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

By

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in memory of my brother-in-law

Md. Abdul Aziz

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.

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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.