Characterisation of *Flavobacterium psychrophilum*, the causative agent of rainbow trout fry syndrome

By

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in memory of my brother-in-law

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DECLARATION

I hereby declare that this thesis has been composed entirely by myself and has not been previously submitted for any other higher degree or qualification.

The work of which it is a record has been performed by myself, and all sources of information have been specifically acknowledged.

Md. Ali Reza Faruk

In the name of Almighty Allah, the compassionate and merciful

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LIST OF ABBREVIATIONS

ABC	Avidin biotin peroxidase complex
AHL	*Arachis hypogaea* peanut
ATCC	American type culture collection
BCWD	Bacterial cold water disease
BGD	Bacterial gill disease
BSA	Bovine serum albumin
cfu	Colony forming units
CLB	*Cytophaga*-like bacteria
Con A	Concanavalin A (*Canavalia ensiformis*) lectin
ddH_2O	Double distilled water
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
ECA	*Erythrina cristagalli* coral tree
ECP(s)	Extracellular product (s)
EDTA	Ethylene diamino tetra-acetic acid
ELISA	Enzyme linked immunosorbent assay
FCS	Foetal calf serum
FITC	Fluorescine isothiocyanate
GML	*Glycine max* soybean
HGL	*Dolichos biflorus* horse gram
HMW	High molecular weight
HRP	Horseradish peroxidase
HSWB	High salt wash buffer
IFAT	Indirect fluorescent antibody technique
IgG	Immunoglobulin G
IM	Intramuscular
IOA	Institute of Aquaculture
IP	Intraperitoneal
IU	International unit
IV	Intravenous
IROMP	Iron regulated outer membrane protein

kDa	Kilodalton
LD_{50}	Lethal dose 50%
LEL	*Lycopersicon esculeulentum* tomato
LPS	Lipopolysaccharide
LSWB	Low salt wash buffer
MAb	Monoclonal antibody
MAOA	Modified Anacker and Ordal agar
MAOB	Modified Anacker and Ordal broth
MIC	Minimum inhibitory concentration
mrbc	Mouse red blood cells
MW	Molecular weight
NCIMB	National Collection of Industrial and Marine Bacteria
OD	Optical density
O-F	Oxidative or Fermentative
OMP(s)	Outer membrane protein (s)
PAb(s)	Polyclonal antibody (ies)
PBS	Phosphate buffered saline
PCM	Percentage of cumulative mortalities
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
RTFS	Rainbow trout fry syndrome
SAPU	Scottish Antibody Production Unit
SC	Subcutaneous
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SLS	Sodium lauryl sarcosinate
TBS	Tris buffered saline
TBST	Tris buffer saline plus Tween-20 (0.001 % v/v)
TEMED	N,N,N, N,- tetramethylethylenediamine
TSA	Tryptone soya agar
TSB	Tryptone soya broth
TVL	*Triticum vulgaris* wheat germ
TYES	Tryptone yeast extract salts
UEA-1	*Ulex europaeus* gorse seed

ABSTRACT

Flavobacterium psychrophilum is the causative agent of rainbow trout fry syndrome (RTFS) and bacterial cold water disease (BCWD) in salmonid fish world-wide. Basic information relating to the antigenic and biochemical characteristics, and pathogenicity of the bacterium are lacking in the literature. Therefore, the aim of this study was to characterise *F. psychrophilum* based on phenotypic and serological differences between isolates. The bacterium was also characterised by means of its extracellular products (ECPs). An attempt was made to develop an experimental challenge model for the bacterium.

Phenotyping of the bacterium was based on growth and biochemical characteristics from which it was found that isolates of *F. psychrophilum* appeared homogenous. Intramuscular (IM) challenge was the most effective route for experimentally challenging rainbow trout fry with *F. psychrophilum*. Virulence of the bacterium was determined by injecting rainbow fry IM with different isolates of *F. psychrophilum*. Variations were found in the virulence of the different *F. psychrophilum* isolates when injected into fish by this route. The levels of protease activity and auto-agglutination characteristics appeared to vary between the virulent and non-virulent isolates.

Electrophoretic analysis of whole cell preparations of *F. psychrophilum* showed that the protein and carbohydrate banding patterns of the different isolates were similar regardless of their origin or their virulence to rainbow trout. A substantial amount of carbohydrate was associated with the bacterium. Using a commercial glycoprotein detection kit, two glycoprotein bands were found at 20 and 23 kDa in whole cell preparations of the bacterium. The electrophoretic protein profiles of the outer membrane protein (OMP) preparations of the bacteria were similar between both virulent and non-virulent isolates.

Characterisation of different *F. psychrophilum* isolates by an enzyme linked immunosorbent assay (ELISA) using rabbit antisera raised against a virulent and non-virulent isolate of *F. psychrophilum*, showed that there may be between three and five different serological groups. No association was detected between serotypes and

geographical origin of the strains, the species of host fish from which they were recovered or the virulence of the isolates. The antisera detected common protein and carbohydrate antigens between the isolates with Western blot analysis.

Antigenic differences were found between different *F. psychrophilum* isolates with ELISA and indirect fluorescent antibody technique (IFAT) using monoclonal antibodies (MAbs) developed against the virulent and the non-virulent *F. psychrophilum* isolates. Two MAbs (9H9 and 5A9) cross-reacted with a related species of bacterium *F. branchiophilum,* in the ELISA. Two MAbs (1E5 and 11B2) recognised high molecular weight material in whole cell preparations of the virulent *F. psychrophilum* in Western blot analysis, which also reacted with rainbow trout anti-*F. psychrophilum* sera raised against the virulent isolate of the bacterium. Due to their lack of specificity or sensitivity, both the rabbit sera and the eight MAbs produced in this study were considered unsuitable as diagnostic probes for screening infected RTFS samples.

F. psychrophilum isolates produced varying amount of ECP proteins after 14 days culture in modified Anacker and Ordal's broth (MAOB), which exhibited substantial protease activity for casein and gelatin. However, the ECPs showed only partial haemolytic activity against rainbow trout erythrocytes. Electrophoretic protein and Western blot profiles were found to be very similar between the ECPs of different isolates. The ECP preparations contain glycoprotein molecules of either 20 or 23 kDa. None of the preparations from the virulent and the non-virulent isolates were found to be toxic to rainbow trout fry.

The study suggests that isolates of *F. psychrophilum* are homogeneous in terms of their biochemical and electrophoretic characteristics, while antigenic characteristics varied between the isolates. The bacterium possesses a substantial amounts of carbohydrate and glycoprotein in its cellular and extracellular products.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

General Introduction

1.1. Historical background of *Cytophaga/Flexibacter/Flavobacterium* sp.

Chromogenic, Gram negative, gliding bacteria, belonging to the family Flavobacteriaceae, have been found to be pathogenic for many freshwater and marine fish species world-wide (Amend, 1982). These include bacteria in the genera *Cytophaga, Flexibacter* and *Flavobacterium.* These organisms have been responsible for sustained high mortality in commercially reared fish, and in turn, have resulted in substantial economic loss to the world-wide aquaculture industry (Bernardet and Grimont, 1989).

According to Bergey's Manual of Systematic Bacteriology Volume 3, bacteria in the genera *Cytophaga, Flexibacter, Flavobacterium* and other less well defined bacteria in the family Flavobacteriaceae are generally referred to as *Cytophaga*-like bacteria (CLB) (Reichenbach, 1989). They were first associated with disease in fish by Davis, in 1922 when he observed serious mortalities among small mouth bass (*Micropterus dolomieui*) and common perch *(Perca fluviatilis*) from the Mississippi river, USA. He reported characteristic column-like masses of bacteria gathered at the periphery of tissue sampled from diseased fish and this led him to name the pathogen *Bacillus columnaris.* He did not succeed in isolating the pathogen responsible for these mortalities, however. In 1943, the aetiological agent of the disease was isolated by Ordal and Rucker (1944) from hatchery reared sockeye salmon (*Oncorhynchus nerka*). They classified the organism amongst the gliding bacteria under the name *Chondrococcus columnaris,* because they thought that

microcysts and fruiting bodies were produced by the organism. In 1945, Garnjobst also isolated this bacterium. He re-classified it as *Cytophaga columnaris*, placing it in the family Cytophagaceae as the organism did not produce either fruiting bodies or microcysts. The disease caused by *C. columnaris* was known as columnaris disease. Subsequently, columnaris disease has been recognised throughout the world in a large range of freshwater fish (Austin and Austin, 1993). In 1974, the name of the bacterium was again changed when Leadbetter called it *Flexibacter columnaris,* placing it in the closely related genus *Flexibacter* (Reichenbach, 1989). Finally, in 1996, the bacterium was transferred to the genus *Flavobacterium* on the basis of DNA-rRNA hybridisation data and fatty acid and protein profiles (Bernadet *et al.,* 1996) and designated as *Flavobacterium columnare.*

Cytophaga-like bacteria have been implicated in cold water disease in fish. Borg (1960) isolated and characterised *Flavobacterium psychrophilum* from fish infected with bacterial cold water disease (BCWD). In the USA and Canada BCWD is a condition responsible for large mortalities in salmonids, particularly in coho salmon (*Oncorhynchus kisutch*) (Holt *et al.*, 1993). More recently, the organism has been associated with a systemic disease of rainbow trout (*Oncorhynchus mykiss*) fry and fingerlings in most European countries, known as rainbow trout fry syndrome (RTFS) (Bernardet *et al.*, 1988; Lorenzen *et al.*, 1991; Santos *et al.,* 1992; Sarti *et al.,* 1992; Toranzo and Barja, 1993; Rangdale, 1995). These two disease conditions will be described in more detail below.

Other members of the family Flavobacteriaceae, principally *Flexibacter maritimus*, cause disease in marine farms in Japan (Wakabayashi *et al.,* 1986), black patch necrosis in Dover

sole (*Solea solea*) in Scotland (Bernardet *et al.,* 1990), turbot (*Scophthalmus maximus*) in Spain (Alsina and Blanch, 1993) and in sea bass (*Dicentrarchus labrax*) in France (Bernardet *et al.,* 1994). Several other commercially important marine fish species have been reported to be susceptible to *F. maritimus.* These include black sea bream (*Acanthopagrus schlegeli*), red sea bream (*Pagrus major*), Japanese flounder (*Paralichthys olivaceous*) (Baxa *et al.*, 1988), white seabass (*Atractoscion nobilis*), Pacific sardine (*Sardinops sagax*) and northern anchovy (*Egraulis mordax*) (Chen *et al.,* 1994). Dungan *et al.,* (1989) reported that disease in cultured juvenile oysters (*Crassostrea gigas*) was associated with *F. maritimus* infection. More recently, *Flexibacter ovolyticus* has been reported to cause disease and high mortality in the eggs and larvae of Atlantic halibut (*Hippoglossus hippoglossus*) in Norway (Hansen *et al.,* 1992). It has been suggested that *Flavobacterium johnsoniae* may be an opportunist skin pathogen in certain fish species. It has been associated with superficial skin erosion in juvenile farmed baramundi (*Lates calcarifer*) in Australia (Carson *et al.,* 1993). *F. johnsoniae* has induced an analogous condition to this and been re-isolated during experimental infectivity trials (Soltani *et al.,* 1994). Opportunist infections can occur in fish when they are immunocompromised by poor environmental conditions or other stress factors. Sudden changes in water temperature appear to be as important in initiating the onset of clinical disease (Carson *et al.* 1993; Soltani *et al.* 1994).

Bacterial gill disease (BGD) is a condition characterised by numerous yellow-pigmented, filamentous, Gram negative bacteria in the genus *Cytophaga, Flexibacter* and *Flavobacterium,* present on the surface of the gill epithelium (Turnbull, 1993; Ostland *et*

al., 1994). The most significant bacterium associated with BGD is reported to be *Flavobacterium branchiophilum* (Wakabayashi *et al.*, 1989; Ostland *et al.*, 1994). According to Turnbull (1993), BGD may be the result of a complex interaction between adverse environmental conditions and variations in the pathogenicity of the bacterium. Reichenbach (1989) isolated *Flavobacterium aquatilis* from the gills of salmon showing signs of BGD at a fish hatchery in Michigan, USA, but its role in the pathogenesis of the disease was not confirmed. *Flavobacterium* which do not correspond to the description of *F. branchiophilum,* are usually designated as *Flavobacterium* spp. [1]*Flavobacterium piscicida* was originally isolated from red tide waters off the coast of Florida, USA and was shown to be pathogenic to fish (Meyers *et al.,* 1959). The name *F. piscicida* did not appear in the "Approved List of Bacterial Names" (Skerman *et al.,* 1980), however. Bacteria assigned to the genus [1]*Sporocytophaga* were reported to be associated with large skin lesions on coho, chinook, sockeye salmon and steelhead trout (*O. mykiss*) (Wood, 1974). In addition, several authors have reported heavy mortalities associated with the presence of unspeciated gliding bacteria (*Cytophaga* sp. or CLB) (Kent *et al.,* 1988; Holliman *et al.,* 1991; Pepin and Emery, 1993; Frelier *et al.,* 1994).

This thesis will only address diseases caused by *Flavobacterium psychrophilum* and will focus mainly on isolates obtained from outbreaks of RTFS, although comparisons will made to isolates obtained from outbreaks of BCWD.

[1] no longer considered to belong to the family Flavobacteriaceae.

1.2. Disease Caused by *Flavobacterium psychrophilum*

The association of *F. psychrophilum* with the disease has now been reported in USA, Japan and Europe, and several species of salmonids have been found to be affected. The bacterium shows lack of host specificity, and has also been demonstrated in a range of non-salmonid fish. The occurrence of *F. psychrophilum* in different fish species and in different geographical regions is detailed in Table 1.2.

Table 1.2. The occurrence of *F. psychrophilum* in various fish species from different geographical regions

Country	Species affected	Reference
USA	Coho Salmon (*Oncorhynchus kisutch*)	Borg, 1960
Germany	Rainbow trout (*O. mykiss*)	Weis, 1989
France	Rainbow trout (*O. mykiss*)	Bernardet *et al.*, 1988
Japan	Ayu (*Plecoglossus altivelis*)	Wakabayashi *et al.*, 1991
Denmark	Rainbow trout (*O. mykiss*)	Lorenzen *et al.*, 1991
Germany	Eel (*Anguilla anguilla*), Common carp (*Cyprinus carpio*), Crucian carp (*Carassius carassius*), Tench (*Tinca tinca*)	Lehmann *et al.*, 1991
UK	Rainbow trout (*O. mykiss*)	Santos *et al.*, 1992.
Italy	Rainbow trout (*O. mykiss*)	Sarti *et al.*, 1992
Spain	Rainbow trout (*O. mykiss*)	Toranzo and Barja, 1993
Finland	Rainbow trout (*O. mykiss*)	Wiklund *et al.*, 1994
Australia	Atlantic salmon (*Salmo salar*)	Schmidtke and Carson, 1995
Chile	Rainbow trout (*O. mykiss*)	Bustos *et al.*, 1994
Japan	Rainbow trout (*O. mykiss*) Ayu (*P. altivelis*)	Wakabayashi *et al.*, 1994
Northern Ireland	Rainbow trout (*O. mykiss*)	McCormick, pers. comm.
Japan	Pale chub (*Zacco plalypus*)	Iida and Mizakami, 1996
Korea	Ayu (*P. altivelis*)	Lee and Heo, 1998
Sweden	Baltic salmon (*S. salar*)	Ekman *et al.*, 1999

1.2.1. Bacterial Cold Water Disease (BCWD)

Bacterial cold water disease is a serious septicaemic infection of hatchery reared salmonids (*Oncorhynchus* spp. *Salvelinus* spp. and *Salmo* spp.) which was initially recognised in North America. Davis (1946) first described a condition or disease occurring at temperatures below 10°C that affected juvenile rainbow trout in the USA. Open lesions occurring on or near the peduncle characterised this disease, and for this reason, it was referred to as Peduncle Disease. Similar signs were observed by Borg (1960) who managed to isolate a bacterium from the kidney and external lesions of diseased juvenile coho salmon and to experimentally reproduce the disease. This isolate was unable to grow at temperatures above 25°C and was described as *C. psychrophila* (Borg, 1960). Borg named the disease low temperature disease as outbreaks usually occurred at temperatures below 10°C. Consequently, the name cold water disease or BCWD was established (Wood and Yasutake, 1956). BCWD is now the most widely used name for the condition.

The characteristics associated with BCWD in salmonids as described by Davis (1946), Borg (1960) and Wood (1974) are the erosion of external tissue, the most notable example being the degeneration of the caudal fin, and in some cases most of the caudal peduncle. External infection can sometimes be seen on the body wall, and this often leads to infiltration and degeneration of underlying muscle tissue (Holt *et al.*, 1993). Lorenzen (1994) reported that BCWD appears to affect all species of salmonid fish, causing up to 50% mortalities in coho salmon.

The severity of the clinical signs of BCWD, as well as the level of mortality which occurs, depend on the size of the fish infected, the stage of their development and the species of fish (Wood, 1974). In coho salmon, which appear to be most susceptible to the disease, the skin covering the yolk sac may be eroded, and mortality as high as 50% can occur. In fingerlings darkening and erosion of the peduncle can occur, with concomitant exposure of the spinal cord and tail loss. Lesions can also occur anterior to dorsal fins, at the lower jaw and near the vent (Holt *et al.*, 1993). Losses due to BCWD are usually around 20% (Wood, 1974). Under yearling fish having survived an outbreak, may develop abnormal swimming behavior and spinal deformities (Kent *et al.*, 1989). Yearlings may show signs of lesions around the peduncle and the lower jaw, and in addition be anaemic and have haemorrhagic gills (Holt *et al.*, 1993). Pacha and Ordal (1970) reported that darkening of the peduncle was a sign of the onset of infection, whereas Bullock *et al.*, (1971) indicated that the adipose fin was the site from which the infection progressed in fingerlings and large fish.

The disease may progress to septicaemia with necrosis observed in internal organs (Wolke, 1975). Wood and Yasutuke (1956) isolated *F. psychrophilum* from the kidney, eye, gill, heart, peritonium and spleen of fish exhibiting severe anaemia. They postulated that the condition had the characteristics of a general systemic infection. The authors reported little inflammatory response in the visceral organs. However, Borg (1960) stated mononuclear infiltration was associated with the disease.

1.2.2. Rainbow Trout Fry Syndrome (RTFS)

Rainbow trout fry syndrome is a systemic bacterial disease affecting hatchery reared rainbow trout fry and fingerlings in many parts of Europe during the last few decades (Bernardet *et al.,* 1988; Lorenzen *et al.*, 1991, Santos *et at.*, 1992; Toranzo and Barja, 1993; Lorenzen, 1994). The syndrome appears to affect rainbow trout in the weight range of 0.2 to 10.0 g at water temperatures between 6 and 16°C (Rangdale, 1994). Fry losses are often between 10 to 30 % within a single batch of fry (Scott, 1989), but can rise to 70% as the disease rapidly spreads amongst stock (Chua, 1991; Santos *et al.,* 1992).

The disease was first recognised in 1984, when a novel systemic disease of unknown aetiology was seen in rainbow trout hatcheries across the UK (Chua, 1991; Casey, 1993; Rangdale, 1995). The condition was characterised by certain behavioural traits include lethargy, cessation of feeding, swimming close to water inlets/outlets and at the side of the tanks. External gross clinical signs comprised exophthalmia, swollen abdomen, darkening of skin, reddening of the vent and occasional raised epidermal lesions. Internal clinical signs included enlarged friable spleen, haemorrhage of the liver and the posterior and anterior kidney, severe anaemia and ascites.

1.2.2.1. Aetiology

Due to the gill pallor of affected fish, and the inability to detect any known viral, bacterial, fungal or parasitic causal agent with any consistency, the condition became known as rainbow trout anaemia syndrome (Rangdale, 1995). It is only recently that *F. psychrophilum* has been associated with RTFS (Bernardet *et al.*, 1988; Baudin-Laurencin *et*

al., 1989; Lorenzen *et al.*, 1991; Santos *et al.*, 1992; Sarti *et al.*, 1992; Bruno, 1992; Toranzo and Barja, 1993). Previously, a nutritional or more complex aetiology was suspected (Austin and Stobie, 1991). At least two different disease conditions were apparently confused with respect to RTFS. The first condition, characterised histologically by pronounced muscle lesions and skeletal and cardiac myopathies, appeared to be caused by vitamin E deficiency. The condition was not responsive to antibiotic therapy, but improved by increasing the vitamin E level of the diet (McCloughlin *et al.*, 1992). The levels of vitamin E required to alleviate or prevent this problem were much higher than the recommended dose of 50 mg/kg (Halver, 1989). Moreover, it was reported by McCloughlin *et al.*, (1992) that vitamin E levels of 484 mg/kg significantly protected fish from this condition, while a commercial diet containing 134 mg/kg, although higher than the recommended level, resulted in myopathy and mortality. Chua (1991) suggested that the incidence of this condition has subsequently decreased possibly due to modification of the vitamin levels in commercial diets. A second condition has been described which is unresponsive to high levels of vitamin E, and has no muscle lesions associated with it. It was, however, responsive to high levels of oxytetracycline (300 mg/kg fish-day/ for 10-14 days) (Chua, 1991).

Other causes for the condition or potential aetiologies have been suggested, for example, infection through contaminated commercial salmonid diets (Scott, 1989). Austin and Stobie (1991) recovered three types of yellow pigmented bacteria from moribund and dead fish from two discrete populations of fry showing clinical disease similar to RTFS. These included coryneforms, *F. columnare* and *Janthinobacterium* sp. In a later study, Austin

and Stobie (1992) recovered the Gram positive cocci, *Micrococcus luteus* and *Planococcus* sp. from the kidney, spleen and ascitic fluid of moribund fry, during an outbreak of RTFS in England. Injection of 10^5 cells $fish^{-1}$ of both bacteria resulted in mortality in 2.0 g fry when administered via intraperitoneal (IP) and intramuscular (IM) routes. It was not suggested that either of the two groups were responsible for RTFS, however. During 1991, pure cultures of *Janthinobacterium lividum*, purple-pigmented Gram negative rods, were isolated from two populations of fry showing clinical RTFS (Austin *et al.,* 1992). Artificial infectivity experiments resulted in morbidity and mortality in 2.0 g rainbow trout. *J. lividum* is, however, ubiquitous within the aquatic environment and its precise role in RTFS was not fully elucidated in these studies.

Rangdale (1995) carried out a comprehensive survey of infected rainbow trout hatcheries, from which she showed that the Gram negative, yellow-pigmented, filamentous bacterium *F. psychrophilum* was implicated in RTFS in the UK and other European countries. Natural and laboratory based infectivity studies with collected isolates of *F. psychrophilum* successfully reproduced clinical signs and gross pathology similar to those observed during field outbreaks of RTFS.

1.2.2.2. Synonyms

There has been considerable confusion surrounding the different names applied to diseases associated with *F. psychrophilum* infection in rainbow trout fry. In Denmark, where it is known as fry mortality syndrome, the disease has been reported to cause up to 60% moralities (Lorenzen *et al.,* 1991). In France, it is known as a visceral form of BCWD

since the gross pathological changes were typically found internally, in contrast to BCWD (Baudin-Laurencin *et al.,* 1989). *F. psychrophilum* has been isolated from these fish and is regarded as the causative organism of the condition (Bernardet and Grimont, 1989; Baudin-Laurencin *et al.*, 1989). The condition has also been diagnosed in Italy where it became known as visceral myxobacteriosis (Sarti *et al.*, 1992), while in the UK it is known as rainbow trout fry anaemia or RTFS (Santos *et al.*, 1992). Other countries with fish affected by the disease include Germany (Lehmann *et al.*, 1991), Spain (Toranzo and Barja, 1993), Finland (Wiklund *et al.,* 1994) and Chile (Bustos *et al.,* 1994). A similar condition has been reported in wild population of trout in Finland (Wiklund *et al.,* 1994). In all the above investigations, *F. psychrophilum* has been repeatedly isolated from external lesions, liver, spleen and kidney tissue of affected fry and fingerlings in the weight range of 0.2-6.0 g.

1.2.2.3. Typical gross pathology

Outbreaks of RTFS usually start 4 to 7 weeks post-first feeding (Lorenzen *et al.,* 1991; Holliman, 1993), but may occur later. Typically, the disease occurs in the spring when water temperatures are below 10°C, but *F. psychrophilum* can also be the cause of clinical disease at temperatures exceeding 10°C. In the UK, RTFS affects fish at water temperatures ranging from 6 to16°C for much of the year, but losses tend to be greatest in the spring and early summer when stocking densities are at their highest (Casey, 1993). The worst months for infection are May, June and July (R.E. Rangdale, personal communication).

Gross pathological signs of the disease from other European countries appeared similar to those observed in UK including lethargy, swimming close to water surface, darkening of the skin, bilateral exophthalmia, abdominal distension and periocular haemorrhaging (Lorenzen, *et al.*, 1991; Toranzo and Barja, 1993; Rangdale, 1995). Mortality patterns in fry are typical of an acute systemic bacterial infection. Fingerlings (≈5.0 g) usually show a broader range of signs. The most pronounced signs are blindness, moderate anaemia, visceral petecheae, skin lesions in the region of dorsal fin or on the flanks and abnormal swimming behaviour (Dalsgaard and Horlyck, 1990; Bruno, 1992; Santos *et al.*, 1992). Internally ascites, swollen spleen, pallor of the liver and anorexia can also be observed (Dalsgaard, 1993; Holliman, 1993; Rangdale, 1996). Larger and older fish are infected with a more chronic form of the disease. Typical gross signs are skin ulcers, in particular behind the dorsal fin and on the peduncle. Ulceration on the peduncle can lead to total erosion and loss of the caudal fin (Bruno and Poppe, 1996). Larger trout and particularly brood fish can be asymptomatic carriers of *F. psychrophilum* and act as a reservoir of infection for younger or previously uninfected fish (Holt *et al.*, 1993; Rangdale *et al.*, 1996).

1.2.2.4. Typical histopathological changes

There are few studies of RTFS describing the histopathological changes, which occur during natural or experimental infection. Rangdale (1995) reported that the histopathology of RTFS varies during the course of the disease, and depends on the methods of infection, i.e. natural or artificial. Chua (1991) reported that the most consistent and prominent changes in naturally affected fish occur in the spleen. The splenic pathology is

characterised by peripheral layering due to fibrous infiltration, loss of border definition, generalised intracellular oedema and the presence of swarms of weakly stained long slender Gram negative bacteria, interspersed throughout the organ. Severe congestion of the splenic stroma, depletion of the haemopoetic tissue components, and pyknosis and karyorrhexis in the sinusoids have also been reported (Chua, 1991; Rangdale, 1995).

Bruno (1992) observed weakly stained, Gram negative, filamentous rods within the spleen, liver, kidney and trabecular layer of the heart. He also reported that lateral skin lesions showed necrosis, collapse, pyknosis and lymphocytic infiltration of the dermis and underlying muscle block. Chua (1991), however, noted intracellular oedema of malpighian cells in the entire thickness of the dermis, but reported that deeper layers of the skin were unaffected in RTFS. Mixed cellular fibrinous pericarditis, spongiosis of myocardium and endocarditis in acutely infected fry have also been observed (Chua, 1991). Rangdale (1995) observed some degree of pericarditis in both naturally and experimentally infected fry, but the changes were not severe and were limited to localised areas of cellular infiltration and vacuolation of the heart muscle. However, no other information on the histological changes in the heart is available in the published literature. Evensen and Lorenzen (1996) reported that *F. psychrophilum* can be detected by immunohistochemistry, and showed localisation of bacteria in the monocyte-macrophage system, in skin lesions, in the retina and the choroid gland of the eye.

1.2.2.5. Prevention and control of RTFS

Currently, the control of RTFS depends on the oral administration of a restricted range of antimicrobial compounds (Rangdale, 1994; Rangdale *et al.,* 1997). A potentiated sulphonamide combining trimethoprim and sulphadiazine, oxolinic acid, oxytetracycline hydrochloride and amoxycillin trihydrate are at present the drugs of choice in the UK (Rangdale *et al.,* 1997). Oxytetracycline incorporated into the diet at levels of up to 300 mg/kgfish/day for 10-14 days has been used in field outbreaks of RTFS with some success (Chua, 1991; Lorenzen *et al.,* 1991; Bustos *et al.,* 1994). Amoxycillin trihydrate (80-100 mg/kg fish/day for 7 days) also reduces mortality (Rangdale, 1994). There is a risk of drug resistance developing after prolonged use, however.

Rangdale *et al.,* (1997) observed significant differences in antibiotic sensitivity between 48 different isolates of *F. psychrophilum in vitro* as determined by a modified micro-broth dilution minimum inhibitory concentration (MIC) method. She suggested that the tetracycline analogue deoxycycline may be effective against RTFS epizootics. Treatment with antibiotics must commence early in an outbreak of RTFS before the fish become inappetant to allow the oral uptake of the drug (Holt *et al.*, 1993). The use of antibiotics is not ideal for the treatment of RTFS, not only because of the development of antibiotic resistant strains, but also because it does not eliminate the source of infection, which is likely to be infected brood stock fish (Rangdale, 1997). Organic deposits within fish tanks might also act as reservoirs of infection (Rangdale, 1995).

The route of transmission of *F. psychrophilum* has not been fully established. Nothing is known about the portal of entry of *F. psychrophilum* in fish during infection, the way it adheres to and penetrates the animal, its spread through the animal to target tissues, and its survival within the animal. It has been suggested that *F. psychrophilum* is transmitted both horizontally and vertically (Brown *et al.,* 1997). The bacterium has been isolated from both brood fish and sexual products from several species of salmonids (Holt *et al.*, 1993; Rangdale *et al.*, 1996; Brown *et al.*, 1997; Ekman *et al.*, 1999) indicating transmission of the bacterium from brood fish to their offspring. The bacterium has also been isolated from the inside of fertilised eggs (Brown *et al.*, 1997).

Disinfection of eggs with iodophors to prevent vertical transmission has been attempted, but was reported to be unsuccessful in preventing BCWD (Holt *et al.,* 1993, Lorenzen, 1994). Rangdale (1997) reported some success in reducing fry mortality attributable to RTFS after treating eggs with either hydrogen peroxide or gluteraldehyde at concentrations of between 100 and 400 ppm for 10 min. Careful hygiene and stock monitoring within a fish farm is vital in preventing cross infection between different holding tanks.

Vaccination is theoretically an ideal way for controlling many fish diseases, however, vaccines are commercially available for only five bacterial fish pathogens at the moment. These offer protection against *Yersinia ruckeri*, *Vibrio anguillarum*, *V. ordalii*, *Aeromonas salmonicida* and *V. salmonicida* (Evelyn, 1997). No commercial vaccine is currently available to protect against *F. psychrophilum* infection. Also, the mechanisms involved in the defence of fish against *F. psychrophilum* infection are poorly understood. Vaccination

as a measure to prevent outbreaks of RTFS in very young fry is unlikely to be completely successful, although protection of juvenile coho salmon against BCWD has been obtained after IP injection of formalin inactivated *F. psychrophilum* mixed with Freund's complete adjuvant (Holt *et al.*, 1993). Vaccination of brood stock may help to prevent vertical transfer of the pathogen.

Future methods for the control of RTFS are likely to include a combination of several methods, including improving the general hygiene of the farms, careful management, disinfection of eggs, vaccination and chemotherapy using antibiotics and the use of immunostimulants (Hofer, 1997).

1.3. Research Objectives

F. psychrophilum, the causative agent of RTFS and BCWD is now recognised as one of the most important salmonid pathogens world-wide. The incidence of RTFS in rainbow trout hatcheries in Europe is considered to be one of the biggest disease problems associated with the production of rainbow trout fry.

The description of the clinical signs and gross pathological changes involved in BCWD of coho salmon in the USA differ to those described for RTFS and BCWD seen in Europe in rainbow trout. As discussed above, besides dark coloration of the skin and bilateral exopthalmia, pathological changes of RTFS are primarily observed in the internal organs. Conversely, signs of BCWD are usually external and include skin and muscle lesions on the flank of the animal, anterior to the dorsal fins or in the peduncle area, and erosion of the

yolk sac in fry. In chronic forms of BCWD, the fish may exhibit spiral swimming behaviour and spinal deformities. BCWD does not appear to affect the sac-fry of rainbow trout in Europe, and clinical and pathological signs associated with RTFS have not been reported in the fry of coho salmon. However, similarities do exist between the two conditions such as the presence of external lesions on fish of varying size, spinal deformities and abnormal swimming behaviour of chronically infected fish. It is not yet fully understood how the same bacterium can cause these two distinct diseases, each with different clinical signs, and affecting different species of fish at different ages.

Information in the literature relating to the biochemical and antigenic characteristics of *F. psychrophilum* and its pathogenicity are severely lacking, and most of the information available has come from isolates recovered from outbreaks of BCWD. Such information is necessary to understand the mechanisms involved in infection by *F. psychrophilum,* and to establish effective control strategies for RTFS. The objectives of this study were therefore to:

- phenotype *F. psychrophilum* isolates obtained from outbreaks of RTFS based on their biochemical characteristics
- establish an effective challenge model for the bacterium, which could be used to identify virulent and avirulent isolates of the bacterium
- characterise *F. psychrophilum* electrophoretically to establish the extent of homogeneity between isolates from different geographical regions

- develop antibody probes (polyclonal and monoclonal antibodies) to virulent and non-virulent isolates of *F. psychrophilum* for use in both serological studies and in diagnostic screening
- examine electrophoretic profiles and enzyme activities of extracellular products of *F. psychrophilum* in an attempt to identify potential virulent factors of the bacterium

CHAPTER 2

General Materials and Methods

The methodology outlined in Chapter 2 consists of a general description of the techniques used in this thesis. Any modifications or variations in individual techniques to those described below will be discussed in the Materials and Methods Section in the relevant Chapters.

2.1. Culture of bacteria

2.1.1. Bacterial isolates

2.1.1.1. Isolates of *F. psychrophilum*

The *F. psychrophilum* isolates used in this study, the fish species and tissue from which they were isolated, the country of origin, the year of isolation and from when they were obtained, are detailed in Table 2.1. Isolates were obtained throughout the course of this work and therefore not all isolates listed in the Table 2.1 were used in every study. Those used for individual studies are indicated accordingly in the relevant Chapters.

2.1.1.2. Isolates of non-*F. psychrophilum* bacteria

A variety of bacteria from different genera was used to test the specificities of rabbit sera and monoclonal antibodies (MAbs) (Table 2.2).

Table 2.1. Isolates of *Flavobacterium psychrophilum* used in this study

Isolates	Fish species	Tissue	Country	Year	Source
NCIMB 1947^T	Coho salmon	Kidney	USA	1955	NCIMB
NCIMB 2282	Silver salmon	-	USA	-	NCIMB
HL313/97	Rainbow trout (RT)	Spleen	Germany	1997	CEFAS
UP193/97	RT	Spleen	England	1997	CEFAS
UP164/97	RT	Spleen	England	1997	CEFAS
B97026	RT	Lesion	Scotland	1997	IOA
B97035 E4	RT	Spleen	Scotland	1997	IOA
59/95	-	-	Chile	1995	UAC
32/97	RT	Kidney	Chile	1997	UAC
34/97	RT	Kidney	Chile	1997	UAC
35/97	Atlantic salmon	Kidney	Chile	1997	UAC
CASO 89/97	RT	Kidney/spleen	Chile	1997	UAC
921/97	RT	Spleen	England	1997	CEFAS
110/97	RT	Spleen	England	1997	CEFAS
10/2	RT	Spleen	England	1997	CEFAS
916/1	RT	Spleen	England	1997	CEFAS
904/10	RT	Spleen	England	1997	CEFAS
B398	RT	Kidney	Canada	1998	IOA
B035	RT	Necrotic myocitis	Canada	1994	IOA
JIP 02/86	RT	Kidney	France	1986	INRA
LPAA P01/88	RT	Spleen	France	1988	INRA
LPAA P03/88	RT	Spleen	France	1988	INRA
LVDL 1456/91	RT	Liver	France	1991	INRA
LVDL 1829/91	RT	Liver	Spain	1991	INRA
LVDL 3077/91	RT	Spleen	Spain	1991	INRA
LVDL 4862/95	RT	Spleen	France	1995	INRA
LVDJ E2047	RT	Spleen	France	1995	INRA
JIP 22/90	Brown trout	Skin lesion	France	1990	INRA
LVDJ G2215	Brown trout	Kidney	France	1997	INRA
LVDI 5/I	Adult carp	Gill lesion	France	1992	INRA
LVDJ XP 189	Adult tench	Kidney	France	1992	INRA
LVDJ D2172	Adult tench	-	France	1995	INRA
JIP 30/88	Adult European eel	Kidney	France	1988	INRA

NCIMB: National Collection of Industrial and Marine Bacteria, Aberdeen, UK; CEFAS: Dr. Rachel E. Rangdale, UK; IOA: Institute of Aquaculture, University of Stirling, Scotland, UK; UAC: Dr. Carlos Farias, Universidal Austral de Chile, Chile; INRA: Dr. J. F. Bernardet, France; Coho salmon (*Oncorhynchus kisutch*), Silver salmon (*O. kisutch*), Rainbow trout (*O. mykiss*), Atlantic salmon (*Salmo salar*), Brown trout (*Salmo trutta*) Carp (*Cyprinus carpio*), Tench (*Tinca tinca*), Eel (*Anguilla anguilla*).

Table 2.2. Non-*Flavobacterium psychrophilum* bacteria used to test the specificity of the rabbit serum and monoclonal antibodies

Bacteria	Origin
Aeromonas hydrophila	NCIMB 1134
Aeromonas salmonicida	NCIMB 1102
Aeromonas sobria	IOA
Bacillus subtilis	ATCC 6633
Bacillus mucoides	ATCC 11778
Corynebacterium aquaticum	NCIMB 9460
Edwardsiella ictaluri	IOA
Edwardsiella tarda	IOA
Escherichia coli	IOA
Flavobacterium aquatilis	NCIMB 2215
Flavobacterium branchiophilum	NCIMB 12094
Flexibacter maritimus	NCIMB 2154
Lactobacillus plantanum	IOA
Listonella anguillarum	NCIMB 6
Micrococcus luteus	NCIMB 570
Nocardia asteroides	IOA
Photobacterium damselae subsp. *piscicida*	IOA
Pseudomonas aeruginosa	ATCC 2753
Pseudomonas anguilliseptica	NCIMB 1455
Pseudomonas fluorescens	NCIMB 1953
Renibacterium salmoninarum	IOA
Serratia sp.	IOA
Streptococcus faecalis	IOA
Vibrio vulnificus	NCIMB 2136
Vibrio ordalii	NCIMB 1953
Yersinia ruckeri	NCIMB 1316

NCIMB: National Collection of Industrial and Marine Bacteria, Aberdeen, UK

IOA: Institute of Aquaculture, University of Stirling, Stirling, UK

ATCC: American Type Culture Collection

2.1.2. Culture of isolates

2.1.2.1. Culture of *F. psychrophilum*

F. psychrophilum was routinely cultured on modified Anacker and Ordal agar (MAOA) or

in modified Anacker and Ordal broth (MAOB) (Anacker and Ordal, 1959) (Appendix 1) at 15°C. The isolates of *F. psychrophilum* outlined in Table 2.1 were grown in MAOA and then immediately placed on cryoprotection beads (Cryoprotect; Technical Service Consultants Limited) according to the manufacturer's instructions and stored at –70°C. When isolates were required, 3 or 4 beads were removed from –70°C, placed into sterile MAOB, and incubated at 15°C. After sufficient growth was obtained (at least 96 h) they were transferred into fresh MAOB or onto MAOA. Throughout the duration of these studies the bacteria were also maintained on MAOA slopes as a temporary stock. Cultures were stored for no longer than two months before they were subcultured onto fresh slopes. Due to their thermo-sensitive and fastidious nature, the slopes were maintained either at 15°C or 4°C, and it was important not to expose the bacteria to long periods at room temperature (22°C). To promote growth upon sub-culturing, the media were held at 4°C prior to use. Preparation of the bacteria for use in different analyses and for immunisation is discussed in the relevant Chapters.

2.1.2.2. Culture of non-*F. psychrophilum* bacteria

Flavobacterium branchiophilum and *F. aquatilis* were cultured in MAOB. *Flexibacter maritimus* was cultured in marine 2216E broth (Difco, USA), *Photobacterium damselae* subsp. *piscicida, Vibrio ordalii, Pseudomonas anguilliseptica* and *Edwardsiella* spp. were cultured in tryptone soya broth (TSB, Oxoid) supplemented with 2% (w/v) NaCl and *Renibacterium salmoninarum* was cultured in selective kidney disease medium. The remaining bacterial genera were grown in TSB. All bacteria were cultured at 22°C, except

R. salmoninarum, which was incubated at 15°C. The recipes for these media are described in Appendix 1.

2.1.2.3. Preparation of bacteria for analysis

Bacteria were harvested, after sufficient growth was achieved, by centrifugation (Mistral 3000i, MES) at 3000 x g for 20 min at 4°C. Bacterial pellets were washed twice with phosphate buffered saline (PBS) (Appendix 2) and then resuspended in PBS. Generally, the absorbance of the suspension was adjusted to 0.4 at 520 nm (CE2041, Cecil) before storing them as aliquots at –20°C. A portion of the harvested bacteria was resuspended in electrophoresis sample buffer (Bollag *et al.,* 1996) (Appendix 2) for electrophoretic studies prior to storing them at –20°C.

2.2. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

2.2.1. Preparing and running the gel

SDS-PAGE was performed following the method of Laemmli (1970) with slight modifications. A discontinuous SDS-PAGE using a mini-vertical apparatus (Hoefer Scientific Instruments, San Francisco, USA) was used.

A separating gel (12%) was prepared (Appendix 2) and degassed. After the addition of cross-linker [ammonium presulphate and N,N,N,N,- tetramethylethylenediamine (TEMED)], the gel solution was introduced into the gel sandwich assembled according to the manufacturer's instruction. Butanol (0.1-0.2 ml) was gently overlaid on top of the separating gel solution and the gel was allowed to polymerise. A 4% polyacrylamide

stacking gel was prepared, degassed and layered on to the separating gel. Combs were inserted and gel was allowed to set.

Whole cells of bacteria, outer membrane protein (OMP) or extracellular product (ECP) were re-suspended in electrophoresis sample buffer (Appendix 2) at a ratio of 4:1. Whole cells were boiled for 5 min and centrifuged at 13000 x g for 5 min in a microcentrifuge prior to loading on to the gel. The OMP and ECP were boiled for 2 min and also centrifuged at 13000 x g for 2 min. Molecular weight markers (BioRad) were boiled but not centrifuged. Twenty µl of each sample was loaded into a well with a micro-syringe. Gels placed at 4°C, were subjected to electrophoresis at 80 V until the dye (contained in electrophoresis sample buffer) reached 0.5 cm from the bottom of the separating gel. The reservoir buffer used during electrophoresis is detailed in Appendix 2.

2.2.2. Staining the gel

Coomassie blue and Silver stains were used to identify protein, and Schiff's reagent was used to identify carbohydrate present on the gel.

2.2.2.1. Coomassie Blue stain

Gels were placed in a fixative solution containing 0.1% Coomassie brilliant blue R-250 (Sigma), 45% methanol and 10% acetic acid overnight with continuous shaking. The gels were then destained in 10% methanol and 10% glacial acetic acid until a good contrast was achieved between the background and the bands.

2.2.2.2. Silver stain

A commercially available Silver staining kit (Sigma) was used. Solutions were prepared according to manufacturer's instructions. The gels were promptly fixed in 30% ethanol/10% acetic acid solution for 30 min with 3 changes of fixative. They were then rinsed 3 times with double distilled water (ddH_2O) for 5 min on each rinse. The gels were equilibrated for 30 min in the silver equilibrium solution supplied in the kit with gentle agitation, followed by a rapid rinse in ddH_2O (10-20 sec). Developer solution was then placed over the gel for 10 min, changing twice during this period. The developer solution was then replaced with stop solution (1 % acetic acid in ddH_2O). The gel was rinsed three times with ddH_2O for 5 min on each rinse, then placed in reducer solution for 10-30 sec, followed by a quick rinse with tap water for 1 min. The gels were finally rinsed three times with ddH_2O.

2.2.2.3. Schiff's reagent

The gels were fixed in 40% ethanol/10% acetic acid for 10 min, then placed in 1% periodic acid/3% acetic acid for 20 min with gentle agitation. They were rinsed twice with distilled water for 5 min on each rinse. Schiff's reagent (Sigma) was added to the gels and incubated for 1 h. The gels were washed with distilled water at least 4 times for 5 min on each wash until the background of the gel was no longer pink. The gels were then placed in 10% glycerol for 15 min. Pre-stained molecular weight markers were used as standards (BioRad).

2.2.3. Determination of molecular weight of bands

The molecular weight of bands on the gel was determined from a standard curve prepared by plotting the $\log_{10}$ of molecular weight of the standard (BioRad) against the relative mobility (R_f). The distance migrated was measured from the beginning of the separating gel to the leading edge of a protein band. The distance of migration of each molecular weight marker in relation to the tracking dye was also measured. The R_f value for each band was calculated according to Equation 2.1.

Equation 2.1:

$$R_f = \frac{\text{Distance of protein migration}}{\text{Distance of tracking dye migration}}$$

The curve was then used to determine the molecular weight of the sample bands (See and Jackowski, 1989).

2.3. Western blots analysis

Samples were electrophoresed as described in Section 2.2.1. Antigens were transferred from SDS-PAGE gels to nitrocellulose membranes according to Wiens *et al.,* (1990) using a wet transblot system (Hoefer TE 22 transfer unit). Briefly, SDS-PAGE gels were placed in transblot buffer (Appendix 2) on a shaker for 10 min. Nitrocellulose paper and filter papers were also equilibrated in transblot buffer for the same period of time. Gels and nitrocellulose paper were then mounted into the transblotting apparatus in the following

order: device envelope (+ve electrode), filter pad, three sheets of filter paper, nitrocellulose paper, gel, three sheets of filter paper, filter pad, device envelope (-ve electrode). The envelope containing the gel and membrane was placed into the transblotting chamber and this was filled with transblot buffer. The transfer was performed at 60 V for 70 min with constant stirring.

After transblotting, the nitrocellulose membrane was removed and placed in 1% (w/v) bovine serum albumin (BSA) in Tris-buffered saline (TBS) (Appendix 2) overnight at 4°C to block the non-specific binding sites on the membrane. It was then washed 3 times with TBS containing 0.1% (v/v) Tween-20 (TBST) (Appendix 2) for 5 min on each wash, after which it was incubated with either anti-*F. psychrophilum* trout sera diluted 1 in 10 in TBS overnight at 4°C, or with rabbit anti-*F.psychrophilum* sera diluted 1 in 1000 in TBS or neat hybridoma supernatants for 2 h at 20°C with gentle agitation. The membrane was washed 3 times with TBST for 5 min on each wash. Membranes incubated with the trout sera were then incubated with neat culture supernatant of MAb 4C10 cell line (Courtesy of Dr. Ann Thuvander, Department of Pathology, Swedish University of Agricultural Science, S-75007 Uppsala, Sweden) for 3 h at 20°C. This MAb recognises the trout IgM molecule. It was washed again three times with TBST, before incubating with donkey anti-mouse immunoblobulin-G labelled with horseradish peroxidase (IgG-HRP) (SAPU: Scottish Antibody Production Unit, Lanark, Scotland, Appendix 2) diluted 1 in 100 in TBS for 1 h at 20°C. Membranes incubated with rabbit serum or with the hybridoma supernatants were washed three times with TBST, 10 min per wash, after which they were incubated with

anti-rabbit IgG-HRP (SAPU) diluted 1 in 200 in TBS or with anti-mouse IgG-HRP (SAPU) diluted 1 in 100 in TBST for 1 h at 20°C, respectively. When avidin-biotin-peroxidase complex (ABC) was applied to amplify the reaction of the MAbs, the membranes were incubated with goat anti-mouse IgG-biotin conjugate (SAPU) diluted 1 in 100 in TBS. After a 1 h incubation at 20°C, the membranes were washed as described above and incubated with 1 in 100 dilution of streptavidin-HRP (SAPU) in TBS for a further 1 h at 20°C. The membranes were washed three times with TBST, 5 min per wash, once with TBS for 1 min and then finally with PBS. The assay was developed using the chromogen described in Appendix 2 until bands became visible. The blots were placed in distilled water for 10 min to stop the reaction.

2.4. Glycoprotein detection

Whole cell preparations of *F. psychrophilum* and their OMP and ECP were subjected to 12% SDS-PAGE electrophoresis according to Section 2.2.1. Samples were transferred from the SDS-PAGE gels on to a nitrocellulose membrane as described in Section 2.3. The glycoproteins on the membrane were stained with a commercially available glycoprotein detection kit (BioRad) according to method outlined by the manufacturer. Gentle agitation was applied to the membrane throughout the procedure, except during the development of the reaction. After transfer of proteins from SDS-PAGE gels, the nitrocellulose membranes were washed with 10 ml PBS (9 mM sodium phosphate, 27 mM sodium chloride, pH 7.2) at 20°C for 10 min. The membranes were then immersed in 10 ml of 10 mM sodium periodate in sodium acetate/ethylenediminetetraacetic acid (EDTA) buffer and incubated in

the dark for 20 min at 20°C. They were washed with 3 changes of 10 ml PBS for 10 min, with fresh buffer on each wash. A biotinylation solution was prepared immediately before use, by adding 2 µl hydrazide solution to 10 ml sodium acetate/EDTA. The membranes were immersed in this solution for 60 min at 20°C, before washing in three changes of 10 ml of TBS (50 mM Tris, 27 mM sodium chloride, pH 7.2) for 10 min. The membranes were then incubated in 10 ml blocking solution at 4°C overnight. The membranes were washed three times with 10 ml TBS at 20°C for 10 min before immersion in the conjugate solution (5 µl streptavidin-alkaline phosphatase conjugate in 10 ml TBS) and incubation for 60 min at 20°C. The membranes were washed again three times with 10 ml of TBS for 10 min. Meanwhile, the developer solution was prepared by adding 50 µl nitroblue tetrazolium and 37.5 µl 5-bromo-4chloro-3-indolyl phosphate provided in the kit, to 10 ml of development buffer [1.21 g Tris, 1.01 g $MgCl_2.H_2O$, 0.58 g NaCl dissolved in 100 ml ddH_2O, pH 9.5]. The membrane was immersed in this solution at 20°C without agitation until bands appeared, after which the membrane was rinsed several times with ddH_2O and allowed to air dry.

2.5. Enzyme linked immunosorbent assay (ELISA)

The antibody titre of anti-*F. psychrophilum* rabbit sera, neat hybridoma supernatants and anti-*F. psychrophilum* trout sera, as well as the specificity of rabbit sera and MAbs were determined using an indirect antibody capture ELISA. Ninety-six-well ELISA plates (Immunon™, Dynatech, USA) were coated with 50 µl $well^{-1}$ of 0.05% poly L-Lysine (Sigma, USA) in 0.5 M carbonate-bicarbonate buffer, pH 9.6, for 1 h at 22°C. The plates

were then washed twice with low salt wash buffer (LSWB) (Appendix I) and 100 µlwell^{-1} of bacterial suspension was added to the plates. The bacteria used in the ELISA (see relevant Chapters for details) was prepared according to Section 2.1.2.3 and adjusted to a concentration of 1 x10^8 ml^{-1} with PBS. A standard curve of concentration against absorbance at 520 nm is described in Section 3.2.3. The bacteria were heat-killed by placing the bacterial suspension in a water bath at 60°C for 60 min before added to the ELISA plates. Plates were incubated overnight at 4°C. Bacteria were then fixed to the plastic by adding 50 µlwell^{-1} glutaraldehyde (0.05% v/v) diluted in PBS for 20 min at 20°C. The plates were washed three times with LSWB. Non-specific binding sites were blocked by incubating with 250 µlwell^{-1}of 1% BSA or 3% dried skimmed milk (Premier Beverages, Stafford, England) in PBS for 2 h at 20°C. The plates were again washed in 3 changes of LSWB. Fish sera, diluted two fold in PBS containing 0.01% (v/v) Tween 20 was added to the wells (100 µlwell^{-1}). Two-fold dilutions of pre-immune fish sera were used as negative control. The plates were incubated overnight at 4°C, washed 5 times with high salt wash buffer (HSWB) (Appendix 2) with a 5 min soak on the last wash to remove unbound antibodies. Neat supernatant of 4C10 cell line was added to the wells (100 µl well^{-1}) for 1 h at 20°C. The plate was washed 5 times with HSWB with a 5 min soak at the last wash before incubating with anti-mouse IgG-HRP (SAPU) diluted 1/1000 in LSWB (100 µl well^{-1}). To determine the antibody response of the rabbit sera, sera was diluted ten fold in LSWB before adding to the wells (100 µlwell^{-1}), while a dilution of 1/100,000 was used to examine the specificity of the rabbit sera. To screen the MAbs or to determine their specificity, neat hybridoma supernatants were added to the plate (100 µlwell^{-1}). Negative

controls (pre-immune rabbit or mouse serum, or LSWB) were also added to the ELISA plate. The plates were incubated at 20°C for 1 h, before washing them five times with HSWB with a 5 min soak on the last wash. Anti-rabbit IgG-HRP and anti-mouse IgG-HRP (SAPU) diluted 1/1000 in PBS was added to the wells (100 $\mu l well^{-1}$) of plates incubated with the rabbit sera or the MAb supernatants respectively, and incubated at 20°C for 1 h. Plates were then washed with 5 changes of HSWB. Chromogen/substrate (Appendix 2) was added at 100 μl $well^{-1}$, and the reaction stopped with the addition of 50 $\mu l well^{-1}$ of 2 M H_2SO_4 after 4 min. The reaction was read with an ELISA reader (MR 5000, Dynatech) at 450 nm.

2.6. Outer membrane protein (OMP) preparation

OMPs were prepared from *F. psychrophilum* cultured in MAOB. The extraction procedure was as described by Bakopoulos *et al.,* (1997a) with slight modifications. Cells were harvested by centrifugation at 2900 x g for 30 min at 4°C. They were then washed twice with 10 mM Tris-HCl, pH 7.8. The bacterial pellet was resuspended to three times its volume with 10 mM tris-HCl/10 mM EDTA, pH 7.8 containing 50 $\mu l ml^{-1}$ phenylmethylsulphonyl fluoride and incubated for 30 min at 45°C. The bacteria were retained on ice during the procedure, except during grinding. The cells were disrupted by vigorously mixing them with glass beads (150-210 μm), added at 2-3 times the volume of bacteria, for 8 min on a rotor mixer. DNAse and RNAse were added to the lysates at 0.2 $\mu g ml^{-1}$ and incubated for 20 min at 20°C. Unbroken cells and cell debris were removed by centrifugation at 2900 x g for 10 min at 4°C and the supernatant collected. The lysates

were centrifuged at 2900 x g for a further 50 min at 4°C to remove remaining cell debris. They were then centrifuged at 47000 x g for 1 h at 4°C. The resulting pellet, containing both the outer and the inner (cytoplasmic) membranes, was selectively dissolved by adding sodium lauryl sarcosinate (SLS) at 1.5 % (w/v) and incubating for 30 min at 22°C (Filip *et al.*, 1973). The preparation containing both OMP and solubilised inner membranes was centrifuged at 47000 x g for 40 min at 4°C. The pellet contained the OMP, was gently washed with the SLS solution as described above, and then resuspended in 10 mM Tris-HCl, pH 7.8. The protein concentration of the OMP was determined using a BioRad protein determination kit following the manufacture's instructions before storage at –70°C.

CHAPTER 3

Basic phenotypic characteristics of *Flavobacterium psychrophilum* and development of an experimental challenge for the bacterium

3.1. Introduction

Over the past decade bacterial fish pathogens of the genus *Flavobacterium* have become of increasing concern to world aquaculture (Michel *et al.,* 1999). The taxonomy of these bacteria have been in a state of confusion since they were first reported, and most have at one time and another been assigned to the genera *Flexibacter* or *Cytophaga* (Bernardet *et al.,* 1996). The taxonomic position of the bacterium *F. psychrophilum* has changed considerably over the past few years, being referred to firstly as *Flexibacter psychrophilus* (Bernardet and Grimont, 1989) and later as *Cytophaga psychrophila* (Reichenbach, 1989). The most recent positioning of the bacterium is in the genus *Flavobacterium,* being designated as *Flavobacterium psychrophilum* (Bernardet *et al.,* 1996).

Taxonomic positioning is based on phenotypic, biochemical and genomic characterisation of the bacterium and there are various reports in the literature relating to these. Cells of *F. psychrophilum* are weakly refractile, Gram negative, strictly aerobic, slender and flexible rods. Reports on the size of the bacterium vary in the literature. Cells from young broth culture (12-48 h) have been reported to be approximately 0.75 μm (Pacha, 1968), 0.3-0.5 μm (Bernardet and Grimont, 1989) and 0.3-0.75 μm (Holt *et al.,* 1993) in diameter. In length, cells have been estimated to be between 1.5 and 7.5 μm (Pacha, 1968), 1.5 –5.0 μm (Bernardet and Grimont, 1989) and 2.0-7.0 μm (Holt *et al.*, 1993). In a 48 h liquid culture,

pleomorphic forms can be found. These include cells that can be longer than 8.0–12 μm, involuted, S and V shaped, rounded at the ends or branched (Holt *et al.,* 1993; Lorenzen,1994). As the age of the culture increases, the cells gradually shorten. No microcysts have been observed.

The bacteria display gliding motility (active movement over surfaces without the aid of flagella) (Soriano, 1973). The nature of the mechanism responsible for gliding is unknown (Gorski *et al.,* 1992). Although there is some strain variation in this ability, it is generally considered that the gliding movement of *F. psychrophilum* is slow and weak, and is only noticed after prolonged observation (Bernardet and Grimont, 1989). Several features associated with the cell envelope of CLB have been correlated with their ability to translate over solid surfaces (Chang *et al.,* 1984; Wolkin and Pate, 1985). For example, slime on the cell's surface appears to play an essential role in the process. This has been demonstrated in many CLB (Dalsgaard, 1993). Dalsgaard (1993) reported that the production of copious amount of extracellular polysaccharides or slime, facilitates this gliding motion. It is probable that the extracellular polysaccharide is implicated in adhesion of the bacterium to its host or to solid surfaces. Bernardet and Kerouault (1989) found that strains of *F. psychrophilum* which had more extensive spreading of colonies exhibit a more rapid gliding movement, although this movement is slower than that observed for most other gliding bacteria. Poor gliding ability is a notable characteristic of *F. psychrophilum* when compared with other fish pathogens of the order Cytophagales, such as *F. columnare* (Bernardet, 1989) and *F. maritimus* (Wakabayashi *et al.,* 1986).

F. psychrophilum is fastidious in its growth requirements, thermo-sensitive and sometimes very slow growing (Bernardet and Grimont, 1989; Lorenzen and Karas, 1992; Lorenzen, 1993; Rangdale, 1995) and this is possibly why the number of *F. psychrophilum* isolates which have been fully characterised is limited (Lorenzen *et al.,* 1997).

A low nutrient medium, devised by Anacker and Ordal (1959) is the most commonly used medium for the isolation and cultivation of *F. psychrophilum.* This medium is also frequently referred to as *Cytophaga* medium (Holt, 1987). Isolates of *F. psychrophilum* incubated for 48-96 h at 15-20°C on this medium produce bright yellow, convex, smooth and glossy colonies with regular margins of 1 to 5 mm in diameter (Bernardet and Kerouault, 1989). Occasionally, spreading colonies with uneven margins are apparent (Holt *et al.,* 1993).

On *Cytophaga* medium, the bacterium usually exhibits slow and fastidious growth. Therefore, several authors have reported improved growth performance by changing the medium formulation e.g. increasing the amount of tryptone (Bernardet and Grimont, 1989), adding 10% foetal calf serum (FCS) (Obach and Baudin-Laurencin, 1991), or 5% FCS and/or 0.5% tryptone (Lorenzen and Karas, 1992). The brand of beef extract used in the medium formulation has been shown to be very important. Lorenzen (1993) examined bacterial growth on standard *Cytophaga* agar supplemented with a semi-solid beef extract supplied by Difco (Cat No. 0126-01) and found augmented growth. Although Holt (1987) reported that tryptone yeast extract salts medium (TYES) or TYES enriched with skimmed milk enhanced the growth of the bacterium, Cipriano and Teska (1994) did not find any

improvement in bacterial growth with the seven different media they tested including that used by Holt (1987). In a recent study, Daskalov *et al.,* (1999) reported an improved growth medium for *F. psychrophilum* by supplementing *Cytophaga* medium with 0.5 gl^{-1} each of D (+) galactose, D (+) glucose, L-ramnose and skimmed milk. Various other media have also been developed (e.g. Shieh, 1980; Hsu *et al.*, 1983; Baxa *et al.*, 1986; Mudarris and Austin, 1988), but no one formulation is currently accepted for use in diagnostic and research laboratories. Generally, growth is limited on tryptone soya agar (TSA) and Lewins medium (Lewin and Lounsbery, 1969).

Growth of *F. psychrophilum* generally occurs between 4 and 23°C (Pacha, 1968), but not at 30°C (Holt *et al.*, 1989). The optimum growth temperature of the bacterium quoted by different authors appears to vary. Pacha (1968) found optimum growth at 20°C. Holt *et al.*, (1993) reported optimum growth at 15°C with a generation time of 2 h, while in a recent study by Uddin and Wakabayashi (1997) optimum growth of the bacterium was reported to be 19.6 ±0.5°C. According to Pacha (1968), salt tolerance of the bacterium generally ranges between 0.8 to 1.0% NaCl, but no growth occurs in the presence of 2.0% NaCl. Bernardet and Kerouault (1989) observed a salt tolerance of 0.5%, and they observed no growth at 1.0% NaCl. This clearly demonstrates that the organism is most suited to low-temperature, freshwater environments.

The fastidious and thermo-sensitive nature of *F. psychrochilum* limits its sub-cultivation even when an improved medium is used. Michel *et al.*, (1999) mentioned that the cells of *F. psychrophilum* are highly susceptible to osmotic conditions. Hofer (1997) suggested that

temperature was the most likely cause for the problems related to the sub-culturing of *F. psychrophilum.* Moreover, Michel *et al.,* (1999) believed that both improvement in the medium formulation and careful handling of the bacteria whilst in isotonic solutions should improve the overall viability and growth of the bacterium.

Isolates of *F. psychrophilum* are unable to utilise either simple or complex carbohydrates, but are actively proteolytic with the capacity to degrade gelatin, casein, and tyrosine (Dalsgaard, 1993; Holt *et al.,* 1993). Using API ZYM galleries (BioMerieux), Bernardet and Kerouault (1989) reported that the bacteria lacked the enzymes necessary for carbohydrate metabolism.

Pacha (1968), Pacha and Porter (1968) and Holt *et al.,* (1993) reported that *F. psychrophilum* was devoid of cytochrome oxidase activity. Other studies have found varying degrees of activity from weakly positive to readily detectable levels (Bernardet and Kerouault, 1989; Bernardet and Grimont, 1989; Bustos *et al.*, 1994; Schmidtke and Carson, 1995). Isolates of *F. psychrophilum* do not possess the ability to produce hydrogen sulfide (H_2S) (Pacha, 1968; Bernardet and Kerouault, 1989; Bernardet and Grimont, 1989; Holt *et al.,* 1993) and the colonies of the bacterium do not absorb Congo red (Bernardet and Grimont, 1989).

Reichenbach and Dworkin (1981), showed that flexirubin pigment is present in most strains of CLB isolated from soil and fresh water, and in *Sporocytophaga* and *Flavobacterium* with a low G+C content. In contrast, most Gram positive bacteria, flagellated bacteria and

nearly all marine CLB bacteria do not contain this pigment. Thus, the presence of flexirubin is considered to be a reliable chemosystematic marker for members of this group (Holt *et al.,* 1993).

Genetic diversity between *F. psychrophilum* isolates has recently been reported (Cipriano *et al.*, 1996; Lorenzen *et al.*, 1997; Chakroun *et al.*, 1998). Cipriano *et al.*, (1996) found that the isolates of *F. psychrophilum* they examined were phenotypically and serologically homogenous, but varied in their ribosomal RNA gene restriction pattern (ribotypes). Chakroun *et al.*, (1998) also reported different ribotypes and plasmid profiles between different *F. psychrophilum* isolates, and found that some ribotypes were associated with a particular fish species from which they were isolated. They did not find any correlation between the ribotype and the geographical origin of the isolate.

Although *F. psychrophilum* is a major source of concern in salmonid hatcheries, little information exists in the published literature describing the mechanisms involved in the virulence of the bacterium. Dalsgaard (1993) reviewed known virulence mechanisms of *F. psychrophilum* in which she reported that the differences seen in the severity of the clinical signs of BCWD outbreaks depends on the virulence of the pathogen. Fish dying from infection by highly virulent strains of the organism appeared to have less extensive signs of lesions. It has also been suggested that the proteolytic activity of *F. psychrophilum* may play a role in the pathogenicity of the bacterium (Dalsgaard, 1993). Several authors have examined the proteolytic activity of different isolates of the bacterium mostly associated with BCWD outbreaks, and different proteolytic activities have been reported for the

bacterium (Pacha, 1968; Otis, 1984; Holt *et al.*, 1993; Bertolini *et al.,* 1994; Madsen and Dalsgaard, 1998). It would appear from these reports that the proteolytic nature of the bacterium varies considerably and this should be further investigated.

Establishing an effective experimental challenge model for *F. psychrophilum* has been difficult and several authors have tried to develop such a model using different routes of infection (Holt, 1987; Chua, 1991; Lorenzen *et al.,* 1991; Bustos *et al.,* 1994; Rangdale, 1995; Madsen and Dalsgaard, 1999). Variations in experimental conditions of challenges, reported by different authors e.g. water temperature, fish size and injection route, make it difficult to directly compare the different challenge models used.

Borg (1960) was able to produce disease in coho salmon when *F. psychrophilum* was injected intramuscularly. Holt *et al.,* (1993) attempted to reproduce the disease in yearling coho salmon using 19 different strains of *F. psychrophilum* by subcutaneous (SC) injection of the bacterium at 2.8 $x10^7$ viable cells per fish. The mortality that resulted ranged from 0 to 100% dependent upon the strain of the bacterium used. Rangdale (1995) performed a comparative study using a number of different *F. psychrophilum* isolates, in which 0.8 g rainbow trout were injected intraperitoneally with approximately 100 µl $fish^{-1}$ of 10^6 cell ml^{-1}. Resultant mortality ranged from between 17 and 74% with the different isolates tested. In a more recent study, Madsen and Dalsgaard (1999) reported a successful IP challenge model using isolates with different elastin-degrading profiles and different sero-types. They could obtain around 60 to 70% mortalities by injecting rainbow trout fry IP with 10^4 cfu $fish^{-1}$.

Rangdale (1995), and Madsen and Dalsgaard (1999) attempted to develop a bath challenge model, whereby groups of rainbow trout fry were dipped into bacterial suspensions. Using 10^5 cfu ml^{-1}, Rangdale (1995), obtained percentage cumulative mortalities (PCM) of 30% and 38% after 14 days post-challenge when fish were dipped into the bacterial suspension for 5 h and 10 h, respectively, while Madsen and Dalsgaard (1999) found PCM between of 27 and 31 % when 10^7 cfu ml^{-1} was applied for only 0.5 h. Infected fish were found to develop typical signs of clinical RTFS.

The aims of this study were firstly, to examine basic growth and morphology of *F. psychrophilum* isolates obtained during outbreaks of RTFS, held in the bacterial culture collection at IOA, and secondly to confirm their biochemical characteristics in relation to those reported by other authors for *F. psychrophilum* isolates obtained from BCWD and RTFS outbreaks. Attempts were made to establish a reproducible challenge model for the bacterium, whereby clinical signs of RTFS were reproduced during the infection. Such a model is necessary in order to establish levels of virulence between the different *F. psychrophilum* isolates held in the collection.

3.2. Materials and Methods

3.2.1. Bacterial isolates

Fifteen isolates of *F. psychrophilum,* 12 of which recovered were from rainbow trout fry during RTFS outbreaks, one isolated from diseased Atlantic salmon and two obtained from NCIMB were used in this study. Details relating to the origin of these isolates are listed in Table 3.1, and their growth conditions are described in Section 2.1.2.1.

3.2.2. Preparation of a growth curve for *F. psychrophilum*

A growth curve was established for *F. psychrophilum* strain NCIMB 1947^T cultured in MAOB. Three to four colonies taken from a 3 day old culture, were inoculated into 500 ml of MAOB in a 1 L conical flask and incubated at 15°C. A 2 ml aliquot of the culture was removed every 8-12 h and the absorbance of the bacterial suspension was measured at 520 nm using a spectrophotometer (CE2041, Cecil). This was repeated for 7 days. When the

Table 3.1. Isolates of *Flavobacterium psychrophilum* used in phenotypic studies

Isolate	Fish species	Tissue	Country	Year
NCIMB 1947^T	Coho salmon	Kidney	USA	1955
NCIMB 2282	Silver salmon	Unknown	USA	Unknown
B97026	Rainbow trout (RT)	Lesion	Scotland	1997
B97035 E4	RT	Spleen	Scotland	1997
HL313/97	RT	Spleen	Germany	1997
UP164/97	RT	Spleen	England	1997
UP193/97	RT	Spleen	England	1997
921/97	RT	Spleen	England	1997
110/97	RT	Spleen	England	1997
59/95	RT	Unknown	Chile	1995
32/97	RT	Kidney	Chile	1997
33/97	RT	Gill	Chile	1997
34/97	RT	Kidney	Chile	1997
CASO 89/97	RT	Kidney/spleen	Chile	1997
35/97	Atlantic Salmon	Kidney	Chile	1997

NCIMB : National Collection of Industrial and Marine Bacteria, ^{T} Type strain;
Coho salmon (*Oncorhynchus kisutch*), Silver salmon (*O. kisutch*), Rainbow trout (*O. mykiss*), Atlantic salmon (*Salmo salar*).

absorbance of the suspension started to increase, colony forming units (cfu ml^{-1}) were determined for the bacterial suspension, prepared according to the drop method described by Miles and Misra (1938) using MAOA plates. Briefly, the bacterial suspension was diluted 10-fold six times with fresh MAOB. Six replicate drops (20 µl $drop^{-1}$) from each dilution was then placed onto a MAOA plate that had been previously divided into six sections. The plates were allowed to dry before incubation at 15°C for at least 96 h until colonies were visible and could be counted. The average number of colonies per drop was counted and cfu ml^{-1} determined for the bacterial suspension using Equation 3.1:

Equation 3.1:

cfu ml^{-1} = number colonies x 20 (volume added) x dilution factor x 50

The absorbance of the suspension at 520 nm and the bacterial concentration (cfu ml^{-1}) of the suspension were then plotted against time.

3.2.3. Preparation of a standard curve for *F. psychrophilum*

A graph was constructed relating the absorbance of a *F. psychrophilum* suspension at 520 nm to the cell concentration, so that the numbers of *F. psychrophilum* cells in any suspension could be determined spectrophotometrically at 520 nm. *F. psychrophilum* type strain NCIMB 1947^T was cultured in MAOB for 96 h at 15°C. The bacterial suspension was centrifuged at 3000 x g for 20 min at 4°C, then washed twice with PBS. The bacteria were finally resuspended in PBS, and the concentration of the bacterial suspension adjusted with PBS to give absorbances between 1.0 and 0.1 at 520 nm. An aliquot of each dilution (20 µl) was placed onto MAOA, and the bacterial concentration of each dilution

determined as cfu ml^{-1} at 520 nm, detailed as above in Section 3.2.2. The bacterial concentration of each dilution (i.e. cfu ml^{-1}) was then plotted against the absorbance of the suspension at 520 nm.

3.2.4. Growth and morphological characteristics

The growth and morphological characteristics of the *F. psychrophilum* isolates shown in Table 3.1 were examined using the tests and procedures previously described by Bernardet and Kerouault (1989) and Lorenzen *et al.,* (1997). Briefly, the bacteria were cultured on MAOA for at least 96 h. Firstly, the morphology of the colonies present on the plates was examined by eye, and with a stereo microscope (x100). The ability of the bacteria to glide was then examined using a hanging drop preparation of a 72-96 h broth culture (Bernardet and Kerouault, 1989). Gram staining of bacteria was performed using cells cultured for 96 h on MAOA. The ability of isolates to grow at different temperatures was investigated by streaking bacterial samples onto MAOA plates, and incubating the cultures aerobically at 4, 20 and 30°C for 10 days. Growth of the isolates on TSA was also assessed, by streaking a bacterial colony from a MAOA plate onto TSA and incubating at 15°C for 10 days. The ability of the bacteria to auto-agglutinate was examined as described by Janda *et al.,* (1987). Fresh *F. psychrophilum* colonies were inoculated into 20 ml of MAOB in a tube and incubated for 7 days at 15°C. Cultures were examined for auto-agglutination at the end of the incubation period, which was apparent as a large aggregations of cells at the bottom of the tube and the greater the agglutination the clear the culture medium.

3.2.5. Biochemical analysis

3.2.5.1. Oxidase test

An oxidase strip (Oxoid) was placed in a clean Petri dish and a substantial bacterial inoculum, taken directly from a 96 h culture, was smeared onto the end of the strip containing the oxidase reagent, N,N-dimethyl-1,4-phenylene di-ammonium chloride. The strip was left for 30 sec and then examined for any colour change; a deep purple/blue colour indicated oxidation of the reagent and a positive reaction.

3.2.5.2. Catalase test

The presence of catalase was tested by the addition of between 100 µl and 200 µl of 15% (v/v) H_2O_2 to 72 to 96 h old cultures for 1 min. Effervescence indicated a positive reaction.

3.2.5.3. Flexirubin-like pigment

The presence of cell-wall-associated flexirubin-like pigments was determined by flooding the Petri dish containing 72 to 96 h old *F. psychrophilum* cultures with 20% KOH (w/v). A positive reaction consisted of a shift in colony colour from yellow to red/brown almost instantly and a return to the original colour after the addition of 1 N HCl (Reichenbach *et al.,* 1981). Samples with negative reactions were incubated for 2 min before being discarded.

3.2.5.4. Oxidative or Fermentative (O-F) test

Oxidative or fermentative glucose metabolism was examined using Hugh-Leifson (O-F) medium (Difco) containing 1% (w/v) glucose. Briefly, a culture of bacteria was inoculated

into freshly prepared tubes of O-F medium by a single stab with a straight wire. One tube was incubated in the presence of air (aerobic tube), the other was covered with a thin layer of liquid paraffin to exclude air (anaerobic tube). The medium also contained bromothymol blue pH indicator to detect acid formation as a result of glucose metabolism. The colour of the medium was examined after incubating the tubes at 15°C for 7 days. If both tubes remained green, this indicated that no reaction had taken place. If the aerobic tube was yellow, but the anaerobic tube was green this indicated than an oxidative reaction had occurred, whereas if both tubes had turned yellow this indicated the presence of a fermentative reaction.

3.2.5.5. Enzyme profile

The semi-quantative micro-method API ZYM (BioMerieux SA, France), consisting of a single strip containing 19 wells, each measuring a different enzymatic activity was used to look at the enzyme profiles of the *F. psychrophilum* isolates. One drop of a bacterial suspension, adjusted to approximately 1 $x10^8$ bacteria ml^{-1} from the standard curve prepared in Section 3.2.3, was inoculated into each cupule, and the strip was then incubated for 24 h at 15°C. Hydrolysis of a given substrate resulted in the production of a specified colour according to the enzyme activity of interest. The intensity of the reaction was scored from 1 to 5 as suggested in the manufacturer's guidelines for the procedure.

3.2.5.6. Degradation of protein substrates

The protease activity of *F. psychrophilum* isolates was examined following the method described by Bertolini *et al.,* (1994) with the exception that the basal medium used in this

study was MAOA. Three protein substrates were incorporated into the MAOA plates. Gelatin, 0.6% (w/v) (BHD Chemical Ltd Pooled, England) and elastin 0.05% (w/v) (Sigma) were added to MAOA during its preparation. A 0.5% (w/v) solution of casein (Sigma) in MAOA was also prepared, but in this case the MAOA was also supplemented with 0.079 % (w/v) $CaCl_2$. H_2O. This was added in order to form white calcium caseinate particles enabling better visualisation of any casein digest. The agar, supplemented with the proteins, was poured into Petri dishes and allowed to set. Six wells of 6 mm diameter were made in each plate. The isolates of *F. psychrophilum* were grown in MAOB for 96 h at 15°C, and the absorbance of the suspension at 520 nm adjusted to 0.4. Four of the wells on each plate were then inoculated with 20 µl of the bacterial suspension and two non-adjacent wells were inoculated with only MAOB as a negative control. Degradation of the substrate was observed as a zone of clearing around the wells and monitored over an eleven-day incubation period at 15°C.

3.2.5.7. Modified elastase assay

Elastase activity of the *F. psychrophilum* isolates was also assayed using a modified elastin medium according to Hsu *et al.,* (1981) with minor modification. Briefly, 1g elastin (Sigma) was added to 20 ml of 0.1N NaOH, heated at 100°C for 1 h and the pH of the solution was then adjusted to 8.0 with 0.1N HCl. The volume of the solution was made up to 400 ml with distilled water, and blended in a food processor at a high speed for 3 min. This solution was autoclaved at 120°C for 15 min. A second solution was prepared containing 600 ml of 0.02 M Tris-HCl buffer, pH 8.9, to which 2 g tryptone, 2 g yeast extract, 0.3 g cysteine hydrochloride, 1 g NaCl, 0.2 g beef extract, 15 g agar and 0.04 M

calcium chloride was added. This solution was also autoclaved at 120°C for 15 min, before mixing the two solutions together and pouring into Petri dishes.

The isolates of *F. psychrophilum* were grown in MAOB for 96 h at 15°C and the absorbance of the resulting bacterial suspension adjusted to 0.4 at 520 nm. Elastin modified agar plates were spot inoculated with 1 µl of bacterial suspension, which contained approximately 10^8 cells ml^{-1} using a multi-point inoculator (Denley). Duplicate plates were prepared, each contained four inocula per plate, MAOB as a negative control and an unspeciated *Aeromonas* sp. (Au 2D8) as a positive control (Pearson *et al.,* 2000). Plates were incubated for up to seven days at 15°C, after which zones of clearing were measured. A zone ratio was obtained by dividing the diameter of the zone of clearing by the diameter of resulting colony. The elastinolytic activity was defined as a negative, intermediate or positive reaction when the ratio was 0, between 0 and 3, and higher than 3, respectively.

3.2.6. Experimental challenge with *F. psychrophilum*

3.2.6.1. Preparation of bacteria

Isolates of *F. psychrophilum* used in this Section are indicated in Table 3.2. They were selected randomly from the bacterial cultural collection at IOA and cultured in either MAOB or MAOA for between 72 and 96 h at 15°C. Bacteria cultured both in MAOB and on MAOA were used in the first challenge. The bacteria were gently scraped off the agar and washed twice with PBS by centrifugation at 3000 x g for 20 min at 4°C. Bacteria grown in the broth were adjusted to an absorbance of 0.4 at 520 nm with fresh MAOB, or

harvested by centrifuging as described above. The bacterial pellet was resuspended in MAOB and the absorbance of the bacterial suspension adjusted to 0.4 at 520 nm with MAOB. Although an absorbance of 0.4 at 520 nm is equivalent to 1 $x10^8$ $cellml^{-1}$, the concentration of bacteria in each bacterial preparation was confirmed by determining the number of cfu ml^{-1}.

3.2.6.2. Fish

Rainbow trout fry/fingerlings were obtained from healthy stocks of fish reared at a commercial farm (Cloan Hatcheries Ltd., Auchterader) with no known history of RTFS. Duplicate groups of rainbow trout (see Table 3.2 for weight and number fish) were placed in 10 l tanks. Each tank was aerated and the water temperature was maintained at between 10 and 12°C. Fish were acclimatised for 4 days, fed with a commercial pellet diet 2-3 times daily. One-third of the water was replaced daily, dead fish were removed and debris siphoned from the bottom of the tank.

3.2.6.3. Challenges

Four different challenges were performed over the course of the study using different routes to administer the bacteria. Details of the set up of each challenge are shown in Table 3.2.

In Challenge 1, *F. psychrophilum* isolate HL313/97 was used in an attempt to infect the rainbow trout fry using different routes (IM, IP, oral and agar implantation) of administration. The bacterial inocula used in the IM and IP injections were prepared from bacteria cultured either in broth or on agar plates to see if there was any effect on mortality

between the two preparations. Only a broth culture of the bacteria was used for oral administration, however. Two groups of 10 fish were used for each challenge route. Prior to injection all fish were anaesthetised with benzocaine solution (0.15 g benzocaine in 3 ml

Table 3.2. Set up of challenge experiments

Challenge no.	*F. psychrophilum* isolate	[a]No. of experimental fish	[a]No. of control fish	Average weight (g)	[b]Challenge dose (cfu fish^{-1})	Routes of administration
1	HL313/97	20	20	3	1.2 x10^7	IM broth
		20	-		1.2 x10^7	IM agar
		20	20		1.2 x10^7	IP broth
		20	-		1.2 x10^7	IP agar
		20	20		1.2 x10^7	Oral
		20	20		-	Agar implant
2	[d]B97026P1	50	50	4.37	1 x10^7	IM
	B97035E4	50	-		1 x10^7	IM
	HL313/97	50	-		1 x10^7	IM
	32/97	50	-		1 x10^7	IM
3	[d]B97021P1	50	50	12	1.1 x10^7	IM
		50	50		1.1 x10^7	IP
		50	50		1.1 x10^7	Oral
		50	50		[c]1.5 x10^5	Bath
4	[d]B97026P1	60	60	2	2 x10^6	IM
		60	-		2 x10^5	IM
		60	-		2 x10^4	IM
		60	60		2 x10^6	Oral
		60	-		2 x10^5	Oral
		60	-		2 x10^4	Oral

[a]Fish were divided into two replicate tanks, [b]Control groups of fish treated with MAOB were included in each challenge using corresponding routes administration and identical numbers of fish , [c]cfu ml^{-1}, [d]Passed through fish once recovered from spleen of infected animal, Abbreviations: IM: intramuscular, IP: intraperitoneal, MAOB: modified Anacker and Ordal broth.

ethanol, added to 10 l water). Fish were injected either IM (below the left dorsal fin of the animal) or IP with 0.1 ml of bacterial suspension contains 1.2 $x10^8$ cfu ml^{-1}. Two groups of 10 fish were exposed to the bacteria by dropping 0.1 ml of 1.2 $x10^8$ cfu ml^{-1} bacterial suspension into the pharynx of the animal using a 1 ml sterile syringe. This was washed down with a further 0.1 ml of water. Bacteria were implanted under the skin of two groups of the animals. A small cut was made in the left dorsal side with a sterile scalpel blade, and a few colonies of *F. psychrophilum* from a 96 h MAOA culture were cut from the agar and placed under the skin of the animal. Two control groups of fish received agar only. Two corresponding groups of control fry were included for each challenge route and these were given 0.1 ml of sterile broth in place of the bacteria.

Challenge 2 was performed in order to identify both a virulent and an avirulent isolate of *F. psychrophilum* to be used in later Chapters. Only the IM route of administration was used to infect fish in this challenge. Four different isolates of *F. psychrophilum* (B97026P1, B97035E4, HL313/97 and 32/97) were used in the challenge. Two groups of 25 fish were used for each isolate. Fish were injected IM with different isolates at 0.1 ml of 1 $x10^8$ cfu ml^{-1} bacterial suspension. Two control groups of 25 fish were injected IM with 0.1 ml broth.

In the third challenge experiment the virulent isolate B97026P1, as established from Challenge 2, was used to infect larger rainbow trout (12 g) by various routes of administration (IM, IP, bath and oral). Two groups of 25 fish were infected with 0.1 ml suspension of 1.1 $x10^8$ cfu ml^{-1} either by IM, IP or oral administration. Two control groups

for each corresponding route of administration were also included, receiving 0.1 ml broth per fish. Two groups of 25 fish were exposed to the bacterium in a bath challenge, for which 100 ml of bacteria at 1.5 $x10^8$ cfu ml^{-1} from a 96 h old MAOB was prepared. The bacterial suspension was diluted to 1.5 $x10^5$ cfu ml^{-1} with tank water (i.e. 5 l of bacterial suspensions) and fish were placed in this suspension for 12 h. Aeration was supplied throughout the procedure, after which the fish were returned to their tanks. Control fish were exposed to MAOB diluted with tank water to the same extent as the bacterial suspension, and fish were exposed to this for the same period of time as in the bacterial challenge.

An attempt was made in a fourth challenge to establish the 50% dose lethal (LD_{50}) for the virulent isolate B97026P1. Intramuscular and oral routes were used since these two routes found to be the most effective from previous challenges. Two replicate groups of 30 fish were used for each challenge dose. Fish were exposed to 1 $x10^8$ cfu ml^{-1}, $1x10^7$ cfu ml^{-1} or $1x10^6$ cfu ml^{-1} in with 0.05 ml of bacteria suspension administrated either orally or by IM injection. Two corresponding control groups of 30 fish for each dose, received 0.05 ml of broth either by IM or by oral administration.

3.2.6.4. Confirmation of *F. psychrophilum* infection

Fish in each challenge were monitored for 28 days, mortality and morbidity were recorded daily and necroscopy involved gross external and internal examinations. Swabs from external lesions, spleen, kidney and ascetic fluid were taken from dead fry and plated onto MAOA. Following incubation (10 days at 15°C) yellow pigmented bacteria were purified

by re-streaking onto fresh MAOA. The presence of *F. psychrophilum* was confirmed from the enzymatic activity pattern (API ZYM) of the bacterium (Bernardet and Grimont, 1989).

3.2.6.5. Statistical analysis

Results from the challenge were analysed using a Chi square test and were considered significant at $P < 0.01$.

3.3. Results

3.3.1. Growth curve of *F. psychrophilum*

A growth curve was prepared for the type strain NCIMB 1947^T in which absorbance of the bacterial suspension at 520 nm was compared to the concentration (cfu ml^{-1}) of the bacterial suspension against time and this can be seen in Figure 3.1.

3.3.2. Standard curve for *F. psychrophilum*

The standard curve of *F. psychrophilum* strain NCIMB 1947^T is shown in Figure 3.2. An absorbance of 0.4 at 520 nm was found to correspond to 1 $x10^8$ cfu ml^{-1}.

3.3.3. Morphological and growth characteristics

F. psychrophilum colonies grown for 96 h on MAOA appeared yellow, circular, convex, smooth and shiny with regular margins. They also had a characteristic cheese-like odour. Bacteria from each isolate exhibited a very slow gliding movement, which was only noticeable after prolonged observation under oil immersion by light microscopy. All of the isolates were Gram-negative rods, and these grew slowly and scantly at both 4 and 20°C, but were unable to grow at 30°C. The optimum temperature for growth appeared to be

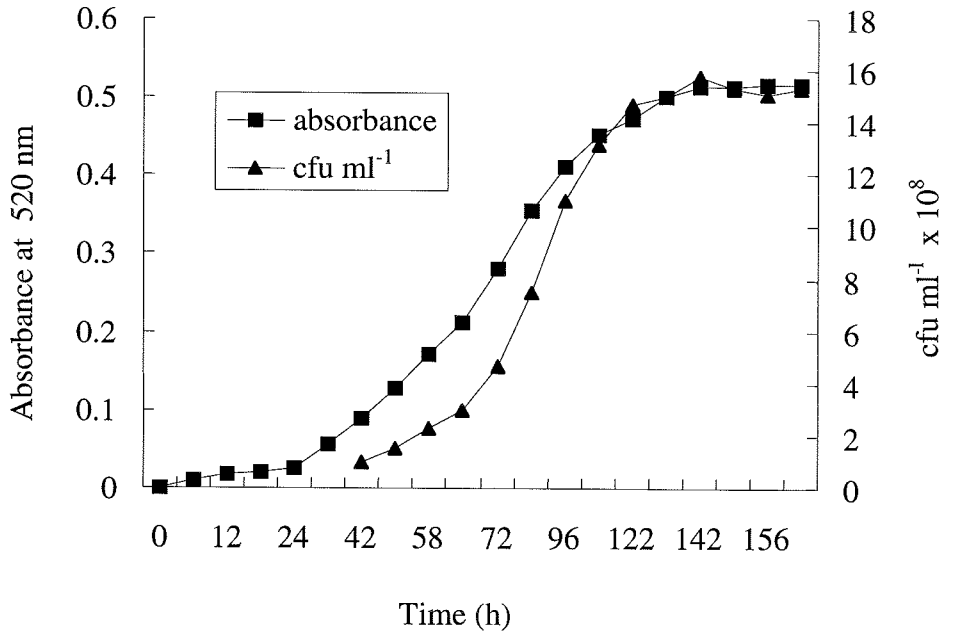

Figure 3.1. Growth curve of *F. psychrophilum* strain NCIMB 1947^T

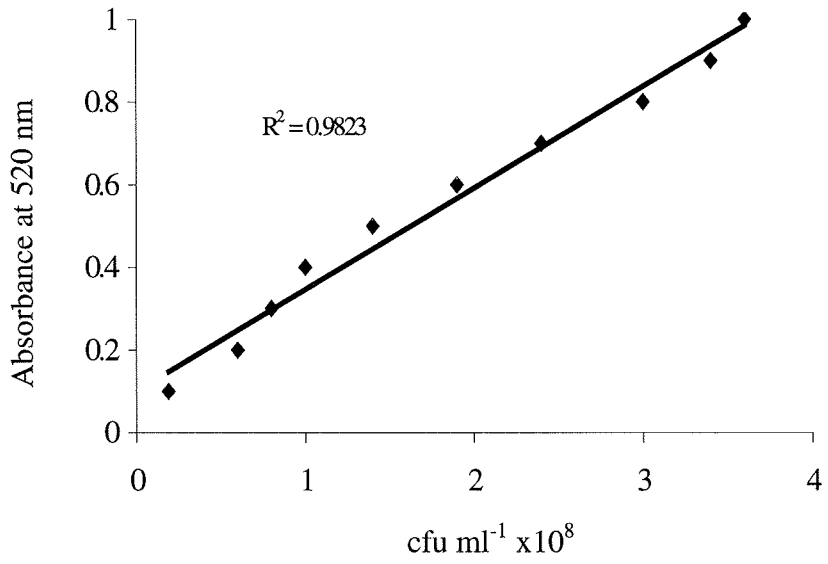

Figure 3.2. A standard curve of the concentration for *F. psychrophilum* (cfu ml^{-1}) at 520 nm (R^2: Coefficient of determination)

15°C. The bacteria were unable to grow on TSA (Table 3.3). All of the isolates spontaneously agglutinated at the bottom of the flask after 7 days incubation, although there appeared to be some variation between the isolates with respect to the degree of auto-agglutination that was observed (Table 3.3). This variation was not quantified.

3.3.4. Biochemical characteristics

All of the *F. psychrophilum* isolates examined had similar biochemical activities (Table 3.3). They all produced flexirubin pigment, were weakly positive for catalase and cytochrome oxidase, and did not utilise glucose in the presence of O-F medium.

The ability of *F. psychrophilum* to digest protein substrates was examined by inoculating live cells into media containing different protein substrates. Protease activity was determined from their ability to produce clear zones on the agar plates. All of the isolates were able to degrade gelatin and casein. An example of the zones of clearing obtained 5 days post-incubation, using casein as substrate, is shown in Figure 3.3, while the size of the zones of clearing obtained with the various substrates over an eleven day incubation period is shown in Table 3.4.

Bacteria cultured on agar containing 0.6% (w/v) gelatin supplementation produced larger zones of clearing at the end of the 11 period day compared to the zones seen on plates containing 0.5% (w/v) casein. Isolate B97026 produced the highest level of protease activity against both gelatin and casein, while isolate 34/97 exhibited the lowest level of activity against these two substrates.

Table 3.3. Phenotypic characteristics of *F. psychrophilum* isolates

	F. psychrophilum isolates														
	NCIMB 1947^T	NCIMB 2282	HL3131/97	UP193/97	UP 164/97	B97026	B97035 E4	32/97	33/97	34/97	35/97	CASO 89/97	59/95	921/97	110/97
Gram staining	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gliding motility	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Auto-agglutination	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 4°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 20°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 30°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth on TSA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Oxidase test	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Catalse test	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
O-F test	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Flexirubin type pigments	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Degradation of casein	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Degradation of gelatin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Degradation of elastin	-	-	(+)	(+)	(+)	(+)	-	-	-	-	-	-	-	-	-
Modified elastase assay	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

-, negative; +, positive; (+), weakly positive

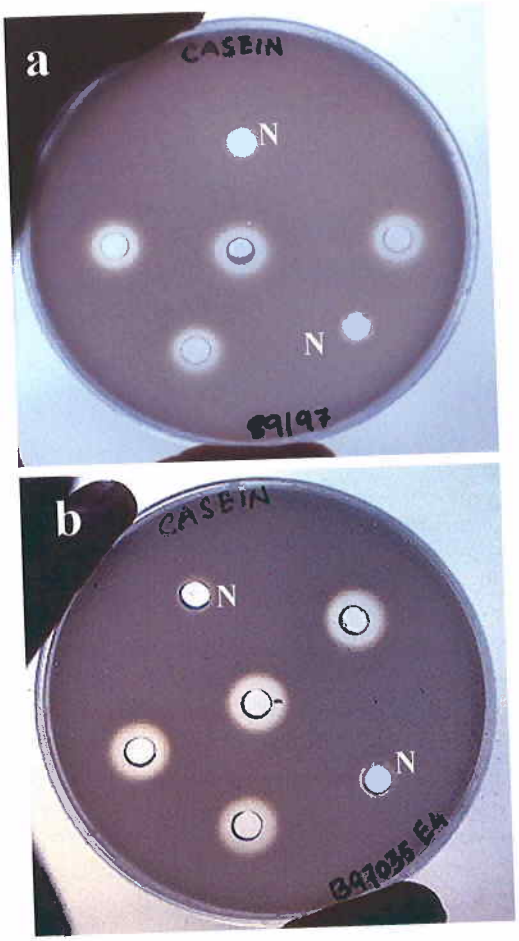

Figure 3.3. Degradation zones produced by *F. psychrophilum* on caseinase substrate media after 5 days of incubation at 15°C (a) isolate CASO 89/97 and (b) isolate B97035E4C.

N: Negative controls filled with sterile MAOB.

Table 3.4a. Degradation of gelatin by *F. psychrophilum* determined as zones of clearing (mm)

Isolates	[a]Zone of clearing (days post-inoculation)									
	2	3	4	5	6	7	8	9	10	11
NCIMB 1947[T]	-	9.3	10.9	13.1	16.0	17.1	19.2	24.7	28.4	31.1
NCIMB 2282	-	9.0	10.1	13.1	16.0	17.8	21.3	25.2	27.4	32.5
HL3131/97	-	13.1	18.2	22.1	26.4	28.2	33.3	35.1	37.4	40.0
UP193/97	-	11.3	13.5	18.3	23.1	25.2	27.3	31.1	33.4	38.1
UP 164/97	-	10.4	12.1	14.8	17.3	22.1	24.3	26.2	29.1	32.4
B97026	-	15.1	18.3	22.1	25.45	28.1	31.3	34.4	37.9	40.1
B97035 E4	-	10.1	14.2	16.3	18.1	21.0	23.6	27.6	29.7	35.0
32/97	-	8.1	10.1	12.0	15.1	16.3	18.5	20.6	25.9	29.1
33/97	-	-	9.6	12.1	14.1	15.5	18.9	21.8	24.7	31.0
34/97	-	-	9.8	11.4	15.4	17.3	19.2	21.5	23.5	25.2
35/97	-	10.4	12.2	15.3	17.3	19.0	19.5	24.6	27.5	34.6
CASO 89/97	-	12.0	14.0	15.8	17.9	19.0	21.0	23.1	28.5	32.3
59/95	-	9.6	11.8	13.6	19.0	19.1	21.6	25.3	29.0	35.7
921/97	-	11.1	14.5	16.3	18.7	22.0	26.3	29.3	32.0	34.5
110/97	-	12.3	15.3	18.5	21.0	23.6	26.7	29.0	32.2	33.7

[a] mean dimeter (mm), - no reaction

Results represent the mean value of four wells

Table 3.4b. Degradation of casein by *F. psychrophilum* determined as zones of clearing (mm)

Isolates	[a]Zone of clearing (days post-inoculation)									
	2	3	4	5	6	7	8	9	10	11
NCIMB 1947[T]	-	9.3	13.1	15.7	18.6	21.4	24.0	26.8	27.2	28.2
NCIMB 2282	-	-	10.1	14.0	16.0	19.0	23.0	25.0	26.6	28.4
HL3131/97		-	10.9	14.9	16.5	19.5	23	25.5	26.9	28.9
UP193/97	-	7.8	11.5	15.6	17.4	20.4	23.6	27.4	30.5	32.0
UP 164/97	-	8.9	11.75	15.5	18.0	21.0	23.3	25.0	29.0	30.0
B97026	-	11.0	14.8	17.5	23.2	24.5	26.0	26.4	30.0	34.8
B97035 E4	-	11.0	15.4	19.3	21.2	24.3	26.9	28.0	31.8	32.2
32/97	-	-	8.5	11.7	12.2	14.5	17.2	18.12	20.0	21.4
33/97	-	12.0	16.2	20.6	23.4	25.5	28.4	30.0	31.1	32.5
34/97	-	-	-	-	-	11.8	13.2	14.25	15.4	17.42
35/97		11.6	15.0	18.5	21.5	23.4	24.5	25.4	31.3	32.3
CASO 89/97	-	-	9.0	11.8	15.6	18.0	24.8	25.0	26.0	28.0
59/95	-	-	8.5	12.2	15.6	18.25	24.5	25.8	26.32	26.3
921/97	-	-	10.1	11.4	13.0	15.4	16.2	19.1	22.0	24.5
110/97	-	9.5	11.98	13.42	15.0	17.6	20.1	24.4	26.4	30.2

[a] mean diameter (mm), - no reaction

Results represent the mean value of four wells

Degradation of elastin was difficult to observe using the same procedure, and only four isolates appeared to produce small zones of clearing with this substrate, which developed much later than the zones seen with the other substrates. Zones of 11.2, 11.4, 12.2 and 14.9 mm were produced by isolates UP164/97, UP193/97, HI313/97 and B97026, respectively after 11 days of incubation. Elastinolytic activity was also assessed *in vitro* using modified elastin agar, and *Aeromonas* sp. (Au 2D8) was included as a positive control to establish that the assay was working. However, no elastinolytic activity was detected for any of the *F. psychrophilum* isolates examined using this particular method, while the zone ratio for the elastinolytic activity of *Aeromonas* sp. (Au 2D8) was 3.8.

The enzyme patterns produced in the API ZYM galleries appeared very similar for all fifteen isolates examined, and only minor variations were noted in the level of the enzyme activities measured (Table 3.5). All isolates were positive for phosphatase alcaline, esterase (C 4), esterase lipase (C 8), leucin arylamidase, phosphatase acid and napthol-AS-BI-phosphohydrolase, while the isolates exhibited weak lipase (C 14) and valine arylamidase activity. All isolates were negative for cystine arylamidase, trypsin and chymotrypsin activity, and also the enzymes involved in carbohydrate metabolism (α galactosidase, β galactosidase, β glucuronidase, α glucosidase, β glucosidase, N-acetyl-glucosaminidase, α mannosidase and α fucosidase)

3.3.5. Challenge experiments

The PCM obtained in Challenge 1 is shown in Figure 3.4. Mortalities commenced on day 3 following IM administration of bacteria grown in broth and continued for 9 days post-

Table 3.5. Enzymatic API ZYM patterns obtaining for variety of *F. psychrophilum* isolates

Enzyme activity	Isolates of *F. psychrophilum*														
	NCIMB 1947^T	NCIMB 2282	HL3131/97	UP193/97	UP 164/97	B97026	B97035 E4	32/97	33/97	34/97	35/97	CASO 89/97	59/95	921/97	110/97
Phosphatase alcaline	[a]5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
Esterase (C 4)	2	2	2	2	2	2	2	2	2	2	3	3	2	2	2
Esterase lipase (C 8)	3	2	3	3	3	3	3	3	3	2	2	2	2	2	3
Lipase (C 14)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Leucine arylamidase	5	5	5	5	5	5	5	5	5	4	5	5	5	5	5
Valine arylamidase	1	1	2	1	1	1	1	2	2	1	2	2	2	1	2
Cystine arylamidase	0	0	0	1	1	0	0	0	0	0	0	0	1	1	0
Trypsin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Chymotrypsin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Phosphatase acid	3	3	2	3	3	3	3	3	3	2	4	3	3	3	4
Naphthol-AS-BI-phosphohydrolase	3	2	3	3	3	3	3	3	3	2	4	3	3	3	3
α galactosidase	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
β galactosidase	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
β glucuronidase	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
α glucosidase	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
β glucosidase	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N-acetyl- glucosaminidase	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
α mannosidase	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
α fucosidase	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

[a]API reaction scores ranging from 0-5: 0 corresponds to a negative reaction, while 5 correspond to a reaction of maximum intensity

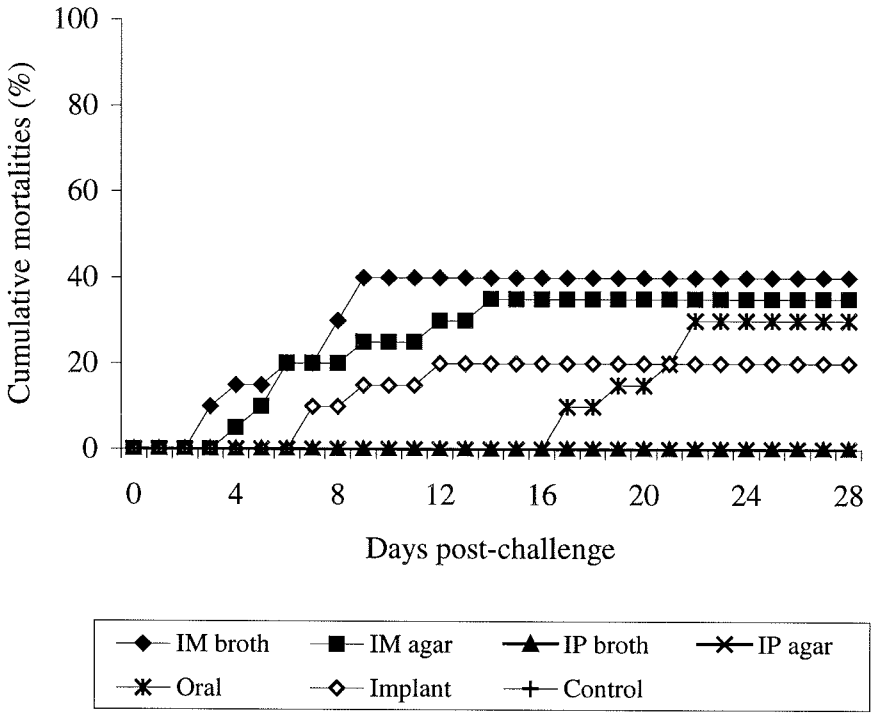

Figure 3.4. Challenge 1: Cumulative mortalities (%) of rainbow trout fry following artificial challenging with *F. psychrophilum* isolate HL313/97 by different routes of administration (n = 20 fish).

The control presented here represents all control groups set up in the challenge since no mortalities occur in any of the control group.

challenge. The mortalities resulting from bacteria cultured on agar plates, administrated IM, started to occur on day 4 and continued until day 14 post-challenge. The PCM of fish which resulted from an IM injection by day 28 post-challenge, the time at which the challenge was discontinued, were 40% and 35% for bacteria from broth and plate cultures, respectively. No significant difference was seen between the two preparations however. The PCM of fish, which received agar implants of the bacterium, was 20% by day 28 post-challenge. Fish infected orally with the bacterium started to die 17 days from the on-set of the experiment and these continued for the next 5 days. On termination of the trial the PCM for this route of infection was 30%. No mortalities were observed in any of the control groups of fish over the course of the trial.

In Challenge 2, the PCM obtained upon termination of the trial at 28 days post-challenge were 100 %, 30 % and 20 % for strains B97026P1, B97035E4 and HL313/97, respectively (Figure 3.5). No deaths occurred in fish following injection with isolate 32/97 or in the control group over the course of the trial. Significant differences were seen with the other three isolates B97026P1, B97034E4 and HL313/97 however when compared with the control group and with isolate 32/97 ($P < 0.01$), but there was no significant difference between isolates B97034E4 and Hl313/97. Based on this trial, isolate B97026P1 was considered virulent and isolate 32/97 as non-virulent.

Figure 3.6 illustrates the results of the third challenge experiment, in which virulent isolate B97026P1 was used to infect larger rainbow trout (12 g) by various routes of administration (IM, IP, bath and oral). Fish began to die on day 1 when bacteria were

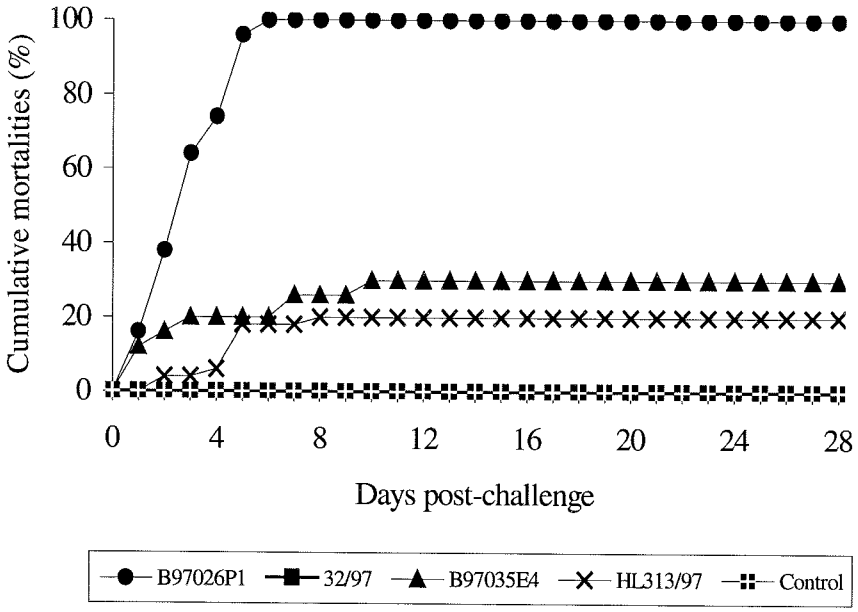

Figure 3.5. Challenge 2: Cumulative mortalities (%) of rainbow trout fry following intramuscular injection with different isolates of *F. psychrophilum* at 1 x10^7 cfu $fish^{-1}$ (n = 50 fish).

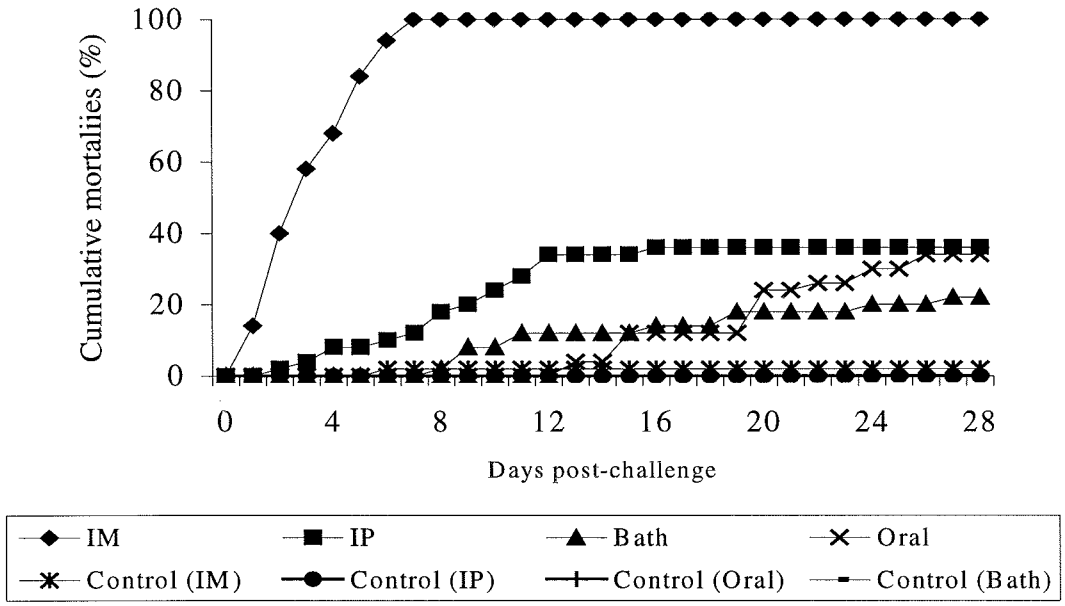

Figure 3.6. Challenge 3: Cumulative mortalities (%) of rainbow trout fry artificially challenged with virulent of *F. psychrophilum* isolate B97026P1 using different routes of administration at 1.1 $x10^7$ cfu $fish^{-1}$ (n = 50 fish).

administrated IM and these continued to die over the next 7 days. A PCM of 100% was obtained by day 28 post-challenge when fish received the bacterium by IM injection. Fish injected IP started to die on day 2 post-challenge and the mortality progressed slowly over the next 17 days with a PCM of 36% obtained by day 28 post-challenge. The levels of mortalities obtained by bath and oral administration were 22 and 34 %, respectively, upon termination of the challenge at day 28. A PCM of 6% was noted in the control group, however, it was not possible to isolate *F. psychrophilum* from any of the dead control fish. Significant differences ($P < 0.01$) were seen with all routes of administration when compared to their corresponding controls. No significant differences were seen in the level of mortalities between the different routes of administration except in the IM challenge ($P < 0.01$).

In Challenge 4, 100% and 46.7% of fry died throughout the course of the trial as a result of IM injection with either 2×10^6 cfu fish^{-1} and 2×10^5 cfu fish^{-1}, respectively when compared to the control group ($P < 0.01$) (Figure 3.7). A dose of 2×10^4 cfu fish^{-1} did not kill any of the fish however. A significantly greater number of fry died (55%) after 28 days post-challenge when fish were infected orally with 2×10^6 cfu fish^{-1}, while only 6.6% of fish died with 2×10^5 cfu fish^{-1}, and a concentration of 2×10^4 cfu fish^{-1} was insufficient to kill any fish.

Fish that died when challenged by an IM challenge route, had muscle erosion around the site of injection generally between 3 or 4 days post-injection and deep skin lesions then occurred. Fish, which survived injection of *F. psychrophilum* IM in Challenges 1 and 4 and

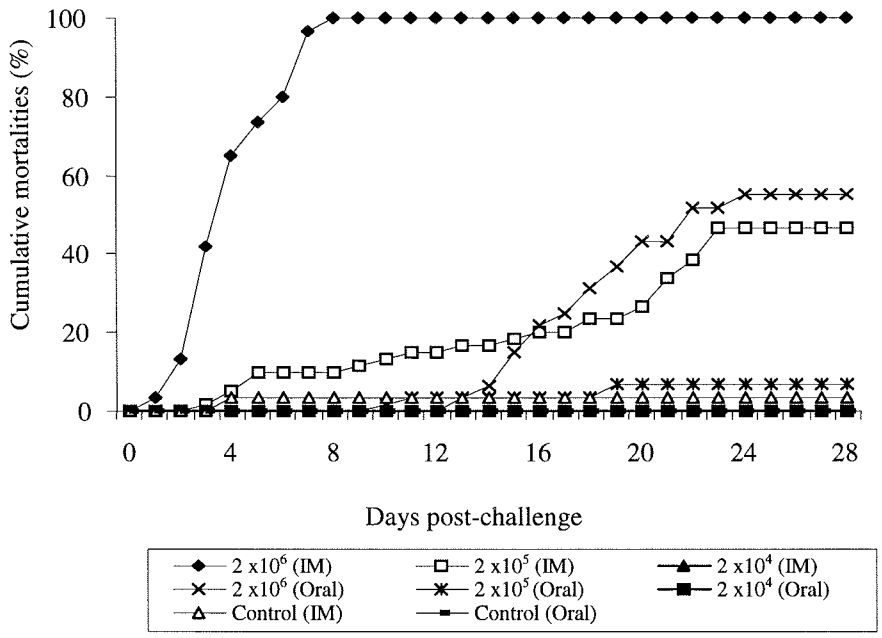

Figure 3.7. Challenge 4: Cumulative mortalities (%) of rainbow trout fry following an artificially challenge with virulent *F. psychrophilum* isolate B97026P1 with different concentrations of bacteria administrated orally or by intramuscular injection (n = 60 fish).

initially showed signs of the disease, made a full recovery. Similarly, fish implanted with the bacterium under the skin, also displayed deep skin lesions at around 5 days after implantation, while fish injected IP showed no signs of any external lesions. Enlargement of the spleen, kidney, swelling ascites and abdominal haemorrhage were observed. Upon post-mortem of dead fry injected either by the IM and IP routes, it was possible to isolate *F. psychrophilum* from all swabs made from the skin lesions, spleen, kidney or ascitic fluid of dead animals. Fish, which died after oral administration of the bacterium, showed varying degrees of peduncle erosion. It was not possible, however, to re-isolate *F. psychrophilum* from all of the dead fish in the oral and bath challenges.

In Challenge 1, *F. psychrophilum* was re-isolated from 4 of the 6 fish which died after oral administration, while the bacterium was re-isolated from only 12 of the 17 dead fish orally challenged in Challenge 3 (Table 3.6). In Challenge 4, 20 of the 33 fish orally infected with 2 $x10^6$ cfu $fish^{-1}$ were positive for *F. psychrophilum* by culture, while only 1 of 4 fish infected with 2 $x10^5$ cfu $fish^{-1}$ that died, was positive for *F. psychrophilum*. Only seven of the eleven fish that died in the bath challenge were established as being positively infected with *F. psychrophilum* (Table 3.6). No *F. psychrophilum* could be isolated from dead fish in any of the control groups.

3.4. Discussion

Fifteen isolates of *F. psychrophilum* most of which were isolated during outbreaks of RTFS in Scotland, England, Germany and Chile, were used in this study. The type strains NCIMB 1947^T and NCIMB 2282, were also included in the study as reference strains of

Table 3.6. Levels of specific fish mortalities which occurred during artificial challenge with *F. psychrophilum*

Challenge no.	*F. psychrophilum* isolate	Routes of administration	[a] Cumulative mortality (%)	No. of dead fish			[b] No. fish positive for *F .psychrophilum*
				Total	Replicates		
					A	B	
1	HL313/97	IM broth	40	8	3	5	8
		IM agar	35	7	4	3	7
		IP broth	0	0	0	0	0
		IP agar	0	0	0	0	0
		Oral	30	6	3	3	4
		Agar implant	20	4	2	2	4
2	[c] B97026P1	IM	100	50	25	25	50
	B97035E4	IM	30	15	9	6	15
	HL313/97	IM	20	10	6	4	10
	32/97	IM	0	0	0	0	0
3	[c] B97021P1	IM	100	50	25	25	50
		IP	36	18	11	7	18
		Oral	34	17	7	10	12
		Bath	22	11	7	4	7
4	[c] B97026P1	[d] IM (2×10^6)	100	60	30	30	20
		IM (2×10^5)	46.7	28	17	11	28
		IM (2×10^4)	0	0	0	0	0
		Oral (2×10^6)	55	33	18	15	20
		Oral (2×10^5)	6.7	4	2	2	1
		Oral (2×10^4)	0	0	0	0	0

[a] 28 day post-challenge, [b] Fish in which *F. psychrophilum* was recovered from the spleen of the dead animal, [c] Passed through fish once recovered from spleen of infected animal, [d] cfu $fish^{-1}$

F. psychrophilum. The growth and biochemical characteristics of these isolates were examined and these are compared here to the characteristics reported for other *F. psychrophilum* isolates associated with RTFS and BCWD outbreaks.

The phenotypic and biochemical characteristics of the isolates examined here were very similar, regardless of their origin, and only minor differences were identified between the different isolates. Initially, difficulties were encountered in trying to culture *F. psychrophilum*. In accordance with Hofer (1997), it was found that over the course of the study it was important not to expose the bacterium to room temperature (>20°C) for extended periods of time. Prolonged exposure to room temperature, as could occur during sub-culturing, was found to restrict the growth of the bacterium. All media were, therefore, routinely maintained at 4°C prior to sub-culturing the bacterium. The growth of the bacterium was found to vary between the different isolates, and large inocula of bacteria were necessary in order to obtain good growth of cultures. It was also necessary to sub-culture the bacterium every week to maintain its viability. The bacterium had to be cultured for at least 72-96 h in MAOB to obtain sufficient yields of cells for harvesting.

It should be noted that the growth of each *F. psychrophilum* isolate differed considerably. Ideally, a growth curve should have been prepared for each isolate to examine individual life cycles of the isolates. The results of the analysis performed here may have represented responses of bacteria in different stages of their life cycle. For example, differences seen in protease activity may have been due to this.

None of the isolates examined here were able to grow at 30°C, which agrees with the findings of Pacha and Porter (1968) and Lorenzen *et al.,* (1997). Bernardet and Kerouault (1989) found that their strains of *F. psychrophilum* grew slowly at 25°C, but not above this temperature. Holt (1987) also observed that 18 of the 28 isolates he examined produced slight growth at 25°C, but none were able to grow at 30°C. Of the 20 Australian isolates of *F. psychrophilum* examined by Schmidtke and Carson (1995) 18 were able to grow at 25°C, but none could grow at 30°C. Four German isolates of *F. psychrophilum*, isolated from eel and cyprinid, were unable to grow above 26°C (Lehmann *et al.,* 1991). Thus it seems that the upper temperature tolerance for the bacterium is close to 25°C (Lorenzen *et al.,* 1997).

The bacteria examined here generally produced round, raised colonies with regular margins on MAOA, and no spreading colony types were observed with any of the isolates examined. This was possibly due to the protein-enriched medium used for their culture. Similar colonies types were reported for the *F. psychrophilum* isolates examined by Lorenzen *et al.,* (1997) who used the same culture medium as in this study (MAOA) however, they supplemented the medium with 5% new born calf serum.

According to the findings of Bullock (1972), the morphology of colonies of Myxobacteria, now referred to as Cytophagales (Reichenbach, 1989), depends on the nutrient content of the agar, i.e. the lower the nutrients present, the greater the spreading of the colonies which was observed. Spreading of *F. psychrophilum* colonies was also reported on very fresh and moist medium by Henrichsen (1972, cited in Lorenzen *et al.,* 1997), whereas Bernardet and

Kerouault (1989) observed both types of colonies on MAOA plates.

The auto-agglutination that was observed in broth culture appeared to be a common feature amongst *F. psychrophilum* isolates examined in this study, although there was some variation between the isolates with respect to the degree of auto-agglutination that occurred. The extent of auto-agglutination between the strain was not quantified here, however. Janda *et al.,* (1987) reported that auto-agglutination of mesophilic aeromonads in broth appears to be a virulence-associated marker of the bacterium. The more virulent strains of either *A. hydrophila* or *A. sobria* shared a common type of auto-agglutination and possessed an extracellular layer peripheral to the cell membrane (Janda *et al.,* 1987). The ability of bacteria to auto-agglutinate in broth culture and the presence of a peripheral extracellular layer has also been used as virulence markers of psychrophilic aeromonads (Udey and Fryer, 1978; Rosenburg *et al.*, 1980). Auto-agglutinating strains of *A. salmonicida*, with an additional layer external to the cell wall (A layer), were found to cause disease in coho salmon, while non-aggregating isolates lacking the A layer did not (Udey and Fryer, 1978). The auto-agglutination of *A. salmonicida* appears to depend upon a hydrophobic protein associated with the A layer. This hydrophobic layer also contributes to the bacterium's resistance to complement-mediated lysis (Evenberg and Lugtenberg, 1982; Evenberg *et al.,* 1982; Munn *et al.,* 1982; Parker and Munn, 1984). No such extracellular layer has been associated with *F. psychrophilum*, but Garcia-Marquez *et al.,* (1998) demonstrated that cells of *F. psychrophilum* possess low hydrophobicity.

It would appear from the challenges performed in this study, that the characteristic of auto-

agglutination may, to some extent, be associated with the virulence of *F. psychrophilum,* since the degree of auto-agglutination which occurred with the virulent isolate appeared greater than that observed with the avirulent isolate. This however would have to be verified by determining the levels of auto-agglutination, which occurred with the different isolates.

The results of the biochemical analysis performed here were generally in agreement with those of Bernardet and Grimont, 1989; Bernardet and Kerouault, 1989; Schmidtke and Carson, 1995; Cipriano *et al.,* 1996 and Lorenzen *et al.,* 1997. Most reactions were slow and weak, and it was important to use cultures that were not older than 2 to 4 days for most of the direct tests such as flexirubin production, and catalase or oxidase activity. Pacha and Porter (1968) and Holt (1987) considered *F. psychrophilum* to be devoid of cytochrome oxidase, as determined by the method of Gaby and Hadley (1957). Bernardet and Grimont (1989) however, reported difficulties in detecting cytochrome oxidase of *F. psychrophilum* by this method and suggested using a more sensitive test such as that of Kovacs (1965, cited in Cowan, 1974), whereby commercial filter papers or discs were used. This method was employed in the present study.

Production of flexirubin pigment is considered a principal characteristic for taxonomical identification of *F. psychrophilum* which gives rise to its characteristic yellow colour (Thoesen, 1994). Holt (1987), however, initially failed to show the presence of flexirubin pigments in his isolates. After Bernardet and Kerouault (1989), and Lehmann *et al.,* (1991) reported to the production of flexirubin pigment by their *F. psychrophilum* isolates, Holt *et*

al., (1993) re-examined their isolates and confirmed the production of the pigment. All the isolates of *F. psychrophilum* used here produced flexirubin pigment.

Most of the enzymatic activities described for *F. psychrophilum* have been established by API ZYM galleries (Bernardet and Grimont, 1989; Bernardet and Kerouault, 1989; Lehmann *et al.,* 1991; Iida and Mizokami, 1996; Ekman *et al.*, 1999). This is a semi-quantitative technique in which nineteen enzymatic reactions are measured and only very small quantities of sample are required for the procedure. The enzyme patterns determined in API ZYM galleries here were identical to the pattern previously described for *F. psychrophilum* with some minor variations in their intensity (Bernardet and Kerouault, 1989). Negative results were obtained for all enzymes involved in carbohydrate metabolism, a characteristic shared with *F. columnare* and *Flexibacter maritimus.* This property clearly distinguishes these three fish pathogens from other members of the family Flavobacteriaceae, commonly associated with the opportunistic or saprophytic microflora of fish, such as *F. aquatilis* and *F. johnsonae* (Lehmann *et al.,* 1991).

All of the *F. psychrophilum* isolates examined here clearly degraded gelatin and casein with strongest activity exhibited against gelatin. Degradation of elastin was difficult to observe using this method, with only four isolates producing small zones of degradation in the elastin-supplemented plates. These zones also sometime appeared after the zones that occurred with the other substrates. Of the four isolates capable of degrading elastin, two isolates (HL313/97 and UP193/97) were previously found to have this activity (Hofer, 1997).

The ability of *F. psychrophilum* to degrade elastin has been found to vary substantially between different *F. psychrophilum* isolates by a number of scientists. Otis (1984), examining 5 strains of *F. psychrophilum* by a method similar to the one described here, found that all strains were able to degrade gelatin and casein, but only four of his isolates were able to degrade elastin. Of the 29 *F. psychrophilum* isolates examined by Bertolini *et al.,* (1994), all were able to degrade gelatin and casein, while 6 of the 29 isolates were unable to digest elastin. Holt *et al.,* (1993) found that only two of the twenty-eight isolates they examined were able to degrade elastin. In contrast, Madsen and Dalsgaard (1998) found 40 elastin-positive isolates amongst the 47 Danish isolates they examined. The type strain NCIMB 1947^T was found to be elastin-negative in this study. This finding is in agreement with Bertolini *et al.,* (1994). The NCIMB strain 2282 was also unable to degrade elastin in the present study.

When modified elastin agar was used here to assess elastinase activity, however, all of the *F. psychrophilum* isolates examined appear devoid of the elastinolytic activity, while *Aeromonas* sp. (Au 2D8), used as positive control, expressed very high activity. One explanation for the lack of activity with this method is that the assay conditions were not optimised for *F. psychrophilum*, although the procedure was found to be very sensitive for *Aeromonas* sp.

The protease activity of *F. psychrophilum* was only assessed against gelatin, casein and elastin substrate. It would also be worth examining the proteolytic activity of the bacterium against other substrates such as collagen, fibrinogen, haemoglobin or fish peptone.

Despite similarities in the phenotypic and biochemical properties of the *F. psychrophilum* isolates in this study, they did display different degrees of virulence when subjected to an experimental challenge. Unfortunately it was only possible to establish the level of virulence of four of the *F. psychrophilum* isolates (B97026P1, B97035E4, Hl313/97 and 32/97). However, marked differences were found in virulence between these, when rainbow trout fry were injected IM with 1 $x10^7$ cfu $fish^{-1}$ with mortalities ranging from 0 to 100%.

The experimental challenges of *F. psychrophilum* presented here clearly demonstrated that the IM route appeared to be the most effective way of establishing RTFS infections in rainbow trout fry under laboratory conditions, although not all of the clinical signs were analogous to those seen during naturally occurring RTFS. High mortalities occurred in fish injected IM, and signs of skin necrosis often progressing to open lesions in the musculature, were apparent. It is suggested that this may have resulted from the proteolytic activity of the bacterium. Holt (1987) found that the pathogenicity of *F. psychrophilum* was greater when administered via the muscle tissue of the animal rather than in the peritoneal cavity. Ostland *et al.,* (1997) found 100% mortality in groups of rainbow trout (7-10 cm total length) injected IM with *F. psychrophilum* at 1.45 $x10^6$ cfu $fish^{-1}$, while only 10% mortality occurred in groups of fish injected IP using the same concentration of bacteria. It was established that many more bacterial cells were needed to kill 50% of the fish injected IP route rather than by SC or IM routes (Holt, 1987). The latter may take advantage of proteolytic activities of the pathogen (Holt, 1987).

The most virulent *F. psychrophilum* isolate (B9702P1) found in this study, was the one recently isolated from the spleen of diseased rainbow trout in Scotland. The isolate appeared to have higher levels of auto-agglutination than that observed with the other isolates used in the challenge. Biochemically, this isolate appeared slightly different to the other *F. psychrophilum* isolates examined. Its protease activity was apparently greater compared to the other isolates, with larger zones of clearing seen on the gelatin and casein plates. This isolate also appeared to have the greatest gelatinase and caseinase activity between the isolates examined, and this activity appeared stronger against gelatin than casein. Elastase activity was also higher in this isolate.

It has been suggested that the virulence of *F. psychrophilum* depends on the serotype and proteolytic nature of the isolate (Madsen and Dalsgaard, 1998). Elastin-degrading isolates appeared to be much more virulent than isolates unable to degrade this protein (Madsen and Dalsgaard, 1999). Moreover, isolate B96026P1 had been passed through a fish once before using it in the challenges, and this may have increased its virulence. Low levels of mortality were observed in fish injected with isolate B97035E4 or HL313/97. The former originated from a Scottish hatchery and did not degrade elastin. The German isolate HL313/97 showed little elastase activity, but was old and had been sub-cultured many times *in vitro* on artificial media. With the exception of isolate HL313/97, all bacteria used in this study had been maintained on cryoprotection beads (Cryoprotect; Technical Service Consultants Ltd) at -70°C immediately after collection. It is speculated that continual sub-culturing of isolate HL313/97 on artificial media reduced its virulence. This appears to be a common phenomenon with many bacterial fish pathogens. Isolate 32/97 was unable to

kill any fish, although it had been recently isolated from the spleen of a diseased rainbow trout in Chile, and suggests that the bacterium may loss its virulence very quickly. This isolate was unable to degrade elastin and had lower protease activities to those seen with virulent isolate B97026P1. Serologically, isolates B97026P1 and 32/97 represented two different groups of *F. psychrophilum* as described in Chapter 5, while isolate B97035E4 appears to be in the same group as B97026P1, and isolate HL313/97 represents a completely separate serological group. The variability in pathogenicity seen between isolates may be related to the expression of different surface antigens. However, the differences in virulence seen in this study are probably not related to serotype differences, as the type strain NCIMB 1947^{T}, which is known to show low pathogenicity in rainbow trout fry (Rangdale, 1995), was placed in the same serological group as virulent isolate B97026P1 (Chapter 5).

Variations in virulence of *F. psychrophilum* isolates have been reported by a number of authors. Holt (1987) compared the ability of 22 isolates of *F. psychrophilum* isolated from BCWD outbreaks to produce disease in yearling coho salmon by injecting them SC with 2.8×10^{7} cells $fish^{-1}$, and found that the percentage mortality caused by each isolate varied between 0 and 100%. Similarly, Bertolini *et al.,* (1994) injected juvenile coho salmon SC with isolates from different groups of *F. psychrophilum* based on their protease activity, and found differences in the virulence of isolates tested. They observed PCM ranging from 0 to 100% over the 21 days period of their challenge. They found that isolates which expressed both caseinases and gelatinases activity in substrate-SDS-PAGE, were more virulent than those lacked such enzyme activities.

It would appear that bacteria cultured in either broth or on agar did not show any significant difference in their virulence when injected IM. Washing the bacterial cells prior to the challenge also did not appear to alter the levels of mortality which were obtained. Rangdale (1995) reported high levels of mortality in fish when challenged with washed *F. psychrophilum* by IP injection.

Oral administration of *F. psychrophilum* appeared to reproduce the symptoms of RTFS in challenged fish. The percentage cumulative mortality obtained by this route was 55 % with 2 x10^6 cfu $fish^{-1}$ when the virulent isolate B97026P1 was used. Mortalities started to occur much later in this model than with IM or IP injection, and tail loss or erosion of the peduncle was a common feature among the fish, which died. It has been shown by Sera and Ishida (1972), that few bacteria are able to survive the adverse conditions of the stomach where pH values of less than 3 are encountered, as well as the presence of digestive enzymes. However, they have been shown to rapidly multiply once they reached the intestine. The results of the oral challenge suggest that sufficient bacterial cells survived and passed into the gut, and the tail necrosis found in the later stages of the challenge may in fact be due to the bacteria passing through the urine or faeces of the animal. It has been suggested that *F. psychrophilum* probably enters the fish via the gills or through the skin, but they may also enter by oral or gastrointestinal routes (Dalsgaard, 1993).

Low levels of mortality were observed when fish were challenged by bath. The PCM obtained here was 22 % when fish were dipped into a suspension of virulent isolate

B97026P1 at 1.5 x10^5 cfu ml^{-1} for 12 h. The reason why the bath challenge was not effective may be that the laboratory conditions under which the challenge was performed were too clean and fish were not sufficiently stressed to succumb to the disease. Another reason for the low mortality in bath challenge may be that the fish secrete of mucus in the bath, which neutralises some of the bacteria, and some of the bacteria stick to the fish. However, Chua (1991), Rangdale (1995) and Madsen and Dalsgaard (1999) were all able to infect fish by bath challenge. Using 10^5 cfu ml^{-1}, Rangdale (1995), obtained 30% and 38% mortalities when fish were dipped into the bacterial suspension for 5 h and 10 h, respectively, 14 days post-challenge, while Madsen and Dalsgaard (1999) found PCM between of 27 and 31 % when 10^7 cfu ml^{-1} was applied for only 0.5 h.

Several authors have reported little success with cohabitation challenges for *F. psychrophilum* using artificially infected fish (Holt, 1987; Madsen and Dalsgaard, 1999). A small cohabitation challenge was also tried here (data not presented), where fish (average weight 5 g) suspected of being infected with *F. psychrophilum* were collected from a hatchery with an ongoing RTFS outbreak and placed with healthy fry collected from a farm with no previous history of the disease. Fish were monitored for 28 days and the water temperature maintained at 10-12°C throughout this period. However, there was no evidence of transfer of infect to the healthy animals under the challenge conditions applied here. Unfortunately, it was later found that the fish had been treated with amoxycillin prior to collecting them from the farm, and also the presence of *F. psychrophilum* was not confirmed in the RTFS-suspected fish, prior to performing the challenge.

In summary, isolates of *F. psychrophilum* from RTFS outbreaks in the UK and in Chile were phenotypically very homogenous. The biochemical characteristics of these isolates were very similar to reference strains obtained from NCIMB and to *F. psychrophilum* isolates reported in the literature obtained from both BCWD and RTFS outbreaks.

Auto-agglutination was seen to be a common feature amongst the of *F. psychrophilum* isolates examined here, although the extent of this appeared to vary between isolates. There are indications that this, together with the protease activity of the isolates may be important in the virulence of the bacterium, and this should be investigated further. Unfortunately, only four isolates of the bacterium were used to examine virulence of the bacterium in this study, and if conclusions about virulence factors are to be made substantially more isolates need to be examined.

The challenge presented here revealed IM to be most effective challenge route for establishing infection in rainbow trout fry under laboratory conditions. However, this did not result in all the clinical signs of RTFS and substantial skin lesions resulted. Oral administration of the bacterium was also reasonably effective, and produced many of the signs typical of RTFS. However, ten-fold more bacteria were required to do so.

CHAPTER 4

Characterisation of *Flavobacterium psychrophilum* by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

4.1. Introduction

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is by far the most reproducible and rapid method for quantifying, comparing and characterising proteins (Bollag *et al.*, 1996). Very little published information is available in the literature describing the SDS-PAGE profiles of *F. psychrophilum.* Cipriano *et al.,* (1996) has examined the protein profiles of whole cell lysates of *F. psychrophilum* isolates obtained from chinook salmon and coho salmon suffering from BCWD and reported a marked homogeneity between the isolates. However, there are no reports in the literature of SDS-PAGE profiles prepared from *F. psychrophilum* isolates obtained from RTFS outbreaks. Bernardet *et al.*, (1994) has also reported similarities between the protein profiles of whole cell preparation of another related species, *Flexibacter maritimus.*

The outer layer of the cell wall of Gram negative bacteria, the outer membrane, is a planar lipid bilayer composed of protein, phospholipid and lipopolysaccharide (LPS). At least half of the outer membrane is composed of proteins, which are covalently linked to the underlying peptidoglycan. The outer membrane of many Gram negative bacterial pathogens plays an important role in the interaction with their host. Examination of the OMPs of the bacterium is often used to study strain variation and relatedness between bacterial isolates and to identify virulence determinants on the bacterium.

Carbohydrates are also known to be associated with the virulence of bacterial pathogens (Wetzler *et al.*, 1992). Lectin staining and a commercially available glycoprotein kit have been used by a number of authors to examine and characterise carbohydrates present on both bacterial (Puttinaowarat, 1999; Jung, 1999) and fungal (Lilley *et al.*, 1997) fish pathogens. Glycoproteins are proteins containing oligosaccharides covalently attached to select amino acid residues. Lectins are carbohydrate-binding proteins of non-immune origin, capable of agglutinating cells or precipitating glycoproteins (Liener *et al.,* 1986; Cuperlovic and HajdukovicDragojlovic, 1996). They are able to bind to carbohydrates with a particular carbohydrate linkage and are widely used to investigate the composition of glyco-congugates and other carbohydrate moieties present on a variety of molecules (Singh *et al.*, 1999). Recently, lectins labelled with fluorescent tags (enzymes or colloidal gold) have been used to detect specific carbohydrate chains on cell surfaces (Oda *et al.,* 1999).

The aim of the present study was to examine whole-cell and OMP preparations of *F. psychrophilum* isolated from fish from different geographical regions during outbreaks of RTFS, and compare their SDS-PAGE profiles using various stains and lectins. This study was performed in order to establish the extent of homogeneity between the isolates with respects to their protein and carbohydrate composition.

4.2. Materials and Methods

4.2.1. Bacterial strains and growth conditions

Fourteen isolates of *Flavobacterium psychrophilum,* used in the current study, are listed in Table 4.1. Of the 14 isolates examined, two isolates were considered as reference isolates

Table 4.1. Isolates of *Flavobacterium psychrophilum* used for SDS-PAGE analysis

Isolate	Fish species	Tissue	Country	Year
NCIMB 1947[T]	Coho salmon	Kidney	USA	1955
NCIMB 2282	Silver salmon		USA	
HL313/97	RT	Spleen	Germany	1997
UP193/97	RT	Spleen	England	1997
UP164/97	RT	Spleen	England	1997
B97026 *(B97026P1)	RT	Lesion	Scotland	1997
B97035 E4	RT	Spleen	Scotland	1997
59/95	RT	Unknown	Chile	1995
32/97	RT	Kidney	Chile	1997
34/97	RT	Kidney	Chile	1997
35/97	Atlantic salmon	Kidney	Chile	1997
CASO 89/97	RT	Kidney/spleen	Chile	1997
921/97	RT	Spleen	England	1997
110/97	RT	Spleen	England	1997

*Passed through fish and recovered from spleen of infected animal,

Coho salmon (*Oncorhynchus kisutch*), Silver salmon (*O. kisutch*), RT: Rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*).

based on their pathogenicity. Isolate B97026P1 was considered virulent and isolate 32/97 non-virulent, as determined by challenging artificially rainbow trout fry (average weight 4.37 g) described in Chapter 3. Bacteria were cultured in MAOB at 15°C as described in Section 2.1.2.1.

4.2.2. Preparation of bacterial samples

Bacteria were prepared for the analysis according to Section 2.1.2.3. The concentrations of the bacterial suspensions were adjusted to approximately 1 x10^9 cell ml^{-1} using the standard curve prepared in Chapter 3. Prior to adding sample buffer, a 100 µl aliquot of the preparation was removed and stored at –20°C for subsequent protein digestion so as to remove protein components from the bacterial preparation (Lilley *et al.*, 1997). Samples for protein digestion were incubated with 10 µl of 10 $mgml^{-1}$ proteinase K solution (Sigma) for 1 h at 60°C before dilution in sample buffer.

4.2.3. Outer membrane protein (OMP) preparation

The OMPs of virulent (B97026P1) and non-virulent (32/97) isolates of *F. psychrophilum* for electrophoretic analysis were prepared according to Section 2.6.

4.2.4. Electrophoresis

SDS-PAGE analysis of whole cell (1 x10^9 cell ml^{-1}) and OMP (1 $mgml^{-1}$) preparations were performed using a 12% separating gel and a 4% stacking gel as described in Section 2.2. Gels were stained with either Coomassie brilliant blue, Silver stain or Schiff's reagent as described in Section 2.2.2.

4.2.5. Lectin staining

Lectins were used to examine carbohydrate moieties present on the bacterial cells according to Lilley *et al.,* (1997). Whole cell preparations of *F. psychrophilum* were subjected to 12% SDS-PAGE according to Section 2.2.1 and the bacterial components were transferred from the SDS-PAGE gels to nitrocellulose membranes using a wet system as described in Sections 2.3. The nitrocellulose membranes were incubated in 1% (w/v) BSA in TBS overnight at 4°C. They were then washed with TBST and incubated for 1 h with lectins labelled with biotin (Sigma) (see Table 4.2) diluted to 20 μgml^{-1} in TBS. The membranes were washed three times with TBST, 10 min per wash, then incubated for 1 h with streptavidin-peroxidase (SAPU) diluted 1/100 in TBS. The membranes were again washed three times with TTBS, 10 min per wash and then give a fourth wash for 1 min with only TBS. The assay was developed with the addition of chromogen and substrate until bands materialised. The blots were placed in distilled water for 10 min to stop the reaction.

4.2.6. Glycoprotein detection

The whole cell preparations of *F. psychrophilum* were screened for the presence of glycoproteins using the detection kit described in Section 2.4.

4.3. Results

4.3.1. SDS-PAGE analysis

After electrophoresis, the SDS-PAGE profiles of whole cell preparations of the *F. psychrophilum* isolates, indicated in Table 4.1, were stained with Coomassie brilliant blue. Although the resolution of the bands in the profiles was not particularly clear, the protein

Table 4.2. Lectins used in this study

Lectin (origin)	Abbreviation	Carbohydrate specificity
Peanut (*Arachis hypogaea*)	AHL	D-galactose
Wheat germ (*Triticum vulgaris*)	TVL	N-acetyl-β-D- glucosaminyl residues and N-acetyl-β-D-glucosamine oligomers
Soybean (*Glycine max*)	GML	N-acetyl-D-galactosamine
Tomato (*Lycopersicon esculeulentum*)	LEL	N-acetyl-β-D- glucosaminyl oligomers
Gorse seed (*Ulex europaeus*)	UEA-1	L-fucose
Concanavalin A (*Canavalia ensiformis*)	Con A	Terminal α-D-mannosyl and α-D-glucosyl residues
Coral tree (*Erythrina cristagalli*)	ECA	D-galactose and D-galactosides
Horse gram (*Dolichos biflorus*)	HGL	Terminal N-acetyl- α-D –galactosaminyl resedues

banding patterns appeared very similar between isolates (Figure 4.1). The major regions stained with the Coomassie blue stain were located at around 32 to 45 kilo Dalton (kDa) and 58 to 73 kDa. A distinct band was also present at 116 kDa. However, faint staining was also noticed with low and very high molecular weight material. Silver stain was also used to examine the SDS-PAGE profiles of the *F. psychrophilum* isolates and again banding patterns were similar between the isolates (Figure 4.2). Staining of the 32-45 kDa and 58-73 kDa region was less intense with the Silver stain than was seen with the Coomassie blue stain. However, very high molecular weight material, >200 kDa, located at the very top of the gel was stained with all isolates. Intense staining was also seen at the bottom of the gel at around 6 to 14 kDa, and with bands between 18 to 35 kDa. Distinct bands were also seen at 42, 45, 70 and 100 kDa. However, a distinct region around 16 kDa remained clear upon staining the gel with the Silver stain. Extremely large molecular weight polysaccharides were visualised by Schiff's reagent, with the majority of stained material remaining in the wells of the 4% stacking gel (Figure 4.3). Weakly stained material was also evident between 18 and 32 kDa with the Schiff's stain.

Very little protein was left after treating the two *F. psychrophilum* reference isolates (B97026P1 and 32/97) with proteinase K, illustrated when the profiles of treated and untreated preparations were stained with Coomassie blue staining (Figure 4.4a, lanes 4 and 5). It could be seen from Silver staining, however, that a large amount of material remained undigested after proteinase K treatment, with two distinct bands at 18 and 30 kDa (Figure 4.4b). The high MW material in the profiles of the treated samples was only weakly stained with Silver stain when compared to the untreated samples. The 18 kDa

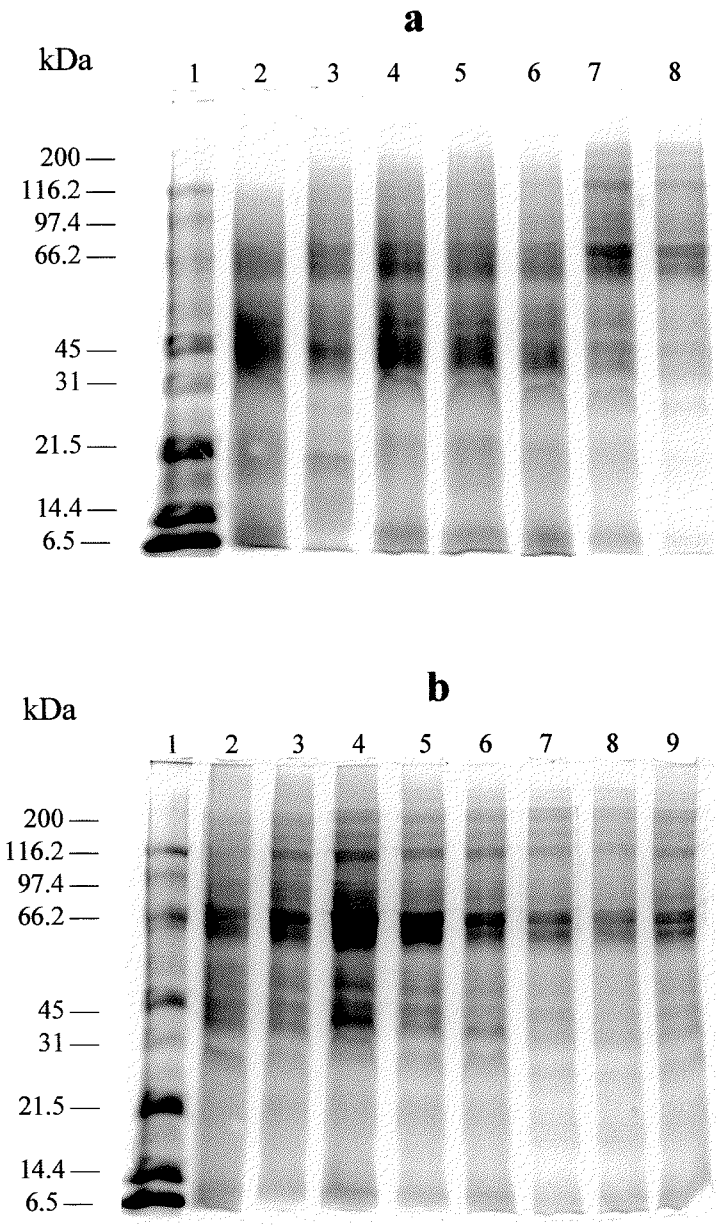

Figure 4.1. Coomassie Brilliant Blue stained SDS-PAGE gels (12%) of whole cell preparation of different strains of *F. psychrophilum*. (a) Lanes: (1) BioRad molecular markers (2) NCIMB 1947^T (3) NCIMB 2282 (4) B97026 (5) B97026P1 (6) B97034E4 (7) HL313/97 (8) UP164/97 (b) Lanes: (1) BioRad molecular markers (2) 32/97 (3) 34/97 (4) 35/97 (5) 59/95 (6) CASO 89/97 (7) 110/97 (8) 921/97 (9) UP193/97.

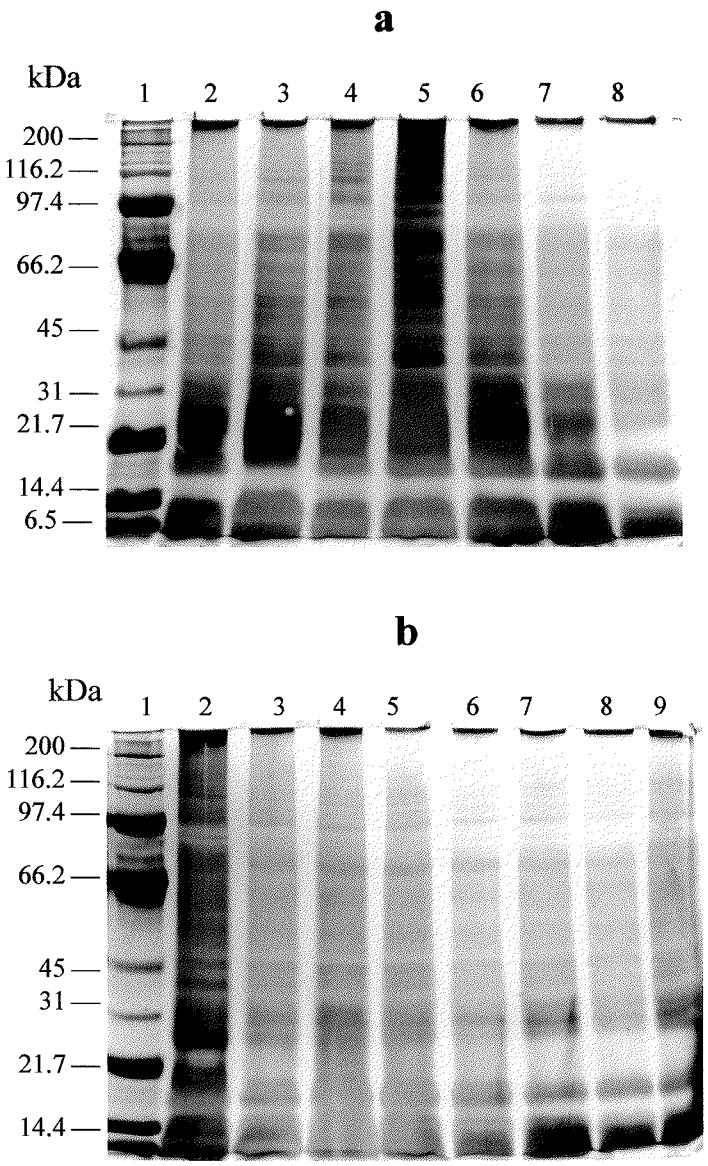

Figure 4.2. Silver stained SDS-PAGE gels (12%) of whole cell preparation of different strains of *F. psychrophilum* (a) Lanes: (1) BioRad molecular markers (2) NCIMB 1947 ^{T} (3) NCIMB 2282 (4) B97026 (5) B97026P1 (6) B97034E4 (7) HL313/97 (8) UP164/97. (b) Lanes: (1) BioRad molecular weight markers (2) 32/97 (3) 34/97 (4) 35/97 (5) 59/95 (6) CASO 89/97 (7) 110/97 (8) 921/97 (9) UP193/97.

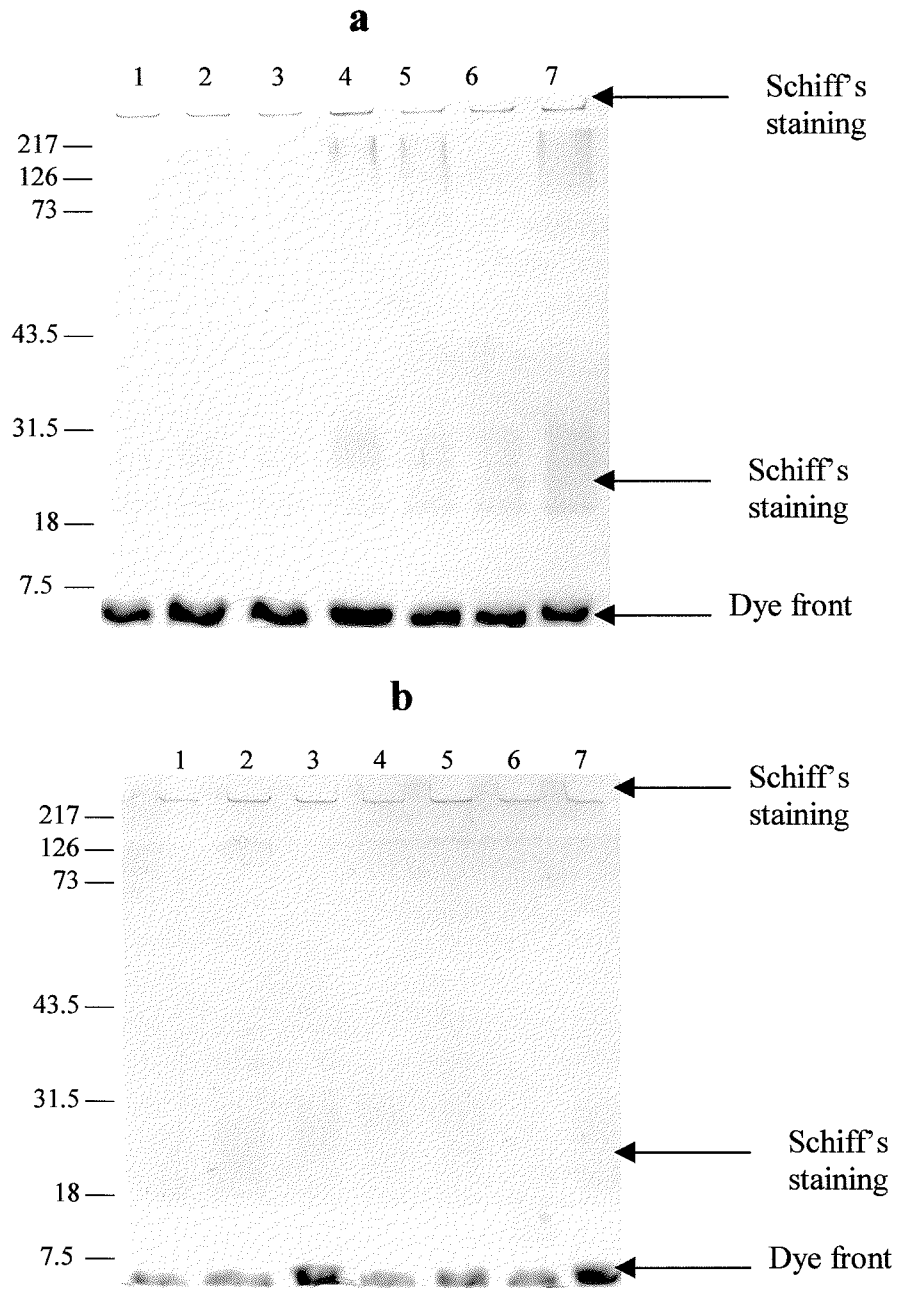

Figure 4.3. Schiff's stained SDS-PAGE gels (12%) of whole cell preparation of different strains of *F. psychrophilum*. (a) Lanes: (1) NCIMB 1947^T (2) NCIMB 2282 (3) B97026 (4) B97026P1 (5) B97034E4 (7) HL313/97, (b) Lanes: (1) 32/97 (2) 34/97 (3) 35/97 (4) 59/95 (5) CASO 89/97 (6) 110/97 (7) 921/97.

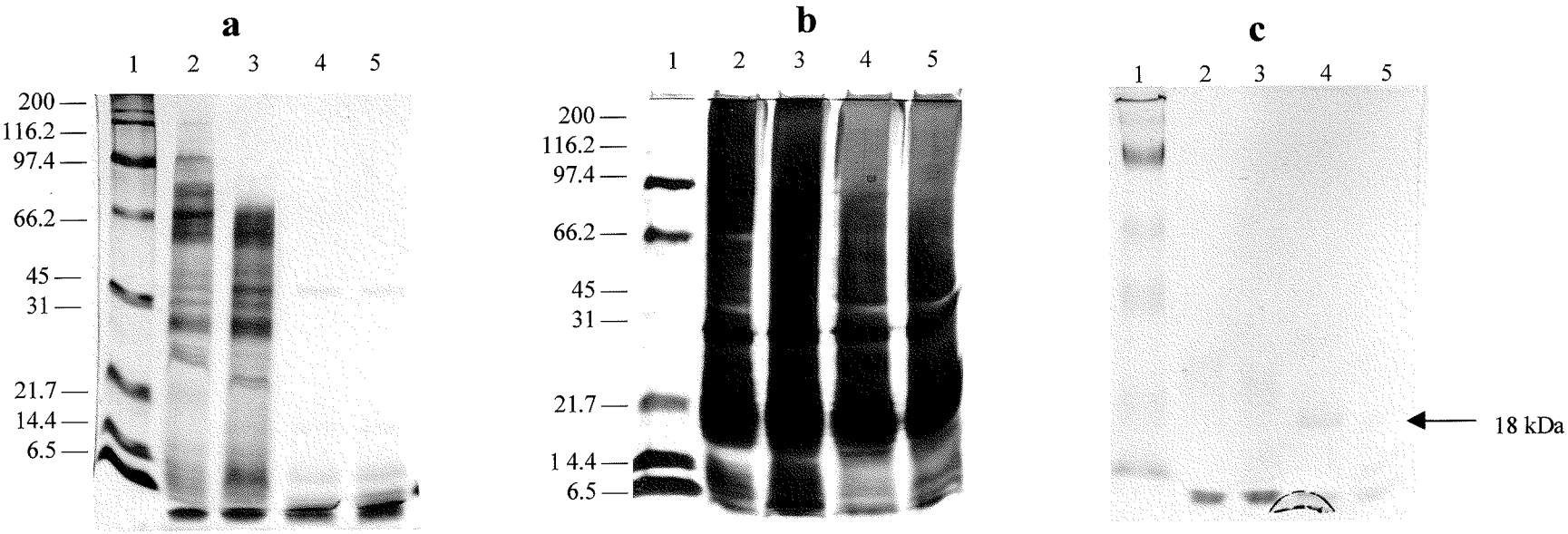

Figure 4.4. SDS-PAGE gels (12%) of proteinase K treated and untreated whole cell preparation of *F. psychrophilum*; (a) Commassie Brilliant Blue stained, (b) Silver stained and (c) Schiffs stained gel, Lanes: (1) BioRad molecular weight marker; (2) Untreated B97026P1 (2) Untreated 32/97 (4) Proteinase K treated B97026P1 (5) Proteinase K treated 32/97.

band together with a very low MW band were seen upon staining the treated samples with Schiff's reagent (Figure 4.4c).

The OMP preparations of the two *F. psychrophilum* reference isolates appeared similar upon staining the SDS-PAGE profiles of the OMPs with Coomassie blue. Dominant protein bands were located at approximately 32, 52 and 116 kDa (Figure 4.5), while no low molecular weight material was evident. Silver staining of the OMP preparations produced very smeared profiles, especially in the low MW region and results are therefore not presented here.

4.3.2. Detection of glycoprotein on nitrocellulose membranes

Whole cell and OMP preparations of *F. psychrophilum* were analysed using a commercially available glycoprotein detection kit to identify the location of glycoprotein molecules in the SDS-PAGE profiles of the bacterial preparations. Two low molecular weight bands at 20 and 23 kDa (Figure 4.6) were detected with the kit, and these were evident in the whole cell preparations of all isolates examined. A few high molecular weight bands around 97 and 110 kDa were also detected in some of the isolates with the kit. The OMPs preparations, on the other hand, did not appear to contain any glycoprotein molecules, and a photograph of the blot is therefore not presented.

4.3.3. Lectin staining

Eight different lectins were chosen to examine the carbohydrate moieties present in the SDS-PAGE profile of whole cell preparations of *F. psychrophilum*. The intensity of

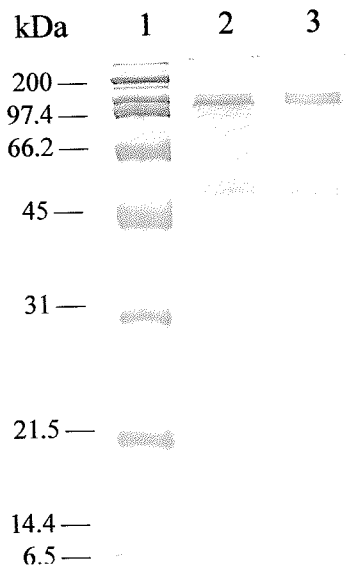

Figure 4.5. Coomassie Brilliant Blue stained SDS-PAGE gels of OMP preperations of *F. psychrophilum.* Lanes: (1) BioRad molecular markers (2) B97026P1 (3) 32/97.

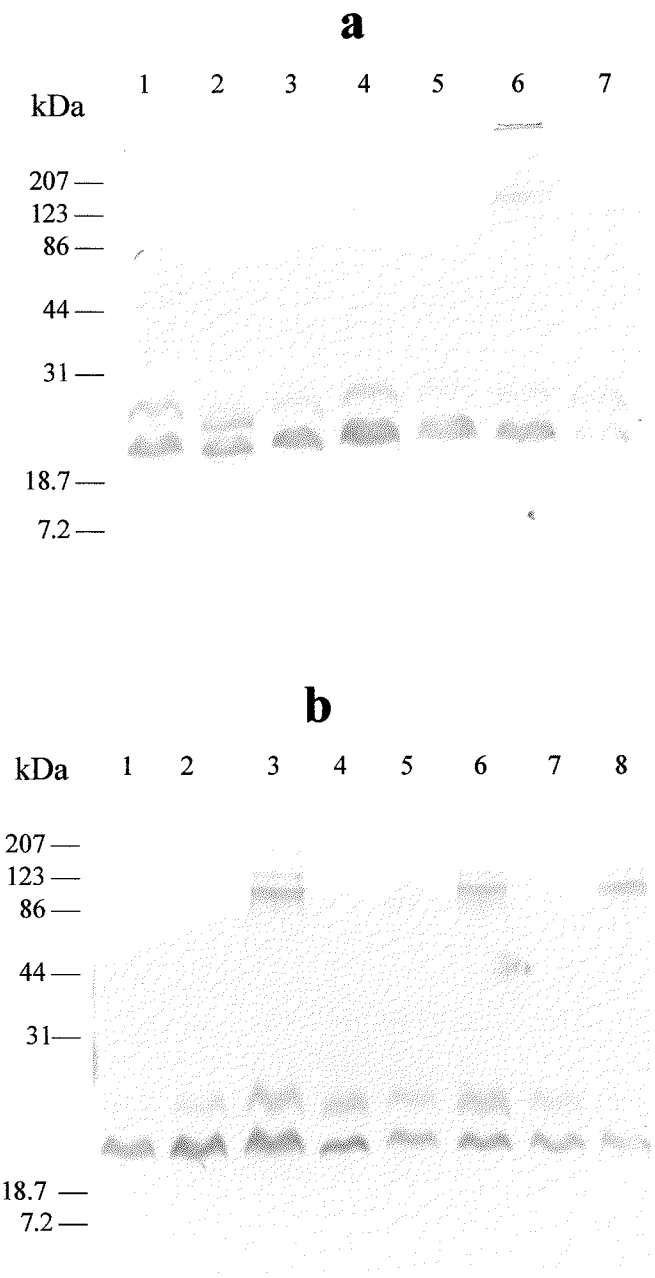

Figure 4.6. Glycoprotein detection of different isolates of *F. psychrophilum,* (a) Lanes: (1) NCIMB 1947^{T} (2) NCIMB 2282 (3) B97026 (4) B97026P1 (5) B97034E4 (6) HL313/97 (7) UP164/97, (b) Lanes: (1) 32/97 (2) 34/97 (3) 35/97 (4) 59/95 (5) CASO 89/97 (6) 110/97 (7) 921/97 (8) UP193/97.

staining varied with each lectin and the reactivity patterns obtained are summarised in Table 4.3. An example of the staining obtained with each of the lectins is presented in Figure 4.7. Of the eight lectins tested, lectins GML, LEL, ECA and Con A had very similar staining patterns to each other (Figure 4.7a, b, c and d, respectively) and generally recognised the same isolates (Table 4.3). They all identified a band around 20 kDa, but did not react with all of the isolates examined (Table 4.3). Isolate B97026 did not react with these four lectins, but when it was passage through a fish (B97026P1) it was found to be reactive with these lectins, with a band appearing at 20 kDa.

Lectin TVL (Figure 4.7e) recognised bands at 10 and 20 kDa, as well as very high molecular weight material, while lectin AHL (Figure 4.7f) detected a region of continuous staining between 10 kDa and 20 kDa with some of the isolates. Only this lectin was able to distinguish between the virulent (B97026P1) and the non-virulent (32/97) isolate, reacting with the region between 10-20 kDa in the SDS-PAGE profile of isolate B97026P1 (Table 4.3).

The remaining lectins (UEA-1 and HGL) recognised a range of bands, including the 20 kDa which was detected in all the isolates examined (Figure 4.7g and h respectively). The bands identified by HGL appeared distinct, while UEA-1 resulted in a smeared staining pattern.

Table 4.3. The reactivity of various lectins with different *F. psychrophilum* isolates

Isolates	Lectins															
	GML		LHL		ECA		Con A		TVL		AHL		HGL		UAE-1	
	kDa	R	kDa	R	kDa	R	kDa	R	kDa	R	kDa	R	kDa	R	kDa	R
NCIMB 1947^T	-	-	-	-	-	-	-	-	20, VHM	+	10-20	+	10, 20,.30, 75,120	++	20	+
NCIMB 2282	-	-	-	-	20	++	-	-	10, 20, VHM	+	-	-	10, 20,.30, 75,120	++	20	+
B97026	-	-	-	-	-	-	-	-	10, 20, VHM	+	10-20	++	20,35,52	+	20	+
B97026P1	20	++	20	+	20	++	20	+	10, 20, VHM	+	10-20	++	20, 35, 30,52	++	20	+
B97034E4	-	-	-	-	-	-	-	-	10, 20, VHM	+	20	+	10, 20,30	+	20	++
32/97	20	++	20	++	20	++	20	++	10, 20, VHM	+	-	-	20, 25, 30	++	20	++
34/97	20	+	20	++	20	++	20	+	20, VHM	++	10-20	+++	20,25	+	20	++
110/97	20	+	20	+	20	+	20	+	20, VHM	++	10-20	+	20,25	+	20	+

Abbreviations: GML: Soybean (*Glycine max*); LEL:Tomato (*Lycopersicon esculeulentum*); ECA: Coral tree (*Erythrina cristagalli*); Con A : Concanavalin A (*Canavalia ensiformis*); TVL: Wheat germ (*Triticum vulgaris);* AHL: Peanut (*Arachis hypogaea*); HGL: Horse gram (*Dolichos biflorus*); UAE-1: Gorse (*Ulex europaeus*); kDa- kiloDaltons; R- reactivity; - No band/ no reactivity, + → +++: Intensity of reaction; VHM Very high molecular weight material

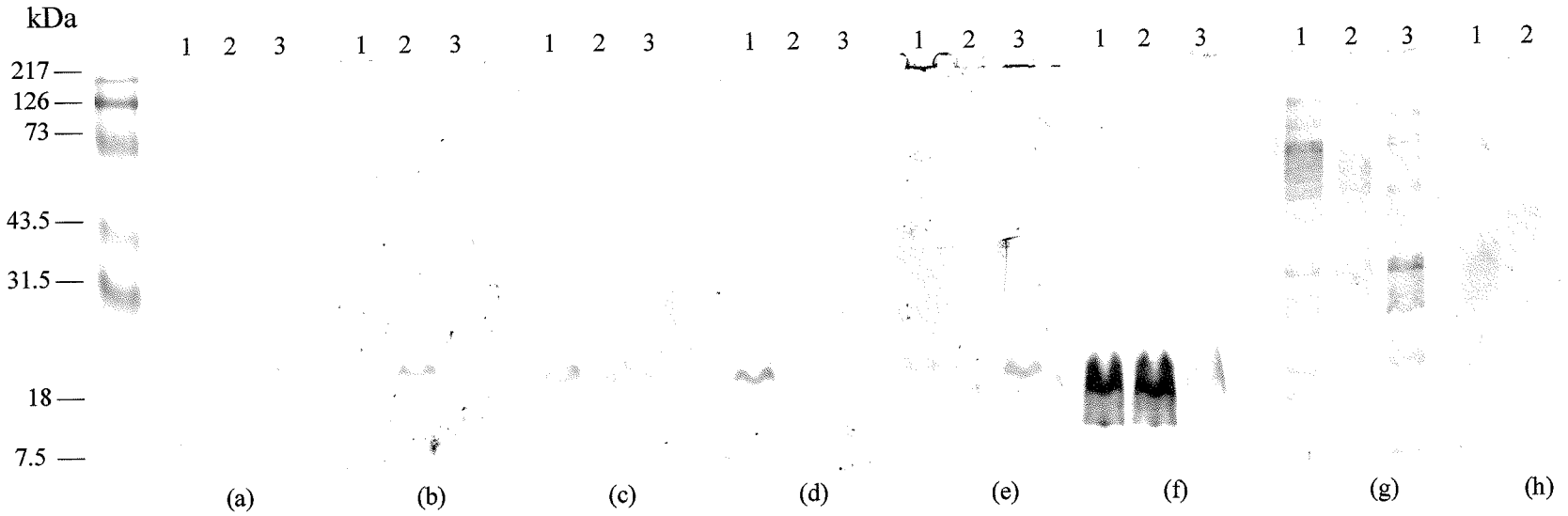

Figure 4.7. Examples of the response of *F. psychrophilum* isolates to lectins. (a) GML with isolate B97026P1, B97034E4 and 32/97; (b) LEL with B97034E4, 32/97 and 34/97 (c) ECA with 32/97, 34/97 and 110/97; (d) Con A with 32/97, 34/97 and 110/97; (e) TVL with B97034E4, 32/97 and 34/97 (f) AHL with B97026, B97026P1 and B97034E4; (g) HGL with B97026P1, B97034E4 and 32/97 (h) UAE-1 with 34/97 and 110/97. Number 1,2,3 correspond to the order of *F. psychrophilum* isolates reacted with each lectin.

GML: Soybean (*Glycine max*), LEL: Tomato (*Lycopersicon esculeulentum*), ECA: Coral tree (*Erythrina cristagalli*) Con A: Concanavalin A (*Canavalia ensiformis*), TVL: Wheat germ (*Triticum vulgaris),* AHL: Peanut (*Arachis hypogaea*), HGL: Horse gram (*Dolichos biflorus*), UAE-1: Gorse (*Ulex europaeus*)

4.4. Discussion

SDS-PAGE profiles of whole cell preparations of *F. psychrophilum* isolated from fish suffering from RTFS were shown to be homogenous in terms of both their protein and their carbohydrate composition, determined using a variety of staining methods, lectins and a glycoprotein detection kit. As found with the isolates in this study, Cipriano *et al.,* (1996) noted a similarity in the banding patterns between whole cell protein lysates of *F. psychrophilum* isolates, regardless of hatchery origin, fish species or the tissue from where they were isolated. The isolates in their study had all been recovered from fish showing signs of BCWD rather than RTFS. Homogeneity of protein profile has also been reported for other *Flavobacterium* species. Bernardet *et al.*, (1994) found similar protein profiles between whole cell preparations of *Flexibacter maritimus,* while Bader *et al.*, (1997) showed that whole cell lysates of pressure and formalin-killed *F. columnare* shared four common proteins at around 100, 80, 66 and 60 kDa.

The protein profiles obtained here with Coomassie blue and Silver staining were complex, smeared and not particularly clear. The running conditions of the SDS-PAGE had to be optimised to allow good separation of bands. The standard running conditions of 180 V at 20°C for 60 min (Jung, 1999) produced very poor resolution of bands. A lower voltage of 80 V at 4°C for 3 h resulted in more reproducible and sharper profiles.

The reason for the problems associated with the SDS-PAGE may have been due to the presence of a large quantity of carbohydrates or glycoproteins in the cellular material. Carlsson (1993) explained that smearing often occurs when glycosylated proteins are

subjected to SDS-PAGE, and suggested that this was due to interference from carbohydrate chains, and that the actual binding of SDS to glycoproteins is lower than seen with other proteins. This, in turn, leads to a slower migration of the glycoproteins through the gel, and results in molecular weight estimations, which are erroneously high. In addition, he mentioned that glycoprotein bands are often broad in shape due to micro-heterogeneity. When glycoproteins display considerable heterogeneity in their carbohydrate chains, e.g. caused by differences in poly-N- acetyllactosamine chain lengths, the glycoproteins appear as smears rather than as distinct bands. Therefore, the problems with streaking experienced when *F. psychrophilum* preparations were subjected to SDS-PAGE may in fact be due to the presence of glycoprotein molecules. As discussed before substantial level of glycoprotein were present in the whole cell preparations of the bacterium.

The presence of carbohydrates in *F. psychrophilum* was demonstrated here using Silver stain and Schiff's reagent. Silver stain reacts with both carbohydrates and proteins, while Schiff's reagent is a general carbohydrate stain. Extremely high molecular weight carbohydrate material was visualised at the very top of the gels by both stains, especially in the wells of the 4 % stacking gel, indicating that this material was too large to migrate into the 12% separating gel. There was also evidence of a 18-32 kDa carbohydrate region with both stains. Glycoproteins were also detected within this region in all isolates examined.

Crump *et al.*, (1999) reported a ladder-like pattern in the SDS-PAGE profiles of *F. psychrophilum*. This was due to the presence of a LPS at around 16 kDa which they identified by its avidity for polymyxin B and resistance to proteinase K. Such laddering

was not evident in the profiles of the *F. psychrophilum* isolates examined here however. The glycoprotein detection kit used here detects carbohydrate attached to the protein moities, confirming the presence of glycoprotein bands at 20 and 23 kDa.

In order to examine the carbohydrates present in whole cell preparations of two reference isolates, B97026P1 and 32/97, a protein digest of the samples was first performed using proteinase K. The treated samples contained very few bands upon staining with Coomassie blue and many of the bands had disappeared after digestion, suggesting that several of the bands were associated with protein. However, some material was still visualised with the Silver stain after digestion, suggesting that there was a large amount of carbohydrate associated with the cellular material, and may have been due to LPS (Crump *et al.*, 1999). The presence of two bands in treated samples upon staining with Schiff's reagent also supported the presence of carbohydrate. The lower molecular weight band seen with the Schiff's reagent at the running front of the dye was also probably LPS.

The OMP profile of two reference isolates of *F. psychrophilum,* as determined by electrophoresis provided further evidence that the species are homogenous. Little protein was observed in OMP profile compared to whole cell preparations, and only three distinct bands were found in the gels upon staining with Coomassie blue. The OMP profiles of the bacteria were heavily stained with Silver stain suggesting the presence of carbohydrate contamination during the OMP extraction procedure. Contamination of OMP preparations with LPS is frequently reported (Lambert, 1988). The OMP of Gram negative bacteria have been associated with their virulence factors (Buchanan and Pearce, 1979; Aoki and

Holland, 1985; Dooley *et al.*, 1986; Biosca and Amaro, 1991). No differences were found between the OMP profiles of the virulent (B97026P1) and non-virulent (32/97) isolates in the present study. Analyses such as substrate gels may highlight differences in enzyme activity between the OMPs of the two isolates.

Whole cell preparations of *F. psychrophilum* contain glycoproteins as detected using the commercially available detection kit. The kit is based on oxidation of specific carbohydrate linkages, which are then labelled with biotin and subsequently with streptavidin-alkaline phosphatase conjugate indicating the presence of the glycoprotein. The kit identified two bands of approximately 20 and 23 kDa on all isolates examined. In addition to these two bands, four isolates (HL313/97, 35/97, 110/97 and UP 193/97) also displayed high molecular weight bands around 97 and 110 kDa. Jung (1999) identified two different groups of *Photobacterium damselae* subsp. *piscicida* based on their glycoprotein content. One group comprised Japanese isolates with a glycoprotein of around 22 kDa and other group included isolates from the Mediterranean region with a glycoprotein molecule of 26 kDa. Six glycoprotein bands were found in whole cell preparations of *Mycobacterium marinum* (Puttinaowarat, 1999) using the same detection method. Bader *et al.*, (1997) reported the presence of a 60 kDa glycoprotein in whole cell lysates of pressure and formalin-killed *F. columnare* using MAbs specific to glycoprotein. The presence of a 60 kDa glycoprotein molecule was also identified in one of two *Cytophaga* spp. isolated from a marine biofilm by Burchard and Sorongon (1998). This glycoprotein is believed to be able to inhibit the adhesion and gliding of other *Cytophaga* species.

Staining of bacterial components blotted on to nitrocellulose membranes and stained with lectins resulted in different binding patterns between the various isolates examined. This provides information on the carbohydrate moieties present on each isolate. The specificity of lectins for carbohydrate moieties depends not only on the position of monosaccharides and the number of branch points, but also on the types of linkages between residues. The high reactivity of TVL, HGL and UEA-1 observed with all the isolates tested suggests that there are significant amounts of saccharides, specifically glucose, galactose and fucose present in the carbohydrate of the *F. psychrophilum* cells. Four (GML, LEA, ECA and Con A) of eight lectins with different carbohydrate specificities produced similar banding patterns with the majority of the strains examined, while other isolates did not react with these lectins. This may be explained by the fact that the bands are composed of different polysaccharides, some of which are specific for the lectins. Only one lectin, AHL, which is specific for D-galactose, was able to distinguish between the virulent and non-virulent isolate. No explanation can be given as to why lectins GML, LHL, ECA and Con A should only react with isolate B97026 after in has been passed through a fish.

It has been reported for many CLB that the production of extracellular polysaccharides or slime potentially facilitates the gliding motion and adhesion of the bacterium to its host or to solid surfaces (Godchuaux *et al.*, 1991; Dalsgaard, 1993). It was shown in the present study that the cells of *F. psychrophilum* contain a large amount of carbohydrate material identified with various staining methods, and this material may, in part, be associated with the slime layer present on the bacterium, especially the very high molecular weight

material which was unable to migrate out of the stacking gel. This suggests that these particular carbohydrates are composed of very large complexes of polysaccharides.

In conclusion, the work presented in this study indicates that whole cell preparations of isolates of *F. psychrophilum* recovered from fish suffering from RTFS are homogenous in terms of their protein and carbohydrate profiles regardless of their origins or pathogenicity in rainbow trout. The protein profiles of OMP prepared from both the virulent and non-virulent isolate were also similar. Glycoproteins located at 20 kDa and 23 kDa were found to be a major cell component of *F. psychrophilum* and a large amount of high molecular carbohydrate material was also evident in profiles of the bacterium. Variations in the intensity and the staining patterns of the carbohydrates present in the SDS-PAGE profiles of the bacterium were observed with the different lectins, thus indicating differences in the carbohydrate composition of the isolates. The significance of the carbohydrate material identified here with respect to pathogenicity such as attachment to its host or movement of the bacterium needs to be established.

CHAPTER 5

Characterisation of *Flavobacterium psychrophilum* using rabbit serum

5.1. Introduction

Serology and serodiagnosis of bacterial fish pathogens have contributed significantly to fish health management in recent years (Busch, 1981). A wide variety of serological procedures have been increasingly used to provide diagnostic techniques for fish pathogens, detect antibodies to specific pathogens in fish sera, evaluate vaccines, and establish serological relatedness of strains of bacterial fish pathogens (Schill *et al.*, 1989).

Despite its importance as a fish pathogen, the serology, antigenicity and pathogenicity of *F. psychrophilum* are poorly understood. Pacha and Porter (1968) were the first to use serological tests as a rapid and useful means of distinguishing between pathogenic and non-pathogenic *F. psychrophilum*. They suggested that somatic antigens might be useful in distinguishing pathogenic from non-pathogenic strains, which share similar cultural and physiological characteristics. None of the non-pathogenic strains reacted with the antiserum prepared against *F. psychrophilum*, whilst pathogenic isolates appeared to be highly specific for the antiserum.

Several authors have reported that common antigens exist between isolates of *F. psychrophilum* (Pacha, 1968; Pacha and Porter, 1968; Wakabayashi *et al.*, 1994). However, analysis with absorbed sera has revealed the existence of different serotypes (Holt, 1987). Depending on the immunological analysis used and the geographical origin of the strains, two (Holt, 1987; Wakabayashi *et al.*, 1994) or three (Rangdale, 1995;

Lorenzen and Olesen, 1997; Izumi and Wakabayashi, 1999) separate serotypes have been reported. It is clear from the literature that serotypic differences exist between the different isolates of *F. pychrophilum*, although the exact number has not yet been fully established.

From the view point of developing an effective vaccine against *F. psychrophilum* infection, the more bacterial serotypes present, both locally and internationally, the less chance there is of developing an effective vaccine. Also, a vaccine containing many serotypes will be more expensive to produce. For the vaccine to be effective in the prevention of both RTFS and BCWD, more work is needed to clarify the antigenic types of *F. psychrophilum* from different geographical regions, present during the two conditions.

Most disease outbreaks caused by *F. psychrophilum* are currently confirmed from bacterial culture after isolation of the pathogen from infected fish. This can be time consuming and is often unsuccessful since the bacterium is particularly fastidious. Rapid and specific identification methods would greatly improve the diagnosis of the conditions caused by *F. psychrophilum*. Rangdale (1995) evaluated the potential of a number of serodiagonistic techniques as a means of rapid detection of *F. psychrophilum* in diseased fish and reported that ELISA technique to be useful for the identification of *F. psychrophilum* from both plate/broth grown cultures and directly from infected fish.

The aim of this study was to produce rabbit sera against a virulent and a non-virulent isolate of *F. psychrophilum* and use them to examine the antigenicity of isolates obtained

from different geographical regions. Their potential use as diagnostic tools is also discussed.

5.2. Materials and Methods

5.2.1. Bacterial isolates and culture conditions

F. psychrophilum isolate B97026P1 (virulent) and 32/97 (non-virulent) were used to produce rabbit sera in this study. The virulence of these isolates was determined by artificially challenging rainbow fry as described in Chapter 3. All *F. psychrophilum* isolates contained in the culture collection (Table 2.1) were used to examine the specificity of the rabbit sera by ELISA. A large number of these had been obtained from RTFS outbreaks, a few from BCWD outbreaks and two from NCIMB including the type strains NCIMB 1947^{T}. Eight *F. psychrophilum* isolates (see Figure 5.2) were used in Western blot analysis, chosen from their reactivity in the ELISA. Twenty-six other bacteria from differing genera, listed in Table 2.2, were used to assess the specificity of the anti-*F. psychrophilum* sera in ELISA, while eight of non-*F. psychrophilum* bacteria which cross-reacted in the ELISA were selected for used in the Western blot analysis. The growth condition of *F. psychrophilum* is described in Section 2.1.2.1, while the growth conditions of non-*F. psychrophilum* bacteria are described in Section in 2.1.2.2.

5.2.2. Preparation of bacterial samples

Bacteria were prepared for analysis according to Section 2.1.2.3. A 100 µl aliquot was taken from each preparation and stored at –20°C for subsequent protein digestion. This involved incubating the aliquots with 10 µl of a 10 mgml^{-1} solution of proteinase K

(Sigma) for 1 h at 60°C before diluting in sample buffer. Bacteria used for immunising rabbits and in the ELISA were heat killed at 60°C by placing the bacterial suspension in a water bath for 60 min.

5.2.3. Immunisation of rabbits

Two female New Zealand White rabbits (*Oryctolayus cuniculus*) were used in the production of polyclonal antisera against isolate B97026P1 (virulent) and 32/97 (non-virulent). Bacteria were grown and prepared as described above, then heat killed at 60°C for 60 min. Prior to immunisation, the rabbits were bled to collect pre-immune serum. The rabbits received 0.8 ml of the bacterial suspensions (1 $x10^8$ $cellml^{-1}$) mixed 1:1 (v:v) with TiterMax® Gold adjuvant (Tratech Scientific Ltd., Luton, England) injected SC into four sites in the shoulders of the animal (0.2 $mlsite^{-1}$). Blood was collected from the marginal ear vein 10 days later, serum was separated by centrifugation at 300 x g for 5 min at 4°C and the antibody response evaluated using the ELISA as described below. Seven weeks after the test bleed, each rabbit was given a secondary boost of a 1:1 (v:v) mixture of bacterial suspension, containing 1 $x10^8$ cell ml^{-1} in TiterMax® Gold adjuvant (0.25 ml into 2 sites SC) into the shoulders as above. Serum antibody levels were again tested 10 days after injection. A final boost of 1 ml heat killed (60 min at 60°C) bacteria at 1 $x10^7$ $cellml^{-1}$ in PBS was administrated intravenously (IV) into the marginal ear vein 4 weeks later. The rabbits were bled by cardiac puncture 10 days later.

5.2.4. ELISA

The antibody titre and the specificity of rabbit sera were determined using the ELISA described in Section 2.5. To determine the antibody titre of the rabbit sera, sera was diluted ten fold in LSWB before adding to the wells (100 $\mu l well^{-1}$), while a dilution of 1/100,000 was used to examine the specificity of the rabbit sera. Pre-immune rabbit sera diluted 1/1000 in PBS was used as negative control. Anti-rabbit IgG-HRP (SAPU) diluted 1/1000 in PBS was used as secondary antibody. The positivity threshold for the reaction was determined as three times the value obtained for the mean negative control.

5.2.5. Western blot analysis

Whole cells (1 $x10^9$ cell ml^{-1}) and OMP preparations (1 mg ml^{-1}) of *F. psychrophilum* were subjected to 12% SDS-PAGE as described in Section 2.2.1. OMPs of *F. psychrophilum* isolate B97026P1 and 32/97 were prepared according to the method described in Section 2.6. Western blot analysis with rabbit sera diluted 1/1000 in TBS, was performed as described in Section 2.3. Anti-rabbit IgG-HRP (SAPU) diluted 1/200 in TBS was used as secondary antibody.

5.2.6. Detection of *F. psychrophilum* in infected tissue by indirect fluorescence antibody technique (IFAT)

5.2.6.1. Experimental infection

Forty rainbow trout fry, with a mean weight of 2.5 g, were held in two 20 l plastic tanks (20 fish per tank) containing static aerated water at a temperature of between 10 and 12°C, and maintained on a commercial diet. Half of the water of each tank was replaced and debris removed daily. After gently anaesthetising the fish with 0.1% alcohol solution of

benzocaine, one group of fish was infected with 50 µl of a suspension of isolate B97026P1 at a concentration of 1.2 x10^6 cfu ml^{-1} by IM injection, while the other group, used as a control, was injected with a corresponding volume (50 µl) of MAOB. One fry from each group was sampled 4 h after injection, and thereafter at approximately 24 h intervals for the next 18 days. Any moribund fry were also sampled. Tissues (muscle, spleen, kidney, and gills) were placed in 10% neutral buffered formalin for at least 24 h prior to cassetting. Tissues were embedded in paraffin wax according to standard procedures described in Appendix 3. Tissue sections (5 µm) were prepared and the presence of the bacterium established in infected tissue using IFAT.

5.2.6.2. IFAT

The IFAT procedure was carried out according to Anderson (1990) with slight modifications. Briefly, the paraffin-embedded tissue sections prepared above, were de-waxed by placing in xylene for 10 min, and then in 100 % (v/v) followed by 70% (v/v) ethanol for 5 and 3 min respectively, and rinsed in distilled water. It was important to keep the sections moist at all times. Tissue sections were encircled with a wax PAP pen, and a 100 µl of rabbit anti-*F. psychrophilum* B97026P1 serum, diluted 1/1000 in PBS, was added to the area containing the tissue section. Pre-immune rabbit serum diluted 1/1000 in PBS and PBS alone were added to each slide as negative controls. The slides were incubated for 30 min at 22°C, before rinsing them with PBS, then washing in a PBS bath for 5 min. A 1:100 dilution of fluorescine isothiocyanate (FITC)-donkey anti-rabbit IgG (SAPU) in PBS was added to the slides as the secondary antibody, incubating for 30 min at 22°C before washing as described above. The slides were mounted with equal parts of 0.5 M

Na_2CO_3 and glycerol, pH 9.0, and cover slips sealed with nail varnish. The slides were stored in the dark at 4°C until they were examined under oil immersion using a fluorescent microscope.

5.3. Results

5.3.1. Anti-*F. psychrophilum* sera

Rabbit anti-*F. psychrophilum* sera produced against isolates B97026P1 and 32/97 had antibody titres of between 1/100 000 and 1/1000 000 by ELISA. The titre of 1/100,000 was than used in ELISA to examine the specificity of the sera. ELISA then compared the response of the antisera to a variety of different *F. psychrophilum* isolates. The response of the antisera to the isolate against which it had been raised was considered as 100 % reactivity. The response of each antiserum differed substantially between the various *F. psychrophilum* isolates. Differences were also found in the recognition pattern between the two antisera (Table 5.1). The anti- B97026P1 serum recognised isolates, which the anti-32/97 serum did not and *vice versa*, and there appeared to be five different groups of bacteria based on their response in the ELISA (Figure 5.1). The first group (G1) contained bacteria which reacted with only the anti-B97026P1 serum. Four of the seven isolates of this group had a relative reactivity of 75% or higher. The second group (G2), comprised 10 isolates, which reacted with only the anti- 32/97 serum. The highest level of reactivity recorded for this group was 36.2 % with isolate LVDI 5/I. The third (G3) and fourth group (G4) of bacteria reacted with both the sera, but stronger reactivity was obtained with anti-32/97 serum for G3 bacteria, and with anti-B97026P1 serum with G4 bacteria. The fifth (G5) group of bacteria did not react with either serum.

Table 5.1. Percentage reactivity of the rabbit anti-*Flavobacterium psychrophilum* sera against a variety of *F. psychrophilum* strains using an enzyme-linked immunosorbent assay (ELISA)

Isolates	[b]Reactivity of serum (%)	
	Anti-32/97 (1/100,000)	Anti-B97026P1 (1/100,000)
NCIMB1947[T]	-	112.2
B97026P1	-	100[a]
B97235E4	-	86.5
NCIMB2282	-	74.9
LVDJG2215	-	32.8
LVDL3077/91	-	27.8
JIP 22/90	-	23.5
110/97	-	0.5
B97026	9.0	91.7
JIP 02/86	17.6	71.8
921/97	37.7	7.5
JIP 30/98	63.7	5.4
UP193/97	25.7	4.9
34/97	49.4	4.4
59/95	29.0	0.5
32/97	100.0[a]	0.2
UP164/97	36.2	-
LVDI 5/I	22.2	-
LVDL1829/91	13.4	-
LPAA P03/88	9.3	-
LVDL4862/95	8.1	-
35/97	6.3	-
LPAA P01/88	4.5	-
916/1	3.0	-
Hl313/97	-	-
LVDL1456	-	-
CASO 89/97	-	-
LVDJ E2047	-	-
LVDJD2172	-	-
LVDJXP.189	-	-
B398	-	-
B035	-	-
10/2	-	-
904/10	-	-

[a]The strain used to prepare the antiserum was considered as the reference strain with 100% reactivity; the positive threshold for the reaction was determined as three times the value obtained for the mean negative controls at 450 nm. Absorbance values for the negative control ranged from 0.09-0.138 and for the reference strains (100% reactivity) between 0.67-0.80. [b] % reactivity were calculated as follows for the mean of duplicate wells:

$$\frac{[OD_{450} \text{ of test sample} - OD_{450} \text{ of background (x3)}]}{[\text{Positive control } (OD_{450} \text{ of reference strain}) - OD_{450} \text{ of background (x3)}]} \times 100$$

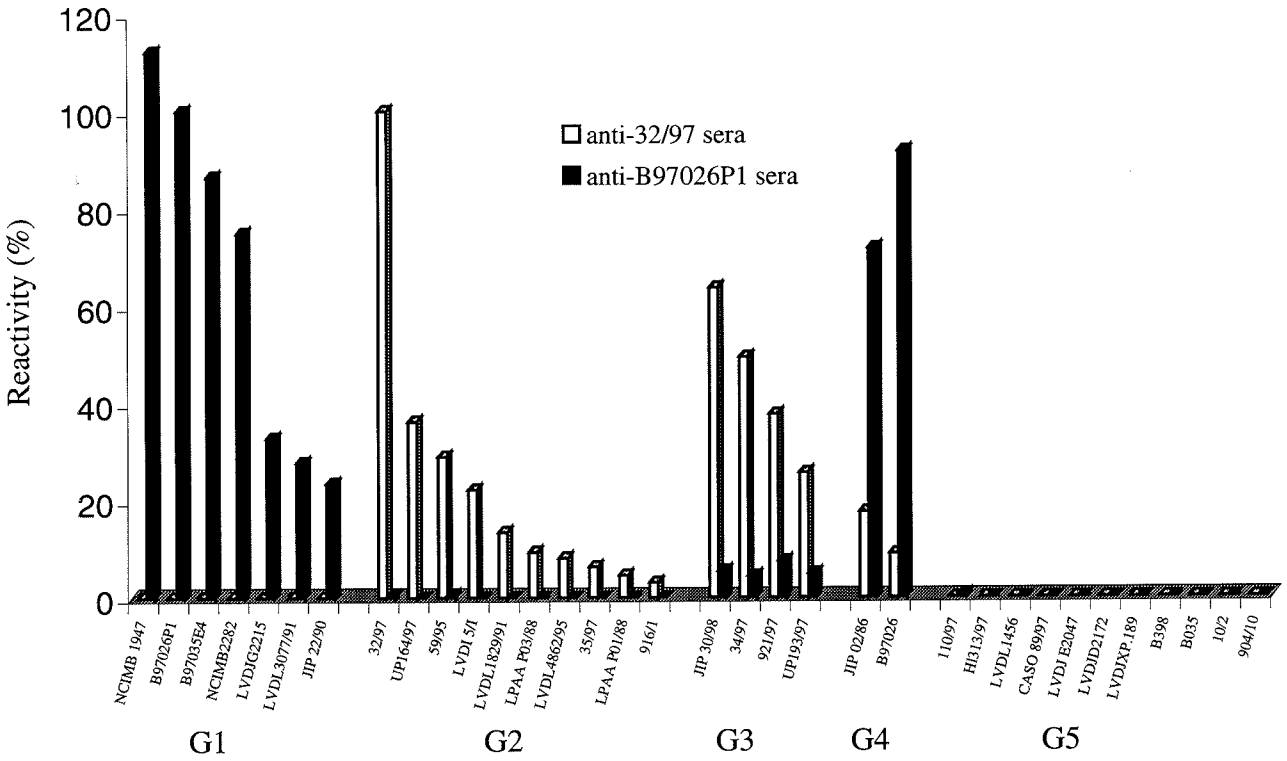

Figure 5.1. Percentage reactivity of the rabbit anti-*Flavobacterium psychrophilum* sera against a variety of *F. psychrophilum* strains using an enzyme-linked immunosorbent assay.

G: Group

5.3.2. Cross-reactivity of antisera with other bacterial genera

Twenty-six non-*F. psychrophilum* bacteria were tested in the ELISA to determine the specificity of the rabbit antisera. The cross-reactivity of the anti-32/97 serum was low, and demonstrated a reactivity of less than 2.9 % (*A. salmonicida*), whereas antiserum against the virulent isolate (B97026P1) had higher levels of cross reactivity. *F. branchiophilum* showed the highest level of cross-reactivity with this serum at 61.3 % (Table 5.2).

5.3.3. Western blot analysis

The response of the rabbit anti-B97026P1 and anti-32/97 sera was examined by Western blot analysis against whole cell preparations of a range of *F. psychrophilum* isolates chosen from each of the five groups based on their reactivity in the ELISA (Figure 5.2a, b). A variety of bands were generally recognised in the profiles of the bacteria by both antisera, with major bands seen at 170, 139, 116, 82, 55, 38, 26, 20 and ≈7 kDa. The 26-kDa band appeared to be part of a doublet with another band around 20 kDa. A region of dark smearing between 10 and 20 kDa was also evident with both antisera.

However, differences were observed in the staining pattern of the two antisera, especially against both high and low molecular weight (MW) material. High and low MW material was heavily stained in the bacteria to which the serum had been raised and which showed high reactivity against the corresponding sera. For example, lane 5 in Figure 5.2a represents staining of isolate 32/97 with rabbit anti-32/97 serum, while lane 1 in Figure 5.2b represents staining of isolate B97026P1 with rabbit anti-B97026P1 serum.

Heavily stained high MW material of isolate 34/97 (G3) and UP164/97 (G2) were also seen

Table 5.2. Cross-reactivity of various bacterial genera with the rabbit anti-*Flavobacterium psychrophilum* sera in an enzyme-linked immunosorbent assay (ELISA)

Bacteria	[a]Cross-reactivity of serum (%)	
	Anti- 32/97	Anti-B97026P1
Aeromonas hydrophila	-	13.0
Aeromonas salmonicida	2.9	37.7
Aeromonas sobria	-	14.9
Bacillus subtilis	-	-
Bacillus mucoides	-	-
Corynebacterium aquaticum	-	-
Edwardsiella ictaluri	-	29.3
Edwardsiella tarda	-	34.3
Escherichia coli	-	37.8
Flavobacterium branchiophilum	0.7	61.3
Flavobacterium aquatilis	-	-
Flexibacter maritimus	-	-
Lactobacillus plantanum	-	-
Listonella anguillarum	-	-
Micrococcus luteus	-	2.2
Nocardia asteriodes	-	-
Photobacterium damselae subsp. *piscicida*	-	-
Pseudomonas anguilliseptica	-	-
Pseudomonas fluorescens	-	7.0
Pseudomonas aeruginosa	-	17.7
Renibacterium salmoninarum	-	-
Streptococcus faecalis	-	-
Serratia sp.	-	-
Vibrio vulnificus	-	-
Vibrio ordalii	-	-
Yersinia ruckeri	-	-

The strain used to prepare the antiserum was considered as the reference strain with 100% reactivity; The positive threshold for the reaction was determined as three times the value obtained for the mean negative controls at 450nm. Absorbance values for the negative control was 0.115 and 1.03 for the reference strains (100% reactivity).

[a] % reactivity were calculated as follows for the mean of duplicate wells:

$$\frac{[OD_{450} \text{ of test sample} - OD_{450} \text{ of background (x3)}]}{[\text{Positive control } (OD_{450} \text{ of reference strain}) - OD_{450} \text{ of background (x3)}]} \times 100$$

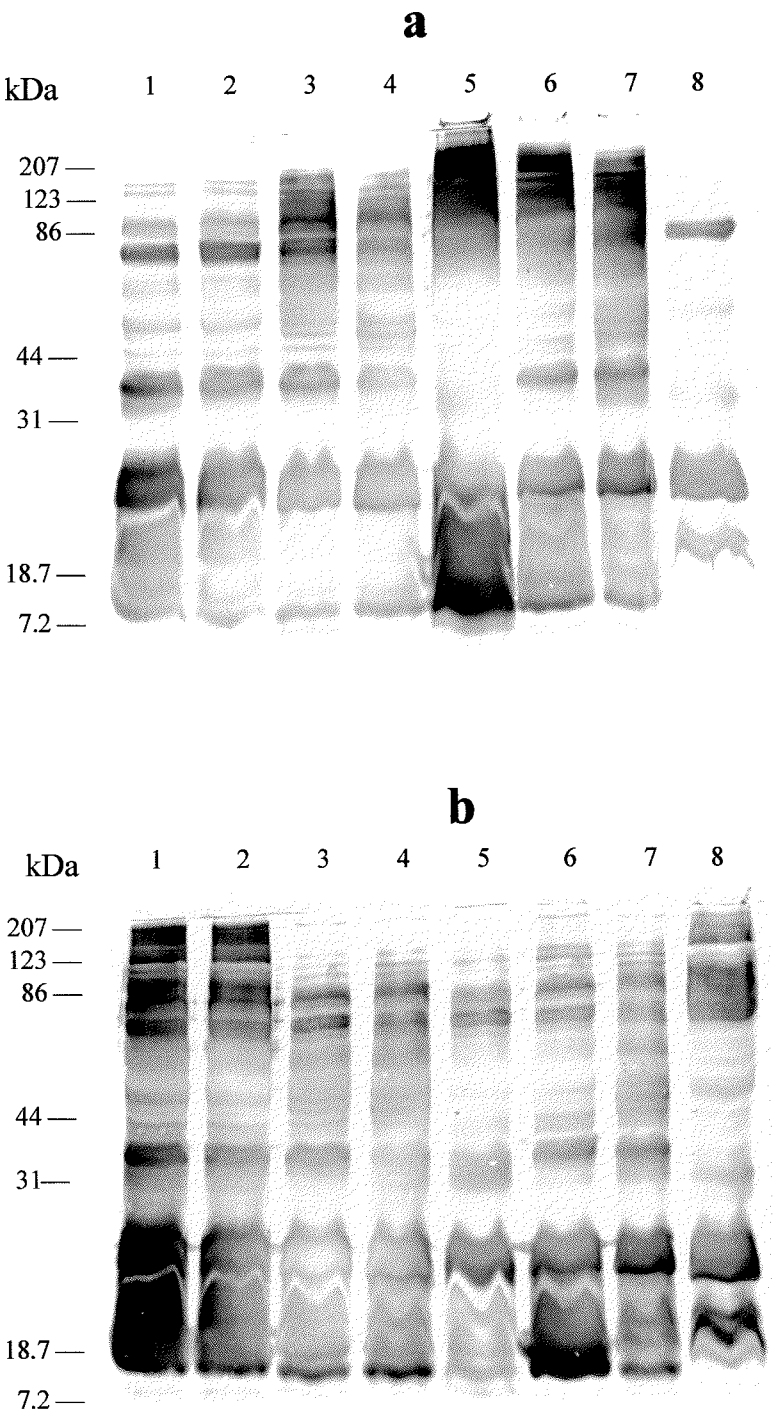

Figure 5.2. Western blot analysis of rabbit anti- *F. psychrophilum* sera against whole cell preparation of *F. psychrophilum,* (a) rabbit anti-32/97 serum and (b) rabbit anti-B97026P1 serum. Lanes: (1) B97026P1 (2) B97026 (3) 59/95 (4) 110/97 (5) 32/97 (6) 34/97 (7) UP164/97 (8) NCIMB 2282.

(lanes 6 and 7 in Figure 5.2a) when reacted with the anti-32/97 serum. These two isolates also had a high level of reactivity in the ELISA with this antiserum. No such staining was evident with isolate B97026P1 (G1) and NCIMB 2282 (G1) with this antiserum (Figure 5.2a lanes 1 and 8), and did not react with this serum in the ELISA. Similar patterns of staining were also evident for the anti-B97026P1 serum (Figure 5.2b).

The reactivity of the sera was also examined against OMP preparations of both isolates. The staining profiles obtained with Western blot analysis (Figure 5.3a, b) were very similar to that seen with the whole cell preparation (Figure 5.2a, lane 5 and Figure 5.2b, lane 1), with heavily stained high and low MW material being recognised in only bacteria to which the serum had been prepared. Not only did the staining intensity of the two OMP preparations differ between the two sera, but differences were also seen in the bands detected. Anti-32/97 serum detected bands at around 32, 52, 65, 81, 96, 116 and 126 kDa in the OMP profile of isolate 32/97 but only clearly recognised the 81 kDa band of isolate B97026P1 and *vice versa* with the anti B97026P1 serum.

When Western blot analysis was performed on proteinase K-digested whole cell preparations the majority of high MW material, previously recognised by the sera in the whole cell preparations, was not detected. Following proteinase K digestion, both antisera only recognised material located in a region between 35 and 10 kDa (Figure 5.4a, b).

A selection of the non-*F. psychrophilum* bacteria, which cross-reacted with the antisera in the ELISA, were also examined by Western blot analysis (Figure 5.5a, b). Bands were

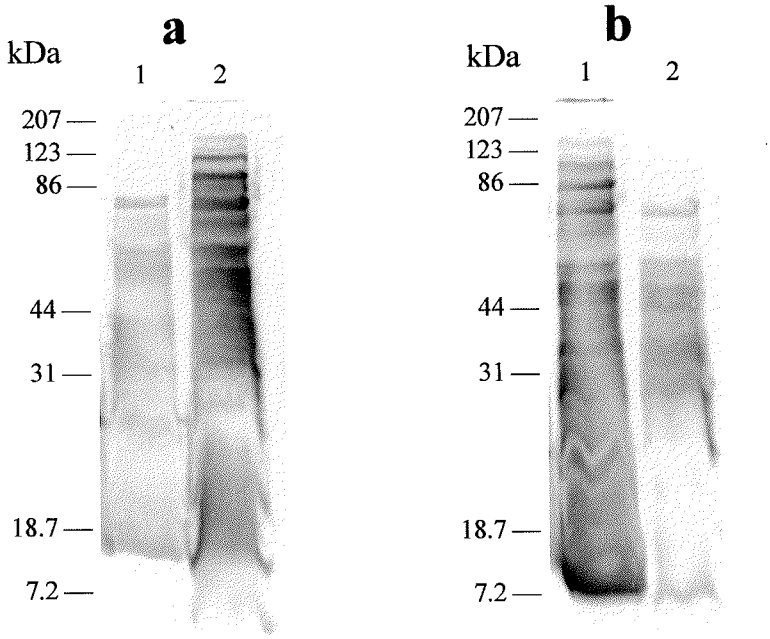

Figure 5.3. Western blot analysis of rabbit anti- *F. psychrophilum* sera against OMP preparations of strains against which they had been raised. (a) anti-32/97 sera (b) anti-B97026P1 sera, Lanes: (1) B97026P1 (2) 32/97.

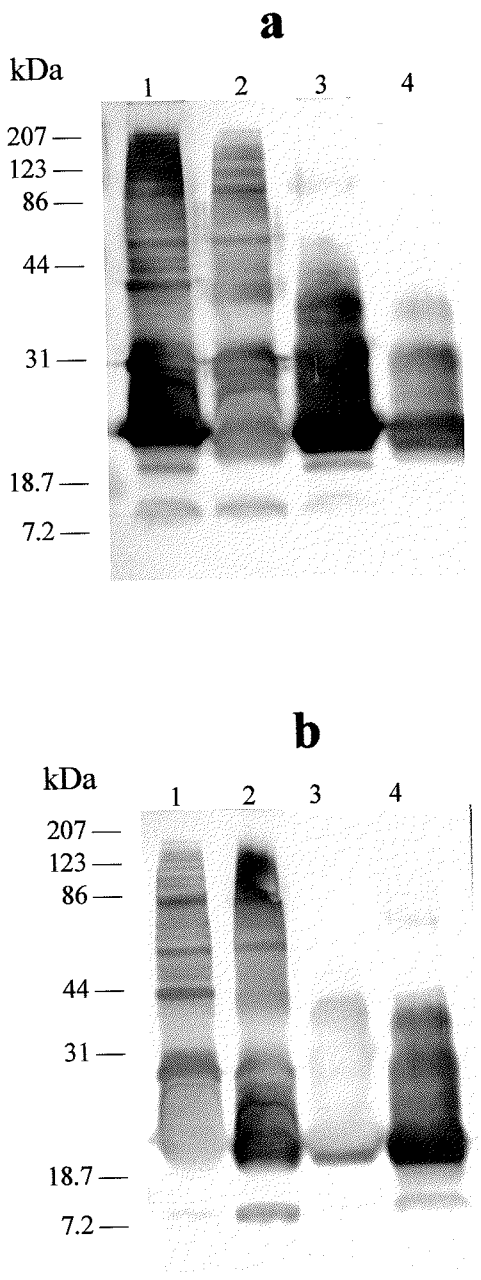

Figure 5.4. Western blot analysis of rabbit anti-*F. psychrophilum* sera against proteinase-K treated and untreated whole cell preparations of the bacteria. (a) rabbit anti-B97026P1 serum and (b) rabbit anti-32/97 serum. Lanes: (1) untreated whole cells of B97026P1 (2) untreated whole cells of 32/97 (3) proteinase K treated whole cells of B97026P1 (4) proteinase K treated whole cells of 32/97.

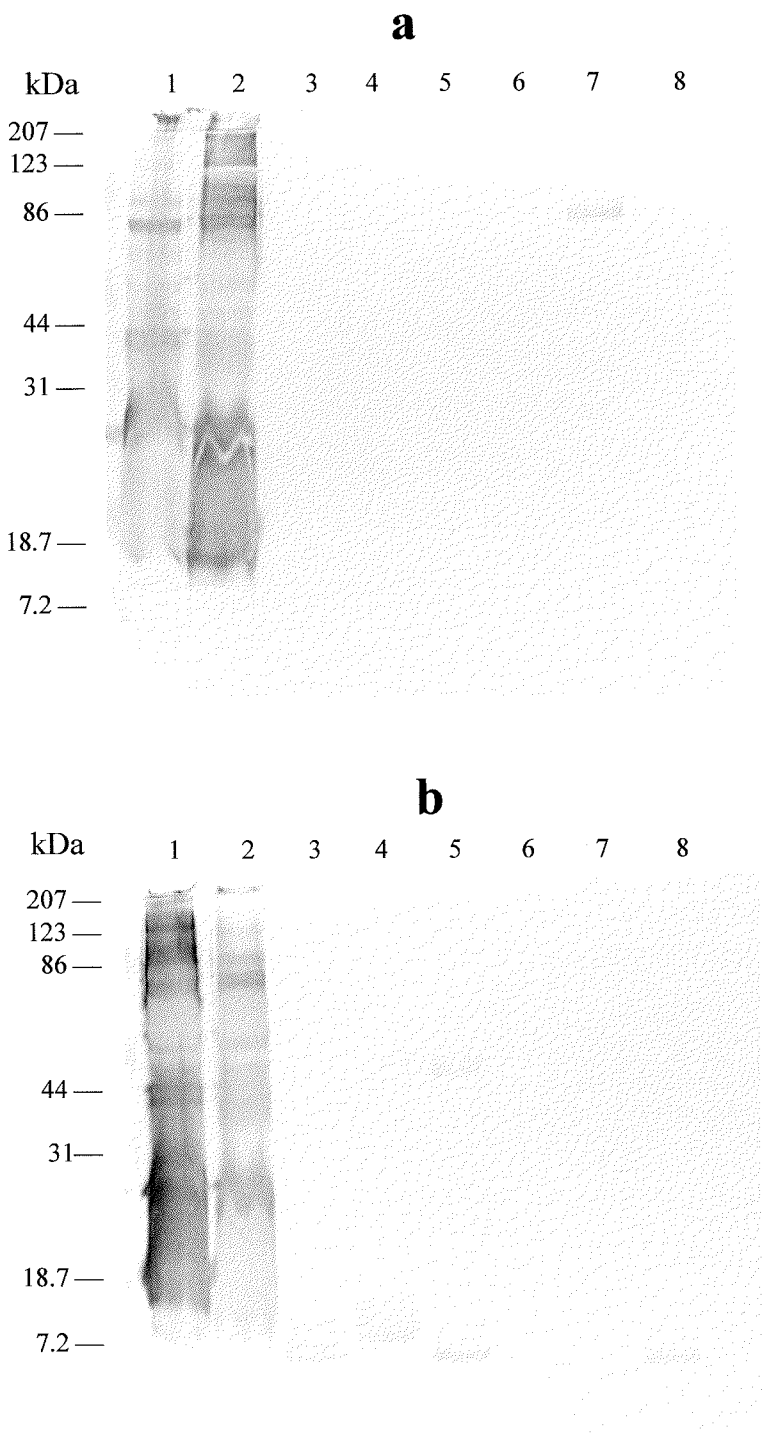

Figure 5.5. Western blot analysis of rabbit anti-*F. psychrophilum* sera with a selection of non-*F. psychrophilum* bacteria which cross-reacted with the sera in ELISA. (a) anti-32/97 sera; (b) anti-B97026 P1 sera. Lanes: (1) *F. psychrophilum* strain B97026P1 (2) *F. psychrophilum* strain 32/97 (3) *Aeromonas hydrophila* (4) *A. sobria* (5) *Edwardsiella ictaluri* (6) *Cyanobacterium aquaticum* (7) *Renibacterium salmoninarum* (8) *Serratia* sp.

seen with several of the bacteria examined, the intensity of which generally reflected the level of cross-reactivity seen in the ELISA. The antiserum raised against non-virulent isolate 32/97 recognised a band of 90 kDa with *R. salmoninarum,* which was not recognised by the antiserum against virulent isolate B97026P1. No cross-reactivity was seen with *R. salmoninarum* in the ELISA.

5.3.4. Detection of *F. psychrophilum* by IFAT

Fish showed signs of infection 3 days after injection, characterised by skin ulcers, lethargy, dark coloration of the skin and a tendency to hang at the water surface. Of the 20 fish injected with *F. psychrophilum*, 10 developed skin lesions around the site of injection after 4 days. The lesions remained for about 8 days, after which time some fish started to recover from the infection, and lesions healed. Only one fish still had a lesion at the termination of the trial, at 18 days post-injection, and only one fish died on day 4 post-injection, with no death occurring in the control group.

Bacteria could be clearly seen in tissues of the rainbow trout fry, injected IM with *F. psychrophilum* using IFAT. An example of the staining seen in the various organs is illustrated in Figure 5.6. Individual bacteria were seen in muscle of fish from 4 h post-injection until the end of the trial 18 days after its on set. Fluorescing bacteria were clearly observed in tissue sections of gills, spleen and kidney from 2 days post-injection for at least 10 day, after which no fluorescing bacteria could be seen in these sections. No fluorescence was evident in any of the negative control tissues at any time interval.

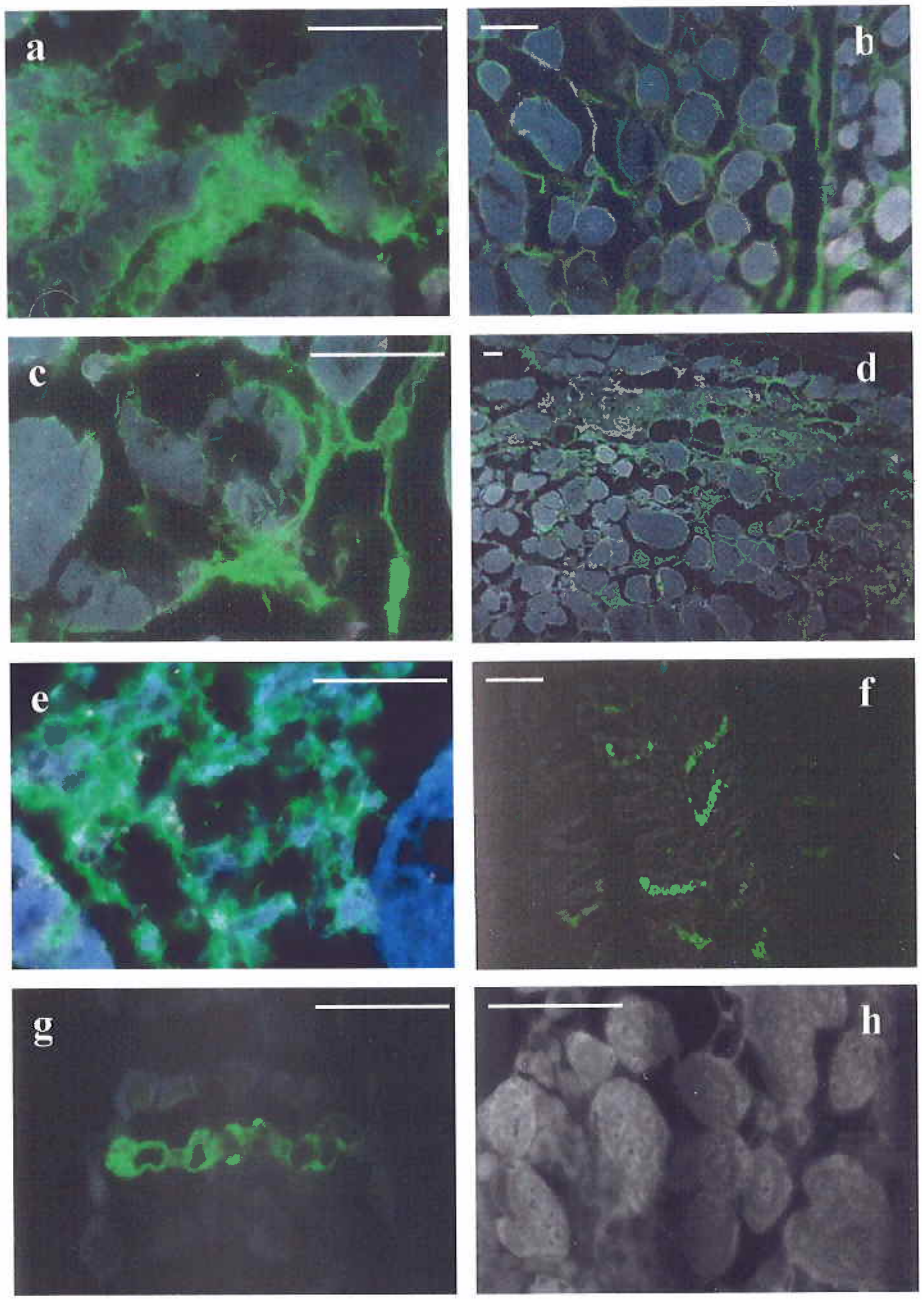

Figure 5.6. Analysis of tissues from rainbow trout fry artificially challenged with an intramuscular injection at 6 x10^4 cfu $fish^{-1}$ of *F. psychrophilum* isolate B97026P1 by indirect fluorescent antibody technique using rabbit anti-*F. psychrophilum* B97026P1 serum: (a) muscle 3 days post-injection, (b) muscle 4 days post-injection, (c) muscle 5 days post-injection, (d) muscle 6 days-post injection, (e) muscle 6 days post-injection, (f) gills 4 days post-injection , (g) gills 4 days post-injection, (h) negative muscle tissue 6 days post-injection (incubated with 1 in 1000 normal rabbit serum in PBS).

Scale bars = 10 μm

5.4. Discussion

Rabbit sera were produced against a virulent and a non-virulent isolate of *F. psychrophilum*. These sera were then used to either examine the antigenicity of isolates mostly obtained during outbreaks of RTFS from different geographical regions by ELISA and Western blot analysis.

The antisera produced different patterns of reactivity in ELISA against the different isolates of *F. psychrophilum*. Although there appeared to be five different groups of bacteria based on their reactivity in the ELISA, three of the groups (G1, G2 and G5) were more distinct then the other two (G3 and G4). Presumably, these groups contain different types of antigens on their cell surface, while G3 and G4 share common antigens between the other groups since they reacted with both sera. There may in fact only be three major groups of *F. psychrophilum,* based on their reactivity pattern in the ELISA, however. The level of reactivity of the antisera between G2 and G3 bacteria was very similar, although the reaction of the anti-32/97 serum was stronger with G3 bacteria. These bacteria may actually belong to G2, since most of the isolates in G3 reacted strongly with anti-32/97 sera. Similarly, the reactivity of G4 bacteria was similar to that of G1 isolates.

Lorenzen and Olesen (1997) identified three different serotypes among the 25 Danish and 20 other European isolates examined by ELISA and slide agglutination. Rangdale (1995) also found at least three distinct serotypes of *F. psychrophilum* examined by an amplified ELISA. In a more recent study, Izumi and Wakabayashi (1999) revealed three host-dependent serotypes among 112 strains of *F. psychrophilum* isolated from different fish

species. Serotype O-1 was reported to be mainly from strains of coho salmon, serotype O-2 was specific for strains from ayu and serotype O-3 represented strains from rainbow trout. The bacteria found in the current study did not appear to show any pattern in their grouping with respect to their geographical origin, culture conditions, age or host species of fish. Techniques used in the interpretation of data vary from laboratory to laboratory, making it difficult to compare results between studies.

The antiserum produced here against a virulent strain of *F. psychrophilum* (B97026P1) showed substantial cross-reactivity with a number of non-*F. psychrophilum* bacteria, suggesting that these bacteria may have antigenic similarities on their surface. Conversely, antiserum produced against a non-virulent strain of *F. psychrophilum* (32/97) showed little cross-reactivity with the same bacteria. No attempt was made here to reduce cross-reactivity of the antisera. Absorption of the antisera with the cross-reacting bacteria may have improved the specificity of the ELISA.

Differences seen in reaction patterns and cross-reactivity between the two antisera may in part be a result of serotype differences between the two strains used to produce the antisera. The differences seen were probably not as a result of differences in their virulence, since some of the members of the putative virulent sero group (G1) were not virulent (data not shown). Rangdale (1995) showed that the reference strain NCIMB 1947^{T} was non-pathogenic to juvenile rainbow trout, however it demonstrated the highest level of reactivity (112%) with the serum against virulent isolate B97026P1. Another non-pathogenic strain NCIMB 2282 also had a high level of reactivity (75%) with this serum.

Very few reports are available relating to differences in the antigenicity of virulent and avirulent *F. psychrophilum.* Among the three serotypes reported by Lorenzen and Olesen (1997), the major serotype (serotype Th) represented most of the Danish and European isolates, while a minor serotype (serotype Fp^T) included the type strain *F. psychrophilum* NCIMB 1947^T, representing isolates from mainly asymptomatic fish or fish species other than rainbow trout. Isolates from clinical outbreaks of RTFS/BCWD did not react with the anti-Fp^T sera and it was therefore postulated that isolates of serotype Fp^T were less pathogenic, at least for rainbow trout. The authors suggested there might be some relationship between serological properties and virulence. They did not find any serological difference between the isolates obtained from clinical outbreaks of RTFS or BCWD.

A range of *F. psychrophilum* isolates, based on their reactivity in the ELISA, was chosen to represent each of the five groups and were examined by Western blot analysis. The intensity of staining seen with the different isolates corresponded to their reactivity pattern with either antiserum in the ELISA. However, the antisera raised against whole cell preparations of isolates B97026P1 and 32/97 resulted in intense staining of both high and low MW material in the profile of bacteria against which the antiserum had been made. The reaction pattern obtained with the two antisera was very similar against the remaining cellular material (170, 139, 82, 55, 38 and 26 kDa). A region around 20 kDa appeared particularly antigenic, with a doublet and a region of dark smearing recognised between 10 and 20 kDa. Crump *et al.*, (1999) identified around five or six major proteinaceous antigens located between 20-100 kDa, in two geographically distinct groups of *F.*

pyschrophilum. Two of the bands at around 16 kDa and 26kDa were highly immunogenic carbohydrates and they believed these to be LPS. The 10-26 kDa bands recognised by both sera in the present study may be associated with highly immunogenic carbohydrate material. Using a variety of lectin stains in Chapter 4, these were identified as carbohydrates. Bands at 20 and 23 kDa were present on all isolates examined and were identified as glycoproteins, using a commercially available glycoprotein detection kit.

Outer membrane proteins from Gram-negative bacteria have been associated with virulence factors of the organisms (Buchanan and Pearce, 1979; Aoki and Holland, 1985; Dooley *et al.*, 1986; Biosca and Amaro, 1991) and protective antigenicity (Granoff and Munson 1986; Heckel *et al.*, 1989; Sengupta *et al.,* 1992; Lutwyche *et al.,* 1995). The protective role of cell surface components of *F. psychrophilum* is unknown. Slight differences were seen in the recognition pattern of the protein bands of the OMP from the two isolates, by the sera. At least three bands (96, 116 and 126 kDa) were missing in the OMP profiles of *F. pyschrophilum* isolate 32/97 when stained with anti-B97026P1 sera and *vice versus.* Only the 116 kDa band was visible with Coomassie blue staining (Chapter 4). The OMP of many more *F. psychrophilum* strains need be examined to be able to correlate their serotype with virulence.

The low MW material stained by both sera, but which was not evident with Coomassie blue staining, may be the result of carbohydrate or LPS contamination during OMP extraction. This is discussed in more detail in Chapter 4.

The low MW material in proteinase-K treated whole cell preparations, recognised by the antisera, suggests that this material is an immunogenic carbohydrate, while the majority of high MW bands recognised in the non-treated sample, are associated with protein. Staining of the 10-35 kDa region in proteinase-K digested samples with the two antisera indicated antigenic difference between the sera. Differences were found in the carbohydrate composition of the isolates of *F. psychrophilum* in Chapter 4 using various lectins.

The antisera against the virulent *F. psychrophilum* isolate cross-reacted with low MW materials of *A. hydrophila, A. sobria, E. ictaluri* and *Serratia* sp., suggesting that they share common antigens with the virulent *F. psychrophilum* isolate. Antisera raised against the non-virulent strain recognised a band at around 90 kDa on *R. salmoninarum,* which was not detected by the antisera against virulent strain.

The specificity of the rabbit anti- *F. psychrophilum* sera appear to differ between the *F. psychrophilum* isolates examined, and the sera were unable to detect all *F. psychrophilum* isolates tested. This indicates that the two anti-sera produced here would be unsuitable as a diagnostic tool for screening infected samples. Although cross reactivity of rabbit anti-32/97 serum with the non-*F. psychrophilum* isolates was generally low, anti-B97026P1 serum cross-reacted with a number of these bacteria, again suggesting that it would be unsuitable for screening.

Bacteria could clearly be seen in the tissues of the rainbow trout fry by IFAT after they had been injected IM with *F. psychrophilum,* using the rabbit anti-*F. psychrophilum* serum

developed in this Chapter. Large numbers of bacteria were seen in muscle surrounding the site of injection from 4 h post-injection until the end of the trial at 18 days. Bacteria could also be seen in sections of gills, spleen and kidney examined 2 days post-injection. Bacteria could be detected in these tissues for at least 10 days post-injection, after which it became much more difficult to visualise clear distinct rods of *F. psychrophilum* within these sections. Presumably the bacteria were being killed by the immune response of its host and then degraded.

It is indicated from this small study that IFAT may be a useful tool to examine the course of infection by *F. psychrophilum* in small fry. A more detailed study should be performed in which histopathology is performed in parallel with the IFAT to assess the pathology of the disease in the various tissues. The rabbit serum used in the study was raised against the *F. psychrophilum* isolate with which the fish had been injected. This method may not therefore be useful for screening fish naturally infected with RTFS because the serum did not react with all strains of *F. psychrophilum* infecting rainbow trout fry.

In conclusion, the number of putative serotype groups still remains to be determined, although this study suggests that there may be between three and five groups. Absorption of the two sera with different isolates may help to indicate antigens, which are common or unique to the different isolates. No correlation was apparent between serotypes and geographical origin of the strains, the species of host fish from which they were recovered or the virulence of the isolates. Although the antisera detected common protein and carbohydrate antigens between isolates, there are indications that the staining seen in the 10

to 35 kDa region is antigenically different between the virulent and non-virulent isolate. It was not confirmed here if this difference actual correlates with the virulence of the bacterium, however. The antisera produced in this study are not suitable for screening infected RTFS samples.

CHAPTER 6

Production and characterisation of monoclonal antibodies (MAbs) against *Flavobacterium psychrophilum*

6.1. Introduction

Flavobacterium psychrophilum is a slow growing and thermo-sensitive bacterium, which is fastidious in its growth requirements (Bernardet and Kerouault, 1989; Lorenzen, 1993; Rangdale, 1995). Like many other *Flavobacterium* species, it appears inert in many biochemical tests (Chakroun *et al.,* 1997). The isolation of the pathogen from fish infected with RTFS is possible by inoculating samples of infected kidney or spleen onto MAOA. Due to slow growth and the fastidious nature of the bacterium, however, diagnosis of RTFS by culture is both difficult and time consuming, and its identification can be confused with other *Flavobacterium* species (Chakroun *et al.,* 1997).

Rabbit polyclonal antibodies have been used for the rapid detection of *F. psychrophilum* in infected fish tissues, but these antibodies can demonstrate low levels of cross-reactivity against *F. columnare* (Lorenzen and Karas, 1992). Rangdale (1995) used rabbit PAbs in a variety of different serodiagnostic tests to detect *F. psychrophilum,* both in infected tissue samples and from culture. In the previous chapter, rabbit PAbs raised against *F. psychrophilum,* failed to react with all of the *F. psychrophilum* isolates examined by ELISA. They also cross-reacted with a number of non-*F. psychrophilum* bacteria. This made them unsuitable as diagnostic tools for screening tissue samples from fish suffering from suspected RTFS. Improved sensitivity and specificity of probes is necessary for

successful diagnosis of *F. psychrophilum.* Hybridoma technology (i.e. the production of MAbs) is a powerful approach for the characterisation of specific antigens, and allows single antigenic determinants on molecules to be identified (Chen, 1996). Due to their specific and homogenous binding characteristics, MAbs are used in the identification of native antigen epitopes or to investigate antigenic structure (Yokota *et al.,* 1992; Kooi *et al.,* 1994). They have also proven useful as diagnostic, imaging and therapeutic probes for clinical medicine (Kuby, 1997). MAbs are currently used in the control of fish disease (Austin *et al.*, 1986; Adams *et al.*, 1995), specifically in diagnosis of pathogens (Adams *et al.,* 1995), environmental monitoring (Hock *et al.,* 1995; Kremer and Hock, 1996) and vaccine development (Adams *et al.,* 1997).

A number of MAbs have now been developed for the diagnosis of a variety of fish pathogens such as *Aeromonas* spp. (Adams and Thompson, 1990; Cartwright *et al.*, 1994; Neelam *et al.*, 1995), *Vibrio* spp. (Espelid *et al.*, 1988; Chen *et al.*, 1992; Miyamoto and Eguchi, 1997), *Photobacterium damselae* subsp. *piscicida* (Bakopoulos *et al.*, 1997b), *Mycobacterium* spp. (Adams *et al.,* 1996; Puttinaowarat, 1999), *Edwardsiella ictaluri* (Plumb and Klesius, 1988), PKX (Saulnier and De Kinkelin, 1996; Morris *et al.*, 1997), *Cryptobia salmositica* (Feng and Woo, 1996) and viruses (Vazquez-Branas *et al.*, 1994).

Establishing the location and distribution of specific antigens on the surface of the bacterium is important for vaccine development. Plumb and Klesius (1988) used MAbs produced against the type strain of *E. ictaluri* in Western blot analysis to show that the 14 different isolates of *E. ictaluri* examined possessed an identical antigenic band at 60 and 36

kDa. The MAbs, conjugated with colloidal gold, were then used to screen the bacterium for the presence of these antigens on the surface of the cells (Klesisus and Horst, 1991). Very little is known about the antigenic composition of *F. psychrophilum.* Crump *et al.*, (1999) identified proteinaceous antigens between 22 and 100 kDa, together with highly immunogenic carbohydrate around 16 kDa and 26 kDa in the SDS-PAGE profiles of the bacterium. In the previous chapter, bands were detected at 170, 139, 116, 82, 55, 38 and 26 kDa in whole cell preparations of *F. psychrophilum* using rabbit anti-*F. psychrophilum* sera.

No literature is currently available on the production and characterisation of MAbs against *F. psychrophilum.* The aim of this study was to produce MAbs against whole cell preparations of the virulent (B97026P1) and the non-virulent (32/97) isolate of *F. psychrophilum* used in Chapter 5, and then to use the MAbs to characterise isolates of the bacterium from diseased fish by ELISA, Western blot and IFAT. Their potential use as diagnostic tools to improve serological screening of infected tissue of rainbow trout fry for the presence of *F. psychrophilum* is also discussed. Moreover, the reactivity patterns of the MAbs are compared to the reactivity obtained with rainbow trout anti-*F. psychrophilum* sera in Western blot analysis.

6.2. Materials and Methods

6.2.1. Bacterial strains and growth conditions

Bacterial strains used in this study have been previously listed in Tables 2.1 and 2.2, while their growth conditions are described in Section 2.1.2.1 and 2.1.2.2 respectively.

6.2.2. Preparation of bacterial samples

Bacteria were harvested by centrifugation at 3000 x g (Mistral 3000i, MES) for 20 min at 4°C, after sufficient growth was achieved (approximately 96 h). Bacterial pellets were washed twice with PBS and then resuspended in PBS. The concentration of the suspension was adjusted to approximately 1 $x10^9$ and 1 $x10^8$ cells ml^{-1} using the standard curve prepared in Chapter 3. Aliquots of the harvested bacteria (100 µl) were resuspended in electrophoresis sample buffer at a ratio of 4:1 before storing at –20°C (see Section 2.2).

6.2.3. Production of MAbs

6.2.3.1. Immunisation of mice

Three nine weeks old balb/c female mice were used for the generation of hybridomas against *F. psychrophilum* isolate B97026P1 (virulent) and two mice (balb/c) were used for isolate 32/97 (non-virulent). The virulence of these *F. psychrophilum* isolates was determined as described in Chapter 3. Bacteria were grown and harvested as described above and resuspended in PBS. The concentration of the suspension was adjusted to 1 $x10^8$ cell ml^{-1}, and the bacteria heat-killed by placing them in a water bath at 60°C for 1 h. The effectiveness of heat-killing was checked by plating the bacterial suspension onto MAOA plate and incubating at 15°C for 7 days.

The inactivated bacteria (1 $x10^8$ cell ml^{-1}) were mixed into an emulsion with 1:1 (v/v) TiterMax® Gold adjuvant (Tratech Scientific Ltd., Luton, England). Mice received 200 µl of the emulsion as an IP injection into two sites (100 $\mu l site^{-1}$). The animals were re-immunised nine weeks later with 100 µl of 1 $x10^7$ bacteria, again mixed 1:1 (v/v) with

TiterMax® Gold adjuvant. The antibody response of the animals was monitored using the ELISA described in Section 2.5 and four weeks after re-immunisation, the mouse with the highest antibody response was injected IV with 100 µl of bacteria (10^7 cells in PBS). Four days later, the mouse was asphyxiated with CO_2, then bled by cardiac puncture, killed and its spleen removed for the fusion procedure.

6.2.3.2. Determination of antibody response

A sample of blood was obtained from the tail vein of the mice prior to the first immunisation. Sampled blood was kept at 20°C for 2 h, then overnight at 4°C. The serum was collected the next day by centrifuging at 300 x g for 5 min. Twelve days after the first injection, blood was again sampled from the tail vein of the animal. Additional blood samples were taken 13 days after the booster injections. The antibody titre of the serum was determined by the ELISA described in Section 2.5. Whole bacteria at 1 x10^8 cells ml^{-1} were heat-killed at 60°C for 1 h, and these were used to coat the ELISA plate. The pre-immune mouse serum diluted 1/1000 in PBS, was used as a negative control. The positivity threshold for the reaction was determined as three times the absorbance value obtained for the mean of the negative controls.

6.2.3.3. Myeloma cell culture

Myeloma cell line, SP2/0-Ag14 (Imperial Laboratories) was cultured in the Dulbecco's Modified Eagle's Medium (DMEM) (Sigma) supplemented with 20% (v/v) heat inactivated FCS, 200 mM L-glutamine, 5000 international unit (IU) penicillin/streptomycin and 100 mM sodium pyruvate at 37°C in a 5% CO_2 atmosphere. Cells were sub-cultured

by diluting the cell suspension with between 1:10 and 1:20 fresh medium every 2 to 3 days. A week before the fusion, the cells were expanded daily to maintain good growth in the log phase.

6.2.3.4. Fusion

The fusion of the myeloma cells with spleen cells isolated from the mouse with the highest antibody response was performed according to Campbell (1984) with modifications. Four days after the final booster injection, the mouse was killed using CO_2, bled out by cardiac puncture and swabbed with 70% ethanol. The spleen was carefully removed and placed in 20 ml of serum-free DMEM medium, supplemented with 20% (v/v) heat inactivated FCS, 200 mM L-glutamine, 5000 IU penicillin/streptomycin and 100 mM sodium pyruvate and warmed to 37°C. Excess fat was removed from the organ before washing it three times with serum-free DMEM warmed to 37°C. Both ends of the spleen were removed, and 5 ml of warmed DMEM was gently passed through the tissue using a needle and syringe to remove the cells from the organ. The cell suspension was placed in a universal and allowed to stand for 2 to 3 min to allow clumps of tissue to settle. The cell suspension was collected and made up to 50 ml with serum-free DMEM, warmed to 37°C, before centrifuging cells at 150 x g for 10 min.

The myeloma cells grown to mid-log phase were removed from the tissue culture flask and centrifuged at 150 x g for 10 min. Both sets of cells were re-suspended in 50 ml of serum-free DMEM, centrifuged at 150 x g for 10 min, then re-suspended in 10 ml of serum-free warmed DMEM. They were counted and mixed together at a ratio of one myeloma cell to

ten spleen cells. The cell mixture was centrifuged at 100 x g for 10 min and the supernatant carefully removed. Polyethylene glycol (PEG 1500, Boehringer Mannheim) [2 ml of 50% (w/v) in warmed DMEM] was gently added to the pellets over 1 min and allowed to stand for 1 min 30 sec with occasional swirling. One ml of warmed DMEM was then added over 30 sec and a further 3 ml of warmed DMEM over 30 sec. Finally, 16 ml of warmed DMEM was added over 1 min and allowed to stand for 5 min before centrifuging the suspension at 100 x g for 10 min. The supernatant was removed before gently re-suspending the cell pellet in 10 ml of DMEM with additives (0.1 mM hypoxanthine, 0.016 mM thymidine, 2 mM glutamine, 0.5 mM sodium pyruvate, 50 $IUml^{-1}$ penicillin/streptomycin and 20% FCS), and incubating at 37°C in a CO_2 incubator for 2 to 3 h. The cell suspension was centrifuged at 100 x g for 5 min. The cell pellet was carefully resuspended in 200 ml of DMEM with additives and mouse red blood cells (mrbc) (3×10^7 cells ml^{-1}). The mrbc, which served as a feeder layer for the hybridomas, were harvested from a non-immunised mouse. The cell suspension was placed into 96 well tissue culture plates (Sero-wel, Bibby Sterilin Ltd., UK) at 180 $\mu l well^{-1}$. Controls of myeloma and non-fused spleen cells were also dispensed. The plates were incubated for 10 days at 37°C in a 5% CO_2 incubator.

6.2.3.5. Screening of hybridomas

Ten days after the fusion, supernatants from wells containing hybridoma clones were screened by the ELISA procedure described in Section 2.5, to establish which clones were producing antibodies against *F. psychrophilum.* Cells from wells where a high optical density was obtained were selected, and these were then expanded and re-screened by

ELISA. Selected hybridomas were cloned in 96 well microtitre plates by limiting dilution as described by Campbell (1984). Clones were re-screened and re-cloned a further two times before they were considered as MAb producing cells.

6.2.3.6. Isotyping of MAbs

MAbs were isotyped using a Sigma Immuno Type Kit ^{TM} following the instructions outlined by the manufacturer. Briefly, a pre-coated isotyping strip was placed into a sample of the cell supernatant. After incubation, the strip was exposed to a highly sensitive biotin-avidin-enzyme detection system to reveal the isotype of the hybridoma immunoglobulin.

6.2.4. Characterisation of the MAbs

6.2.4.1. Specificity in ELISA

The reactivity of the MAbs against a variety of *F. psychrophilum* isolates (Table 2.1), and their cross-reactivity with other bacterial genera (Table 2.2), was determined using the ELISA described in Section 2.5. The ELISA plate was coated with heat-killed whole bacteria at 1 x10^8 cell ml^{-1}. The pre-immune mouse serum diluted 1/1000 in PBS, was used as a negative control. The positivity threshold for the reaction was determined as three times the value obtained for the mean negative controls.

6.2.4.2. Western blot analysis

Whole cells (1 x10^9 $cellml^{-1}$) and OMP preparations (1 $mgml^{-1}$) of *F. psychrophilum* were subjected to 12% SDS-PAGE as described in Section 2.2.1. OMP preparations of *F.*

psychrophilum isolate B97026P1 and 32/97 were prepared according to the method described in Section 2.6. Western blot analysis with different MAbs was performed as described in Section 2.3 using 1% anti-mouse IgG-HRP (SAPU), or ABC by applying 1% anti-mouse IgG-biotin (SAPU) followed by 1% streptavidin-HRP (SAPU) diluted in TBS. Pre-immune mouse serum diluted 1/1000 in TBS, and TBS alone were used as negative controls, while immune mouse sera diluted 1/1000 in TBS was used as positive control. All incubations were carried at 20°C for 1 h.

6.2.4.3. Indirect fluorescent antibody technique (IFAT)

The IFAT procedure was similar to that described in Section 5.2.6.2. Bacterial suspensions were used here rather than tissue sections, and were placed on the wells of slides (DYN-EX) containing 21 wells, 4 mm in diameters. The slides were cleaned with acetone, and approximately 5 µl of bacterial suspension (see Table 6.3) was added to each well of the slides. The slides were dried in an oven to fix the bacteria to the glass. A 20 µl drop of each MAb supernatant was then added to the wells of the slide. The same volume of rabbit anti-*F. psychrophilum* serum, prepared in Chapter 5 (1/1000 in PBS), and pre-immune mouse serum diluted 1/1000 in PBS and PBS alone, were added to each slide as positive and negative controls respectively. A 1/100 dilution of either FITC-donkey anti-rabbit IgG (SAPU) or FITC-donkey anti-mouse IgG (SAPU) prepared in PBS, was added to the slide as the secondary antibody. Incubation times, washing and mounting procedures were as described in Section 5.2.6.2.

6.2.5. Production of rainbow trout anti- *F. psychrophilum* sera

Fish serum raised against *F. psychrophilum* was prepared to compare the responses of the mouse sera with that of the fish. One hundred and twenty rainbow trout (average weight 25.5 g) were randomly placed in four 100 l tanks (30 fish $tank^{-1}$) supplied with flow-through water and constant aeration. The water temperature was maintained between 10 and 12°C. Fish were maintained on a commercial diet (EWOS). They were anaesthetised with benzocaine (ethyl *p*-aminobenzoate, Sigma) during injection or bleeding procedures. They were injected IP with 0.1 ml of either a live or a heat-killed (60 min at 60°C in a water bath) bacterial suspension (1 $x10^8$ cells ml^{-1}) of isolate B97026P1 or 32/97.

Fish from each group were randomly sampled at 0, 2, 4, 6 and 8 weeks post-injection by bleeding them from their caudal vein. The blood was kept at 20°C for 2 h, then overnight at 4°C. Serum was collected the next day by centrifuging at 1500 x g for 10 min, and storing at –20°C. The antibody titre of the serum was determined by the ELISA described in Section 2.5. The ELISA plates were coated with either a heat-killed suspension of isolate B97026P1 or 32/97 at 1 $x10^8$ cells ml^{-1}. Non-specific binding was blocked by 3% dried skimmed milk (Premier Beverages, Stafford, England) in PBS for 2 h at 20°C. Pre-immune fish serum, diluted two fold in PBS or PBS alone, were used as negative controls. The positivity threshold for the reaction was determined as three times the absorbance value obtained for the mean of the negative control. Western blot analysis of the whole cell preparations of isolate B97026P1 and 32/97 was performed with the fish sera according to Section 2.3, using pre-immune trout sera diluted 1 in 10 in TBS and TBS alone, as negative controls.

6.3. Results

6.3.1. MAb production

Seven MAbs (1E5, 9G7, 5G6, 3A11, 13B11, 11B2 and 9H9) were produced against *F. psychrophilum* isolate B97026P1 (virulent by experimental challenge) and one MAb (5A9) was produced against isolate 32/97 (non-virulent by challenge). The isotype of all MAbs was of an IgM subclass, except 1E5 and 5G6, which were Ig3 and Ig2b, respectively.

6.3.2. Characterisation of the MAbs

6.3.2.1. By ELISA

The responses of the different MAbs with the *F. psychrophilum* isolates indicated in Table 2.2 when examined by ELISA, are shown in Table 6.1. The reactivity of the MAb is expressed as a relative reactivity to that of the isolate against which the MAb had been prepared. The reactivity of the latter isolate was considered as 100% reactivity. It was found that the MAbs did not react with all *F. psychrophilum* isolates examined, and they appeared to differ in their patterns of reactivity. However, the reactivity of MAbs 1E5, 5G6 and 11B2 were similar to each other with only minor exceptions. MAbs 1E5 and 11B2 reacted with nine of the thirty-four isolates examined, while MAb 5G6 reacted with seven these. They did not react with any of the Chilean (32/97, 34/97, 35/97, 59/97 and CASO 89/97) or Canadian (B035 and B398) isolates. Four of the isolates (NCIMB 1947^T, NCIMB 2282, B97035E4, B97026) were positive with all of the MAbs, with which they had reactivities of 95 % or greater. One of the MAbs (9H9) showed a reactivity of 80% or greater with 30 of the 34 isolates tested. MAbs 13B11 and 9G7 reacted with a number of the isolates, but their profiles differed to those of the other MAbs. MAb 13B11 reacted

Table 6.1. The reactivity of the anti-*F. psychrophilum* monoclonal antibodies against a variety of *F. psychrophilum* isolates in an enzyme linked immunosorbent assay (ELISA)

F. psychrophilum	[b]Reactivity of MAbs (%)							
	1E5	5G6	3A11	9G7	9H9	11B2	13B11	5A9
NCIMB1947[T]	117.6	138.5	-	145.1	99.64	98.25	124.1	106.5
NCIMB2282	116.3	94.67	-	-	83.33	104.73	-	100.1
B97034E4	114.0	102.8	-	133.1	95.16	109.96	112.6	98.64
B97026	128.4	119.9	-	151.8	104.8	114.69	141.4	101.5
[a]B97026P1	[a]100	[a]100	[a]100	[a]100	[a]100	[a]100	[a]100	110.9
Hl313/97	-	94.50	-	-	90.86	-	106.9	102.7
UP164/97	-	-	-	93.0	83.15	-	116.9	103.6
[a]32/97	-	-	-	-	84.44	-	-	[a]100
34/97	-	-	-	122.3	100.0	-	124.1	111.6
35/97	-	-	-	117.9	80.00	-	140.2	106.7
59/95	-	-	-	-	102.0	-	-	87.79
CASO 89/97	-	-	-	-	103.8	-	-	110.1
921/97	-	-	-	124.9	106.8	-	151.7	103.3
110/97	-	-	-	-	89.06	-	-	107.2
UP193/97	-	-	-	-	88.71	-	113.4	103.7
LVDJ E2047	-	-	-	-	93.82	-	-	102.2
LVDJD2172	-	-	106.8	-	110.4	-	125.5	110.9
LVDJXP.189	-	-	-	-	89.72	-	-	110.7
LVDJG2215	43.41	-	-	-	81.32	94.35	-	105.0
LVDL4862/95	-	-	-	-	86.86	-	-	98.02
LVDL1829/91	-	-	-	-	104.9	-	-	93.89
LVDL3077/91	59.34	-	-	-	90.98	98.07	-	101.8
LVDL1456	-	-	-	-	-	-	-	-
LVDI 5/I	-	-	-	-	86.55	-	-	104.8
LPAA P01/88	-	-	-	-	95.72	-	-	120.3
LPAA P03/88	-	-	-	-	89.39	-	-	-
JIP 22/90	49.75	-	-	-	82.43	88.57	-	115.5
JIP 02/86	84.85	110.00	72.46	-	90.66	93.35	-	102.5
JIP 30/98	-	-	-	-	107.1	-	83.2	120.5
B035	-	-	-	-	77.85	-		-
B398	-	-	-	-	-	-	-	-
10/2	-	-	-	-	67.72	-	-	103.6
916/1	-	-	-	-	92.40	-	-	100.7
904/10	-	-	-	-	88.13	-	-	116.9
OD_{450} of the negative control	0.151	0.151	0.151	0.085	0.085	0.088	0.088	0.088
OD_{450} of the reference isolate	0.732	0.527	0.313	0.257	0.558	0.803	0.261	0.811

[a]The strain used to prepare the MAbs was considered as the reference strain with 100% reactivity; Three times the absorbance value of the negative control at 450 nm was considered as the positivity threshold of the ELISA.

[b] % reactivity were calculated as follows for the mean of duplicate wells:

$$\frac{OD_{450} \text{ of test sample}}{\text{Positive control } (OD_{450} \text{ of reference isolate})} \times 100$$

with 11 isolates of the 34 isolates, with reactivities ranging from between 83.2 and 151.5 %, while MAb 9G7 reacted with 7 of the isolates with reactivities ranging from between 93 and 151.8%. MAb 3A11 only reacted with 3 of the isolates tested. It should be noted that MAbs 3A11, 9G7 and 13B11 all had low levels of absorbance with the reference isolate in the ELISA. In fact, MAb 3A11 had absorbance levels below that which was considered as the positivity threshold for the ELISA (i.e. three times the absorbance of the negative control). The only MAb to be produced against the non-virulent isolate 32/97 was MAb 5A9, and this MAb had reactivities ranging from 87.8-120.5% with 30 of the 34 isolates examined. Its pattern of reactivity in the ELISA was similar to that obtained with MAb 9H9.

The reactivity of the MAbs with a variety of bacteria other than *F. psychrophilum* was also examined using the ELISA, the results of which are shown in Table 6.2. MAb 9H9 cross-reacted strongly with *F. branchiophilum, A. hydrophila, Photobacterium damselae* subsp. *piscicida* and *C. aquaticum,* exhibiting reactivities of greater than 90%. MAb 5A9 on the other hand, cross-reacted with *F. branchiophilum* (127.4%), *A. sobri* (46.5%), *Y. ruckeri* (51.7%) and *E. ictaluri* (63.7%). In fact, the reactivities of *F. branchiophilum* with both of the MAbs were greater than that obtained with the reference isolates (B97026P1 and 32/97). The other MAbs did not appear to cross-react with any of the bacterial genera tested.

6.3.2.2. Western blot analysis

Western blot analysis of whole cell preparations of *F. psychrophilum* isolates B97026P1

Table 6.2. The cross-reaction of the monoclonal antibodies raised against *F. psychrophilum* with non-*F. psychrophilum* bacteria in an enzyme linked immunosorbent assay (ELISA)

Bacteria	[b]Cross-reactivity of the MAbs (%)							
	1E5	9G7	5G6	3A11	13B11	11B2	9H9	5A9
Aeromonas salmonicida	-	-	-	-	-	-	-	-
Aeromonas sobria	-	-	-	-	-	-	-	48.5
Aeromonas hydrophila	-	-	-	-	-	-	89.4	-
Bacillus mycoides	-	-	-	-	-	-	-	-
Bacillus subtilis	-	-	-	-	-	-	-	-
Corynebacterium aquaticum	-	-	-	-	-	-	95.7	-
Edwardsiella tarda	-	-	-	-	-	-	-	-
Edwardsiella ictaluri	-	-	-	-	-	-	-	63.7
Escherichia coli	-	-	-	-	-	-	-	-
Flavobacterium aquatilis	-	-	-	-	-	-	-	-
Flexibacter maritimus	-	-	-	-	-	-	-	-
Flavobacterium branchiophilum	-	-	-	-	-		127.4	127.4
Lactobacillus plantinarum	-	-	-	-	-	-	-	-
Listonella anguillarum	-	-	-	-	-	-	-	-
Micrococcus luteus	-	-	-	-	-	-	-	-
Nocardia asteriodes	-	-	-	-	-	-	-	-
Photobacterium damselae subsp. *piscicida*	-	-	-	-	-	-	95.3	-
Pseudomonas aeruginosa	-	-	-	-	-	-	-	-
P. fluorescens	-		-	-	-	-	-	-
P. anguilloseptica	-	-	-	-	-	-	-	-
Renibacterium salmoninarum	-	-	-	-	-	-	-	-
Serratio sp.	-	-	-	-	-	-	-	-
Streptococcus faecalis	-	-	-	-	-	-	-	-
Vibrio ordalii	-	-	-	-	-	-	-	-
Vibrio vulnificus	--	-	-	-	-	-	-	-
Yersinia ruckeri	-	-	-	-	-	-	-	51.7
OD_{450} of the negative control	0.124	0.124	0.111	0.111	0.115	0.148	0.148	0.121
OD_{450} of the reference isolate	0.732	0.257	0.527	0.313	0.261	0.803	0.558	0.811

The strain used to produce the MAbs were considered as the reference strain with 100% reactivity; Three times the absorbance value of the negative control at 450 nm was considered as the lower threshold of the ELISA.

[b] % reactivity were calculated as follows for the mean of duplicate wells:

$$\frac{OD_{450} \text{ of test sample}}{\text{Positive control } (OD_{450} \text{ of reference isolate})} \times 100$$

and 32/97 were performed using both the anti-B97026P1 and anti-32/97 mouse PAbs from the immunised mice and the MAbs produced in this study (Figure 6.1). The mouse anti-B97026P1 PAbs reacted with whole cell material of B97026P1 ranging from 160 to 88 kDa, with intense staining occurring in the profile of the bacterium at 116 kDa (Figure 6.1a lane 10). The PAbs also recognised a band around 28 kDa. Mouse anti-32/97, serum, on the other hand, reacted with whole cell material of isolate 32/97 in the range of 124 to 55 kDa, with a strong reaction occurring between 55 and 116 kDa (Figure 6.1a, lane 21). A region between 7 and 28 kDa was also recognised by these PAb.

Of the seven MAbs produced against virulent isolate B97026P1, only two MAbs (1E5 and 11B2) recognised the region between 160 and 88 kDa (Figure 6.1a, lanes 1 and 7 respectively) as recognised with the mouse PAbs (Figure 6.1a lane 10). These MAbs also recognised the band at 116 kDa in the whole cell material of isolate B97026P1. The MAbs did not recognise any material in the whole cell preparations of isolate 32/97 (Figure 6.1a, lanes 12 and 18 respectively), however. The other five MAbs (9G7, 5G6, 3A11, 13B11 and 9H9) produced against the virulent isolate did not react with the whole cell material of either the virulent or the non-virulent isolate in Western blot analysis. The only MAb developed against the non-virulent isolate 32/97, MAb 5A9, also failed to recognise any material in whole cell preparations of either of the two isolates (Figure 6.1a lane 9 and 20).

Avidin-biotin-peroxidase complex was applied to the Western blot to amplify the reaction of MAbs, with the result that a much more intense staining pattern was obtained (Figure 6.1b). The mouse anti-B97026P1 serum recognised cellular material of isolate B97026P1

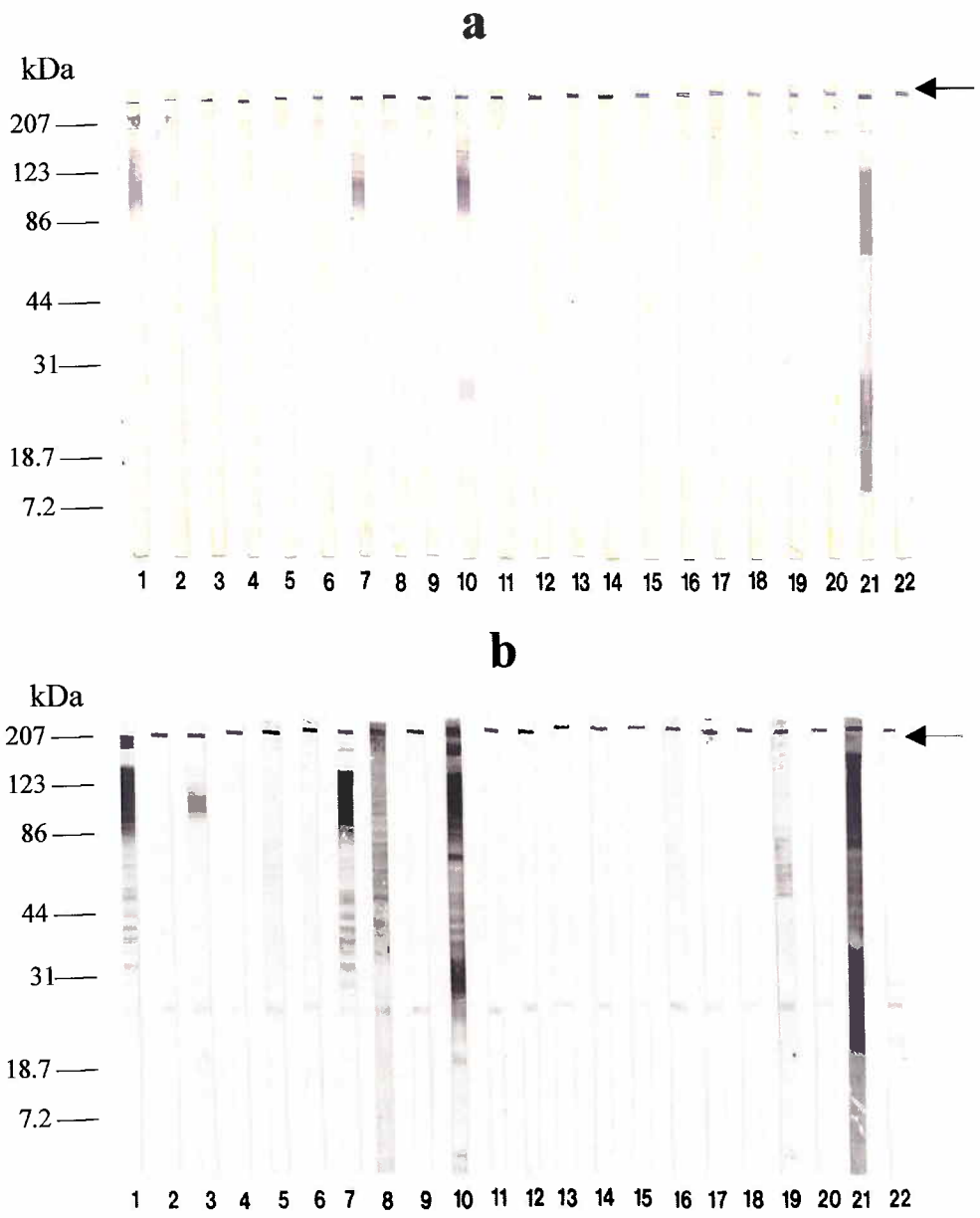

Figure 6.1. Western blot analysis of whole cell preparations of *F. psychrophilum* isolates B97026P1 and 32/97 with mouse monoclonal and polyclonal antibodies; (a) using goat anti-mouse IgG-HRP conjugate (b) using avidin-biotin-peroxidase complex. Lanes: (1-11) isolate B97026P1; (12-22) isolate 32/97. Lanes containing supernatant from hybridoma (1)1E5 (2) 9G7 (3) 5G6 (4) 3A11 (5) 13B11 (6) TBS (7) 11B2 (8) 9H9 (9) 5A9 (10) mouse anti-B97026P1 serum (11) pre-immune mouse sera (12) 1E5 (13) 9G7 (14) 5G6 (15) 3A11 (16) 13B11 (17) TBS (18) 11B2 (19) 9H9 (20) 5A9 (21) mouse anti-32/97 serum (22) pre-immune mouse sera.

Arrow: Line indicating top of gel

at 188, 160 to 88, 72 and 35 to 21 kDa (Figure 6.1b, lane 10), while the mouse anti-32/97 serum appeared to react with almost all of the material present in the profile of strain 32/97 (Figure 6.1b, lane 21). MAbs 1E5 and 11B2 (Fig. 6.1b, lanes 1 and lane 7, respectively) reacted strongly with antigen around 188 kDa, and between 160 and 88 kDa, as well as some less intense bands between 30 and 88 kDa. MAb 5G6 only recognised a doublet around 116 kDa (Figure 6.1b, lane 3). This was not identified by MAb 5G6 when it was used together with the anti-mouse-HRP conjugate (Figure 6.1a, lane 3). MAb 9H9 reacted with a range of high MW bands of both isolates (Figure 6.1b, lane 8 and 19 for isolate B97026P1 and 32/97 respectively). In addition, all MAbs recognised a band around 26 kDa in both isolates, which was also evident in the negative controls (Figure 6.1b, lanes 6, 11, 17 and 22).

The reactivity of the MAbs with the OMPs of isolates B97026P1 and 32/97 in Western blot analysis is shown in Figure 6.2. The reactivity pattern obtained with the MAbs in the analysis was found to be similar to those obtained with whole cell preparations (Figure 6.1a). MAbs 1E5 and 11B2 (Figure 6.2, lanes 1 and 7 respectively) reacted with bands at 160, 116, 95 kDa, and a variety of bands in the region between 95 to 77 kDa of strain B97026P1. They did not recognise any material in the OMP of non-virulent isolate 32/97 (Fig 6.2, lanes 12 and 18). MAbs 5G6 and 5A9 faintly recognised a band at 160 kDa on isolate B97026P1 (Figure 6.2, lane 3 and 9 respectively). In addition, MAb 5G6 also recognised the 95 kDa on isolate B97026P1. The other MAbs examined did not appear to react with the OMPs from either isolate. The mouse anti-B97026P1 serum reacted with a band around 160 kDa and a variety of bands between 27 and 116 kDa in the OMP

preparation of isolate B97026P1 (Fig 6.2, lane 11), while the mouse antiserum against isolate 32/97 reacted strongly with almost all the OMP material present on the nitrocellulose membrane (Fig 6.2, lane 22).

6.3.2.3. Examination of *F. psychrophilum* by IFAT

Different isolates of *F. psychrophilum* were examined with the eight MAbs by IFAT (Table 6.3). Examples of the immunofluorescence staining obtained with the different MAbs is illustrated in Figure 6.3, with apparent difference being observed between the isolates with the MAbs. Some isolates were not recognised by the MAbs. MAbs 9H9 and 5A9 showed strong fluorescence with 14 of the 18 isolates examined. MAb 1E5 reacted with 7 of the isolates with varying intensity, while MAb 9G7 faintly stained 13 of the 18 isolates.

The intensity of the staining was also found to vary between individual isolates with the different MAbs (Table 6.3), and differences could also be seen in the actual staining of the bacterium. MAb 9H9 generally appeared to stain the whole cell and material surrounding the bacterium (Figure 6.3 a), while MAb 5A9 and 3A11 stained the outer edges of the cell (Figure 6.3 b and c). Two of the MAbs, 1E5 and 11B2, showed similarities in their staining of the bacterium (Figure 6.3 d and e respectively). MAb 5G6 reacted with molecules situated at the surface of the bacterium, as well as material, which appeared to be secreted by the bacterium, resulting in what appeared to be aggregates of cells (Figure 6.3 f). The staining obtained with MAb 13B11 appeared as discrete particles on the surface of the bacterium (Figure 6.3 g), but the actual shape of the bacterium could not be distinguished with this antibody. The positive control (rabbit anti-B97026P1 or rabbit anti-

Table 6.3. Indirect fluorescent antibody test of monoclonal antibodies (MAbs) against a variety of *F. psychrophilum* isolates

Isolates	MAbs							
	1E5	5G6	9G7	3A11	11B2	13B11	9H9	5A9
NCIMB 1947	++	++	+	++	+++	+++	++	+++
NCIMB 2282	++	++	+	++	++	++	+++	+++
B97034E4	+	++	+	++	++	++	+++	++
B97026	++	++	+	++	++	++	+++	++
B97026P1	+++	++	+	++	++	++	+++	+++
HL313/97	+	++	+	++	+	+	+++	+++
UP193/97	-	+	+	++	+	+	++	++
32/97	-	++	+	++	+	+	++	+++
34/97	-	-	-	-	-	-	-	-
35/97	-	+	+	+	++	++	++	+++
59/95	-	-	+	-	+	+	++	+
CASO 89/97	-	+	+	-	+	+	++	++
LVDJ 2172	-	+	++	++	+	+	++	++
LVDJG2215	-	-	-	-	-	-	-	-
LVDL3077/91	++	-	-	++	++	++	+++	-
LPAA P01/88	-	-	-	+	++	++	++	+
LVDL1456/91	-	-	-	-	-	-	-	-
LVDJ E2047	-	+	+	++	++	++	++	++

- → negative fluorescence

+→ +++ weakly fluorescent to strongly fluorescent

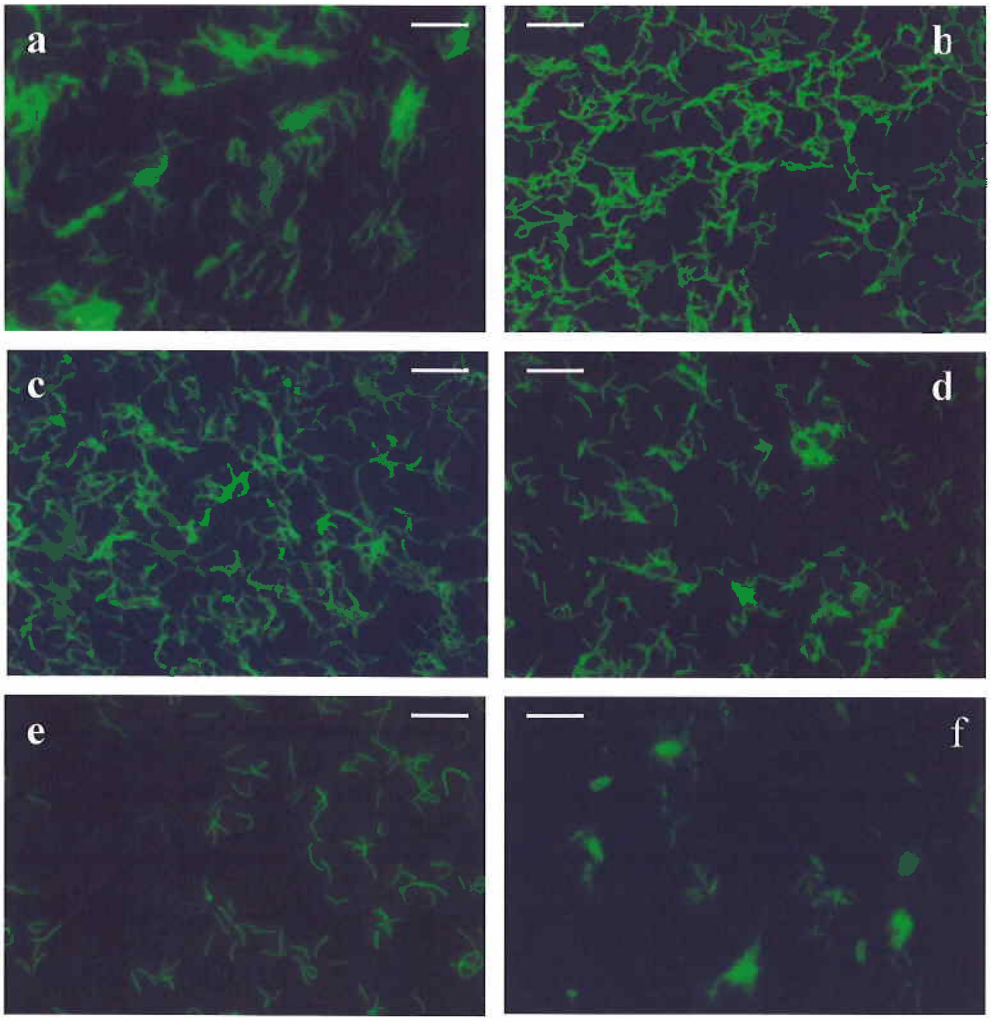

Figure 6.3. Reaction of MAbs with *F. psychrophilum* by an indirect fluorescence antibody test. (a) MAb 9H9 with isolate B97026P1 (b) MAb 5A9 with isolate NCIMB 1947^{T} (c) MAb 3A11 with isolate B97026P1 (d) MAb 1E5 with isolate B97026P1 (e) MAb 11B2 with isolate 32/97 (f) MAb 5G6 with isolate NCIMB 1947^{T}.
Scale bars = 5 μm

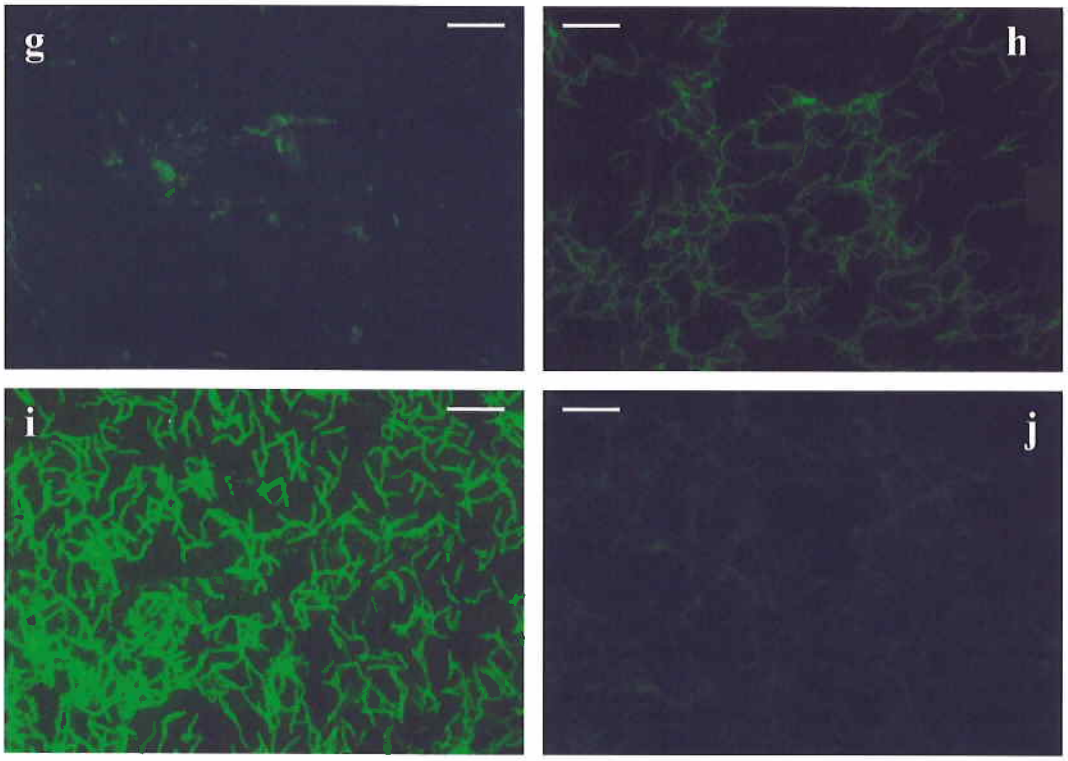

Figure 6.3. (continued) Reaction of MAbs with *F. psychrophilum* by an indirect fluorescence antibody test. (g) MAb 13B11 with isolate HL313/97 (h) MAb 9G7 with isolate LVDJ E2047 (i) Positive control (reaction of 1/1000 dilution of rabbit anti-*F. psychrophilum* B97026P1 serum with isolate B97026P1) (j) Negative control (reaction of 1/1000 dilution of pre-immune mouse sera with isolate B97026P1). Scale bars = 5 μm

32/97 sera) was strongly stained with little background fluorescence occurring (Figure 6.3 i). No fluorescence was observed with the negative controls (pre-immune mouse sera) (Figure 6.3 j).

6.3.3. Response of rainbow trout anti-*F. psychrophilum* sera by Western blot analysis

The response of rainbow trout anti-*F. psychrophilum* sera in Western blot analysis was compared to the response seen with the mouse sera and the MAbs against whole cell preparations of isolates B97026P1 and 32/97. Sera collected 4 weeks post-injection were used, since a peak antibody response was obtained at this time. The mean antibody titres ($\log_2 +1$) at week 4 was 6.3 ±1.0 and 6.3 ±0.5 for fish injected with live and dead cells of isolate B97026P1, respectively, while the mean titres of sera from fish injected with live and dead cells of isolate 32/97 was between 5.3 ±1.1 and 5.1 ±1.3, respectively. The mean titres of sera from fish injected with live cells of isolate B97026P1 were 2.2 ±2.4, 1.3 ±2.1 and 2.4 ±3.1, at week 2, 6 and 8 respectively, while the mean titre of sera from fish injected with dead cells was 2.0 ± 2.6, 3.1 ±3.1 and 3.9 ±3.1. Fish injected with live cells of isolate 32/97, had mean antibody titres of 2.1 ±2.5, 3.6 ±2.6 and 0.6 ±1.9, at week 2, 6 and 8, while sera from fish injected with dead cells, were 0.8 ±1.7, 2.6 ±3.0 and 0.6 ±1.3, respectively. The mean titre of the pre-immune serum was 1.93 ±2.3. Values are expressed as average titres (±SD) of 10, fish including the non-responding fish.

Individual sera from four fish, sampled from each group at week 4 post-injection, were used in Western blot analysis. Sera from two of the four fish injected with live B97026P1 cells reacted with bands at 116 and 95 kDa in the whole cell preparations of isolate

B97026P1 (Figure 6.4a, lane 1 and 3). Bands at 130 and 26 kDa were also weakly recognised by the serum from one of these fish (Figure 6.4a, lane 3). Bands at 200 and 116 kDa were also faintly recognised by the serum of a third fish (Figure 6.4a, lane 2), while the serum of the fourth fish did not react with any of the B97026P1 material (Figure 6.4a, lane 4). A weak response was generally obtained with sera raised against heat-killed cells of isolate B97026P1 when reacted with the whole cell preparations of the same isolate in Western blot analysis (Figure 6.4a, lanes 5 to 8). Bands at 116 and 95 kDa, recognised by the sera from fish immunised with live cells of isolate B97026P1, were also faintly stained by sera from fish immunised with heat-killed cells (Figure 6.4a, lane 7 and 8), and a band at 130 kDa was also faintly recognised by serum from another fish (Figure 6.4a, lane 6 and 8). Serum from the four fish of this group did not react with any material of this isolate (Figure 6.4a, lane 5). Rainbow trout sera raised against live and heat-killed cells of the non-virulent isolate 32/97, on the other hand, faintly recognised a low MW band at around 10 kDa in the whole cell preparations of isolate 32/97. Three of the four fish immunised with live cells of isolate 32/97 and two of the four fish immunised with heat-killed cells, recognised this band (Figure 6.4b, lanes 1, 2, 4, and 6 and 7, respectively). The pre-immune fish serum also recognised the 10 kDa band, but it was not seen when TBS was used as negative control (Figure 6.4b, lane 10 and 9, respectively).

6.4. Discussion

The reactivity of the MAbs produced in this study, against whole cell preparations of both a virulent (B97026P1) and an avirulent (32/97) isolate of *F. psychrophilum,* was compared

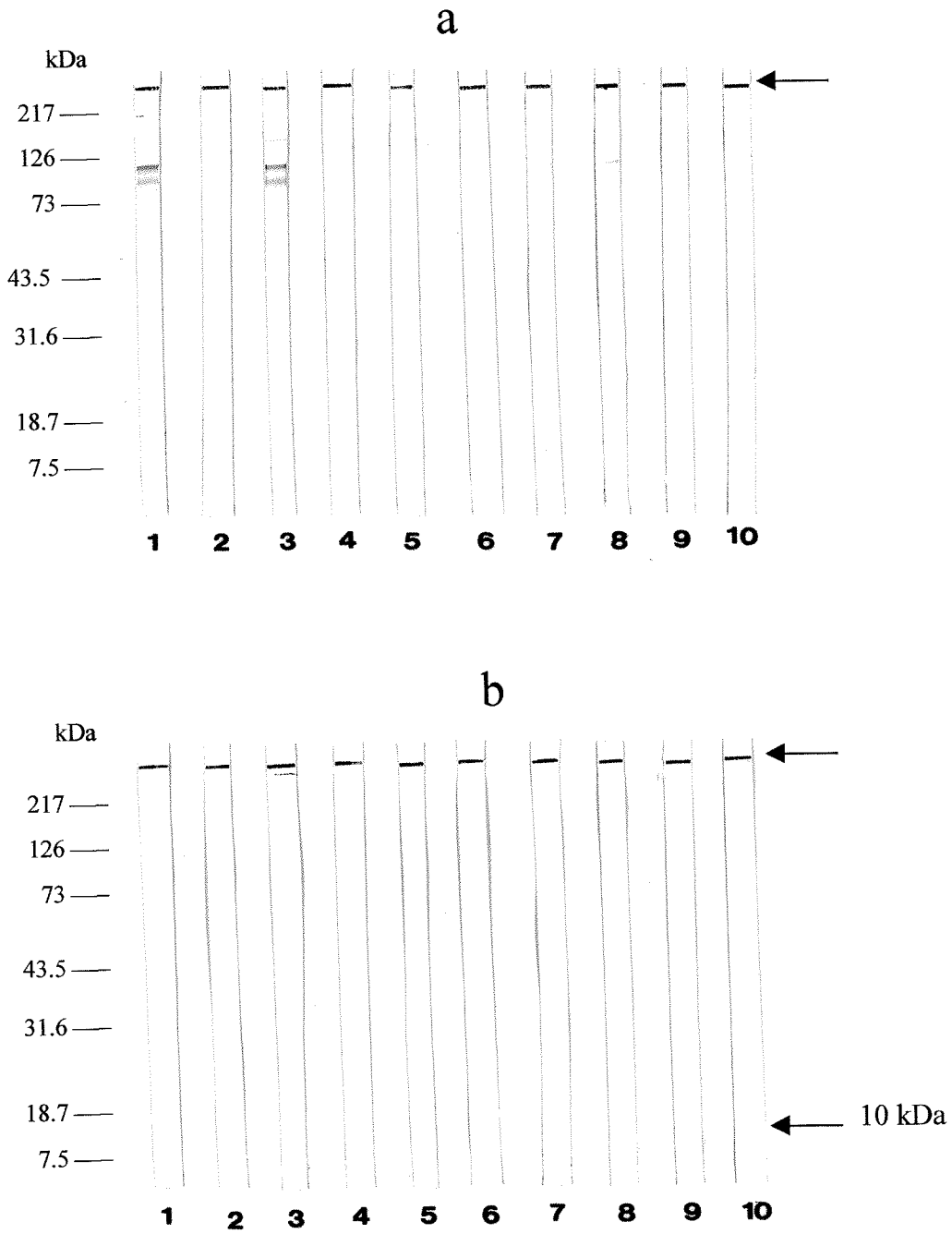

Figure 6.4. The response of rainbow trout anti-*F. psychrophilum* sera, sampled 4 weeks after immunisation, in Western blot analysis. (a) reaction of anti-*F. psychrophilum* B97026P1 serum with isolate B97026P1 (b) reaction of anti-*F. psychrophilum* 32/97 serum with isolate 32/97. Lanes: (1 to 4) sera from individual fish immunised with live cells, (5 to 8) sera of individual fish immunised with dead cells, (9) TBS (10) pooled sera of several pre-immune fish.

Arrow: Line indicating top of gel

by ELISA, Western blot and IFAT. This reactivity was then compared with the response seen with fish sera raised against these bacteria.

MAbs 1E5, 5G6 and 11B2 exhibited similar patterns of reactivity in the ELISA, while MAbs 9H9 and 5A9, and 9G7 and 11B2 appeared to be similar to each other. All eight MAbs failed to react with all of the *F. psychrophilum* isolates examined, although MAbs 9H9 and 5A9 reacted with many of them. The MAbs did not appear to show any particular pattern of reactivity against the 34 isolates of *F. psychrophilum* tested in the ELISA, with respect to their geographical origins, the species of host fish from which they were recovered or the virulence of the isolates. The different reactivities exhibited by the MAbs with the various *F. psychrophilum* isolates is more than likely due to differences in the antigens present on the surface of individual isolates. The composition of the antigen is known to be important to its ability to bind to the polystyrene plate (Andersen *et al.,* 1986). Another possibility for the difference in reactivity may therefore be that the binding potential of each isolate differed as a result of a difference in the antigens expressed on their surface. ELISA plates were coated with poly-L-lysine to enhance the binding of the bacterium.

From the viewpoint of using the MAbs for diagnosis, MAbs 9H9 and 5A9 reacted strongly with most of the isolates examined, but they also cross-reacted with a number of non-*F. psychrophilum* bacteria, thus making them unsuitable as diagnostic probes. MAbs 3A11, 9G7 and 13B11 were not particularly sensitive and gave very low absorbance levels with the reference isolate B97026P1. The reactivity of MAb 3A11 was so low that absorbance

values obtained with this MAb were below that of the positivity threshold of the assay i.e. three times the absorbance of the negative control. The three remaining MAbs, 1E5, 11B2 and 5G6, all exhibited similar patterns of reactivity patterns in the ELISA, but unfortunately they were unable to detect all *F. psychrophilum* isolates examined and were therefore, also unsuitable as diagnosis tools.

Substantial cross-reactivity occurred with *F. branchiophilum* in the ELISA with MAbs 9H9 and 5A9. In fact, the reactivity of these MAbs was greater with this bacterial species than with the bacterial isolate to which they had been raised. This bacterium also showed a high level of cross-reactivity with rabbit anti-*F. psychrophilum* B97026P1 serum in Chapter 5. MAb 9H9 also cross-reacted with *A. hydrophila*, *C. aquaticum* and *P. damselae* subsp. *piscicida,* while MAb 5A9 cross-reacted with *A. sobria*, *E. ictaluri* and *Y. ruckeri*. Among these bacteria, *A. hydrophila*, *A. sobria* and *E. ictaluri* all cross-reacted with the rabbit anti-*F. psychrophilum* B97026P1 serum as described in Chapter 5, indicating that there are common surface antigens between these bacterial species.

When Western blot analysis was performed on whole cell and OMP preparations of the virulent (B97026P1) and non-virulent (32/97) isolates, it was found that the MAbs produced against the virulent isolate failed to recognise material in the SDS-PAGE profiles of the non-virulent isolate. The only MAb derived against the non-virulent isolate, 5A9, failed to recognise bands in the profiles of either isolate. The reason why the MAbs did not appear to react with the non-virulent isolates in the Western blot analysis is unknown. It may possibly be due to differences in the virulence between isolates, or due to serotype

differences between the two isolates, but as discussed with the rabbit sera in Chapter 5, MAbs used here against the virulent isolate also reacted with known avirulent strains (e.g. NCIMB 1947^{T}). MAbs 1E5 and 11B2 reacted with high MW material including the band at 116 kDa in both the whole cell and OMP preparations of B97026P1. The protein band seen at 116 kDa was also observed in the SDS-PAGE profiles of whole cell and OMP preparations of virulent isolate B97026P1 (Chapter 4), and which was recognised by both rabbit anti- *F. psychrophilum* sera in Western blot analysis in Chapter 5.

The OMPs of aquatic pathogenic bacteria play an important role in the pathogenicity of the organism (Aoki and Holland, 1985; Biosca and Amaro, 1991) and can also act as protective antigens in vaccine development (Sengupta *et al.*, 1992; Lutwyche *et al,*. 1995; Suzuki *et al.,* 1996; Tu and Kawai, 1998). The OMPs of two reference isolates (B97026P1 and 32/97) were used in this study to examine whether the material recognised by the MAbs in whole cell preparations of the bacterium constituted part of the OMP of the organism. Generally, the intensity of staining obtained with the OMP was stronger than that seen with whole cell preparations of the bacterium for MAbs 1E5 and 11B2. This increase is most likely due to a concentration of antigen during the OMP extraction procedure. MAb 5G6 faintly recognised bands at 160 kDa and 95 kDa in the OMP profile of isolate B97026P1. However, this MAb did not react with these two bands in the whole cell preparations of the bacterium.

Amplification of antibody or molecular-based reactions with ABC peroxidase is frequently used in both research and for diagnosis (Wilchek and Bayer, 1990). Biotin is a water

soluble Vitamin B6 and acts as a co-enzyme (Bonjour, 1991). Avidin, a glycoprotein-containing carbohydrate isolated from egg whites or streptavidin, a protein isolated from the bacterium *Streptomyces avidinii*, has a high affinity for biotin (Chait and Wolf, 1964; Diamandis and Christopoulos, 1991). Due to the relatively small size of biotin, antibodies can be conjugated with several molecules of biotin, each of which, in turn can bind a molecule of streptavidin or avidin, thus increasing the sensitivity of the assay system. However, a non-specific reaction sometimes results when the avidin binds to tissue or serum rather than the biotin (Jones *et al.*, 1987).

When the ABC amplification system was used in the Western blot with whole cell preparations of *F. psychrophilum*, the sensitivity of the reaction was greatly increased. MAbs 1E5 and 11B2 reacted strongly with antigens around 188 kDa, and between 160 and 88 kDa. MAb 5G6 recognised a band of around 116 kDa in the whole cell preparation of the virulent isolate when ABC was applied to nitrocellulose membrane, which was not detected with anti-mouse-HRP conjugate alone. MAb 9H9 also reacted with a range of high MW bands in both isolates with ABC amplification which were not apparent with the anti-mouse-HRP conjugate. All MAbs reacted with a band at around 26 kDa in both strains. This band appeared to be non-specific since it is was also present in the negative control lanes. The band had a very similar MW to that of the glycoprotein identified in Chapter 4. The glycoprotein detection kit used in Chapter 4 detected a doublet at 20 and 23 kDa in the profiles in the whole cell preparations of all *F. psychrophilum* isolates examined. However, the glycoproteins stained with the kit appeared as two distorted bands, while the band detected here by ABC amplification appeared as single straight band.

Jung (1999) also reported the presence of a non-specific band at 26 kDa in the Western blot profiles of *P. damselae* subsp. *piscicida* when analysed using ABC amplification, and this band also corresponded to a glycoprotein at 26 kDa. MAb 9H9 produced against *F. psychrophilum* in this Chapter, cross-reacted strongly with *P. damselae* subsp. *piscicida* in the ELISA (95.3%). The ELISA used here did not include ABC peroxidase amplification, and so was not the result of a non-specific reaction with the glycoprotein. It would have been interesting to examine the profiles of *P. damselae* subsp. *piscicida* with this MAb (9H9) in Western blot analysis to see if the resulting cross-reactivity occurred at the 26 kDa band.

IFAT is widely used in the detection and diagnosis of disease in both human and animal medicine (Bullock and Stuckey, 1975; Jones *et al.,* 1978; Ristic *et al.,* 1986). The technique is also used for the diagnosis and monitoring of different fish pathogens (Anderson, 1990). IFAT, for example, has been developed to diagnose BGD (Huh and Wakabayashi, 1987; Ostland *et al.,* 1994), bacterial kidney disease (Bullock and Stuckey, 1975; Mitchum *et al.,* 1979; Laidler, 1980) and enteric red mouth disease (Johnson *et al.,* 1974). Lannan *et al.,* (1991) reported IFAT to be very sensitive and specific method for detection of *Piscirickettsia.* Lorenzen and Karas (1992) have used the technique to detect *F. psychrophilum* in spleen imprints of rainbow trout suffering with RTFS. MAbs have also been used in IFAT, for example in the recognition of *E. ictaluri* (Rogers, 1981; Ainsworth *et al.,* 1986).

Analysis of *F. psychrophilum* isolates by IFAT, using the MAbs prepared in this Chapter, showed that the MAbs did not react with all of the eighteen isolates examined. This appears to be a common feature of MAb against bacterial fish pathogens. Plumb and Klesius (1988) also observed that of the seven MAbs produced against *E. ictaluri,* only one of the MAbs reacted against all of the seventeen *E. ictaluri* isolates examined in IFAT. The intensity of fluorescent staining seen here between the different MAbs was found to vary with individual isolates. Differences were also detected in the staining of the bacterium by the different MAbs. For example, MAb 9H9 appeared to stain the whole cell, while MAb 5A9 and 3A11 stained the outer edges of the cell. These differences indicate that different epitopes are being recognised by the different MAbs. Immuno-gold staining would have been useful to identify the different cell surface epitopes recognised by the different MAbs. Unfortunately, due to time constraints, it was not possible to perform such work in the present study.

One advantage of the ELISA over other immunological methods is that it is quantitative and sensitive, especially if ABC amplification is applied, and large numbers of samples can be screened (Adams *et al.,* 1994). Western blot analysis is qualitative rather than quantitative, and has an advantage over the ELISA in that it allows individual antigens to be detected (Wiens *et al.,* 1990). There are differing opinions about the levels of sensitivity obtained in IFAT for the diagnosis of fish disease compared with other immunological assays such as ELISA (Saki *et al.,* 1989; Anderson, 1990). IFAT is sensitive, rapid and easily accomplished in any laboratory with a fluorescence microscope (Lannan *et al.*,

1991), but the method is restricted by the number of samples that can be processed in a day.

The reactivity patterns obtained with individual MAbs in ELISA, Western blot and IFAT did not really correspond to each other. Only two MAbs, 1E5 and 11B2, showed similar reactivity in both the ELISA and in Western blot analysis, suggesting that these MAbs were able to detect the presence of the antigen against which they were specific in both techniques. Their staining pattern was quite different in the IFAT, however. Although these two MAbs exhibited a similar reactivity to that of MAb 5G6 in the ELISA, MAb 5G6 did not recognise any band in Western blot analysis using anti-mouse-HRP conjugate, indicating that in fact, MAb 5G6 recognised different epitopes than those recognised by MAbs 1E5 and 11B2. However, upon amplification with ABC-peroxidase two bands were recognised by MAb 5G6, which were also recognised by 1E5 and 11B2. This is probably the reason why MAb 5G6 showed a similar reactivity to these MAbs in the ELISA. The profile obtained with the OMP of isolate B97026P1 with this MAb was however quite different to 1E5 and 11B2. Conversely, MAbs 9H9 and 5A9 were positive with almost all isolates in the ELISA and IFAT, but were unable to detect any bands in Western blot analysis when mouse anti IgG-HRP conjugate was used. These two MAbs appeared to be able to bind to surface antigens present on the bacterium in ELISA and IFAT, but were unable to recognise these antigens in Western blot analysis. MAb 3A11 reacted with 13 of 18 isolates examined in IFAT and only with 3 of 34 isolates in ELISA. This MAb also did not react at all in the Western blot analysis. This phenomenon has also been observed with other fish pathogens. MAbs against *Photobacterium damselae* subsp. *piscicida* (Jung,

1999) and *Mycobacterium* spp. (Chen, 1996) both shared substantial differences in their reactivity between ELISA and Western blot analysis.

The difference in the response of the MAbs in the ELISA, IFAT and Western blot in this study may possibly be due to the destruction of antigens during the processing of bacteria for SDS-PAGE. Live cell suspensions were used in the IFAT, while bacteria were heat-killed at 60°C for 60 min for the ELISA, and boiled in the presence of 2-mercaptoethnol and SDS for 5 min for the electrophoresis procedure. Madsen and Dalsgaard (1998) reported the possible existence of thermo-labile antigens in the OMP of different *F. psychrophilum* isolates and antigens such as these may have been affected by the processing procedure performed for the ELISA and SDS-PAGE in this study.

Another explanation for the differences that were seen could be that different antigens are present in the different procedures. It was shown in Chapter 4 that a large amount of carbohydrate material was retained in the wells of the stacking gel. So therefore carbohydrate present on the whole cell bacteria in the ELISA and IFAT procedures may not be present in the Western blot analysis. It was seen from the lectin studies in Chapter 4, that different lectins were able to recognise some isolates, but not others, indicating differences in the composition of carbohydrates present of the various isolates. This may be reflected in the reactivities obtained with the MAbs. No attempt was made here to try to classify the epitopes to which the MAbs reacted. Proteinase K digests of both whole cell bacteria and OMPs would have helped to indicated if they were reacting with proteins or carbohydrates. Heat killing of the bacteria for use in the ELISA may have removed the

slime layer, which surrounds the bacterium, whereas it would have been present during IFAT procedure. Some MAbs e.g. MAb 5G6 and 9H9 appeared to react with material surrounding the bacterium in the IFAT, and may have been reacting with the slime layer.

The antibody response of fish to *F. psychrophilum* was compared to the response of the MAbs. No literature is currently available relating to the antibody response of fish to *F. psychrophilum* involved in RTFS. The major bands identified here by the fish sera in whole cell preparations of *F. psychrophilum* (116 and 95 kDa) were fewer than those detected by the mouse polyclonal sera (160 to 88 kDa, 55, 28, 28 to 7 kDa) and by the rabbit sera in Chapter 5 (170, 139, 116, 82, 38, 55, 26 and 20-10 kDa). The responses of the fish antibodies varied between the two isolates examined. Sera from fish immunised with the live cells of virulent isolate B97026P1, elicited a stronger reaction against this isolate compared with the sera raised against dead bacteria. Bands recognised by these sera on isolate B97026P1 at 116 and 95 kDa correspond to the bands recognised by MAbs 1E5 and 11B2. It should be noted that some of the pre-immune sera reacted with both isolates in the ELISA, while it only reacted only with isolate 32/97 in Western blot analysis.

A better understanding of how the immune response of fish recognises and reacts with different antigens present on the surface of *F. psychrophilum* is required. Some antigens may be particularly immunogenic to the fish, but do not result in a protective response against the bacterium, while other antigens may be immunosuppressive. From the viewpoint of vaccine development it is necessary to characterise the antigens recognised by the immune response of fish. It would have been interesting to use the different MAbs

produced here in passive immunisation to see if they affected the onset of the disease in same way. This may indicate if any of the antigens recognised by the MAb can potentially elicit a protective response in the fish.

Hastings (1988) showed that while rabbit serum recognise 25 extracellular components of *A. salmonicida*, rainbow trout could only identify four or five. Similar species differences were noted by Chen (1996) with ECP and whole cell sonicates of *Mycobacterium* spp. where fewer bands were recognised with fish sera in Western blot analysis compared with mouse and rabbit sera. Bakopoulos *et al.,* (1997c) also found this when comparing the reaction of fish, rabbit and mouse anti-*P. damselae* subsp. *piscicida* sera against whole cells of *P. damselae* subsp. *piscicida* in Western blot analysis. The differences seen between species is probably simply due to the fact that the immune system of the rabbit is able to produce a larger repertoire of antibodies against different antigens compared to mice and fish.

In conclusion, an array of MAbs was produced against *F. psychrophilum.* The reactivity patterns observed with the different MAbs against different isolates of the bacterium in ELISA varied and did not react with all isolates examined, although MAbs 9H9 and 5A9 did recognise all but four isolates. These two Mabs also cross-reacted with a number of non-*F. psychrophilum* bacteria in ELISA, while the other MAbs showed no cross-reactivity with other bacterial species. Only two of the MAbs (1E5 and 11B2) were found to react in Western blot analysis against whole cell and OMP preparations of the virulent isolate of *F. psychrophilum*, but they did not recognise any material in the non-virulent isolate. A non-

specific band of 26 kDa was evident in Western blot analysis after applying ABC amplification. The MAbs recognised different surface antigens on the bacterium by IFAT, and the intensity of the reaction varied between the different isolates examined.

Reactivity of different *F. psychrophilum* isolates with the MAbs in IFAT, ELISA and Western blot analysis appeared to vary between techniques, suggesting that different antigens were present during the different procedures. This is possibly due to differences in the processing procedure used to prepare the bacteria for the different techniques. No attempt was made here to characterise the antigens recognised by the MAbs. This is important if the MAb are to be used to identify potential vaccine candidates.

The use of the eight MAbs produced in this study is limited by the fact that they do not recognise all *F. psychrophilum* isolates and some cross-react with non-*F. psychrophilum* strains. This makes them unsuitable as diagnostic probes for screening infected RTFS samples. Further MAbs need to prepare against additional bacterial preparations, in the search for MAb, which recognise species-specific antigens for use in diagnostic screening and vaccine development.

CHAPTER 7

Characterisation of extracellular products (ECP) from *Flavobacterium psychrophilum*

7.1. Introduction

One strategy employed by successful pathogenic bacteria is the production of extracellular and metabolic products. These help to protect the organism from the defence mechanisms of its host and allow the organism to use host tissue as a source of nutrition, thus allowing it to survive and replicate within its host (Ellis, 1991; Falkow, 1991; Stanley *et al.,* 1994). A great deal of information is already available in the literature relating to the ECPs of a variety of bacterial fish pathogens such as *Aeromonas* sp., *Vibrio* spp., *Y. ruckeri* and *R. salmoninarum.* Most reports focus on the identification of factors responsible for lethal effects and pathology caused by the bacterial ECPs.

A variety of proteases, haemolysins and cytotoxins have been shown to be produced in the ECPs of *A. salmonicida* (Ellis *et al.,* 1981; Gudmundsdóttir *et al.*, 1990; Lee and Ellis, 1991; Gudmundsdóttir, 1996; Gudmundsdóttir and Gudmundsdóttir, 1997) and *A. hydrophila* (Allan and Stevenson, 1981; Shotts *et al.,* 1985; Pansare *et al.,* 1986; Nietio and Ellis, 1991; Khalil and Mansour, 1997). Extracellular products, notably proteases, haemolysins, cytotoxins and LPS have also been identified from *Vibrio harveyi* (Liu *et at.,* 1996; Montero and Austin, 1999), while phospholipase and haemolysins (Magarinos *et al.,* 1992), and capsular polysaccharides (Bonet *et al.,* 1994) have been found to be important in the pathogenicity of *Photobacterium damselae* subsp. *piscicida.* Barton *et al.,* (1997) reported that *R. salmoninarum* released high concentrations of extracellular protein into the

tissues of infected fish, which was found to be almost entirely composed of a 57 kDa protein and its breakdown products. Electrophoretic separation of this particular protein indicated the presence of a number of proteolytically active bands ranging from >100 to 18 kDa. Soluble polysaccharide-like material was also detected in the ECP of *R. salmoninarum* (Barton *et al.,* 1997). A 37 kDa toxin with a lethal proteolytic effect has been purified from the ECP of a virulent strain of *E. tarda* (Suparato *et al.,* 1996), while Chen *et al.,* (1997) identified a 65 kDa and a <14 kDa protein band in the of ECP of *Mycobacterium* spp. isolated from fish suffering from mycobacteriosis.

Despite the importance of *F. psychrophilum* as a world-wide pathogen of farmed fish, the determinants of its pathogenicity are poorly understood. Little information is available in the literature on the biological activities of its ECP. Extracellular protease has been repeatedly proposed as a modulator of this pathogenicity (Hofer, 1997). Proteases (47, 40, 34 and 32 kDa) have been identified in the ECP of a related species *F. columnare* by Bertolini and Rohovec (1992) using substrate gel electrophoresis. Using the same method Bertolini *et al.,* (1994) identified proteases in the ECP of a reference isolate of *F. psychrophilum* (SH3-81) with molecular weights of around 114 and 152 kDa and these were found to be active against both casein and gelatin. From the analysis of a further 29 isolates, they were able to divide the bacteria into four different groups based on their protease activity, and the activity of these proteases appeared to be related to the virulence of the bacterium. In contrast, Hofer (1997) failed to identify the presence of any protease activity in the ECP of *F. psychrophilum* using substrate gel electrophoresis.

In the present Chapter, the enzymatic activities and electrophoretic profiles of ECPs from 12 isolates of *F. psychrophilum* were examined. The glycoprotein detection kit described in Chapter 4 was also used to screen the ECPs for the presence of glycoprotein molecules. The antigenicity of the ECPs was examined by Western blot analysis using the rabbit sera produced in Chapter 5. Furthermore, the toxicity of the ECP preparations was tested in rainbow trout, and an attempt to identify any extracellular protease activity in the preparations was performed using substrate gel electrophoresis.

7.2. Materials and Methods

7.2.1. Bacterial isolates and preparation of extracellular products (ECPs)

Twelve different *F. psychrophilum* isolates were used in this study, two of which were know reference strains, and the remaining ten were obtained from fish suffering from RTFS (Table 7.1). Colonies were taken from 96 h plate grown cultures and inoculated into 500 ml of MAOB in 1 l flasks. The cultures were incubated at 15°C for 14 days to allow expression of sufficient amount of ECPs in the medium. The bacterial suspension was centrifuged at 3500 x g for 45 min at 4°C, and then remaining bacteria were removed from the suspension by successive filtration through a Whatman No. 1 filter paper and a 0.2 μm Millipore filter. The filtered supernatant was further concentrated using PEG (MW 8000, Sigma). Dialysis tubing (12-14 kDa MW cut-off, Medicell, London) was boiled for 10 min in 10 mM EDTA and washed several times with distilled water. The cell-free supernatants containing the *F. psychrophilum* ECPs were then placed within the dialysis tubing, the ends of the tubing were sealed with a double knot. The tubing was placed in a plastic tray and covered generously with a layer of PEG. The dialysis tubing was incubated overnight at 4°C. The following morning, the dialysis tubing was rinsed with

Table 7.1. Isolates of *Flavobacterium psychrophilum* used for the production of extracellular products

Isolate	Fish species	Tissue	Country	Year
NCIMB 1947[T]	Coho salmon	Kidney	USA	1955
NCIMB 2282	Silver salmon	Unknown	USA	Unknown
HL313/97	Rainbow trout (RT)	Spleen	Germany	1997
UP164/97	RT	Spleen	England	1997
B97026	RT	Lesion	Scotland	1997
B97035 E4	RT	Spleen	Scotland	1997
59/95	RT	Unknown	Chile	1995
32/97	RT	Kidney	Chile	1997
33/97	RT	Gill	Chile	1997
34/97	RT	Kidney	Chile	1997
35/97	Atlantic Salmon	Kidney	Chile	1997
CASO 89/97	RT	Kidney/spleen	Chile	1997

NCIMB : National Collection of Industrial and Marine Bacteria

[T] Type strain; Coho salmon (*Oncorhynchus kisutch*), Silver salmon (*O. kisutch*), RT: Rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*).

sterile distilled water to remove the PEG. The concentrated ECP was resuspended in 1 ml of PBS, and then dialysed against PBS for 24 h at 4°C with two changes of the buffer. The protein concentration of the ECPs was determined with a BioRad protein determination kit using BSA as a standard. These were then stored at –70°C until required.

7.2.2. Analysis of ECPs activities

A plate assay, used to evaluate the enzyme activity of the ECPs was performed according to Chen *et al.,* (1997) with modifications. Briefly, a solution of agarose (Sigma) supplemented with the different enzyme substrates indicated below was poured into 85 mm diameter Petri dishes (25 ml per dish). The agarose was stabbed with a 6 mm diameter sterile biopsy punch (Stiefel Laboratories Ltd, England) to form six wells per plate. Four wells were inoculated with the ECPs (20 µl) and two non-adjacent wells were inoculated with the same volume of MAOB as a negative control.

7.2.2.1. Mucinase activity

Twenty µl of each ECP sample was added to the wells of a 1% (w/v) agarose plate supplemented with 0.28% (w/v) porcenic stomach mucin (Sigma) and incubated for 24 h at 22°C. After the incubation, the plates were flooded with 1% (w/v) calcium chloride, where upon mucinase activity was revealed as a zone of clearing within the gel.

7.2.2.2. Protease activity

Protease activity was measured by incubating 20 µl of ECP overnight at 22°C in wells of a 1% (w/v) agarose plate containing either 1% (w/v) gelatin (BHD Chemical Ltd Poole,

England), 1% (w/v) casein (Sigma) or 0.5% (w/v) elastin (Sigma), prepared in Tris-HCl buffer (25 mM, pH 8.5). After incubation, the plates were flooded with saturated ammonium sulphate solution to reveal the presence of protease activity, apparent as a zone of clearing within the gel.

7.2.2.3 Lipase activity

Lipase activity was detected by incubating 20 µl of ECP overnight at 4°C in wells in a 1 % (w/v) agarose plate supplemented with 1% (v/v) Tween-80 and 0.01% (w/v) $CaCl_2.H_2O$. A positive reaction was indicated by the presence of an opaque halo around the wells.

7.2.3. Haemolytic activity

Haemolytic activity of the ECPs was examined using rainbow trout red blood cell as substrate. Rainbow trout were anaesthetised with 10 ml of 5% benzocaine per 500 ml of water, and the blood was withdrawn from the caudal vein of the fish into a heparin-coated syringe. Sampled blood (1 ml) was placed in 9.5 of ml sterile physiological saline (0.9%) containing 20 IU of heparin. The blood samples were centrifuged at 1000 x g for 10 min. The supernatant was discarded and the blood cell pellet was resuspended 1/200 (v/v) with sterile physiological saline.

The ECPs of an unspeciated *Aeromonas* spp. (Au 2D8) and *Aeromonas veronii biovar veronii* 2N1, characterised by Pearson *et al.,* (2000) were used as positive and negative control respectively. They were cultured in 10 ml of TSB for 24 h at 30°C. Bacteria were

removed by centrifugation at 3000 x g for 20 min at 4°C, and the supernatant was then filtered through a 0.45 μm Millipore filter.

The blood cell suspension (100 μl) was added to 100 μl of ECP, which had been diluted two fold with sterile saline in the wells of a microtitre plate (U-bottomed wells, Nalge Nunc Intl. Denmark). Each test was performed in duplicate. ECPs of Au 2D8 and *Aeromonas ver. bv. veronii* 2N1 were also added to each plate as positive and negative controls, respectively. The plates were incubated at 37°C for 1 h, and then placed at 4°C for 12 h. Haemolytic titres were expressed as the reciprocal of the highest dilution of ECP needed to produce partial haemolysis of the erythrocytes.

7.2.4. Gel electrophoresis

SDS-PAGE of the ECPs was performed using 12% separating gel and 4% stacking gel, as described in Section 2.2. Broad range molecular weight markers (BioRad) were used as a reference to determine the molecular weight of resulting bands. ECP samples were diluted 4:1 in electrophoresis sample buffer (Appendix 2), heated for 2 min at 96°C and centrifuged at 13000 x g for 5 min prior to loading (20 μl) on to the gels. The gels were run at 80 V for approximately 3 h at 4°C, then stained with Coomassie blue or Silver stain as described either in Section 2.2.1 or in Section 2.2.2.

7.2.5. Substrate SDS-PAGE

Protease activity of the ECPs was examined using substrate gel electrophoresis according to Bertolini *et al.,* (1994) with modifications. The electrophoresis procedure was conducted as described by Laemmli (1970), however, either gelatin (BHD Chemical Ltd

Pooled, England) or casein (Sigma) was added to the 12% separating gel at 0.01 % (w/v) from a 1% (w/v) stock solution prepared in water (Appendix 2) prior to pouring the gel. The ECP samples were diluted 4:1 with sample buffer (Appendix 2), centrifuged at 13000 x g for 5 min, but were not boiled prior to performing the electrophoresis. Before adding sample buffer, a 100 µl aliquot was removed and stored at –20°C for subsequent lysozyme treatment. This involved incubating the aliquots with 10 µl of a 10 $mgml^{-1}$ solution of lysozyme (Sigma) for 1 h at 60°C before diluting with sample buffer. ECP of *A. hydrophila,* characterised by Petchinda (1999) was used as positive control for the substrate gel electrophoresis. Twenty µl of each sample was loaded onto the gel before they were subjected to electrophoresis, initially 150 V for 1 h at 4°C and then 50 V for the remainder of the procedure, approximately 4 h.

After electrophoresis, the gels were incubated for 2 h at 4°C in a solution of 2.5% (v/v) Triton X-100 in order to remove the SDS, and re-inactivate enzyme activity. The gels were incubated overnight at 15°C in 0.1 M glycine-NaOH buffer, pH 8.0, after which they were fixed and stained with Coomassie blue for 1 h. They were destained until the molecular weight markers became visible, and protease activity was indicated by clear bands within the blue-stained gel.

7.2.6. Western blot analysis

Western blot analysis of the ECPs with the rabbit sera produced in Chapter 5 was carried out according to the method described in Section 2.3. The sera were diluted 1/1000 in TBS, and the membrane was incubated with this primary antibody for 60 min at 20°C. The

secondary antibody (goat anti-rabbit IgG-HRP, SAPU) was diluted 1/200 in TBS. The remainder of the procedure is as described in Section 2.3.

7.2.7. Glycoprotein detection

The ECP samples were screened for the presence of glycoproteins using the detection kit described in Section 2.4.

7.2.8. ECP toxicity

A total of 90 rainbow trout fry (average weight 3.5 g) was divided into 9 groups (10 fish group^{-1}) in separate tanks and fed daily with a commercial diet. Each tank was aerated, and the water temperature was maintained at 15 ±2°C throughout the experimental period. One-third of the water was changed daily and debris at the bottom of the tank removed. Six of the nine groups of fish received an IM injection of 0.1 ml of ECP from either *F. psychrophilum* isolate B97026P1 or 32/97 at a dose of 50, 25 or 5 µg protein ml^{-1}. The remaining three groups of fish were used as the controls. Control fish were injected with concentrated broth diluted to the same extent as that containing the ECPs. Fish were monitored daily.

On termination of the experiment, 18 days post-injection, three fish from each tank were sampled for histopathological analysis. Samples (muscle, spleen, kidney, liver and gills) were placed in fixative (10% neutral buffered formalin) for at least 24 h prior to preparation. Tissues were embedded in paraffin wax according to standard procedures, and 5 µm sections were prepared, and stained with haematoxylin and eosin (H & E). These

were then examined using a light microscope. The full histological procedure is provided in Appendix 3.

7.3. Results

The amount of protein produced in the ECPs of the different *F. psychrophilum* isolates shown in Table 7.2 was between approximately 0.5 and 0.9 mg protein ml^{-1}. The ability of these ECP preparations to digest different enzyme substrates was examined using a variety of agarose plate assays. Levels of protease activity obtained from each preparation is displayed in Table 7.2, and protease activity against both casein and gelatin was detected in the ECPs of all *F. psychrophilum* isolates examined using this technique. An example of the zones of clearing obtained against gelatin can be seen in Figure 7.1. The level of protease activity was seen to differ between the isolates examined, and the protease activity against gelatin substrate was higher in the ECP preparation than that obtained against casein. Of the 12 isolates examined, only two isolates demonstrated weak elastase activity, and four isolates exhibited a weak activity against mucinase. Only two *F. psychrophilum* isolates, B97026P and HL313/97, appeared to have both elastase and mucinase activity (Table 7.2). No lipase activity was detected in any of the isolates. Very little haemolytic activity against the rainbow trout erythrocytes was observed in the ECP preparations from the different *F. psychrophilum* isolates examined. A haemolytic titre of 1/2 was obtained with all isolates compared to a positive control of ECP from an unspeciated *Aeromonas* strain, Au 2D8 which had a titre of 1/1024. No haemolytic activity was noted in the ECPs of *A. veronii biovar veronii* 2N, the negative control.

Table 7.2. The amount of protein produced in the extracellular products of different *F. psychrophilum* isolates and their corresponding enzyme and haemolytic activities

Isolates	Protein ($mgml^{-1}$)	[a]Enzyme activity					[b]Haemolytic titre
		Caesinase	Gelatinase	Elastinase	Mucinase	Lipase	
NCIMB 1947^T	0.62	+	+++	-	-	-	1/2
NCIMB 2282	0.45	+	+++	-	-	-	1/2
HL313/97	0.56	++	++++	+	+	-	1/2
UP164/97	0.73	++	++++	-	+	-	1/2
B97026	0.70	++++	++++	+	+	-	1/2
B97035 E4	0.45	+++	++	-	-	-	1/2
59/95	0.88	+++	++++	-	+	-	1/2
32/97	0.50	++	++	-	-	-	1/2
33/97	0.55	++	+++	-	-	-	1/2
34/97	0.70	+	++	-	-	-	1/2
35/97	0.90	+	+	-	-	-	1/2
CASO 89/97	0.71	++	+++	-	-	-	1/2

[a] Mean diameter (mm) of zones of clearing: ++++ (12-15), +++ (10-12), ++ (8-10), + (8), - (no reaction)

[b] Haemolytic titre of 1:1024 was found with the unspeciated *Aeromonas* spp. (Au 2D8) (positive control), while no haemolytic activity was detected for *A. veronii biovar vironii* 2N1 (negative control)

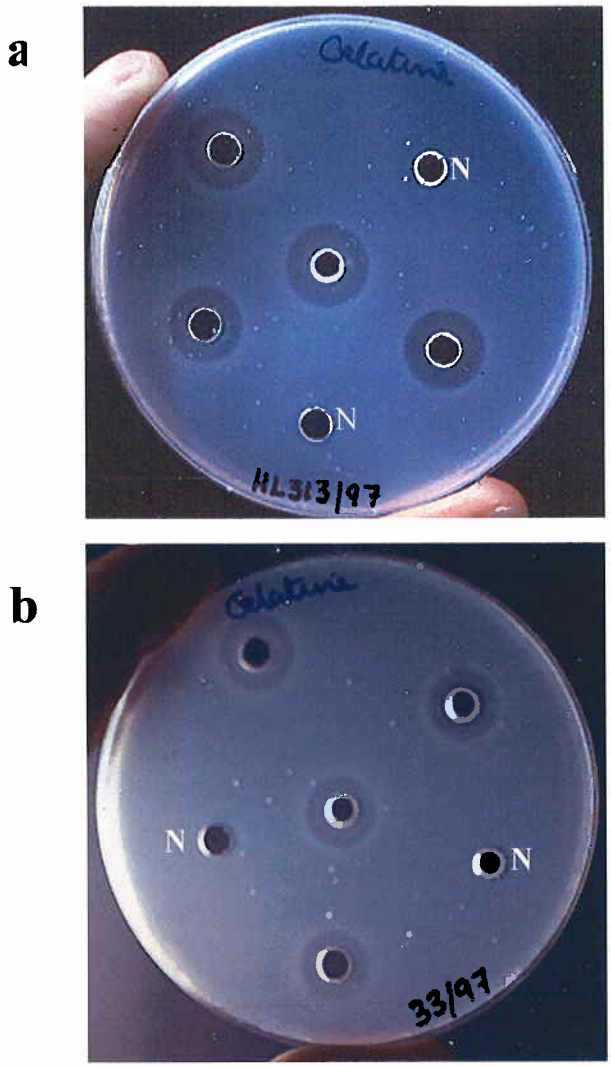

Figure 7.1. Degradation of gelatin (1% w/v) by the extracellular products of *F. psychrophilum* using an agarose plate assay. (a) isolate HL313/97 (b) isolate 33/97.

N- Negative control (broth)

Concentrated ECP preparations from 14 day old cultures of *F. psychrophilum* isolates were examined by SDS-PAGE.The profiles of the different preparations were found to be very similar to each other, with major bands found at 88, 62, 40-55, 20 and 15 kDa upon staining the gels with Coomassie blue (Figure 7.2). There did appear to be some difference between isolates in the intensity of the 15 kDa band. A substantial amount of diffuse staining was seen throughout the profiles after staining the gels with Silver staining, but major bands could be seen at 98, 77-90, 43-58 and 14 kDa (Figure 7.3). Low molecular weight material was also apparent upon staining with both Coomassie blue and Silver staining.

Analysis of the ECP preparations was performed by Western blot analysis using the rabbit sera raised in Chapter 5 against whole cell preparations of virulent (B97026P1) and non-virulent (32/97) isolates of *F. psychrophilum*. Both sera recognised a range of bands in the profiles of the different ECP preparations, although the bands were not clearly defined (Figure 7.4). The region of staining obtained with both sera generally ranged from between 19 to 120 kDa, but more distinct bands were evident at around 116, 87, 72 and 61 kDa with both sera (Figure 7.4a and b). The serum raised against the non-virulent isolate, 32/97, reacted strongly with the ECP obtained from this isolate (Figure 7.4a, lane 2), while the serum raised against B97026P1 recognised a substantial amount of materials in the ECP profile of isolate B97026 (Figure 7.4b, lane 1). Both sera generally failed to recognise very high or low MW material in the ECP of most isolates examined. However, the anti-32/97 serum did recognise high MW material in isolate 32/97 and B97035E5 (Figure 7.4 a, lanes 2 and 4, respectively) and low MW material in the ECP of isolate 34/97 (Figure 7.4a

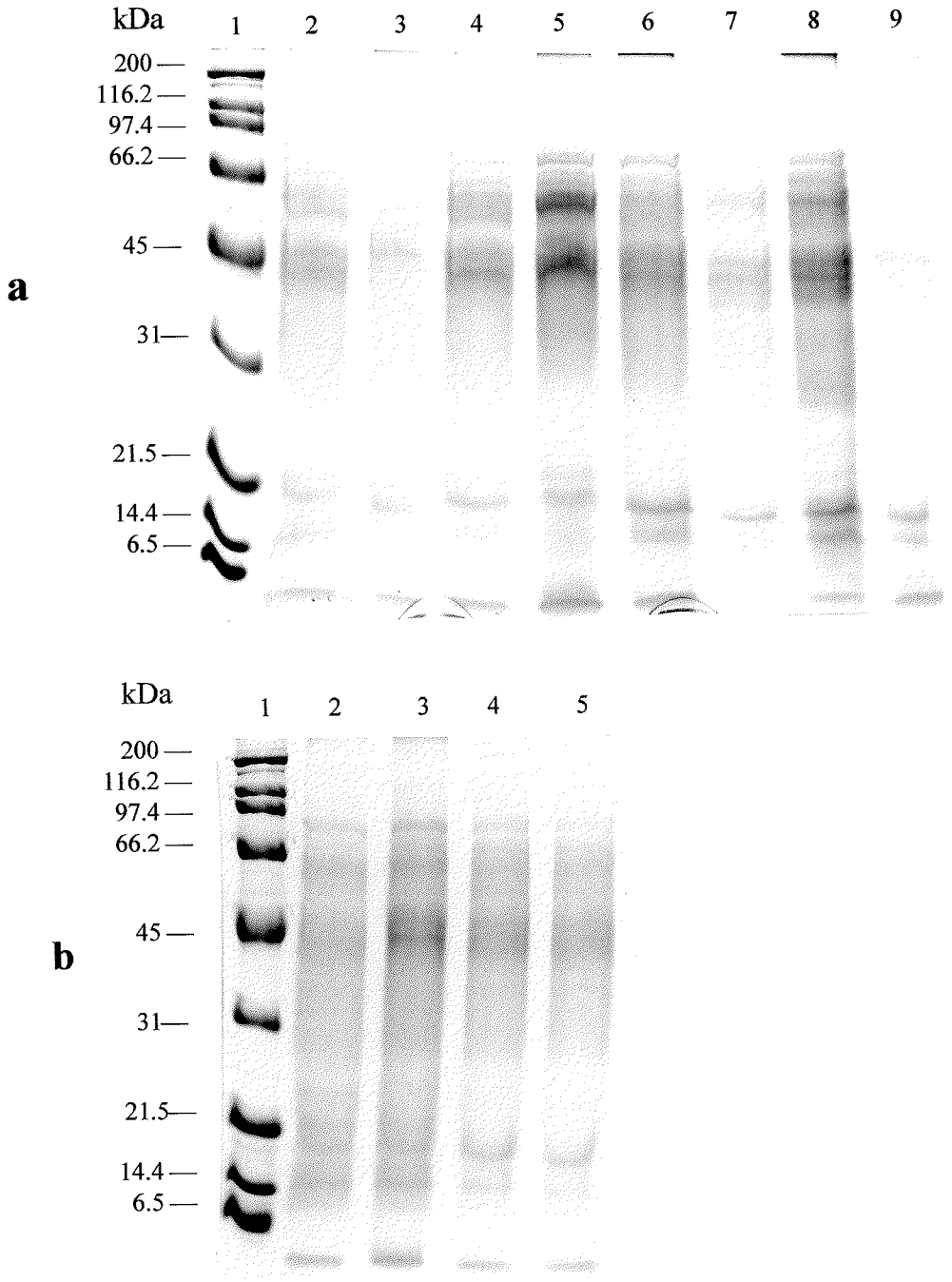

Figure 7.2. SDS-PAGE (12%) of the ECP preparations of *F. psychrophilum*, stained with Coomassie blue, (a) Lanes: (1) MW marker (2) NCIMB 1947^T (3) NCIMB 2282 (4) B97026 (5) B97035E4 (6) HL313/97 (7) UP 164/97 (8) 32/97 (9) 33/97. (b) Lanes: (1) MW marker (2) 34/97 (3) 35/97 (4) 59/95 (5) CASO 89/97.

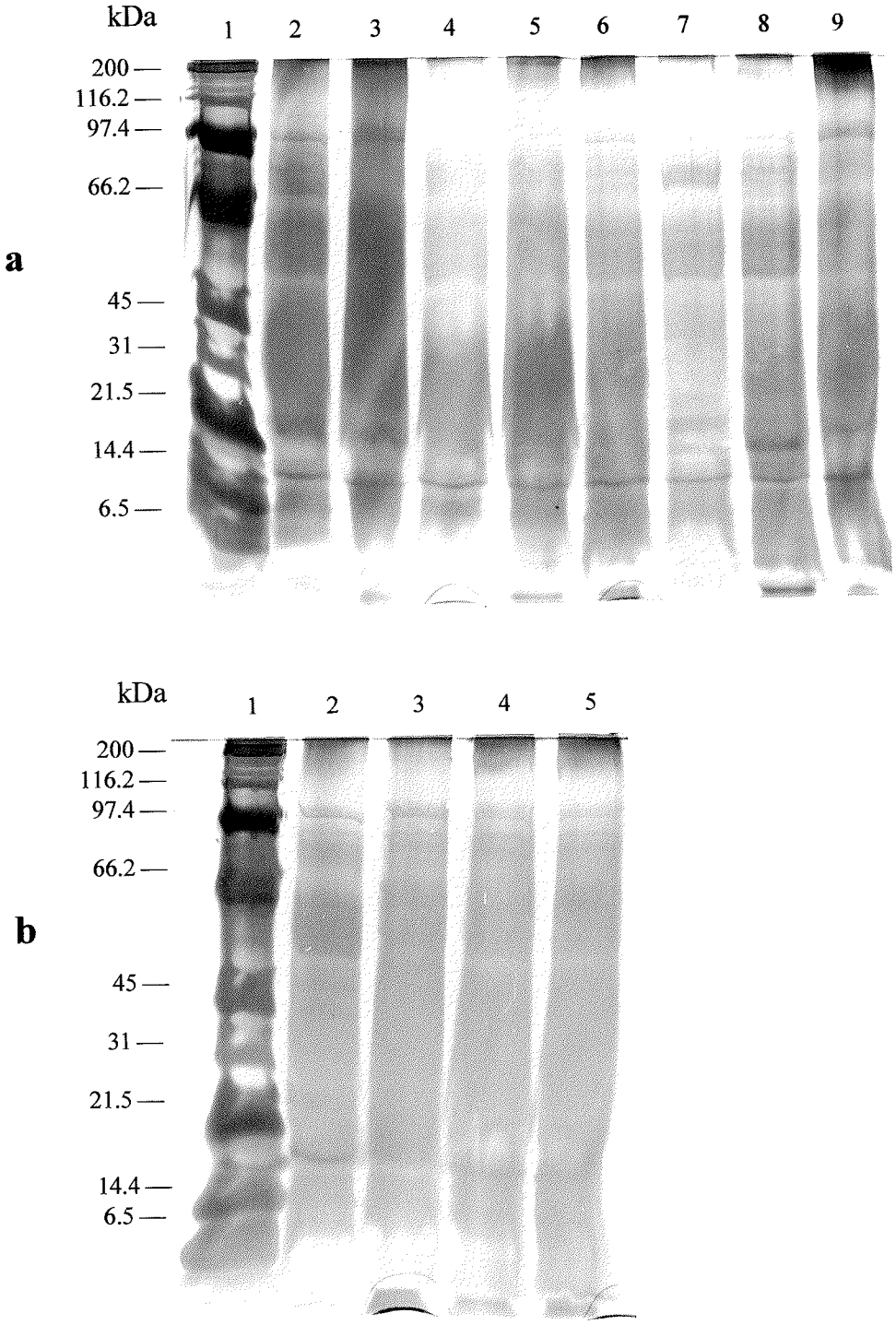

Figure 7.3. SDS-PAGE (12%) ECP preparations of *F. psychrophilum*, stained with silver stain. (a) Lanes: (1) MW marker (2) NCIMB 1947^{T} (3) NCIMB 2282 (4) B97026 (5) B97035E4 (6) HL313/97 (7) UP 164/97 (8) 32/97 (9) 33/97. (b) Lane: (1) MW marker (2) 34/97 (3) 35/97 (4) 59/95 (5) CASO 89/97.

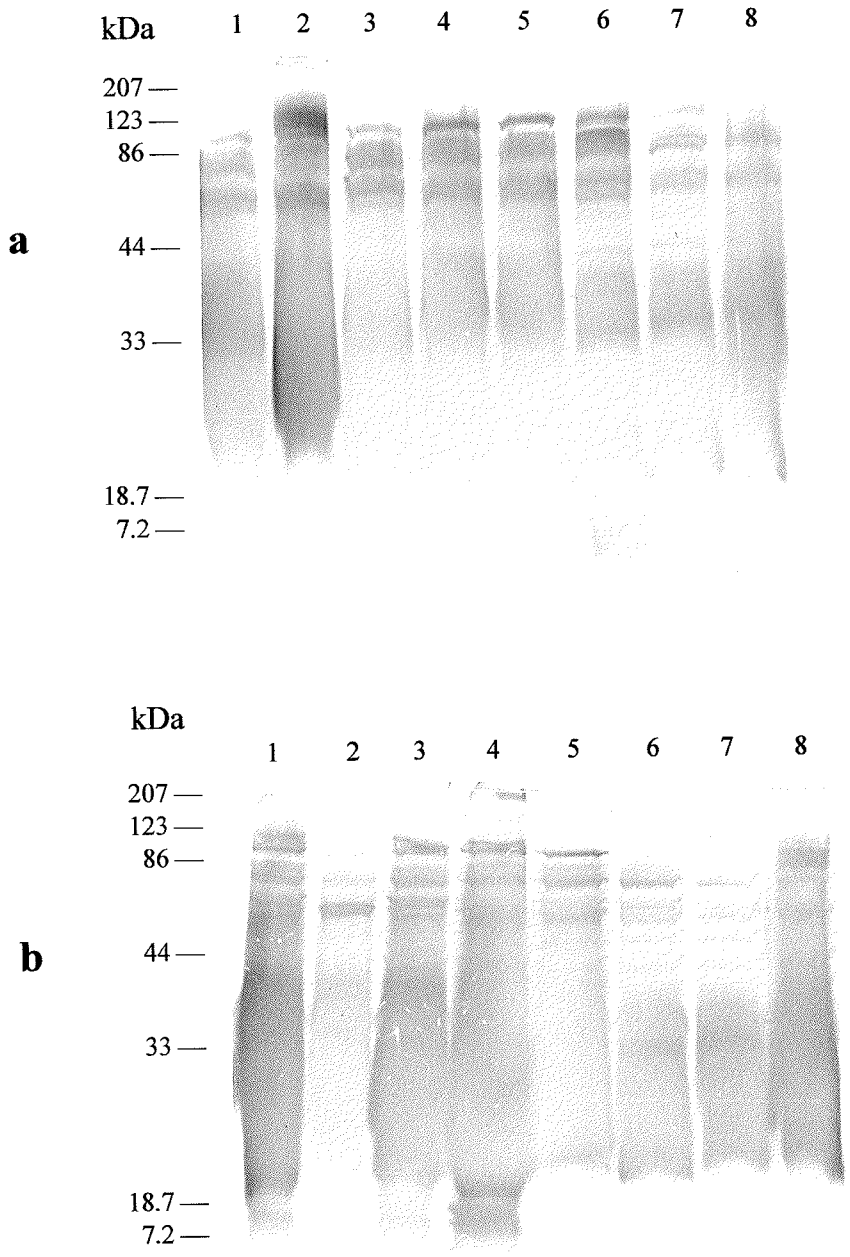

Figure 7.4. Western blot analysis of ECP preparations of different *F. psychrophilum* isolates. (a) rabbit anti-32/97 sera (b) rabbit anti B97026P1 sera. Lanes: (1) B97026 (2) 32/97 (3) NCIMB 1947^T (4) B97035E4 (5) 35/97 (6) 34/97 (7) UP 164/97 (8) NCIMB 2282.

, lane 6). Anti-B97026P1 sera, recognised both high and low MW material in the ECP of isolates B97026P1 and B97035E4 (Figure 7.4b, lanes 1 and 4 respectively). It also recognised some low MW material the ECP of isolates NCIMB 1947^T and NCIMB 2282 (Figure 7.4b, lanes 3 and 8 respectively).

When the glycoprotein detection kit was used to screen the nitrocellulose membrane containing electrophorised ECP materials, a low MW band could be seen at around 20 kDa in most of the isolates examined (Figure 7.5). However, this band appeared slightly higher at approximately 23 kDa with isolates NCIMB 1947^T (Figure 7.5a, lane 1), 34/97 (Figure 7.5b, lanes 1) and 35/97 (Figure 7.5b, lanes 2) or as a doublet with isolates HL313/97, UP164/97 and 32/97 (Figure 7.5a, lanes: 5, 6 and 7, respectively) with bands at both 20 and 23 kDa. The staining of the glycoprotein bands of isolate NCIMB 1947^T, B97026, 32/97 and 34/94 were more intense than seen the with other isolates (Figure 7.5a, lanes 1, 3, 7, and Figure 7.5b, lane 1, respectively). The region between 123 and 20 kDa appeared lightly stained, and may have been due to a non-specific reaction.

The ECPs from the virulent (B97026P1) and non-virulent (32/97) isolates, as determined by the artificial challenge of rainbow trout fry as described in Chapter 3, were used to assess proteolytic activity in the ECP preparations *in vitro,* using substrate SDS-PAGE. A clear region, although very faint, was evident between 200 and 116 kDa in the gels containing 1% gelatin (Figure 7.6, lanes 2 and 3), while no such region was apparent in gels containing casein as substrate.

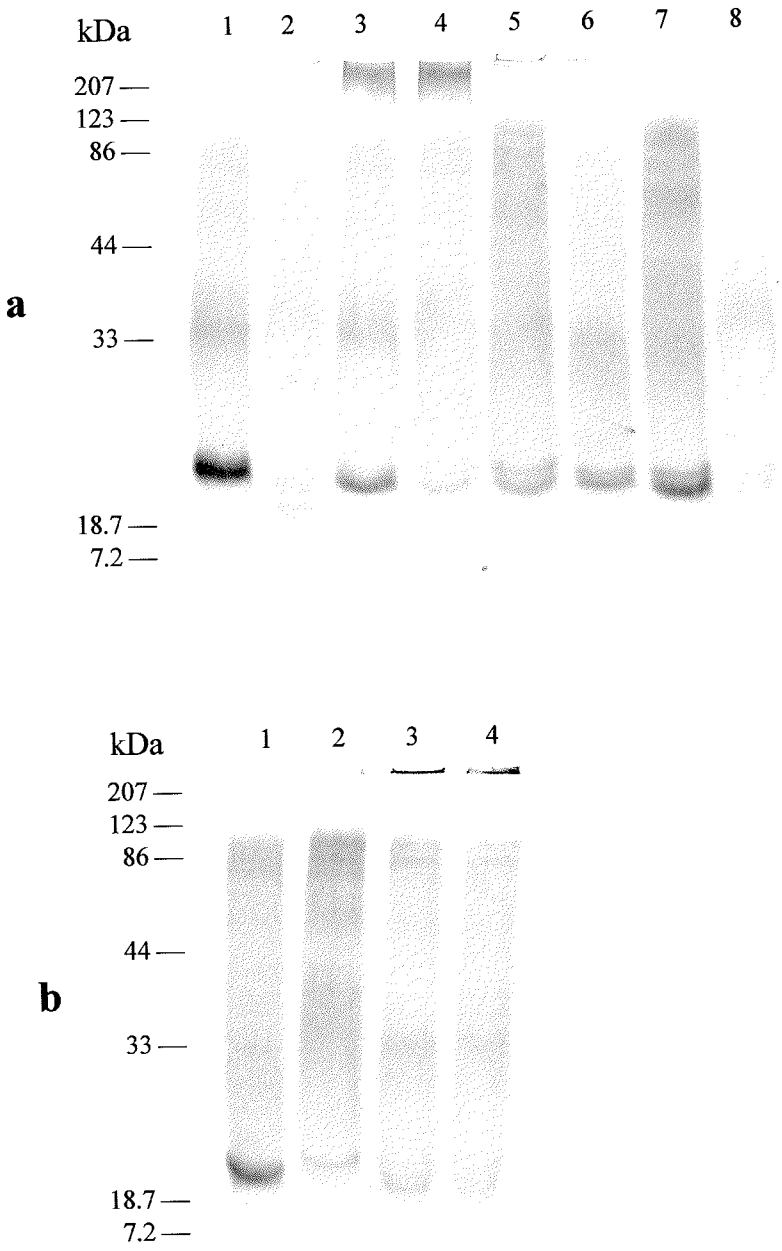

Figure 7.5. Glycoproteins present in the ECP preparations of different *F. psychrophilum* isolates (a) Lanes: (1) NCIMB 1947^T (2) NCIMB 2282 (3) B97026 (4) B97035E4 (5) HL313/97 (6) UP 164/97 (7) 32/97 (8) 33/97, (b) Lanes: (1) 34/97 (2) 35/97 (3) 59/95 (4) CASO 89/97.

The toxicity of the ECPs from either isolate B97026P1 or 32/97 was examined by injecting concentrated ECPs preparations in rainbow trout fry. No mortalities or external lesions occurred in experimental fish over the course of the trial and histological examination of the muscle and internal organs showed tissues to be free of lesions or tissue damage due to proteolytic activity.

7.4. Discussion

The amount of protein produced in the ECP of the isolates of *F. psychrophilum* used in the present study, over the course of 14 days when the bacteria were cultured in MAOB, was between 0.5-0.9 mg of protein ml^{-1}. As mentioned in Chapter 3, the growth rate of the different *F. psychrophilum* isolates varied considerably, and at the time of ECP collection each isolate may have been at a different stage in its life cycle. Therefore, the different yields of protein obtained here may be due in part to variations in the growth rate of individual isolates. No attempt was made to compare ECP production with bacterial concentration, so it was not possible to determine if there was any correlation between bacterial concentration and protein content of different ECP preparations.

The amount of protein secreted by the *F. psychrophilum* isolates is similar to that reported for *V. harveyi* (0.3-0.9 mg protein ml^{-1}) by Montero and Austin (1999), while *Mycobacterium bovis*, on the other hand, was found to produce between 125-150 mg of protein l^{-1} in its ECPs (De Bruyn *et al.*, 1987). Substantially lower amounts of protein (1.5-2.5 mg protein l^{-1}) were found in the ECPs of *Mycobacterium* spp. isolated from fish, which were believed to be either *M. marinum* or *M. fortuitum* (Chen *et al.*, 1997).

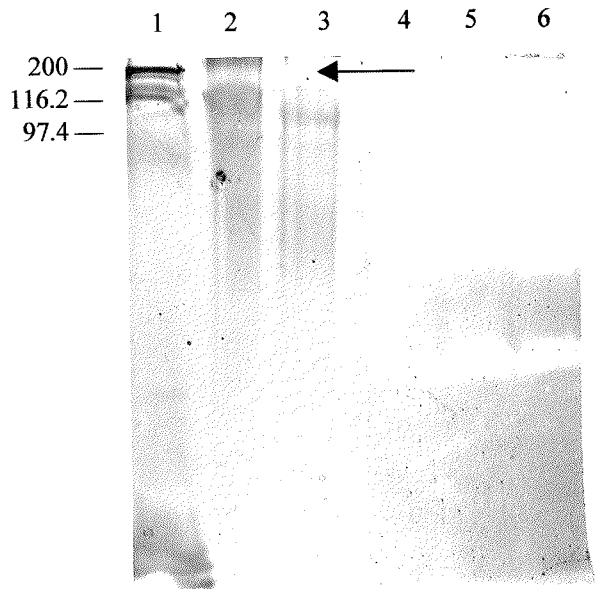

Figure 7.6. Substrate SDS-PAGE of ECP preparations from *F. psychrophilum* and *A. hydrophila* (positive control). Lanes: (1) MW marker (2) *F. psychrophilum* isolate B97026P1 (3) *F. psychrophilum* isolate 32/97 (4, 5 and 6) *A. hydrophila* isolate 98140, 98141 and T4, respectively.

Arrow: Protease activity

The SDS-PAGE profiles of ECPs concentrated from 14 day old cultures of *F. psychrophilum* were very similar between the isolates examined with both Coomassie blue and Silver staining. Differences could be found between the ECP profiles presented here when compared to the whole cell preparations shown in Chapter 4. A 20 kDa band was only evident in the ECP preparations, suggesting this molecule was secreted by *F. psychrophilum* over the course of the 14 day culture period. It is difficult to compare the profiles of ECP and whole cell preparations directly however, since some proteins will have been cleaved or glycosylated upon secretion, thus changing their molecular weight. Differences were seen by Chen *et al.,* (1997) when they examined the protein profiles of ECPs and whole cell sonicates of *Mycobacterium* spp., with a <14 kDa protein found to be present only in the ECP preparations of the bacterium. Up to seven different protein components have been reported to be secreted into the ECPs of *A. hydrophila* (Allan and Stevenson, 1981; Thune *et al.,* 1982). Suprato *et al.,* (1996) identified a 37 kDa protein in the SDS-PAGE profile of purified ECP of *E. tarda*, which showed proteolytic activities and lethal toxicity in Japanese eel with LD_{50} of 1.6 μg per g body weight. They found that this protein was present only in the ECP of virulent isolates of *E. tarda,* but not in those of avirulent isolates.

The rabbit anti-*F. psychrophilum* sera recognised a similar range of bands in the ECP profiles of all *F. psychrophilum* isolates examined by Western blot analysis. As with the whole cell preparation, the anti- B97026P1 sera reacted strongly with ECP from isolate B97026 and the anti-32/97 reacted strongly with ECP from isolate 32/97. Both sera recognised major bands around 116, 87, 72 and 61 kDa, but generally failed to recognise

the very high and low MW material stained in whole cell preparations of the bacterium in Chapter 5. The rabbit anti-32/97 serum detected some low MW material in the ECP of isolate 34/97, however, while rabbit anti-B97026P1 serum identified low MW material in the ECP of isolates B97026P1, B97035E4 and NCIMB 2282.

It was possible to detect two distinct glycoprotein bands at 20 and 23 kDa in whole cell preparations of all *F. psychrophilum* examined in Chapter 4. However, glycoprotein molecules detected here were present at either 20 kDa or 23 kDa in the ECPs profiles of the different *F. psychrophilum* isolates examined with the glycoprotein detection kit. The kit generally was only able to detect one of these bands in the ECP preparations of the bacterium, although in some instances two bands could be seen. The staining seen with Silver stain may be due to in part to the secretion of the slime layers of *F. psychrophilum.* This material is composed of carbohydrate (Dalsgaard, 1993). The glycoprotein seen here may also be associated with this layer since glycoproteins are involved in adherence of other fish bacterial pathogens, e.g. *Photobacterium damselae* subsp. *piscicida* (Magarinos *et al.,* 1996).

As discussed above in Section 7.1, extracellular enzymes have been reported to play an important role in the pathogenesis of various fish pathogens. Proteases may contribute to the pathogenicity of the bacterium by causing damage directly to the tissues of its host or by enhancing invasiveness of the bacterium (Dalsgaard, 1993). Bertolini *et al.,* (1994) reported that the virulence of *F. psychrophilum* may be associated with its protease production.

The presence of protease activity in the ECP of *F. psychrophilum* isolates, examined in this Chapter, was indicated using an agarose plate assay. Both caseinase and gelatinase activity was detected in the ECP of all isolates examined. Weak elastase activity was evident in only two of twelve ECP preparations examined, however. Uddin and Wakabayashi (1997) reported that optimal temperature for *in vitro* protease production of *F. psychrophilum* is between 13.3 ±1.9°C, which is somewhat lower than the optimal growth temperature (19.6 ±0.5°C). The protease activity assay in this study was performed at 22°C, following the procedure of Chen *et al.,* (1997), and this may have affected enzyme production. It would be worth repeating the assays at 13 to 15°C, the culture temperature of the bacterium, to see if a decrease in temperature enhances protease production.

The results of this work agrees with the work of Bertolini *et al.,* (1994), who reported both gelatin and casein degradation by protease in the ECP of all 29 *F. psychrophilum* isolates they tested, but only six of the isolates were capable of digesting elastin. Holt *et al.,* (1993) also found that only two of 28 *F. psychrophilum* isolates they examined were able to degrade elastin. Some authors, however have reported a possible link between the virulence of the *F. psychrophilum* isolate and its ability to degrade elastin (Madsen and Dalsgaard, 1998). They found that elastin-degrading isolates were more virulent than isolates that were unable to degrade elastin, which they established by artificially challenging rainbow trout fry with both elastin-degrading and non-elastin degrading isolates. A relationship between high elastase activity and virulence has been previously reported for *Aeromonas* spp. (Hsu *et al.,* 1981, Santos *et al.,* 1988, Esteve *et al.,* 1995). Mucinase may be involved in the actual penetration of pathogens into host tissue, as seen in

the case of *V. cholera,* which has been shown to invade via the intestine of its host (Freter *et al.,* 1981).

Different concentrations of proteins were produced in the ECPs preparations by the various *F. psychrophilum* isolates, although all ECPs preparations were prepared under exactly the same conditions. There did not appear to be any correlation between protein concentration of the ECP preparation and the level of protease activity obtained. For example, the ECP preparation of isolate 35/97 had highest protein concentration of all isolates examined (0.90 $mgml^{-1}$), but it had the lowest level of proteolytic activity on the agarose plate. Conversely, the ECP of isolate B97035E4 had the lowest protein content (0.45 $mgml^{-1}$), but had a higher level of protease activity than seen with the ECP of isolate 35/97. On the other hand, isolate B97026 had the highest level of protease activity with a protein content of 70 $mgml^{-1}$.

As mentioned above, the level of ECP produced by the different isolates was not compared to the concentration of bacteria in the suspension and different isolates appeared to have different rates of growth, and thus may have been at different growth phase at the time of ECP harvest. This may been have reflected in the levels and activities of ECPs obtained here.

Haemolysins are cytolytic toxins produced by a wide diversity of organisms, and obtained their name from their ability to lyse erythrocytes. The ability of certain bacteria to produce haemolysins was first detected by their ability to cause haemolysis around colonies

growing on blood agar (Rowe and Welch, 1994). Microbial haemolysins are generally considered as virulence factors of the organisms, although the relative contribution of haemolysins to disease is variable among different microbes and different host species (Rowe and Welch, 1994). A notable clinical sign of RTFS is severe anaemia, and in the UK the condition was originally known as rainbow trout fry anaemia (Rangdale, 1995). The characteristic signs of anaemia, which develop during *F. psychrophilum* infection in rainbow trout fry, may be explained to some extent by the bacterium's ability to partially lyse rainbow trout erythrocytes (Lorenzen *et al.,* 1997). According to Rangdale (pers. comm.), anaemia found in fry suffering from RTFS is due to the massive sequestration of red blood cells in the spleen, and hence this explains the swollen friable nature of this organ upon post-mortem. Otis (1984) noticed a toxic effect of the ECPs *from F. psychrophilum*, on trout erythrocytes and macrophages. It has proven difficult to assess the extent of haemolysin activity by *F. psychrophilum,* as this bacterium fails to grow on blood agar (Dalsgaard, 1993). The haemolytic activity of ECPs of *F. psychrophilum* was assessed in the present study by examining their ability to lyse rainbow trout erythrocytes in a microtitre plate assay. The ECP of *F. psychrophilum* were shown to elicit only a very weak haemolytic activity however, with haemolysin titres of 1/2 being observed. Lorenzen *et al.,* (1997) believed that *F. psychrophilum* possesses some kind of specific haemoagglutinin, as they observed partial haemoagglutination (titres of 1/2) of rainbow trout erythrocytes by the ECPs of the bacterium. However, Garcia-Marquez *et al.*, (1998) found that regardless of their degree of virulence, all isolates of *F. psychrophilum* examined were unable to agglutinate erythrocytes from humans or from fish. The production of haemolysins has also been demonstrated in a related species, *Flexibacter*

maritimus (Baxa *et al.,* 1988). Intraperitoneal injection of ECP from this bacterium in both black and red sea bream fry, showed that it possessed haemolysin activity, and which was responsible for the pathological effects observed under histopathological examination of infected fish.

The lethal toxicity of ECPs from a number of bacterial fish pathogens is reported in the literature. ECP from *V. harveyi*, for example, exhibits toxicity with a lethal dose 50% (LD_{50}) of 4.4 μg protein per prawn (Montero and Austin, 1999), while the ECP of *V. anguillarum* has an LD_{50} of 80 μg $fish^{-1}$ in gold fish (Inamura *et al.,* 1984). The LD_{50} of ECP from *A. hydrophila* was found to around 19 μg g^{-1} when injected into channel catfish (Thune *et al.,* 1982). Chen *et al.,* (1997), however, reported low levels of toxicity of ECP from *Mycobacterium* spp. when 400 μg of protein $fish^{-1}$ was injected into either rainbow trout or Nile tilapia. No toxicity was reported here when the ECPs of the two *F. psychrophilum* isolates were injected into fry, not even at the highest dose of 50 μg $fish^{-1}$. Otis (1984) found gross and microscopic lesions when steelheads were injected with the ECP of *F. psychrophilum.*

Bertolini *et al.,* (1994) identified proteases of 114 and 152 kDa in the ECP of a reference *F. psychrophilum* isolate (SH3-81) associated with BCWD by substrate SDS-PAGE, and both of these bands were active against casein and gelatin. They were also able to divide the 29 isolates they examined into four groups based on the presence of certain protease activity in the substrate SDS-PAGE. Of these isolates, five did not show any protease activity in the substrate SDS-PAGE. The authors were unable to detect the caseinase bands, found at 45

and 114 kDa, after eight days of having set up the bacterial culture, while this activity was clearly visible in the ECPs harvested after 1 or 2 days of culture. The protease bands found in the present study between 200 and 116 kDa on the gel containing 1% gelatin were not particularly clear, and in accordance with Bertolini *et al.,* (1994), no caseinase activity could be detected in concentrated ECP preparations prepared from 14 day old cultures by substrate SDS-PAGE. This suggests that the caseinase is unstable, it is not produced in later stage of bacterial growth, or perhaps the gelatinase has degraded this enzyme. Hofer (1997) however, failed to identify any protease activity in the ECP of *F. psychrophilum* isolated from outbreaks of RTFS in Europe and BCWD in Japan, using the same substrate SDS-PAGE procedure as described for the current study. Very little gelatinase activity was obtained here compared to the study of Bertolini *et al.,* (1994). One explanation may be that the growth medium used by Bertolini *et al.,* (1994) was different to that used in the current study and by Hofer (1997). The different growth media that were used may have influenced the activity of the ECPs, when examined by substrate SDS-PAGE. A simpler explanation for the lack of activity is that the sample buffers used here and by Hofer (1997), contained 2-mercaptoethanol, whereas that used by Bertolini *et al.,* (1994) was free of this agent. This breaks the sulphur bond within the protein, thus possibly reducing protease activity.

Dalsgaard (1993) reported the production of copious amount of extracellular polysaccharides or slime by many *Flavobacterium*-like bacteria. It was thought that the presence of some carbohydrate material, in the ECPs might have interfered with the protease activity. The ECPs preparations were therefore treated with lysozyme to remove

some carbohydrate material and increase proteolytic activity (results not presented). However, this treatment was not effective and did not improve the levels of protease activity obtained.

In conclusion, *F. psychrophilum* produced between approximately 0.5 to 0.9 mg protein ml^{-1} in the ECPs preparations made here after 14 days of culture in MAOB. Strong caseinase and gelatinase activity was detected in the ECPs, while very little elastase and mucinase activity and no lipase activity could be detected. Only partial haemolytic activity against rainbow trout erythrocytes was observed, but the ECP preparations made here were not found to be toxic to rainbow trout fry. Protease activity measured in the ECP of the two reference isolates by substrate SDS-PAGE was inconclusive and analysis of additional isolates is necessary to verify the link between enzyme activity and virulence. SDS-PAGE profiles of the ECPs of different isolates were found to be very similar, and little difference was noted in the Western blot profiles of the ECP preparations using antiserum raised against a virulent and an avirulant isolate *F. psychrophilum*. Glycoprotein molecules of either 20 or 23 kDa were detected in the ECPs of the bacterium. Further work is required to establish if there is any clinical significance in these molecules to the pathogenicity of the bacterium.

CHAPTER 8

Summary

A lack of basic information relating to *F. psychrophilum* infection associated with RTFS and the problems associated with working with the organism exist. The major aims of this thesis were to characterise *F. psychrophilum* isolates obtained from RTFS outbreaks based on phenotypical and serological differences between isolates. Additionally, a challenge model was produced to enable RTFS to be reliably replicated in susceptible fish under laboratory conditions.

A number of serological studies have already been performed (Holt, 1987; Wakabayashi *et al.*, 1994; Lorenzen and Olesen, 1997; Izumi and Wakabayashi, 1999). However, the antigenic characteristics of *F. psychrophilum* have not been fully elucidated, and therefore antibody probes were produced to characterise the antigenic profile of the bacterium.

The rabbit sera raised against both the virulent and non-virulent isolates of *F. psychrophilum,* recognised a number of similar protein and carbohydrates bands on the whole cell profiles of all the isolates examined. However, differences were seen in the staining of high and low MW material in the Western blot profile of the bacterium with these two antisera. This material was recognised more strongly in bacteria to which the serum had been raised. Although the protein profiles of the OMP preparations of the virulent and non-virulent isolates of *F. psychrophilum* were very similar in this study, their reactivity pattern in Western blot analysis with the rabbit sera revealed some differences. Not only the intensity of staining varied between the two sera, but some distinct bands were missing in the OMP profiles of virulent isolates of *F.*

psychrophilum when stained with the sera raised against the non-virulent isolate and *vice versa*. The OMP of more isolates of *F. psychrophilum* need to be examined to be able correlate their serotype with pathogenicity of the bacterium.

Characterisation of different isolates of *F. psychrophilum* with the MAbs in IFAT, ELISA and Western blot analysis showed differences in surface antigens between isolates. The reactivity patterns obtained here with individual MAbs in ELISA, Western blot and IFAT did not fully correspond to each other. The difference in the response of the MAbs in the ELISA, IFAT and Western blot in this study may possibly be due to the presence of different antigens being present as a result of different processing procedures or destruction of antigens during the processing of bacteria for SDS-PAGE. Madsen and Dalsgaard (1998) reported possible existence of thermo-labile antigens in the OMP of different *F. psychrophilum* isolates and antigens such as these may have been affected by the processing procedure performed for the ELISA and SDS-PAGE in this study.

A comparison of the response of fish, rabbits and mice sera to whole cell antigens indicated differences between the species by Western blot. More bands were recognised by rabbit and mouse serum than with fish serum. This phenomenon has been seen with other fish pathogens (e.g. *A. salmonicida*, Hastings and Ellis, 1988; *Mycobacterium* spp. Chen, 1996; *P. damselae* subsp. *piscicida*, Bakopolous *et al.*, 1997c), and is probably due to the fact that the immune system of rabbits and mice are more developed than that of the fish. It may be necessary to exploit such differences in the development of an effective vaccine for fish.

The studies performed here showed that isolates of *F. psychrophilum* are very similar in terms of their protein and carbohydrates profiles regardless of their origins or their pathogenicity in rainbow trout. However, the rabbit sera and MAbs produced here did indicate antigenic differences between isolates of *F. psychrophilum.* In addition, it was established that biochemical characteristics are very similar between the different *F. psychrophilum* isolates. Enzyme patterns obtained by API ZYM test were homogenous, although differences were noted in protease activity between different isolates assessed using the agar plate assay.

Diagnosis of RTFS is usually confirmed by isolation of the bacterium from internal organs, particularly the spleen of fry and fingerlings showing characteristic signs of RTFS (Rangdale, 1995). Isolation and identification on culture are both time consuming and may be confused with other *Flavobacterium* species. Obtaining accurate viable bacterial counts for this fastidious organism has proved problematic (Michel *et al.*, 1999). Standard procedures for quantifying *F. psychrophilum* in infected tissues or environmental samples by culturing *in vitro* have not yet been established. It would however, appear that no more than 25% of the *F. psychrophilum* cells present in the cultures are able to produce colonies on agar media (Michel *et al.*, 1999). The difficulty in culturing *F. psychrophilum* may partly explain why few studies were performed related to the pathogenesis of the bacterium or its relationship with its host.

Serodiagnostic methods using antibody probes are not always useful for diagnosis because of the presence of several serotypes of the bacterium. A number of serotypes have been previously reported for *F. psychrophilum* (Holt, 1987; Wakabayashi *et al.*, 1994; Rangdale, 1995; Lorenzen and Olesen, 1997; Izumi and Wakabayashi, 1999). In

this study, characterisation of a number of *F. psychrophilum* isolates by ELISA using the rabbit sera indicated that there may be between three and five different groups of bacteria based on their antigenicity. However, no correlation was apparent in this study between serotypes and geographical origin of the strains, the species of host fish from which they were recovered or the virulence of the isolates.

The rabbit antisera and the different MAbs produced against *F. psychrophilum* were not suitable for use as diagnostic tools because they failed to recognised all the *F. psychrophilum* isolates examined, moreover, they cross-reacted with a number of non-*F. psychrophilum* bacteria. Since MAbs are directed towards a single epitope on an antigen, they generally have high levels of specificity for the immunising antigen. However, the MAbs prepared here showed substantial cross-reactivity with a related bacterium *F. branchiophilum* in the ELISA. This bacterium also showed a high level of cross-reactivity with the rabbit sera suggesting the presence of common antigens between the bacteria. If cross-reacting antigens between *F. psychrophilum* and *F. branchiophilum* are protective to fish, they may potentially be useful as a bivalent vaccine for RTFS and BGD. The chemical composition of these antigens still needs to be determined.

It was found to be difficult to produce species-specific MAbs for *Mycobacterium* sp. isolated from fish (Adams *et al.*, 1996) possibly due to the thick cell wall of the bacterium, constructed from a complex mixture of mycolic acids, glycolipids, mycosides and phospholipids (Goren *et al.*, 1972). The MAbs developed in this study against whole cell preparations of *F. psychrophilum* failed to recognise all *F. psychrophilum* isolates examined. This may be due to the presence of the extracellular

polysaccharide material (slime layer) that surrounds the bacterium. This material may mask strain-specific antigens so that the mouse is unable to respond to them upon immunisation. Further MAbs should be prepared against additional bacterial preparations in the search for MAb, which recognise species-specific antigens for use in diagnostic screening.

It is concluded that more specific and sensitive methods are necessary to confirm infection due to *F. psychrophilum*. Recently, molecular techniques have been developed for the diagnosis of *F. psychrophilum*. Polymerase chain reaction (PCR) has been developed to amplify *F. psychrophilum* DNA and has proven useful in diagnostic application (Toyama *et al.*, 1994; 1996). Urdaci *et al.*, (1998) reported a PCR-based method to identify and detect *F. psychrophilum* in infected tissue. Ribotyping and plasmid profiling of *F. psychrophilum* showed that these methods, used alone or in combination with other typing techniques, are powerful tools in epizootiological studies of *F. psychrophilum* (Chakroun *et al.*, 1998). These techniques have limited application, as they do not establish if the bacterium is viable and furthermore the presence of the bacterium does not assure a diseased state in the animal.

A reproducible challenge model is important for examining the virulence of *F. psychrophilum* during studies relating to the pathogenesis of the organism (Madsen and Dalsgaard, 1999). Variations in experimental challenge protocols reported by different authors such as the injection route, water temperature and fish size, make it difficult to directly compare the different challenge models used (Panigua *et al.*, 1990). In this study, intramuscular injection of *F. psychrophilum* was the most effective route for producing mortality under laboratory conditions, and this finding is in accordance with

Borg (1960), Holt (1993) and Ostland *et al.*, (1997). The intramuscular challenge route for *F. psychrophilum* was optimised in this study, and used as the challenge model for *F. psychrophilum.* This model was used to establish which isolates were pathogenic for rainbow trout fry. The biochemical and serological characteristics of a virulent isolate were then compared with an avirulent isolate. Considerable variations were observed in the pathogenicity of the four isolates of *F. psychrophilum* examined following IM injection. These differences were less apparent using the IP injection method, which did not reproducibly result in disease.

Wide variation in pathogenicity has been noted between isolates of other types of bacterial fish pathogens when administered by IM and IP routes. Following injection of 3 $x10^7$ cfu $fish^{-1}$ in rainbow trout, 72% of *A. hydrophila* isolates and 63% of *A. sobria* were pathogenic for fish by IM challenge, while a virulent rate of 39% and 45%, respectively, were noted when an IP challenge route was used (Panigua *et al.*, 1990).

The presence of external and internal clinical signs of disease varied depending on the route used to administer infection. The signs observed were not necessarily the same as those reported during natural outbreaks of RTFS following injection of the pathogen. Injection of infectious agents is not an entirely appropriate method of testing pathogenicity and assessing the potency of vaccines, as this method circumvents non-specific immune mechanisms located in the skin, such as mucus, lysozyme and skin-associated phagocytes. These non-specific immune mechanisms may be important in preventing infection, so bath and cohabitation challenges may represent a more natural exposure to the pathogen (Bricknell, 1995; Nordmo, 1997).

In the present study, oral administration of *F. psychrophilum* reproduced clinical signs of RTFS in challenged fish. This agrees with the findings of Lorenzen (1994) who reported that bacteria and bacterial products could be detected in the gut lumen and mucosa/ submucosa of naturally infected fry. Rangdale (1995) found filamentous bacteria in the gut of intraperitoneally-infected fish. Dalsgaard (1993) suggested that *F. psychrophilum* probably enters the fish via the gills or through the skin, but they may also enter by oral or gastrointestinal routes.

In larval fish, a wide range of infections are thought to occur via the gastrointestinal tract, especially since gastric secretions present in the stomach of these young animal may still largely be absent, and appear later in the development of the animal (Olafsen, 1994). In older fish, however, infection via the gastrointestinal tract presents a problem for some pathogens. The gastrointestinal tract is a hostile environment for *A. salmonicida,* for example (Bogwald *et al.*, 1994), as it appears to have a limited ability to cross the intestinal epithelium (Evelyn, 1996), and a poor survival rate in the presence of stomach secretions (Hiney *et al.*, 1994). On the other hand, the intestinal tract is believed to be the primary portal of entry for *E. ictaluri* (Shotts *et al.*, 1986; Baldwin and Newton, 1993) and *Y. ruckeri* especially since oral administration of *Y. ruckeri* successfully reproduced the disease (Ross *et al.*, 1966). Once the bacterium has gained access to the intestine, it apparently can persist there and then initiate active infection at a later date (Busch and Lingg, 1975).

Bacterial adhesion to the surface of its host via appropriate receptors, is one of the first steps in microbial pathogenesis (Dawson *et al.*, 1981; Daly and Stevenson, 1987). The adherence of *Photobacterium damselae* subsp. *piscicida* to various cell lines seemed to

be mediated by a glycoprotein receptor of the bacterial cell surface (Magarinos *et al.*, 1996). However, little is known about the components involved in the adhesion of *F. psychrophilum* to the surface of its hosts. Santos *et al.*, (1991) found that with bacteria such as *A. salmonicida, A. hydrophila, A. sobria, V. anguillarum* and *Y. ruckeri,* molecules other than proteins are responsible for bacterial attachment to inanimate surfaces or to host cells. Dooley *et al.*, (1986) reported that all highly pathogenic *A. hydrophila* contained a polysaccharide O antigen and an uniform protein array on their cell known as S protein. A capsular outer glycocalyx composed of mucopolysaccharide has been associated with *F. columnare*, and with its ability to adhere to its host (Plate and Ordal, 1967). DelCorral (1988, cited by Shotts and Starliper, 1999) found that it had an important role in cellular adherence and correlated the presence of this layer with the haemoagglutinating activity of the bacterium. A relationship was also noted between the adherence of the bacterium to epithelial cell *in vitro* and the virulence of the isolates.

Substantial carbohydrate material was associated with the bacterium, as identified in the present study using various staining methods such as lectins, Schiff's reagent and Silver stain. The lectins generally identified a band around 20 kDa in the whole cell preparation of *F. psychrophilum.* The presence of glycoproteins was also detected in both the cellular and extracellular components of *F. psychrophilum* using a commercially available glycoprotein detection kit. The kit detected two distinct glycoprotein bands at 20 and 23 kDa in whole cell preparations of all *F. psychrophilum* isolates examined. Western blot analysis with rabbit anti-*F. psychrophilum* sera performed here on proteinase K-digested whole cell preparation of *F. psychrophilum* showed that the remaining carbohydrate material was very immunogenic. Crump *et al.*,

(1999) also found highly immunogenic carbohydrates around 16 and 26 kDa in *F. psychrophilum* preparations, and suggested these to be LPS. It has been reported that many CLB produce extracellular polysaccharides or a slime layer, and this is believed to facilitate the gliding motion of the bacterium and its adhesion to host or to solid surfaces (Godchaux *et al.*, 1991; Dalsgaard, 1993). In electron microscopic studies, Rangdale (1995) reported the existence of this slime layer in *F. psychrophilum.* The significance of the carbohydrate material and the glycoprotein molecule in the attachment of *F. psychrophilum* to its host was not examined here and remains to be elucidated.

The virulence mechanisms associated with the pathogenicity of *F. psychrophilum* are poorly understood. It is speculated that components present in the ECPs, and in the cell wall or on surface of the bacterium, such as OMPs, carbohydrates, LPS, proteases and iron acquisition systems are involved with infection and virulence (Dalsgaard, 1993). Some potential virulence factors have already been identified for the bacterium, for example extracellular protease activity (Bertolini *et al.,* 1994), although their role and significance in the disease process is not fully understood. In this study, the virulence of only a few isolates was examined and data comparing virulence factors with the pathogenicity of isolates would have been strengthened had more isolates been examined. In the present study, the levels of protease activity appeared to vary between the virulent and non-virulent isolates examined. The zones of degradation on gelatin and casein substrate by the virulent isolate were much larger than those obtained with the non-virulent isolate. Elastin was also degraded by the virulent isolate, while the non-virulent isolate did not show any activity against elastin. These activities may be significant in the pathogenicity of the organisms. Further studies are needed whereby

the virulence factors of more isolates are examined and correlated with the pathogenicity of the isolates.

Conflicting reports exists as to the relationship between the ability of bacteria to auto-agglutinate, the virulence of the strain, and its hydrophobicity (Trust *et al.*, 1980; Larsen *et al.*, 1988). In the present study, isolates of *F. psychrophilum* were found to auto-agglutinate in broth culture, and it would appear that auto-agglutination may be an indicator of virulence, with strong auto-agglutinating *F. psychrophilum* having higher levels of virulence than the less auto-agglutinating strains. Garcia-Marquez *et al.*, (1998) evaluated the ability of different strains of *F. psychrophilum* isolated from different fish species in distinct geographic areas to adhere to fish tissues with respect to their virulence. They generally observed low levels of hydrophobicity in *F. psychrophilum* cells. Further studies are needed to examine the relationship between cell surface hydrophobicity, auto-agglutination and virulence.

The financial impact of *F. psychrophilum* infection on world aquaculture is hard to quantify. In the UK alone, the recent estimates of actual losses of rainbow trout fry and fingerlings due to *F. psychrophilum* infection are as high as 10 million fry per annum. These fish have a potential value of nearly £500,000 and represent up to 22% of all fry produced (Rangdale, 1997). The bacterium and the response it induces in fish are poorly understood, and as a result no effective control methods for RTFS are currently available to fish farmers. The control of RTFS largely depends on the use of antibiotics, such as oxytetracycline and amoxycillin, either for treating diseased fish or as a preventive measure. Alderman and Barker (1997) highlighted the problems associated with the treatment of *F. psychrophilum* infection due to the emergence of

resistance to these two compounds. There has been a decrease in bacterial susceptibility to these drugs over recent years, and the numbers of resistant *F. psychrophilum* isolates are increasing (Lorenzen, 1994; Rangdale, 1996). There are very few alternatives to the drugs currently used, since very few antibiotics have been registered for use in aquaculture in the European Community. A long-term aid for the control of RTFS and one that would reduce the increasing use of antibiotics, is the development of an effective vaccine. No commercial vaccine is currently available to protect against *F. psychrophilum* infection.

The development of vaccines against RTFS is complicated by the fact that mortality due to *F. psychrophilum* generally occurs in juvenile fish ranging from 0.5 to 5.0 g in weight. Firstly, it has not yet been fully established exactly when the immune system of rainbow trout fry is sufficiently mature for vaccination to be successful and it may not be possible to vaccinate small fry against the disease (Rangdale, 1999). Secondly, a reproducible and standardised model of experimental infection is currently difficult to achieve and this is necessary to investigate the potency of vaccine preparations. Thirdly, there are technical problems associated with the production of large amounts of this fastidious bacterium under laboratory condition (Bernardet, 1997). Finally, it has been shown in this study that serotype differences exist between the different isolates of *F. psychrophilum,* although the exact number have not yet been fully established.

The work presented in this thesis characterising the phenotypic and serologic properties of a diverse collection of isolates will form the basis of future vaccine studies. The production of a successful challenge model will be of tremendous value to other researchers and is fundamental to advances in the area of disease control and treatment.

Further studies should be conducted to establish the clinical significance of molecules such as the glycoprotein and carbohydrate. Additional studies to clarify the role of OMP, iron regulated outer membrane protein (IROMP), LPS, plasmids or major ECPs of *F. psychrophilum* in virulence are required. The number of serotypes of the bacterium also remains to be confirmed. Immuno-gold staining should be performed with the MAbs developed here against whole bacteria to identify the cell surface epitopes recognised by the different MAbs. Further species-specific antibodies are also required for immunohistochemistry screening of fish tissue. A standardised protocol for a reproducible experimental challenge model, by bath or orally, is required to examine the portal of entry of *F. psychrophilum*. The roles of specific and non-specific immune mechanisms in fish against *F. psychrophilum*, as well as establishing a protective response against the bacterium also need to be determined. It is important to find the age and the size of fish, which successfully respond to immunisation and produce a protective immune response against *F. psychrophilum* infection.

In conclusion, description of biochemical and electrophoretic properties of *F. psychrophilum*, isolated from RTFS outbreaks and showed them to be homogeneous. It has also shown antigenic variations between different isolates of the bacterium. Furthermore, it has described enzymatic activity and protein profiles of the ECPs of the bacterium. Finally, it has highlighted further avenues of investigation for future studies.

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APPENDIX 1

Culture media

1. Modified Anacker and Ordal Medium (MAOA)

(Per litre)

Tryptone	5.0 g
Yeast extract	0.5 g
Sodium acetate	0.2 g
Beef extract	0.5 g
Agar bacteriological (agar No.1)	15.0 g
(pH 7.2)	

Method: Suspended in 1 litre distilled water. Sterilised by autoclaving at 121°C, 15 min (0.72 kg/cm^3)

2. Selective kidney disease medium

(Per litre)

Trypton	10 g
Yeast extract	0.5 g
Distilled water	900ml
Foetal calf serum	100ml
L-Cysteine hydrochloride	12.5g
Cycloheximide (1.0g in 100ml distilled water)	5.0ml
D-Cycloserine (0.1g un 10.0ml)	1.25ml
Polymyxin B (0.1g in 10.0ml)	2.5ml
Oxolinic acid (0.1ml in 0.5ml)	0.25ml
(pH6.8)	

APPENDIX 2

Buffers

1. 0.02 M phosphate buffered saline (PBS) (Per litre)

$NaH_2PO_4.2H_2O$	0.37 g
$Na_2HPO_4.2H_2O$	2.561 g
NaCl (0.15M)	8.77 g

Dissolved in 1 L distilled water, adjusted pH to 7.2

2. Electrophoresis

2.1. Sample buffer (5 times concentration) (10 ml)

60 mM Tris-HCl	0.61 ml 1 M Tris-HCl (pH 6.8)
25% glycerol	5.0 ml 50% glycerol
2% SDS	2.0 ml 10% SDS
14.4 mM 2-mercaptoethanol	0.5 ml 2- mercaptoethanol
0.1% bromophenol blue	1.0 ml 1% bromophenol blue
Distilled Water	0.9 ml

Stable for weeks in the refrigerator or months at -20°C. Samples were diluted 1:4 in sample buffer.

2.2. Polyacrylamide gel formulation

The following stock solutions were used for the preparation of polyacrylamide gels.

2.2.1 Separating gel buffer (Per 500 ml)

1.5M Tris	91 g
0.4% SDS	2 g

Adjusted to pH 8.7 with HCl

2.2.2. Stacking gel buffer (Per 100 ml)

0.5M Tris	6.05 g
0.4% SDS	0.4 g

Adjusted pH to 6.8 with HCl

2.2.3. 30% Acrylamide store at 4°C

2.2.4 10% w/v ammonium presulfate in DW (prepared fresh for every electrophoresis run)

2.2.5. TEMED (N',N',N', N',- tetramethylethylenediamine)

2.3. Stacking gel (4% polyacrylamide)

Separating gel buffer	2.5 ml
Acrylamide	1.34 ml
Distilled water	6.1 ml
Ammonium persulfate	50 µl
TEMED	10 µl

Degassed for 15 min under vacuum before adding ammonium persulfate and TEMED . Recipe for two mini-gels.

2.4. Separating gel (12% polyacrylamide)

Separating gel buffer	5 ml
Acrylamide	8ml
Distilled water	7 ml
Ammonium persulfate	70 µl
TEMED	15 µl

Degassed for 10 min under vacuum before adding ammonium presulfate and TEMED. Recipe for two mini-gels

2.5. Reservoir buffer (x5) (Per litre)

Tris	15g
Glycine	43.2 g
SDS	5 g

Adjusted pH to 8.3.

3. Western blot buffers

3.1. Transblot Buffer, pH 8.3 (Per litre)

Tris-base	14.4 g
Glycine	3.03 g

Prepared in 100 distilled water and then 200 ml methanol was added and finally brought to 1 litre with distilled water

3.2 Tris-buffered saline (TBS), pH 7.5 (Per litre)

Tris base(10mM)	2.42 g
NaCl (0.5 M)	29.24 g

Dissolved in 1 litre distilled water. Adjusted to pH 7.5

3.3 Tween-20 TBS (TBST)

1ml Tween-20 (Sigma) in 1 litre of TBS

3.4 Substrate

Stock solution :	4-chloro-napthol (0.3% w/v in methanol), stored at –20°C
Working solution:	2 ml 4-chloro-napthol stock solution with 10 ml PBS and 10 µl of H_2O_2

4. Buffer used for ELISA

4.1 Coating buffer (Per litre)

Carbonate-bicarbonate solution

Na_2CO_3	1.59 g
$NaHCO_3$	2.93 g

Dissolved in 1 l distilled water. Adjusted pH 9.6 and made freshly.

4.2. Low salt washing buffer (LSW), pH 7.4 (Per litre)

Tris-base (0.02M)	2.42 g
NaCl,(0.38M)	22.2 g
Tween-20 (0.05%)	0.5 ml

Dissolved in 1 litre distilled water and pH adjusted to 7.4 with conc. HCl

4.3. High salt washing buffer (LSW), pH 7.8 (Per litre)

Tris-base (0.02M)	2.42 g
NaCl,(0.5M)	29.2 g
Tween-20 (0.05%)	1 ml

Dissolved in 1 litre distilled water and pH adjusted to 7.8 with conc. HCl

4.4. Substrate buffer, pH 5.4 (Per litre)

Citric acid	19.2 g
Sodium acetate	8.2 g

Dissolved in 1 litre distilled water, adjusted to pH 5.4 with 1 M NaOH

5 µl of H_2O_2 was mixed with 15 ml substrate buffer.

4.5. Substrate

Tetramethyl-benzidine dihydrochloride (TMB) (42 mM) was added to 1:2 acetic acid: distilled water. 150 µl of this solution was added to 15 ml substrate buffer.

4.7. Stop solution

2M H_2SO_4 in distilled water.

APPENDIX 3

Histology

1. Fixation

Materials for histological examination was placed in fixative (10% neutral buffered formalin saline) for at least 24 h prior to cassetting.

2. Cassetting

The allocated case number was entered on the cassette using a pencil. Tissue samples were trimmed to a suitable size taking care not to place too many tissue blocks in the cassettes as this can lead to ineffective dehydration and ultimately difficulty in sectioning. Small samples were wrapped in tissue paper before placing in the cassette. Soft and hard tissues were kept separate. Cassetted samples were not allowed to dry out and were left in a bowl of water or fixative until loading onto the processor.

3. Tissue processing

This procedure was carried out by placing the cassettes into a basket, which was moved round automatically by a tissue processor at the appropriate time interval schedule

1. 50% Methylated sprit	1 h
2. 85% Methylated sprit	2 h
3. 100% Methylated sprit	2 h
4.100% Methylated sprit	2 h
5. 100% Methylated sprit	2 h
6. 100% Alcohol	2 h
7. 100% Alcohol	2 h
8. Chloroform	2 h
9. Chloroform	2 h
10. Molten wax	1 h
11. Molten wax	2 h
12. Molten wax	2 h

Cassettes were removed from the processors and placed in molten wax until ready to block out. Tissues were trimmed and sections cut by microtomy

4. Staining

Haematoxylin and Eosin

1. Xylene	5 min
2. Alcohol	2 min
3. Methylated sprits	1.5 min
4. Running tap water	10 min wash
5. Haematoxylin	5 min
6. Wash in tap water	3 min
7. Acid Alcohol	3 quick dips
8. Wash in tap water	3 min
9. Scott's tap water	30 sec
10. Wash well	
11. Eosin	5 min
12. Quick wash in Tap water	10 sec
13. Methylated sprit	30 sec
14. Alcohol II	2 min
15. Alcohol I	1.5 min
16. Xylene	5 min
17. Xylene	10 min

Sections were coverslipped after the last xylene in the staining series and mounting fluid allowed to dry before examination.