[3] Diseases Caused By Bacterial Pathogens In Inland Water

genomovar I isolates. Olivares-Fuster et al. (2011) demonstrated that the cells of a genomovar II strain adhered to channel catfish gill in higher numbers within 1 h post-challenge. It is plausible that genomovar II strains could more efficiently adhere to the epithelial tissues and mucus coverings of catfishes. However, further research is needed to confirm if all genomovar II strains are indeed more effective at colonizing gills of channel catfish than genomovar I strain (Olivare-Fuster et al. 2011).

5. BACTERIAL COLD-WATER DISEASE

Hisatsugu Wakabayashi

5.1. Synopsis

Bacterial cold water disease (BCWD) caused by *Flavobacterium psychrophilum* is a serious disease in freshwater fish, particularly salmonid fish and ayu, worldwide. The epizootics are most prevalent at low temperature. The bacterial cells are Gram-negative, slender rods measuring 0.5×2-7 m, exhibiting weak gliding motility. The clinical signs of BCWD depend on the age of affected fish species. In coho salmon fingerlings, the erosion of tissue in peduncle area is a classic characteristic early in the epizootics. Chemotherapy with antibiotics is still the most effective treatment method, but acquired resistance of *F. psychrophilum* is a major challenge. Currently, there are no vaccines commercially available to prevent BCWD.

5.2. Introduction

Bacterial cold-water disease (BCWD) is caused by *Flavobacterium psychrophilum* (formerly *Flexibacter psychrophilus* and *Cytophaga psychrophila*). Davis (1946) described it as ‘peduncle disease’ based on the characteristic pathology that was associated with the peduncle of the diseased rainbow trout (*Oncorhynchus mykiss*) in West Virginia, USA. Although Davis could not isolate the causative agent, he observed a number of long thin bacteria within the lesions of affected fish. The etiologic bacterium was originally isolated from diseased coho salmon (*Oncorhynchus kisutch*) in Washigton, USA, in 1948 by Borg (1960). He proposed the name *Cytophaga psychrophila* for this organism. The disease became known as ‘bacterial cold-water disease’ or ‘low-temperature disease’ because epizootics were most prevalent at low water temperature.

BCWD was believed to be limited to North America until its outbreaks occurred among rainbow trout in Germany (Weis 1987) and France (Bernardet et al. 1988). In Europe, the disease is called as ‘rainbow trout fry syndrome’ (RTFS) (Lorenzenn et al. 1997), ‘visceral myxobacteriosis’ (Baudin-Laurencin et al. 1989), or ‘fry mortality syndrome’ (FMS) (Lorenzenn et al. 1991). *F. psychrophilum* has been isolated in USA, Canada (Lumsden et al. 1996), Germany, France, UK (Santos 1992), Northern Ireland (Lorenzen et al. 1991), Denmark (Lorenzen et al. 1991), Spain (Toranzo and Barja 1993), Swizterland (Lorenzen and Olesen 1997), Finland (Wiklund et al. 1994), Norway (Lorenzen and Olesen 1997), Sweden (Madetoja et al. 2001), Estonia (Madetoja et al. 2001), Turkey (Kum et al. 2011) , Japan (Wakabayashi et al. 1991), Korea (Lee and Heo 1998), Australia (Schmidtke and Carson 1995), Chile (Bustos et al. 1995), Peru (Lindstrom et al. 2009). Although outbreaks commonly occur among salmonids, BCWD

also affects carp (*Cyprinus carpio*), crucian carp (*Carassium carassius*), eel (*Anguilla anguilla*), and tench *(Tinca tinca*) (Lehmann et al. 1991), ayu (*Plecoglossus altivelis*) (Wakabayashi et al. 1994), chub (*Zacco platypus*) (Iida and Mizokami 1996), gobies (*Chaenogobius urotaenia* and *Rhinogobius brumneus*), and dace (*Trybolodon hakonensis*) (Amita et al. 2000).

The importance of BCWD has led to a significant volume of publications, which have been adequately reviewed by various authors (Wood 1979, Holt et al. 1993, Nematollahi et al. 2003a, LaFrentz and Cain 2004, Cipriano and Holt 2005, Barnes and Brown 2011, Starliper 2011).

5.3. Disease Agent

Cytophaga agar (CA) (Anacker and Ordal 1959) is the most commonly used medium for isolation of *F. psychrophilum* from diseased fish. Colonies grown for 2-5 days at 15-20°C on CA are moist, yellow, circular, convex, smooth and non-adherent, 1-5 mm in diameter. Most strains produce colonies with a thin spreading irregular edge. Some strains produce colonies with a regular edge or a mixture of the two types (Figure 5.1).

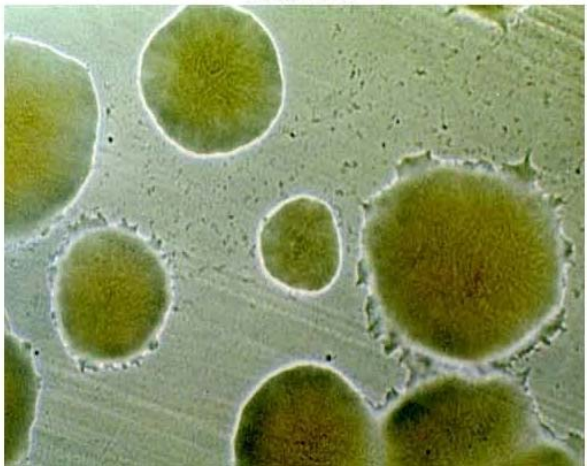

Figure 5.1. Colonies of a *F. prychrophilum* strain on Cytophaga agar, showing a mixture of regular and irregular periphery.

The cells are Gram-negative, slender rods measuring 0.5×2-7 µm (Figure 5.2). The bacteria exhibit gliding motility on wet mount, but gliding is slow and difficult to observe. Holt et al. (1993) described the physiological characteristics of 28 strains of *F. psychrophilum* isolated from various salmonids in USA as follows. All 28 strains grew in supplemented TYE broth at 5-23°C, 18 strains grew slowly at 25°C, and no growth occurred at 30°C. All 28 strains grew in the presence of 0.5 and 1.0% NaCl but none grew in 2.0%. Catalase is positive, and cytochrome oxidase is negative. Flexirubin pigment is present in cells. Ammonium is produced, and hydrogen sulfide, indole, acetylmethl carbinol are not produced. Nitrate is not reduced. Casein, gelatin, albumin, and collagen are degraded. Agar, cellulose, carboxymethyl cellulose, starch, and chitin are not degraded. Degradation of elastin and tyrosine are variable. No acid is produced from simple or complex carbohydrates. Bernardet and Kerouault (1989) reported the presence of cytochrome oxidase but the reaction was weak. The DNA base composition (G+C

content) were reported 33.2-35.3 mol% with a mean of 34.3% for 13 strains (Holt et al. 1993), and 32.5-33.8mol% with a mean of 33.4% for 3 strains (Bernardet and Kerouault 1989). Duchaud et al. (2007) reported the complete genome sequence of the virulent strain JIP02/86 (ATCC49511) of *F. psychrophilum* that contained 32.54% G+C content.

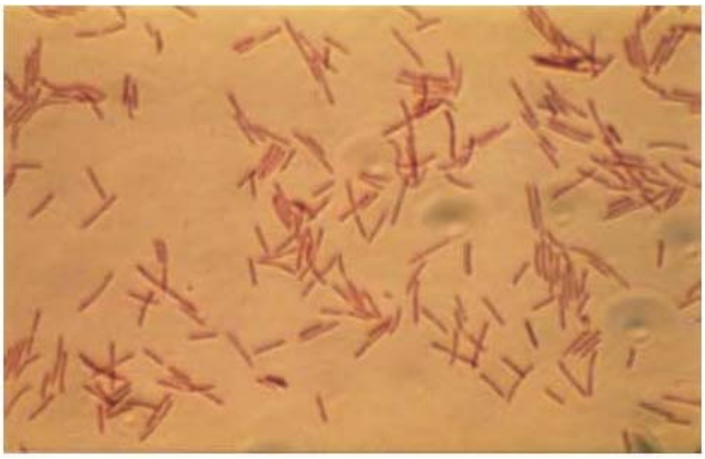

Figure 5.2. Gram-stained *F. prychrophilum* cells, measuring 0.5 ×2 – 7 μm.

Strains of *F. psychrophilum* from salmonids in USA were reported to share common antigen(s) by various authors (Pacha 1968, Pacha and Porter 1968, Bullock 1972, Holt et al. 1993). On the basis of the absorption analysis with thermo-stable antigens, Wakabayashi et al. (1994) and Izumi and Wakabayashi (1999) demonstrated the existence of antigenic diversity within the species, and established three O groups (O1, O2 and O3). The typing system developed by Lorenzen and Olesen (1997) recognized the Tp^T, Th (subtype Th-1 and Th-2) and Fd serotypes. Mata et al. (2002) found seven host-dependent serovars from 34 isolates worldwide. Serovar 1, previously described as O1 or $Fp^{T,}$ was only found in strains isolated from salmon. Serovars 2 and 3, previously described as O3 or Th and Fd, were only found in rainbow trout. Serovars 4, 5, 6 were found in isolates from eel, carp, and tench, respectively. Serovar 7 was equivalent to serotype O2 previously only found in strains from ayu in Japan. Using *Cla*I, *Hae*III and *Pvu*II restriction enzymes in ribotyping analyses 13 different genotypes were demonstrated and a possible relationship between serotype Fd and genotype F1 was determined (Madetoja et al. 2001). Izumi et al. (2003) reported that *F. psychrophilum* could be divided into two genotypes, A and B, by the polymorphism in an anonymous product of 290 bp that was amplified with universal primers for gyrase subunit B gene. Genotype A was found only in isolates from ayu, (*n*=109), while genotype B was found in isolates from coho salmon (*n*=11), ayu (*n*=35), rainbow trout (*n*=43) and other fishes (*n*=44). Yoshiura et al. (2006) identified the 290 bp fragment as a part of coding region of peptidyl-polyl cis-trans isomerase C (*ppiC*) gene. A 326 bp DNA fragment that differentiated genotypes A and B was amplified with a new PCR primers designed for *ppiC* gene (Yoshiura et al. 2006). The evidence that ayu has its own peculiar type of *F. psychrophilum* provoke a question where the type is originated. A hypothesis is that selection for the adapted mutants of *F. psychrophilum* might have occurred in Lake Biwa, the largest lake in Japan, because the single outlet of the lake, Seta River, is dammed and all the population of ayu are landlocked in the lake (Wakabayashi 2009). Nicolas et al. (2008) examined the nucleotide polymorphisms at 11 protein-coding loci of the core genome in a set of 50 strains from 10

different host fish species and four continents. The analysis provided no clues that the initial range of the bacterium was originally limited to North America, and suggested that human activities might enable the main two clonal complexes (CC1 and CC2) spread worldwide (Nicolas et al. 2008).

Strains of *F. psychrophilum* differ widely in virulence (Holt et al. 1993, Madsen and Dalsgaard 2000). Dalsgaard (1993) reviewed various reports concerning the factors determining virulence of *F. psychrophilum.* Nematollahi et al. (2003b) made a comparison between the adhesion capacity of a high and low virulence *F. psychrophilum* strain by using a gill perfusion model and demonstrated that the high virulent strain attached more ready to the gill tissue than did the low virulence. Furthermore, the adhesion of the high virulent strain to the gill tissue was influenced by environmental factors such as organic material, nitrite and temperature. Analysis of 29 isolates of *F. psychrophilum* indicated that the isolates formed four groups based on the presence or absence of certain proteases visualized by substrate SDS-PAGE. *In vivo* infectivity experiments with juvenile steelhead and coho salmo indicated some association between protease group and virulence (Bertolini et al. 1994). Ostland et al. (2000) shown that a crude extracellular preparation from a strain of *F. psychrophilum* had proteolytic activity in that it could degrade gelatin and type II collagen *in vitro* and can produce severe muscle necrosis in experimentally injected rainbow trout after 24 h at 8°C. Secardes et al. (2001) purified an extracellular protease, designated Fpp1 (*F. psychrophilum protease* 1), that cleaved gelatin, laminin, fibronectin, fibrinogen, collagen type IV, and to a lesser extent, collagen types I and II. Production of Fpp1 depended on factors such as calcium concentration, growth phase of the culture, and temperature. Nematollahi et al. (2005) reported that high virulence in *F. psychrophilum* appeared to be correlated with higher macrophage cytotoxicity and resistance to reactive oxygen species (ROC) and, therefore, with enhanced resistance to bacterial killing by rainbow trout macrophages. Nagai and Nakai (2011) demonstrated that the *in vitro* growth of *F. psychrophilum* isolates in host fish serum correlated well with their pathogenicity to host fish, particularly in ayu. All isolates (n=19) from ayu grew well with a 9- to 116-fold increase of colony forming unit (CFU) in ayu serum, while CFU decreased markedly in amago salmon (*Oncorhynchus masou ishikawae*) serum. Experimental infection by intraperitoneal injection showed that ayu isolates examined were all pathogenic to ayu but not to amago salmon.

5.4. Diagnostic Methods

Epizootics of BCWD commonly occur when water temperatures range between 4 and 10°C, but mortality generally abates as temperature approach 15-18°C (Cipriano and Holt 2005). In feral ayu, the disease occurs mostly at water temperature between 12 and 20°C, and a sudden drop in water temperature after heavy raining may have a major impact on the outbreaks (Wakabayashi 2009).

The clinical signs of BCWD differ with the age of affected fish. In coho salmon alevins, the skin covering the yolk sac becomes eroded and the sac may rupture. In fingerlings, the erosion of tissue in peduncle area is observed early in epizootics of BCWD, and later in the outbreak these lesions are found at various locations such as anterior to the dorsal fin, on the lateral side, ventrally, near the vent or on the lower (Cipriano and Holt 2005). In some outbreaks, moribund coho salmon with no external skin lesions, display dark

pigmentation on one side of the body, exhibit dorsal swelling just posterior to the skull, and swim in spiral motions when agitated (Kent et al. 1989). Juvenile coho salmon with aberrant spinal columns occurred several months after symptoms of BCWD disappeared, and the incidence is always greatest at hatcheries where BCWD was most severe (Conrad and DeCew 1967). Such fish often have to be discarded and can result in significant economic loss.

In case of 'rainbow trout fry syndrome' (RTFS), fish weighing 0.2-1 g are the most frequently affected. They exhibit dark coloration of the skin, ascites and exophthalmia. The fry suffered from a severe anaemia causing extremely pale gills. The most consistent internal lesion is spleen hypertrophy often associated with liver discoloration (Berma; det et al. 1988, Lorenzen et al. 1991). *F. psycrophilum* infection at the fry stage may result in an increased occurrence of vertebral column deformities in farmed rainbow trout (Madsen and Dalsgaard, 1999, Madsen et al. 2001).

The clinical signs of ayu infected with *F. psychrophilum* are similar to those of salmonid species. Skin and muscle peduncle lesions are observed first in juvenile ayu (Figure 5.3). In epizootics of BCWD among feral adult ayu, deep dermal ulcerations with necrosis of underlying musculature are found at various locations on the lateral side (Figure 5.4). Most of the affected feral ayu show pale gills, liver discoloration, and spleen hypertrophy (Iida and Mizokami, 1996).

Figure 5.3. Peduncle lesions caused by *F. psychrophilum* in juvenile ayu.

Figure 5.4. Deep dermal ulceration with necrosis of the underlying musculature caused by *F. psychrophilum* in feral adult ayu

For presumptive diagnosis, a microscopic examination of an imprint of spleen tissue that have been air dried and stained with safranin for one minute often will reveal many cells with typical *F. psychrophilum* morphology (Cipriano and Holt 2005). For definitive diagnosis, bacteria should be isolated on an appropriate medium and identified as *F. psychrophilum.* Cytophaga agar (Anacker and Ordal 1959) is the most commonly used, but there have been several reports of improved culture media for *F. psychrophilum* (Holt et al. 1993, Lorenzen 1993, Daskalov et al. 1999, Michel et al. 1999, Cepeda et al. 2004, Alvarez and Gijarro 2007). Other sensitive diagnostic techniques than bacterial culture have been employed to detect *F. psychrophilum* in fish and its surroundings. These include serological methods such as immuno-fluorescence method (Lorenzen and Karas 1992, Izumi and Wakabayashi 1997, Amita et al. 2000, Vatsos et al. 2002, Lindstrom et al. 2009, Long et al. 2012), immuno-enzyme method (Evensen and Lorenzen 1996, 1997, Aikawa 1998) and enzyme-linked immune-sorbent assay (ELSA) (Rangdale and Way 1995, Mata and Santos 2001, Lindstrom et al. 2009, Long et al. 2012). Molecular techniques have also been employed for non-culture based detection of *F. psychrophilum*, including restriction fragment length polymorphism (Nilson and Strom 2002), *in situ* hybridization (Liu et al. 2001), and polymerase chain reaction (PCR) (Toyama et al. 1994, Bader and Shotts 1998, Urdaci, et al. 1998, Cepeda and Santos 2000). Nested PCR assays have been adopted to detect low levels of *F. psychrophilum* from fish tissues and particularly from its surroundings (Izumi and Wakabayashi 1997, Wiklund et al. 2000, Baliarda et al. 2002, Taylor and Winton 2002, Izumi et al. 2005, Crumlish et al. 2007). Suzuki et al. (2008) compared the sensitivity and specificity of PCR methods targeting 16S rDNA, DNA gyrase subunit genes (*gyr A, gyrB*) and *ppiC* for detection of *F. psychrophilum*, and concluded the PCRs targeting gyrB and ppiC seem to be preferable because of no false-positives. Del Cerro et al. (2002a) and Altinok (2011) developed maltiplex PCR methods for the simultaneous detection of three and four major fish pathogens including *F. psychrophilum*, respectively. Del Cerro et al. (2002b) also developed a new detection method for *F. psychrophilum* based on a TaqMan PCR assay. Orieux et al. (2011) described quantification of F. psychrophilum in rainbow trout tissues by qPCR.

5.5. Control

Chemotherapy with antibiotics is still an important method of BCWD control. Oxytetracycline (OTC), amoxicillin (AMS), oxolinic acid (OXA) and florfenicol (FLO) have been widely used around the world (Bruun, 2000, Lumsden et al. 2006). In USA, OTC and FLO are approved for treatment of BCWD in captive-reared fish (Starliper 2011). In Japan, the approved drugs are OTC, OXA, FLO, and sulfuisozole for freshwater-cultured rainbow trout, and OXA, FLO, and sulfuisozole for ayu. However, it has been reported that acquired resistance of *F. psychrophilum strains* exists to various antibiotics (Rangdale et al. 1997, Soule et al. 2005, del Cerro et al. 2010, Kum et al. 2008, Henriquez-Nunez et al. 2012).

Although no licensed vaccines are currently available for prevention of BCWD, several attempts to vaccinate fish against *F. psychrophilum* have been published. Various levels of protection were demonstrated in immunization trials with whole-cell bacterins administered by immersion and/or injection routes (Holt 1993, Obach and Laurencin 1991, Rahman et al. 2000, LaFrentz et al. 2002, Madetoja et al. 2006). Kondo et al.

(2003) demonstrated the effectiveness of oral vaccination against BCWD in ayu. Recent studies have aimed at the development of subcellular vaccines. Rahman et al. (2002) reported that the outer-membrane fraction of *F. psychrophilum* induced significantly higher protection against BCWD in both rainbow trout and ayu compared to the whole-cell bacterin. Plant et al. (2009) demonstrated high antibody resposes in rainbow trout to heat shock proteins 60 and 70. LaFrentz et al. (2011) identified 15 proteins of *F. psychrophilum* by immunoproteomics and suggested that antibodies apecific for outer membrane protein OmpA, trigger factor, ClpB, elongation factor G, gliding motility protein GldN and a conserved hypothetical protein may be important for protective immunity from BCWD. A few studies have dealt with live attenuated vaccine against *F. psychrophilum* infection. LaFrenz et al. (2008) demonstrated that the immersion delivery of the rifampicin resistant 259-93B.17 strain stimulated protective immune responses in fish at 10 weeks post-immunization. Gliniewicz et al. (2012) described that the 259-93B.17 strain havoured a mutation in the *rpoB* gene consistent with resistance to rifampicin. Alvarez et al. (2008) reported that a mutant in one of two *exbD* loci of a TonB system in *F. psychrophilum* showed attenuated virulence and conferred protection against BCWD.

Because *F. psychrophilum* has been detected in fluid surrounding the eggs in sexually mature salmonids, iodophore treatment of eggs is routinely practiced to reduce microbial contamination of the egg surface (LaFrentz and Cain 2004, Cipriano and Holt 2005). However, *F. psychrophilum* presents within egg contents, not just in the surrounding fluids or on the egg surface (Brown et al. 1997, Kumagai et al. 2000, Taylor 2004, Cypriano 2005). Broodstock or egg culling and segregation programs can reduce the probability of BCWD epizootics in progeny at select aquaculture facilities, and the ELIZA is an appropriate tool to screen broodstock and provides an indication of infection severity (Lindstrom et al. 2009, Long et al. 2012).

5.6. Recent Topics

Recent researches suggest that selective breeding for innate resistance may offer a promising tool to control BCWD. Nagai et al. (2004) showed that amphidromous stock of ayu was significantly lower in susceptibility to *F. psychrophilum* challenges than domesticated and land-locked stocks. Henryon et al. (2005) demonstrated additive genetic variation for resistance to *F. psychrophilum* in a Danish rainbow trout population. These studies indicated a favorable potential for selective breeding for increased resistance. Haddi et al. (2008) characterized the phenotype of *F. psychrophilum* resistance and susceptible families of fish as they increased in size > 300-fold, and they showed a positive correlation between disease resistance and normalized spleen weight. Silverstein et al. (2009) demonstrated that rainbow trout survival after *F. psychrophilum* injection challenge was a moderately heritable trait in their broodstock population, indicating favorable implictions for selective breeding for increased disease resistance. More recently, a paper entitled as ‘Selective breeding of food sized rainbow trout against Flavobacteriosis’ was presented in the 3rd International Conference on the Members of the Genus *Flavobacterium* (LaPatra et al. 2012).