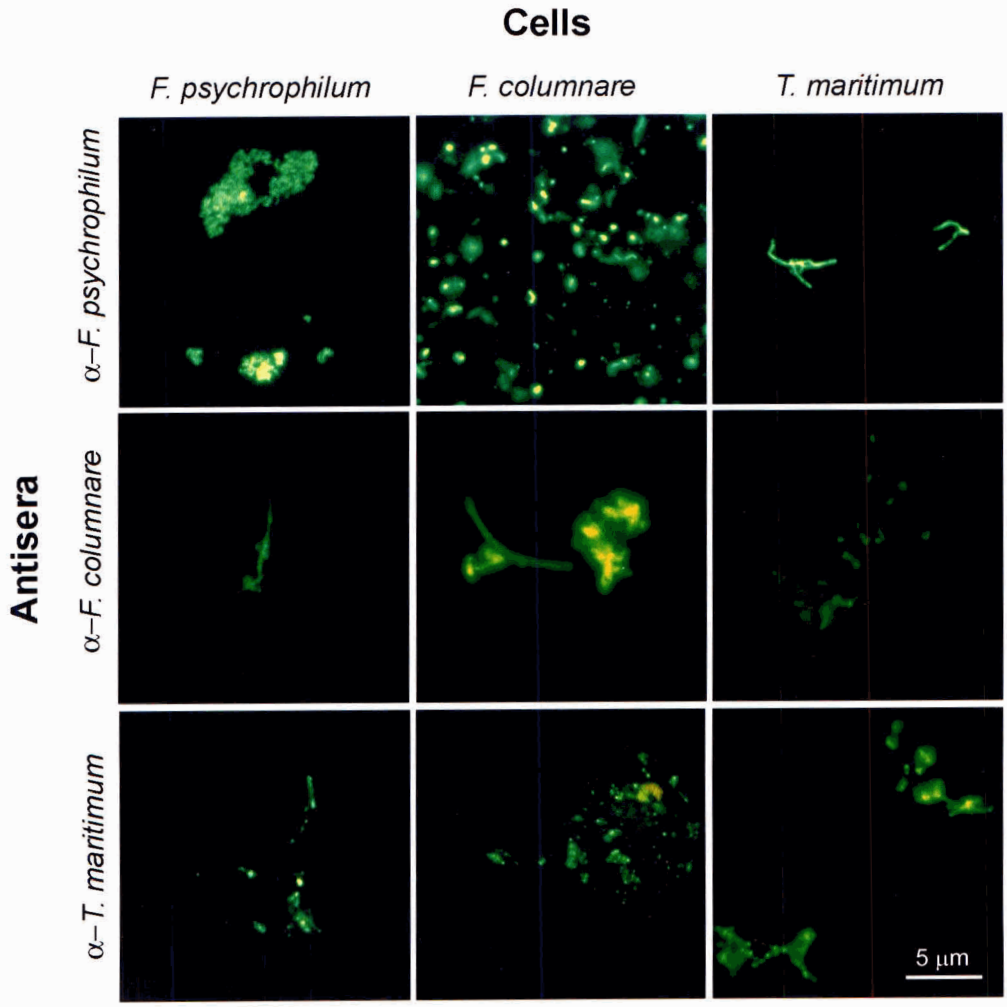

Figure 42. Immunofluorescent labeling of *F. psychrophilum*, *F. columnare* and *T. maritimum*. Rabbit antisera were raised against formalin fixed whole cells of either *F. psychrophilum*, *F. columnare* or *T. maritimum*. Each strain was subsequently reacted with each of the three antisera. Goat anti-rabbit secondary antibodies conjugated to fluorescein isothiocyanate (FITC) were used to visualize the cells under a fluorescence microscope.

Detection of F. psychrophilum by anti- F. p. OPS serum

The ability of the rabbit anti-*F. psychrophilum* OPS serum to differentiate *F. psychrophilum* from other related organisms was tested in an ELISA based assay (Figure 43). 96 well plates were coated with live whole cells (10 μg (wet wt) per well) of *F. psychrophilum*, *F. columnare*, *F. aquatile*, *F. johnsoniae* and *T. maritimum* and reacted with the anti-*F. p.* OPS serum. The results show that the anti-OPS serum can readily distinguish *F. psychrophilum* from the other species tested. Controls were carried out with pre-immune serum as well as no secondary antibody, no primary antibody and no antigen controls. All control reactions were negative.

Development a of latex bead agglutination assay for F. psychrophilum.

An agglutination assay using coloured latex beads was developed for *F. psychrophilum*. Rabbit anti-OPS antibodies were purified from whole serum and adsorbed onto latex beads. Non-specific binding of bacterial cells, in particular *F. columnare* binding to blocked, antibody-free latex beads, was overcome by prior heating of the cells (50 °C 10 min or boiling 1 min). Heating of bacterial cells did not adversely affect the specific binding of *F. psychrophilum* to the antibody-adsorbed beads. Agglutination occurred with all *F. psychrophilum* strains tested but was negative for *F. columnare*, *T. maritimum*, *F. johnsoniae*, *F. aquatile* and all other unknown, non-*F. psychrophilum* species isolated from diseased fish (Table 20). Examples of typical positive and negative agglutination reactions are shown in Figure 44 below.

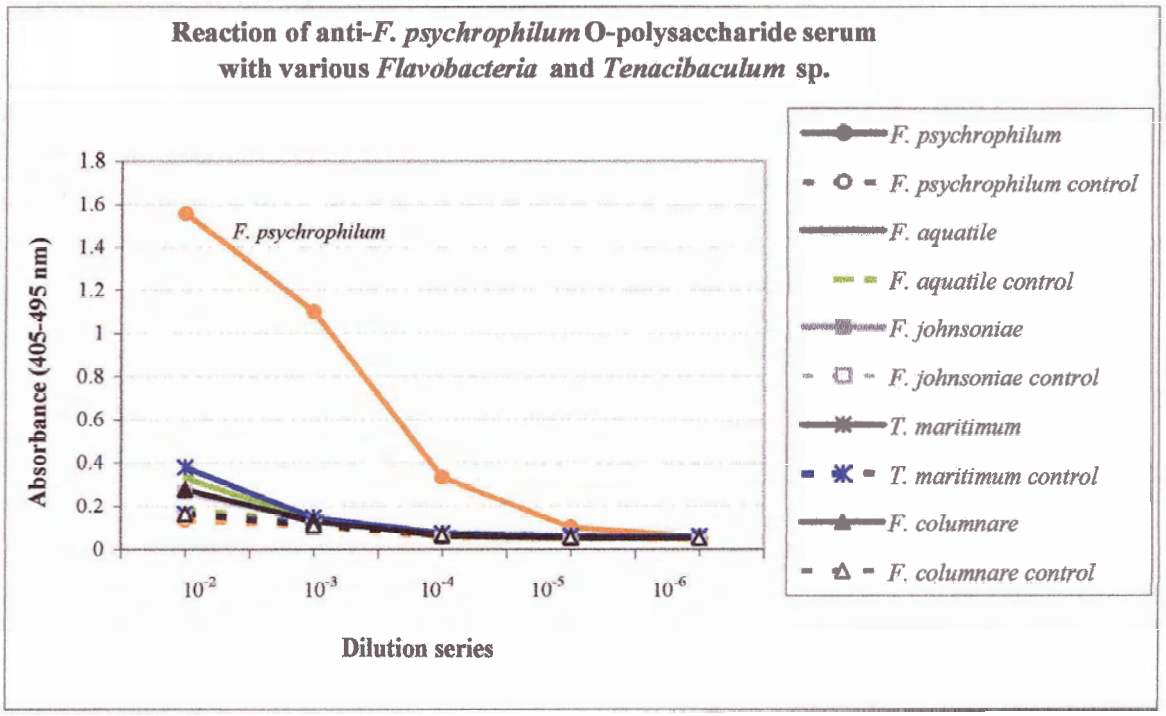

Figure 43. Reaction of anti-*F. psychrophilum* O-polysaccharide (OPS) serum with *F. psychrophilum* and related species. 96-well plates were coated with whole cells of *F. psychrophilum*, *F. columnare*, *T. maritimum*, *F. johnsoniae* and *F. aquatile*. Cells were reacted with anti-*F. psychrophilum* OPS serum at a series of dilutions followed by goat anti-rabbit conjugated alkaline phosphatase.

Table 20. Specificity of latex bead agglutination assay for *F. psychrophilum*.

Strain	Agglutination	Strain	Agglutination
F. psychrophilum UP97/01	+	*F. psychrophilum* 911126-3	+
F. psychrophilum 259-93	+	*F. psychrophilum* 910614-3	+
F. psychrophilum ATCC 49418	+	*F. psychrophilum* 910614-5	+
F. psychrophilum PBS9701	+		
F. psychrophilum PBS9702	+	unknown PBS9704	-
F. psychrophilum PBS9703	+	unknown PBS9705	-
F. psychrophilum PBS9708	+	unknown PBS9706	-
F. psychrophilum PBS9709	+	unknown PBS9707	-
F. psychrophilum PBS9710	+	unknown PBS9711	-
F. psychrophilum 970522-1	+	unknown PBS9712	-
F. psychrophilum 960104-1	+	unknown PBS9713	-
F. psychrophilum 950824-1	+	unknown PBS9714	-
F. psychrophilum 950920-1	+	unknown PBS9715	-
F. psychrophilum 951027-1	+	unknown PBS9716	-
F. psychrophilum 911209-2	+		
F. psychrophilum 911209-1	+	*F. aquatile* ATCC 11947	-
F. psychrophilum 910619-1	+	*F. columnare* ATCC 43622	-
F. psychrophilum 910614-2	+	*F. johnsoniae* ATCC 29585	-
F. psychrophilum 911126-2	+	*T. maritimum* ATCC 43397	-

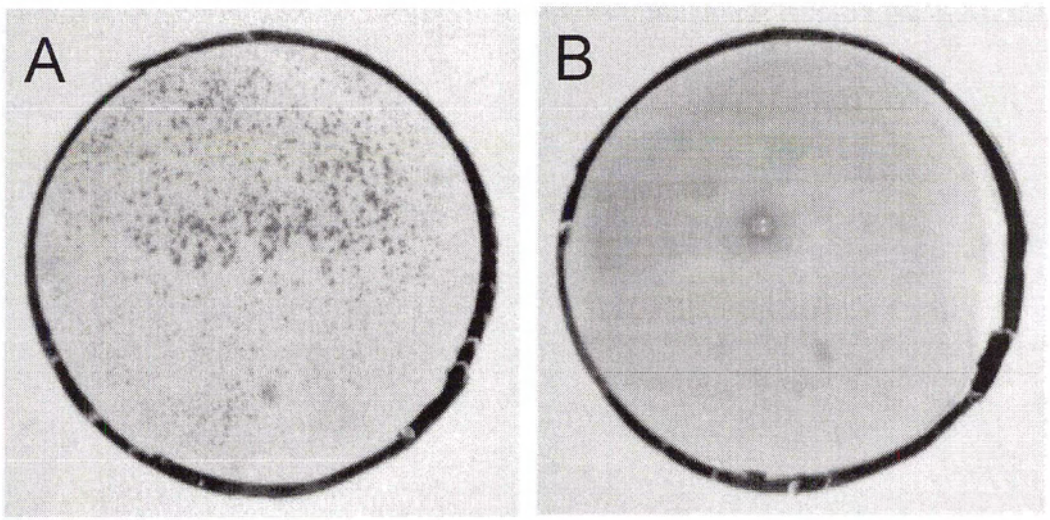

Figure 44. Latex bead agglutination assay for *F. psychrophilum*. Typical results with anti-*F. p.* OPS antibody adsorbed latex beads. A) positive agglutination test following reaction of heated *F. psychrophilum* cells B) negative agglutination test following reaction with heated *F. columnare* cells. PBS control tests were negative for agglutination.

DISCUSSION

The highly cross-reactive nature of flavobacterial antigens was demonstrated by immunofluorescence and led to the development of a specific antibody-based diagnostic. The different strains tested shared numerous cross-reactive antigens which can hinder accurate antibody-based identification. Our goal was to bypass the need for extensive cross-absorption with a variety of bacteria by raising polyclonal serum against an antigen unique to *F. psychrophilum*. The O-antigen of LPS was selected due to the uniqueness of O-antigens in nature.

Several reports in the literature exist citing the use of polyclonal antibodies in ELISA and IFAT procedures to identify *F. psychrophilum* from fish tissue. In one published procedure the rabbit polyclonal antiserum was not cross-adsorbed and only tested against one other related organism (153). Other reports address the need for cross-adsorption (62, 113, 119). However, cross absorption requires the selection of all possible organisms that may react. A bacterial fish pathogen, *Aeromonas salmonicida*, not belonging to the *Flavobacterium-Cytophaga* group was also found to cross react with anti-*F. psychrophilum* serum (62), however, this may be due to non-specific antibody absorption by the S-layer. Due to the high level of cross-reaction of anti-*F. psychrophilum* polyclonal serum with other fish pathogenic species, it would seem that use of pAb in an effective diagnostic would first require significant prior cross adsorption to prevent false positives.

In addition to other potential pathogens that may result in false positive reactions, normal flora of fish may also be reactive. The genera *Pseudomonas*, *Aeromonas*, *Vibrio*

and *Cytophaga* and *Flavobacterium* contain fish pathogens and are also commonly isolated from diseased fish. When isolating bacteria from diseased fry, given their size, it is possible that normal microflora of fish eggs, skin, gills and intestine may also be isolated. Intestinal microflora of fresh water salmonids consists mainly of *Aeromonas*, *Enterobacteriacae*, *Flavobacterium* and *Pseudomonas* sp. (140, 188, 216). Microflora of salmonid eggs, gills and skin has been found to comprise mainly of *Pseudomonas*, *Aeromonas*, *Cytophaga*, *Flavobacterium* and *Vibrio* sp. (20, 84, 140, 187). Conceivably, members of the natural microflora of fish, especially *Flavobacterium* and *Cytophaga* species, could also cross-react with PAb raised against *F. psychrophilum.*

The immunofluorescent labeling results (Figure 42) demonstrate the unreliability of using polyclonal immune serum to detect *Flavobacteria.* In order to differentiate these species reliably, extensive cross-adsorption of the immune serum against the different strains present would be needed. Otherwise, monoclonal antibodies or polyclonal serum raised against a unique antigen must be used.

To get around the problem of cross-reactivity of anti-*F. psychrophilum* serum, and to avoid the need to cross absorb the serum against other potentially cross reactive species, a unique antigen, the LPS O-polysaccharide, was sought. Rabbit immune serum raised against the OPS was clearly able to differentiate *F. psychrophilum* from the other species tested in an ELISA based assay. A greater antigen load was required, compared to ELISAs performed with anti-whole cell serum (10 μg instead of 1 μg per well). However, this is not surprising given that the polyclonal serum was raised against just one molecule.

The ease of identification of *F. psychrophilum* using the ELISA was duplicated in the development of a latex bead agglutination test for *F. psychrophilum.* Over a range of cells densities, only *F. psychrophilum* tested positive. The non-specific binding of *F. columnare* to blocked, antibody-free latex beads may have been due to hydrophobic interactions between surface molecules and the beads. Heating the cells may have denatured proteins involved in such hydrophobic interactions. Charge is not believed to be a factor in non-specific binding because *F. columnare* cells bound the beads over a wide range of pH (data not shown).

Here we have shown that using polyclonal antiserum raised against the O-antigen of *F. psychrophilum*, we were able to easily and specifically identify *F. psychrophilum.*

General Discussion

The goal of this work was to further our understanding of the important fish pathogen *F. psychrophilum,* the etiological agent of rainbow trout fry syndrome (RTFS) and bacterial cold water disease (BCWD) in teleost fish. As a comprehensive study, this work covered aspects of growth, speciation and the identification and characterization of several antigens, culminating in recombinant vaccine trials in rainbow trout fry.

Over the last decade, considerable effort has been made to re-organize the taxonomic branch of the family *Flavobacteriaceae.* Previously, much confusion surrounded their taxonomy, making identification of pathogens a difficult and confusing task. This work has contributed several methods for the identification of *F. psychrophilum*, based on PCR, Congo red sensitivity and unique antigens.

Our work on growth of *F. psychrophilum* revealed the misconception that this micoorganism was non-fermentive. Several reports demonstrate that *F. psychrophilum* does not metabolize carbohydrates (27, 24, 39, 86, 114), however this is likely due to the methodology for testing carbohydrate metabolism. We have shown that at least two sugars are metabolized. Further work in this area may identify other carbohydrates metabolized by *F. psychrophilum*, although in the literature it is described as not degrading complex carbohydrate. The discovery that *F. psychrophilum* utilizes maltose led to the ability to increase the growth rate *in vitro*. With improved growth rate came the opportunity to attempt large scale growth in a fermentor at reduced risk of contamination and cost. In turn, the ability to grow large quantities of *F. psychrophilum*

led to the purification of sufficient LPS to enable structural analysis of the O-antigen of *F. psychrophilum.*

Western blot analysis of *Flavobacterial* fish pathogens revealed extensive cross-reactivity between species. The cross-reactive nature of these microorganisms led to the search for a unique antigen of *F. psychrophilum* that may be used for diagnostic purposes. The unique antigen was determined to be carbohydrate and a collaboration was initiated to further elucidate the unique carbohydrate antigen of *F. psychrophilum.* This investigation led to the first structure determination of O-polysaccharide from *Flavobacterium* sp. By exploiting the unique nature of the *F. psychrophilum* OPS, an antibody based, latex bead agglutination assay was developed for the rapid and easy identification of *F. psychrophilum.* The discovery of unusual sugars in the *F. psychrophilum* OPS led to further collaborations to elucidate OPS structure of related fish pathogens *Tenacibaculum maritimum* (198) and *Flavobacterium columnare* (197). In turn, these will likely result in latex agglutination assays for diagnosis of these pathogens.

Through the construction and immunological screening of a DNA expression library, over 15 kb of *F. psychrophilum* genomic DNA was sequenced. Analysis of the DNA sequence resulted in the identification of 13 hypothetical proteins, including a putative ribosomal protein and a histone-like protein, which were cloned and expressed in *E. coli* for protection studies in fish. These comprise the first known proteins from *F. psychrophilum*, from which the only prior DNA sequences determined were rRNA genes and a partial gyrase B gene sequence used for phylogenetic analysis and identification (89, 134).

Although rabbit antiserum has been successfully employed in the past to identify protective antigens in fish, we sought to identify an antigen recognized by the host immune system. Limited quantities of convalescent rainbow trout serum prevented its use in the screening of the *F. psychrophilum* DNA expression library. Instead, a proteomics approach was taken to identify a 20 kDa antigen strongly recognized by the host. Degenerate PCR (dPCR) was performed to identify a partial gene sequence, based on peptide sequence obtained from Q-TOF mass spectrometry analysis. Ultimately, DNA encoding 179 aa of the 20 kDa protein was sequenced and cloned for the purposes of *F. psychrophilum* protection studies in fish.

Paramount to the testing of any potential vaccine candidate is the prior establishment of a reproducible disease model. We have developed a reliable injection challenge model for RTFS in 1-2g rainbow trout fry. The high numbers of bacteria required to affect 70 % mortality in fry highlight the inadequacy of *in vitro* propagation and infection. More work is required to better understand the pathogenic nature of *F. psychrophilum* in wild fish stocks and the effects of temperature and stress in the onset of RTFS.

The three recombinant *F. psychrophilum* proteins tested as potential vaccine candidates in rainbow trout fry were all shown to confer protective immunity. The immunity varied depending on the size of the fish at vaccination, highlighting the difficulties associated with working with such immature fish. Despite this, the protection data were extremely encouraging. Interestingly, the incorporation of a T-cell epitope from measles virus effected each of the vaccines differently.

The cross-reactive nature of *Flavobacterial* fish pathogens, considered a hindrance for diagnosis, may be of benefit if exploited in terms of vaccinology. One of the protective protein antigens cloned from *F. psychrophilum*, FP91, was found to be highly cross-reactive with serum raised against *F. columnare* and *T. maritimum,* suggesting that FP91 may also confer protective immunity against these two related fish pathogens. In the future, this work may lead to the development of a vaccine against a variety of *Flavobacterial* pathogens. However, further work is needed to develop disease models for each of these bacterial pathogens before suitable candidates for a broad-based vaccine can be tested.

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Appendix

F. psychrophilum DNA Sequences

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 151  CCACCTTTTG GTTTTCAATT TTTTTATCTG TTTTGGGAGA ATAAAAAGCA
 201  ACGGGTACTA CTCTATTTCT CTCTTTGTCA AATAAATTTA CAGTATCTAG
 251  TTTAACTTTA AATGTCGGAA TAGTGTTTTG AAGATGTACG GCGTCCGTGT
 301  TTTTAATACA ACTTATTAAA AATAAGCTTA CTAGAATTTT AAAATAGATT
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 401  TTCCACATGA TCGTTTACCA TTCCTGTGGC TTGCATGTAT GCGTACATCA
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3501  TTTGGCGTTG GTTTGGCTCG TATGGTTAGA ACTATTCGTG AGAGAATGAA
3551  CGTTAGAGAT AACGAGGTGT TTACACCTAT CGATTTGATT AATGCTAAAA
3601  CATTATCATC GGTAATCAAC TCTTTCTTTG GTACAAACCA GTTGTCTCAA
3651  TTTATGGATC AAACCAATCC ATTAGCCGAG ATTACACACA AGCGTCGTTT
3701  ATCTGCCCTA GGACCAGGTG GTCTTTCTCG TGAAAGAGCT GGTTTTGAGG
3751  TACGTGACGT TCACTACACG CACTACGGAA GACTTTGCCC AATTGAAACT
3801  CCTGAGGGAC CAAACATTGG ATTAATTTCA TCTTTGGGTG TTTACGCAAA
3851  AGTAAACGGA ATGGGTTTTA TCGAAACGCC GTATCGTAAA GTAACAAATG
3901  GTGTTGTTGA TTTAACTTCT GTCCCTAAAT ATTTAAGTGC CGAAGAAGAA
3951  GAAGGAATGT TAATTGCACA AGCAAACATT CAAATGGATG AAAATGGTAA
4001  AATTACTGCC GATAACGTTA TTGCTCGTCA AGAAGGTGAT TTTCCAGTAA
4051  TAGACCCAAC TGCTGTTCAT TATACCGATG TTGCGCCTAA TCAAATTGCA
4101  TCTATCTCAG CTTCATTAAT TCCTTTCTTG GAACATGATG ATGCGAATAG
4151  AGCCTTGATG GGATCTAACA TGATGCGTCA AGCTGTTCCT CTTATTCGCC
4201  CTGAAGCACC AATTGTTGGT ACAGGTTTAG AGCGTCAAGT AGCCTCAGAT
4251  TCTAGAGTAT TAATAAATGC TGAAGGAGCT GGAGTTGTAG AATATGTTGA
4301  TGCAGATATG ATTACCATCA AATACGATAG AACGGATGCC GAAAGAGCAG
4351  TAAGTTTCGA ATCTGATGAG AAGACATATA AATTAATTAA ATTTAGAAAA
4401  ACGAATCAAG GAACTTCTAT CAACTTAAAA CCTATCGTAA GAAAAGGGGA
4451  TAAAGTAATA AAAGGACAAG TGCTTTGTGA AGGATATGCA ACACAAAACG
4501  GAGAGTTAGC TTTAGGTCGT AACCTAAAAG TAGCCTTTAT GCCTTGGAAA
4551  GGGTATAACT TCGAGGATGC GATTGTAATT TCTGAAAAAG TAGTTCGTGA
4601  TGATATTTTT ACCTCAATCC ACGTAGATGA TTATTCATTA GAAGTTAGAG
4651  ATACAAAATT AGGTAACGAA GAGTTGACTA ATGATATTCC TAATGTTTCT
4701  GAAGAAGCTA CCAAAGATTT AGATGAAAAC GGAATGATTA GAATTGGTGC
4751  CGAAGTTAAA CCTGGCGATA TTCTAATAGG AAAAATTACA CCAAAAGGAG
4801  AATCAGATCC AACTCCAGAA GAGAAATTGC TTCGTGCCAT CTTCGGGGAT
4851  AAAGCTGGTG ATGTAAAAGA TGCTTCATTA AAAGCTTCTC CATCACTTCA
4901  TGGTGTTGTT TTAGATAAAA AACTATTCGC AAGAGCAGTA AAAGACAAAC
4951  GTAAACGTAC ACAAGATAAA GACGCATTGT CTGATCTGGA AATGGAATTT
5001  GAAGTTAAAT TTGTTGAATT AAAAGACAAA TTAGTAGACA AATTATTTAC
5051  CATAGTAAAC GGAAAAACAT CGCAAGGTGT GATGAATGAT TTGGGTGAAG
5101  AAGTTTTACC AAAAGGTAAA AAATATACTC AAAAAATGTT ATATGCAGTT
5151  GAAGATTTTG CTCACTTAAC AAGAGGACAA TGGGTTGCTG ACGATGAAAC
5201  CAATAAAATG GTGAACGACT TAATTCATAA CTACAAAATT AAGTTAAACG
5251  ACCTTCAAGG CGCATTAAGA AGAGAGAAAT TTACCATTAC AGTTGGAGAT
5301  GAATTGCCAG CAGGAATCTT GAAATTAGCC AAAGTTTATA TCGCCAAAAA
5351  ACGTAAGTTA AAAGTAGGTG ATAAAATGGC GGGTCGTCAC GGAAATAAAG
5401  GAATCGTTGC ACGTATTGTT CGTCACGAAG ACATGCCTTT CCTTGAAGAC
5451  GGAACACCAG TAGATATTGT ATTGAATCCA CTTGGAGTAC CTTCTCGTAT
5501  GAACATTGGT CAGATTTACG AAACCGTTCT AGGATGGGCA GGACAAAACT
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5551    TGGGACGCAA ATATGCAACA CCAATTTTCG ACGGAGCATC ATTAGATCAA
5601    ATAAACGCAA TTACTGACGA AGCTGGGGTA CCACGTTTCG GACACACACA
5651    TCTTTATGAT GGTGGTACAG GAGAGCGTTT CCACCAAGCC GCAACAGTTG
5701    GAGTTATCTA TATGTTGAAA CTTGGACACA TGGTTGATGA CAAAATGCAC
5751    GCACGTTCTA TCGGACCATA CTCACTTATT ACACAACAAC CATTGGGTGG
5801    AAAAGCTCAA TTTGGAGGGC AGCGTTTTGG AGAGATGGAG GTTTGGGCAC
5851    TTGAAGCTTA TGGAGCATCA AGTACACTAC GTGAAATTTT GACAGTAAAA
5901    TCTGATGATG TTATTGGTAG AGCAAAAACT TACGAAGCAA TCGTGAAGGG
5951    AGAAACTATG CCAGAACCAG GATTACCTGA ATCGTTCAAT GTATTAATGC
6001    ATGAACTAAA AGGTCTTGGA TTAGATATTC GTTTAGAAGA ATAAAAAATC
6051    TCGAAAGTCG AAAGTCAAAA GACTTTCGAC TTTCGATTTT TCAACTAATT
6101    TTAATAAGTT TTTAGGATTT ATACCCGTAA TCCGTTCCGA TTTTTTATTA
6151    ATATTTAATA ATTAGCACTT CAATATTTAT TTGAAGTTTA GAGAAGTAAT
6201    CCGAGGTAGT GTTTAAGTCA TAAAGTCAAA TGTCATAAAG TTTTAAAGTC
6251    TCGAATGACT TTTCGACCTT AGACTTTCAA ACTTTCGACG TAAATAAAAT
6301    CAATTTTTAA TTGCAAATAA ATCAATAGTA AAAACTATGA TGGAGAATAG
6351    AAACAATAAT AATAACAGAG ACAAGAATCC AGTTAAAAGA TTCAATAAAA
6401    TCTCTATAGG ATTAGCTTCA CCAGAATCTA TCTTGGCAGA GTCAAGAGGA
6451    GAGGTGTTAA AGCCGGAAAC TATCAACTAT AGAACGCATA AGCCAGAACG
6501    TGATGGTCTT TTCTGTGAAC GAATTTTCGG ACCTGTAAAA GATTTCGAAT
6551    GTGCTTGTGG AAAATACAAA AGAATCCGAT ACAAAGGAAT CGTTTGTGAC
6601    CGTTGTGGTG TAGAAGTCAC TGAGAAAAAA GTACGTCGTG ATAGAGTAGG
6651    ACACATTAAT CTTGTTGTGC CAATCGCTCA TATCTGGTAT TTCCGTTCAC
6701    TTCCAAACAA AATTGGGTAC ATTCTTGGAT TGCCATCTAA GAAATTAGAT
6751    ATGATTATTT ACTACGAAAG ATATGTAGTA ATTCAAGCGG GTATTGCTCA
6801    AAATGCTGAA GGAGAATCTA TCAAAAGATT AGATTTCTTA ACAGAAGAAG
6851    AGTACTTAAA TATTCTTGAT ACTCTTCCAG CTGATAACCA ATATTTAGAT
6901    GATTTTGATC CTAATAAATT TGTTGCCAAA ATGGGAGCAG AGTGTATTAT
6951    GGACTTATTA GCACGTATCA ATCTAGACGA ATTGTCTTAC GATTTGAGAC
7001    ACAAAGCAAA TAACGAAACA TCTAAGCAAA GAAAAACAGA AGCCTTAAAA
7051    AGACTTCAAG TGGTTGAATC TTTCCGTGAG TCTAACAAGA ACAGAGAGAA
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7151    TACGCCCGCT TGTACCACTA GACGGTGGTC GTTTTGCAAC TTCAGATTTA
7201    AATGACTTAT ACCGTCGTGT AATTATACGT AACAACCGTC TAAAAAGATT
7251    AATGGAAATT AAAGCTCCAG AAGTAATCTT AAGAAACGAA AAACGTATGT
7301    TGCAGGAATC AGTAGATTCA CTTTTCGATA ATACTCGTAA AGCTTCTGCT
7351    GTAAAAACGG AATCTAACAG ACCGTTAAAA TCCCTTTCTG ATTCCTTAAA
7401    AGGAAAACAA GGTCGTTTCC GTCAAAACTT ACTAGGAAAA CGTGTCGATT
7451    ATTCTGCTCG TTCGGTAATT GTTGTTGGAC CTGAATTAAG ATTATTCGAA
7501    TGTGGTATCC CAAAAGATAT GGCGGCCGAA TTATACAAAC CATTCGTTAT
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7751    TGCACCCTTT AGTTTGTACA GCTTTTAATG CCGATTTCGA TGGAGATCAG
7801    ATGGCAGTTC ACTTGCCACT TGGACCAGAG GCTATTCTAG AAGCACAATT
7851    GTTAATGTTG GCTTCACACA ATATCTTAAA TCCAGCAAAT GGTGCGCCAG
7901    TTACTGTACC TTCTCAAGAC ATGGTACTTG GTCTGTATTA CATGACCAAG
7951    GAACGTTTAA CAACCGAAGG ACACATTATT CTAGGACAAG ATTTAGTTTT
8001    CTATTCTGCC GAAGAAACAA ATATTGCGCT CAACGAAGGA CGAGTAGAAC
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8101    TTAGTATATA AAATTATACA AACCACTGCC GGTCGTGTAT TATTTAATGA
8151    AGTAGTACCA GAAGCAGCAG GTTATATAAA TGACGTATTA ACTAAGAAAA
8201    ACTTACGTGA TATTATTGGG AAAATCCTTG CAGTAACAGA TGTTCCTACA
8251    ACGGCAGCTT TCCTTGATAA CATTAAAGAT ATGGGTTATA AATTTGCCTT
8301    CAAAGGTGGA TTATCATTCT CTCTTGGTGA TATTAGAATT C
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P8 SEQUENCE

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  51  AGCATCGGGT AGAGCTAGAA TTACTGTTTT TAGAAAAGAT GGCGGTTATT
 101  ACTTACCCGA AAACAACAAT GTAGCTTATG ATATTTCAGT TTCTAAAATT
 151  GAAAAACCGC TTTACGCAAT TCAAAAAGCA AATTATGAAG TCGATTTATA
 201  TAAAGATTTT TATATCTCGA AACAGCTATT TTCTACACTG AAAACAACAA
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 301  GATAATTGCA TTCTAATTAA TGAAGATAAG AATGTAATCG AAGCTTTAAA
 351  CGGGAATATT TTTATGCTAA AAGAAGGGGT TTTATATACC CCTCCAATTT
 401  CTGAAGGTTG CTTAAATGGT ATCATGCGAA AGCAAATTTT ATCTTTAGCA
 451  AAAACGATTG AAAATATTGA AATTACTGAA GCGGTAATTT CCCCTTTCGA
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 801  CAATTGGGCT GCATCCCAGC CCGAATATCC TAGGAAAAAG CGAATATTAT
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1701  CTTTATTTTT TTGTTAATAA ATAGGCTTTT TTAATTTAAA AAAAACAAGT
1751  TACGCCTTTA AAATACCATA GCAAACTCAG AAAAAGTGAG CCCATTTAGT
1801  AATTCGTGAG CCATCATTTT CTTTTTTCCT GGAAATTGTA AAGACAATAC
1851  CTGAATAAAA CCGCCAAGAA CTGCAATTTT TATTTCTTTC TTGGTCGTAA
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2101  TTTATATCTG ACGTATCTTC CTGAATTGTG GTGACCACAT TACCATGGGC
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2201  TATCATGAAG GTCGCCTAAG TTTTCAGATT CGGAAATTTC TAATTCTTTA
2251  CTTAAAATCA TGGCACCAGT ATCTATTTTG TCATCAATAA AAAAAGTAGT
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2651  CTAGAATTC
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